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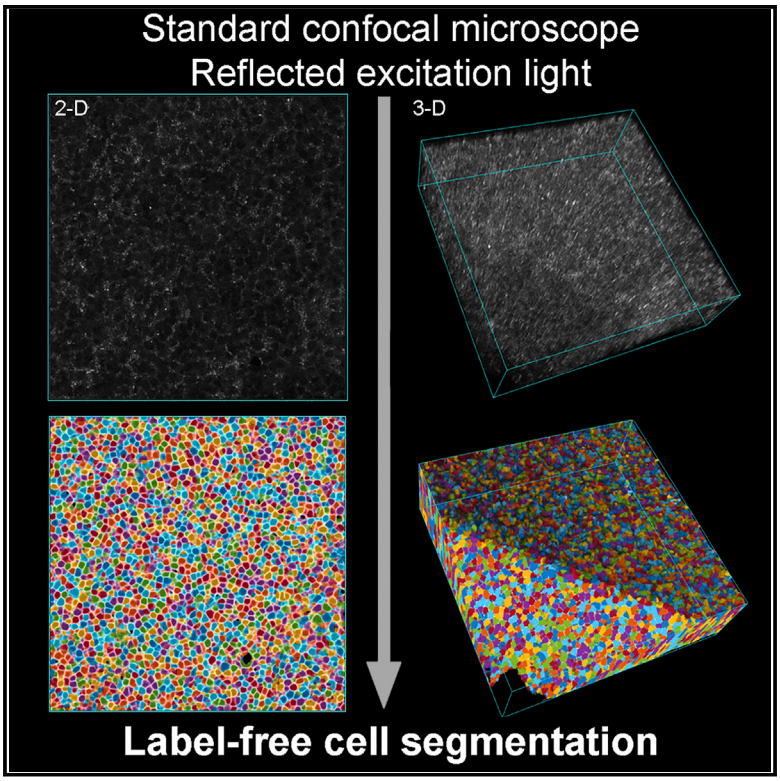
Report

Label-free cell segmentation of diverse lymphoid tissues in 2D and 3D

Graphical abstract



Highlights

- Cell segmentation of tissues can be achieved from reflected laser excitation light
- Single-cell information is freely established for many tissue microscopy studies
- Windows software is provided alongside extensive video tutorials and data

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In brief

Cell segmentation is an essential step in quantitative tissue microscopy. Wills et al. show this can be achieved simply using the reflected laser light always present during routine imaging by confocal microscopy. This frees up microscope channels and establishes single-cell information as an attainable start point for many tissue microscopy experiments.

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Report

Label-free cell segmentation of diverse lymphoid tissues in 2D and 3D

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MOTIVATION Across the biomedical sciences, there is an urgent need to move beyond qualitative imaging to quantitative, cell-based reporting of tissue microscopy data. Typically, cell segmentation requires fluorescent labeling of nucleus and cytoplasm, which limits the spectral bandwidth available for other reporter molecules. However, recent advances in deep-learning algorithms have transformed automated image classification, and this raises the possibility of proceeding with reduced image information. Here, we show that 2D and 3D cell segmentation of lymphoid tissues can be freely established from the reflected laser excitation light always present during routine confocal microscopy using entirely standard equipment.

SUMMARY

Unlocking and quantifying fundamental biological processes through tissue microscopy requires accurate, *in situ* segmentation of all cells imaged. Currently, achieving this is complex and requires exogenous fluorescent labels that occupy significant spectral bandwidth, increasing the duration and complexity of imaging experiments while limiting the number of channels remaining to address the study's objectives. We demonstrate that the excitation light reflected during routine confocal microscopy contains sufficient information to achieve accurate, label-free cell segmentation in 2D and 3D. This is achieved using a simple convolutional neural network trained to predict the probability that reflected light pixels belong to either nucleus, cytoskeleton, or background classifications. We demonstrate the approach across diverse lymphoid tissues and provide video tutorials demonstrating deployment in Python and MATLAB or via standalone software for Windows.

INTRODUCTION

The analysis of tissues using fluorescence labeling and confocal microscopy represents a mainstay biomedical technique that is used worldwide to understand the biology of cells *in situ*.[1–5] However, despite the accessibility of confocal microscopy and its ability to provide sensitive, quantifiable data with subcellular resolution in both 2D and 3D, the number of channels that can be successfully imaged is often limited in practice.[2,6] Moving beyond qualitative observations to cell-based quantifications for every cell in a tissue specimen requires fluorescent staining (e.g., nuclei, cell membrane, or cluster of differentiation [CD] markers) to enable cell segmentation.[2–5] However, these stains occupy channels that are often needed to fully address the bioclinical question.[2,3,5] At the same time, as the number of fluorescence channels increases, so does the complexity, time requirement, and potential for channel cross-talk.[5,7] Correcting this complicates analysis and downstream data processing, increasing the expertise required and the risk of error.[3,5,7,8]

Recognizing these complexities in addition to the need to avoid phototoxicity and temporal errors during live-cell experiments with monolayer cells *in vitro*, microscopy techniques that harness endogenous contrast (i.e., label free) have been developed (e.g., phase/differential interference contrast, etc.).[8–11] However, a recognized difficulty for subsequent, cell-based image analysis is that label-free cell segmentation accuracy decreases as cultures become confluent and cell-to-cell contact is established.[9,10] In this regard, tissue environments are inherently complex and challenging as they are almost entirely comprised of contacting cells in 3D, layer-upon-layer arrangements.[2,3,5]

An often overlooked capability of nearly all laser scanning confocal microscopes is the ability to capture reflected laser excitation light. Importantly, unlike transmitted light, this label-free signal is filtered by the confocal aperture enabling capture

as 3D "z stacks" of optically isolated sections that can be simultaneously acquired alongside fluorescence information.[7,8] Using diverse lymphoid tissues as an exemplar, here we demonstrate how this signal can be harnessed to provide accurate, label-free cell segmentation in 2D and 3D. We show the approach can be deployed in conjunction with the user-friendly, open-source CellProfiler software,[12] enabling single-cell data extraction for image-based cell profiling in addition to reproducible workflow dissemination. The approach provides every cell and nearest-cell neighbor relationship *in situ* with high precision, leaving a spectrally unencumbered landscape for subsequent interrogation. To support uptake, we provide extensive video tutorials and data, demonstrating deployment using Python, MATLAB, or standalone software for Windows.

RESULTS

Figure 1 shows our strategy using mouse splenic tissue. Training data, exemplifying the cellular structure of the tissue, are collected from parallel tissue sections using simple, antibody-independent fluorescent staining for cell nuclei and cytoskeletal F-actin (Figures 1A and 1B). During imaging, both fluorescence data and the backscattered reflectance signal from one of the excitation lasers are captured (Figures 1C and 1D; reflectance imaging setup shown in Method S1; laser invariance demonstrated in Figure S1). From these training data, ground-truth pixel-classification labels representing "background," "nuclei," and "cytoskeleton" classes are easily assembled by binary thresholding the training slide's fluorescence information (Figure 1E). These labels are then used to train a simple U-Net neural network[13] (Method S1; Figure S2) to output the probability that pixels in the reflectance image belong to each of the classifications (demonstrated using MATLAB, Python, or standalone Windows software; Method S1). Because of pixel-wise averaging of any error in the binary representations of staining used as the ground truth, the probability maps outputted by the network exhibit smooth intensity gradients that flow between classifications (Figure 1F). The nature of these images enables them to serve directly as inputs for segmentation of individual cell objects (Figures 1G and 1H; Video S1). This process can be achieved using the user-friendly CellProfiler software,[12] providing a flexible and accessible route to bioimage analysis, feature extraction, and reproducible workflow dissemination. After training, subsequent experimental samples only require the reflectance image to obtain the cell segmentation, leaving the fluorescence spectrum entirely available (Figure S3) for any form of spectral interrogation or combination of fluorescent markers (Figure 1H).

To probe the accuracy of the cell segmentation achieved while exploring the compatibility of the approach with different tissue types, we moved on to tissue sections from intestinal Peyer's patches (Figure 2A), which are key players in the orchestration of mucosal and systemic antibody responses for the microbiome, food, and oral vaccines. To do this, the cell segmentation achieved in CellProfiler using either the fluorescence information (Figure 2B) or the label-free probability maps from the reflectance data (Figure 2C) was compared against the results of careful manual annotation (Figure 2A) using the intersection-over-union (IOU) metric. The label-free approach outperformed (Figures 2D–2F) the results obtainable direct from the fluorescence information (median IOU 0.72 versus 0.61, respectively) while additionally saving two channels.

Using a parallel tissue section to Figure 2 (i.e., with cell segmentation accuracy established), we next considered the ability of the approach to simplify image-based cell profiling. As such, we replicated a recently published experiment[2] that had previously required dedicated nuclei and actin fluorescent stains to achieve accurate single-cell and nearest-cell-neighbor measurements. Mouse Peyer's patch tissue sections were dual immunolabeled for CD11c (identifying mononuclear phagocytes, typically antigen-presenting cells) and for CD3 as a pan T lymphocyte marker. Alongside this, using tissue-matched serial sections, secondary-only controls, isotype controls, and fluorescence-minus-one controls were prepared to inform on background, non-specific antibody binding and fluorescence cross-talk, respectively (see STAR Methods). In half of the previously required imaging time, each tissue section was tile scanned for fluorescence information with concomitant collection of reflected light. Figure 3A exemplifies the outcome, with a region of interest placed around the lymphoid tissue. Guided by the control data, $CD11c^+$ and $CD3^+$ cell populations were built using single-cell fluorescence measurements and simple, flow cytometry-type gating (Figures 3B–3E). As found previously,[2] a second sequential gate on the area occupied by fluorescence within each cell (Figures 3C and 3E) helped to reduce "bystander-positive" events caused by the surface-located fluorescence spanning segmented cell outlines into immediately adjacent cell objects (Figure 3F). From this simplified experiment—now using just two labels instead of the four previously required—diverse information regarding cell location, expression, and nearest-cell-neighbor relationships was obtainable (Figures 3G–3L). Interestingly, a population of highly juxtaposed, CD11c-CD3 neighboring cells that still identified positive for both markers after bystander removal were identifiable, suggesting a high likelihood of cell-cell interaction (Figure 3G). Visually intuitive cell expression maps for CD11c and CD3 could also be assembled (Figures 3H and 3I). Use of the label-free approach also enabled identification and segmentation of all of the unlabeled (i.e., $CD11c^-/CD3^-$) cells, which, in the Peyer's patch environment, predominantly represent B lymphocytes.[14] Hence, the spatial distribution of antigen-presenting cell (APC)-T, APC-B, and T-B lymphocytes that were within interactive distances of one another as nearest-cell neighbors could also be isolated and mapped from the label-free objects (Figure 3J–3L). In this regard, comparing Figures 3J and 3K, a predominance of APC-B interactions, as opposed to APC-T interactions, were observed within the immunoactive subepithelial dome region of the tissue.[15]

In addition to frozen samples, tissue specimens are also commonly archived in formalin-fixed paraffin-embedded (FFPE) format. As a final 2D experiment, we therefore considered if the approach was transferable to this section type. Of note, F-actin staining using phalloidin conjugates is known to fail in FFPE sections because the actin cytoskeleton is degraded by solvent exposures incurred during FFPE processing.[2] The cell outline ground-truth fluorescence labeling on the training slide was therefore switched to cell membrane (i.e., phospholipid) staining using

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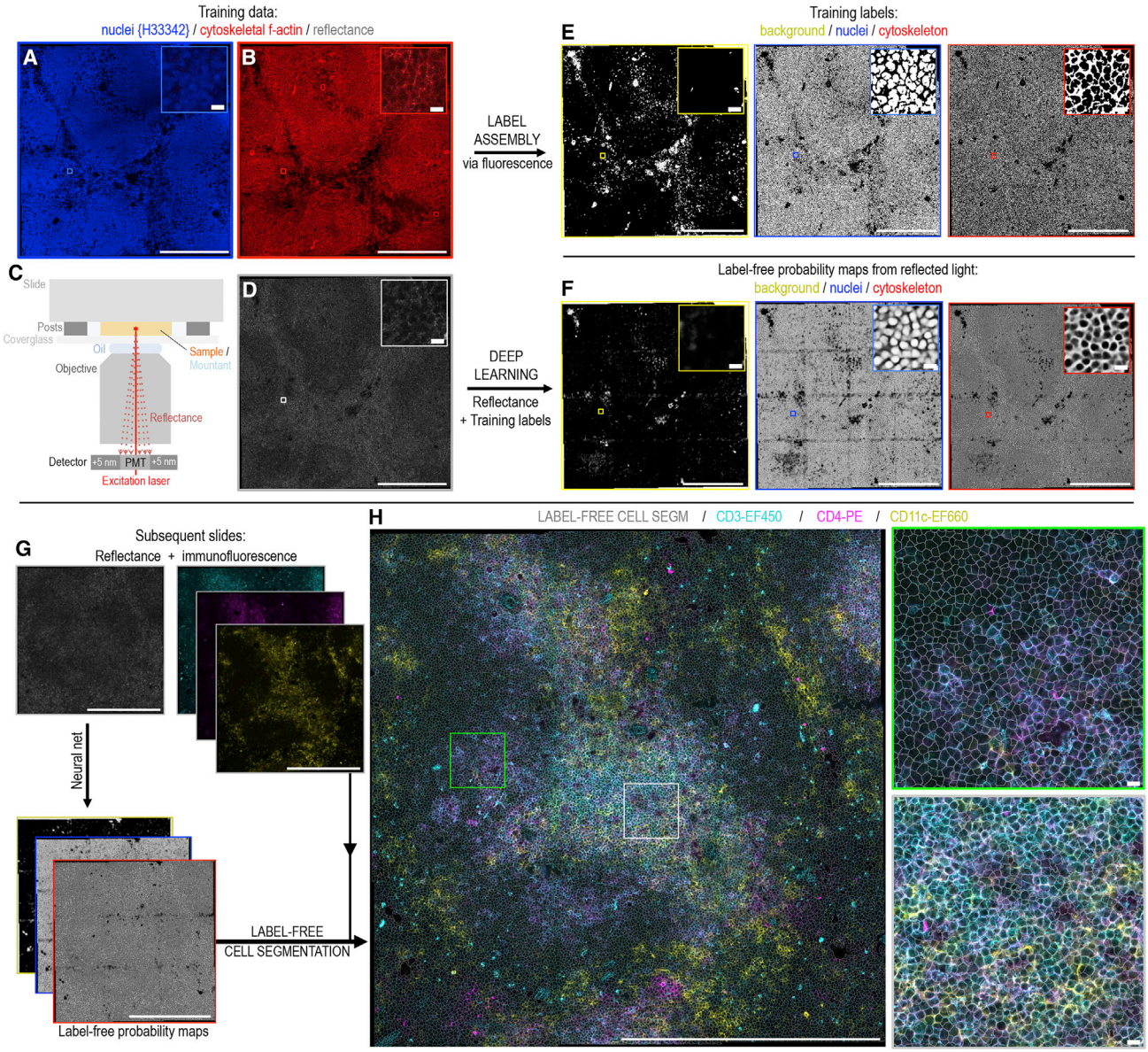

Figure 1. Label-free cell segmentation of tissue microscopy image data collected by routine confocal microscopy

(A–D) Image data (here, mouse splenic tissue) for initial network training are obtained from serial tissue sections stained for (A) nuclei (Hoechst 33342) and (B) cytoskeletal f-actin (phalloidin-AlexaFluor 647) while simultaneously collecting (C and D) reflected laser excitation light by detector placement close (± 5 nm) to the excitation wavelength.

(E) Binary pixel-classification labels representing "background," "nuclei," and "cytoskeleton" classes are created by thresholding the fluorescence data.

(F) A neural network using a simple U-Net architecture is trained to output the probability that pixels in the reflectance image belong to each of these classes. (A)–(F) show zoomed insets of the exact same image region. Comparing across these insets, the outputted probability maps (F) exhibit consistent intensities across each image field, with clear gradients that flow between the individual classifications. This enables easy, consistent instance segmentations of individual cell objects using routine watershed approaches.

(G and H) For subsequent slides, nuclei and actin stains are no longer required as the cell segmentation is achieved direct from the reflectance information via the probability map images. This establishes the cell segmentation while leaving the entire detection spectrum free for fluorescence-based analyses. For example, (H) shows the approach operating with CD3-eFluor450, CD4-PE, and CD11c-eFluor660 immunofluorescence conjugates utilizing the spectral bandwidth previously occupied by the nuclei (Hoechst 33342) and actin (phalloidin-AlexaFluor 647) stains. The label-free cell segmentation is overlaid.

(H) Insets demonstrate successful label-free cell segmentation of both CD marker-stained and entirely unstained cells in both red (green inset) and white (gray inset) pulp tissue regions.

(A–H) Main image scale bars: 250 μm, and inset image scale bars: 10 μm.

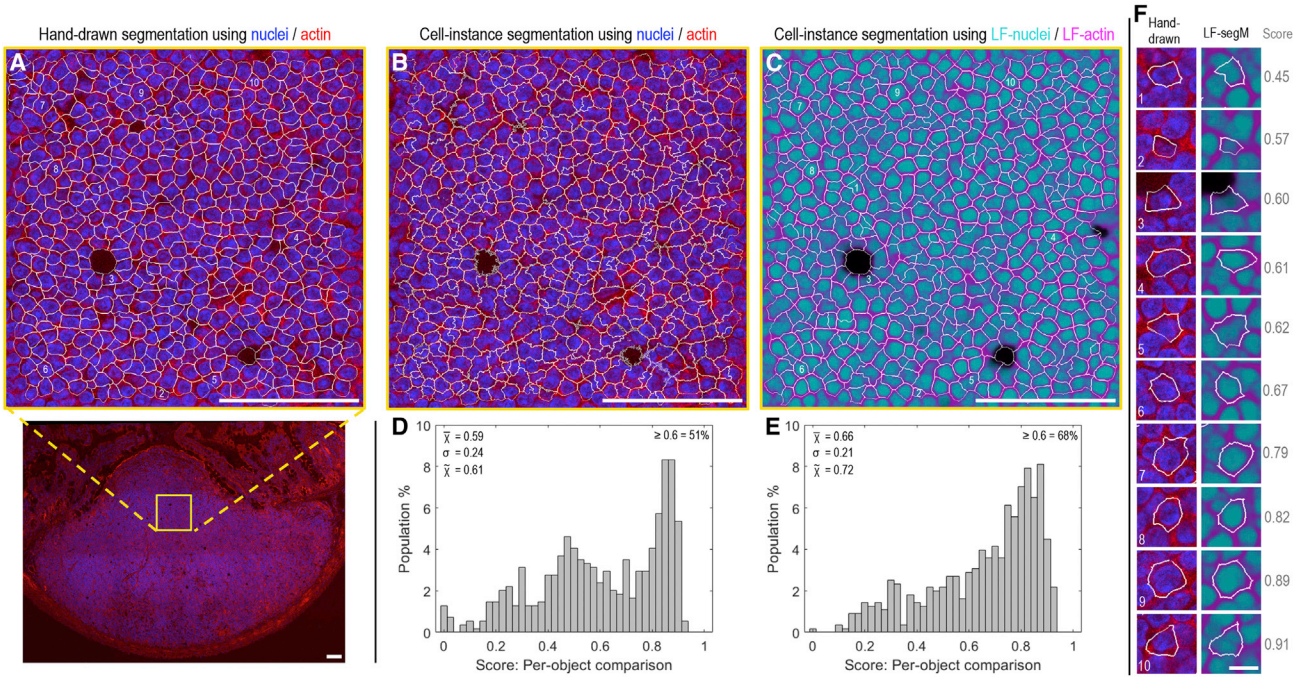

Figure 2. Assessing cell segmentation accuracies using mouse Peyer's patch tissue

(A) Hand-drawn cell segmentation performed using nuclei/actin fluorescence information for the tissue region indicated by the yellow box in the wider, tile-scanned image.

(B and C) Automated cell instance segmentations for the same image-region as (A) using either (B) the raw nuclei and actin fluorescence data or (C) the label-free probability maps obtained from the neural network using reflectance alone as input (image data from this tissue section were unseen during training).

(D and E) Cell-object intersection-over-union (IOU) score distributions comparing the hand-drawn cell segmentations shown in (A) against the automated cell segmentations shown in (B and C) using either (D) fluorescence or (E) label-free information.

(F) Example hand-drawn versus label-free cell segmentation comparisons and IOU scores.

The positions of each cell in the source images are shown by the cell-object numberings in (A), (C), and (F). An IOU score of 1 represents perfect per-pixel overlap between hand-drawn and automated cell segmentations. (F) Within the comparison presented here, scores $\geq$0.6 are seen to represent a good match, approaching the limit of hand-drawing accuracy given the relatively low resolution of the source image data. (A–C) Scale bars, 100 μm. (F) Scale bar, 10 μm.

fluorescently conjugated wheat germ agglutinin (WGA). Despite a notably different reflectance signal (presumably due to cytoskeletal degradation), a segmentable relationship between the reflectance information and the WGA staining was learnable (Figure S4). Encouragingly, similar IOU scores (FFPE median score = 0.74) were attainable to those achieved using frozen sections (0.72) (Figures S4 and 2). In this way, segmentations based on training exemplifications from a different fluorescence label unlocked this important section type to the label-free technique.

With the capability of our approach established in 2D, we moved forward to 3D imaging with the goal of retrieving entirely label-free segmentations for all cells in imaged volumes (Figure 4). Previously, T cell clustering in secondary lymphoid tissues (lymph nodes) and the role that FOXP3$^+$ regulatory T cells play in suppressing potentially autoreactive T cells were demonstrated using 3D imaging and the "histocytometry" approach.[4] In that work, segmentations were achieved for cells expressing a fluorescent marker—but not for unlabeled cells. Here, a simple extension to a 3-D U-Net architecture (Method S1) enabled the generation of probability maps from z stacked reflectance information that were easily segmentable into 3D cell objects using CellProfiler 4 (Figures 4A–4H). As before, cell segmentation accuracies in the xy, zy, and xz dimensions were assessed against manual annotations using the IOU approach (Figure S5). Encouragingly, use of a 3D network leveraging data across multiple z planes simultaneously improved the segmentation accuracies (median IOU scores xy = 0.84, zy = 0.74, xz = 0.78) achievable relative to the 2D network results (xy = 0.72) (Figures S5 and 2).

To test the ability of the approach to retrieve single-cell and nearest-neighbor relationships in 3D, immunofluorescence data for FOXP3 or isotype control were collected (Figure 4I). As in 2D, flow cytometry-type gating established the FOXP3$^+$ cell population *in situ* (Figures 4J–4L). FOXP3$^+$ cells were, indeed, isolatable as cell populations of independent events (Figure 4M), and harnessing the segmentation information of the unlabeled cells further allowed 3D identification and visualization of all nearest-cell neighbors with interactive potential (Figure 4N). In this way, individual cells, with or without "touching" nearest-cell neighbors, could be isolated and examined as independent populations in a manner similar to imaging flow cytometry,[16,17] with the additional ability to rotate, cut away, and consider objects and their contents from any angle (Figure 4M). Moreover, with tissue-relevant localization retained, the full *in vivo* cell-cell environment incorporating all nearest neighbors in 3D was

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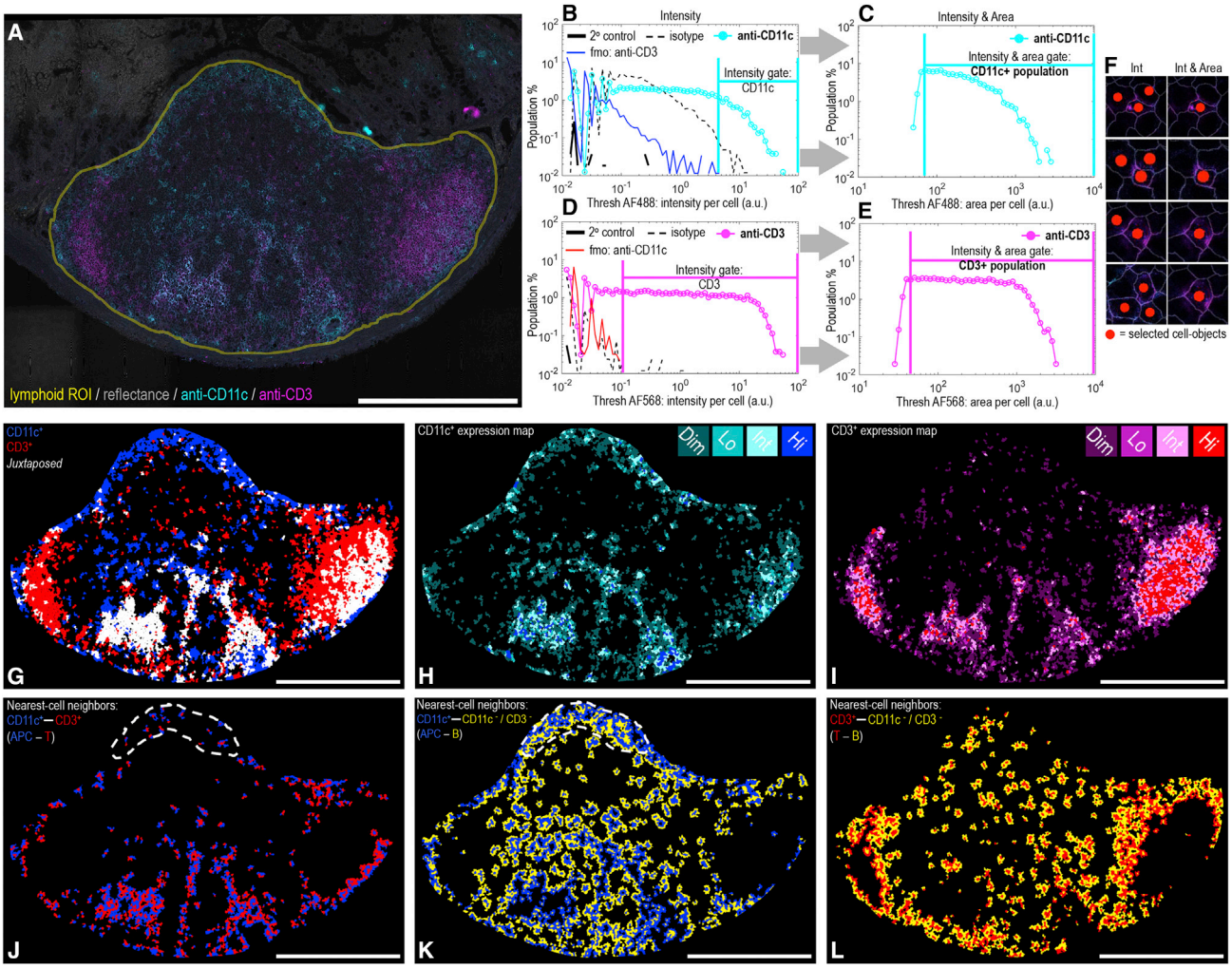

Figure 3. Label-free cell segmentation enables image-based cell profiling

(A) Tile-scanned mouse Peyer's patch tissue section imaged for reflectance in addition to immunofluorescence markers for CD11c (i.e., mononuclear phagocyte antigen-presenting cells) and CD3 (T lymphocytes). The yellow region of interest (ROI) represents the lymphoid tissue upon which the label-free cell segmentation approach was deployed (~16,000 cells). Outside of the ROI, the reflectance image is seen to still provide interpretable histological context.

(B–E) Flow cytometry-type gating to establish $CD3^+$ and $CD11c^+$ cell populations informed by secondary only, fluorescence-minus-one (fmo) and isotype single-cell fluorescence distributions obtained from label-free cell object data collected from adjacent, serial tissue sections. Due to the dense cellular packing of lymphoid tissue, (C and E) second sequential gates on the fluorescence area occupied per cell object helped to reduce (F) bystander-positive events caused by fluorescence overlap into neighboring cells.

(G) Cell map view showing the gated cell populations *in situ* using flood filling of label-free cell-objects. Juxtaposed CD11c-CD3 neighboring cells that still identified positive for both CD markers after bystander removal are shown in white.

(H and I) CD11c and CD3 expression maps with cell objects shaded into four levels (dim, low, intermediate, high) according to each segmented cell's level of immunofluorescence.

(J–L) Nearest-cell-neighbor maps simplifying the view shown in (A) to only show touching groups of cell objects according the combinations (J) $CD11c^+$-$CD3^+$ (i.e., APC-T), (K) $CD11c^+$-$CD3^-$/$CD11c^-$ (i.e., APC-B), and (L) $CD3^+$-$CD3^-$/$CD11c^-$ (i.e., T-B). In this way, the views give a sense of key cell types within interactive distances of one another. The dashed line in (J) and (K) indicates the subepithelial dome tissue region.

Scale bars: 500 μm.

revealed (Figures 4L–4N). The approach was tested with tissue sections up to ~100 μm in thickness. At this depth, the fluorescence from typical blue nuclear labels (e.g., Hoechst 33342 or DAPI) is attenuated by the tissue thickness and cannot serve as an input for accurate segmentation, whereas the label-free strategy still operated effectively (Figure S6).

DISCUSSION

There is a growing need for accessible means to obtain *in situ*, single-cell information from tissue images across the bioclinical sciences. A major barrier is the relatively few channels, for separate biomarkers, that conventional microscopy allows for

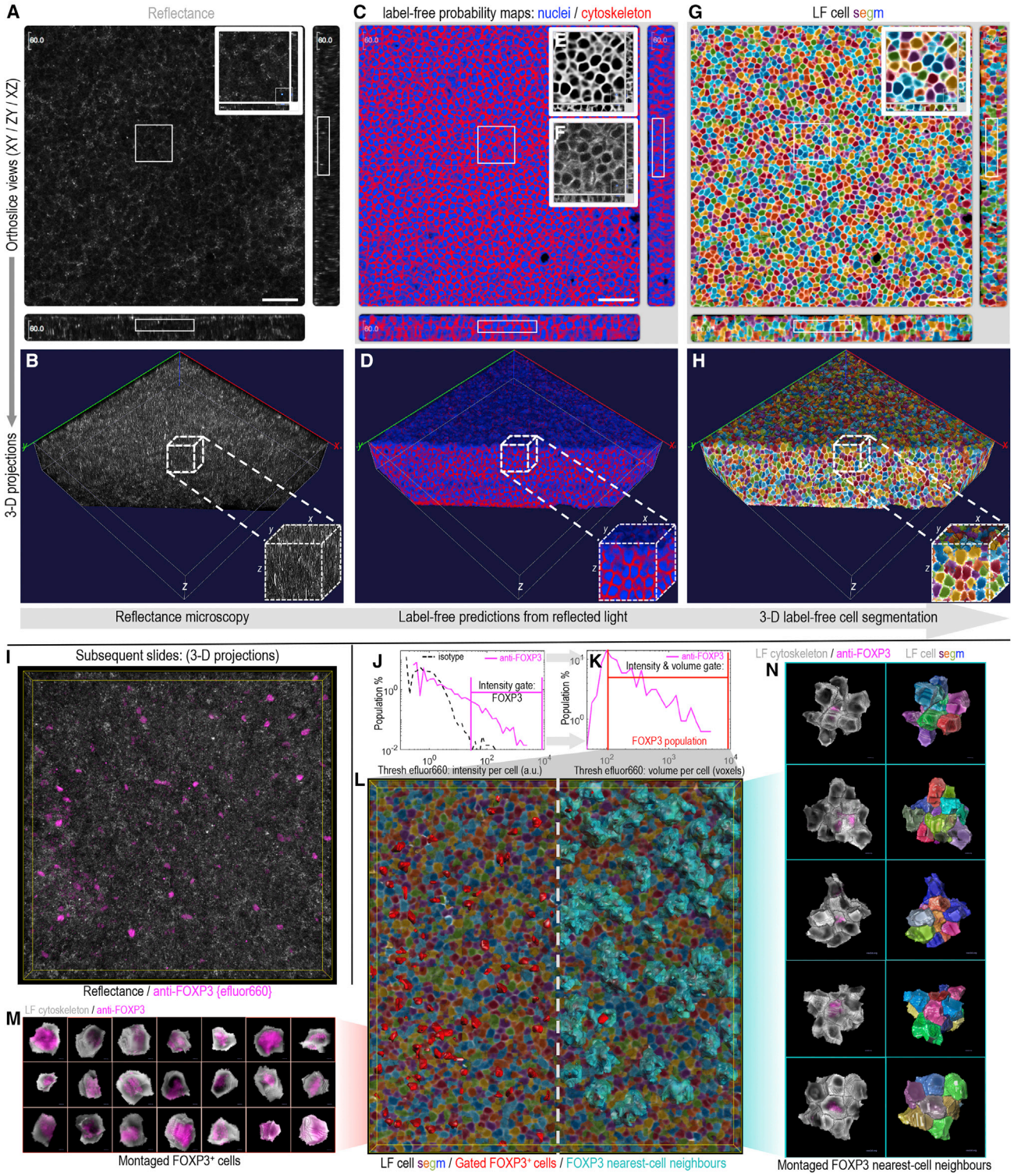

Figure 4. 3D label-free cell segmentation of tissue microscopy image data collected by routine confocal microscopy

(A–H) Stepwise exemplification of the 3D strategy using z stack image data of mouse mesenteric lymph node tissue. Outcomes at each step are displayed by (A, C, and G) orthoslice and (B, D, and H) 3D volumetric projection views, with the latter cut away to better display outcomes along the z dimension.

(A and B) 3D reflectance signal.

(legend continued on next page)

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experimentation (often ≤6 in practice). This becomes compounded when two of these channels are required to achieve accurate cell segmentation. Imaging mass spectrometry systems, which are capable of resolving many metal-conjugated antibodies, may partially obviate these issues, but instrumentation is not widely available. In practice, to move bioclinical research beyond "representative image" reporting, 2D and 3D cell-based quantitation of tissues with standard confocal microscopy equipment must become routine, and the workflow from image data to cell features must be disseminable.[2,4,5]

Previously, fluorescence image restoration[18] and virtual *in vitro* cell labeling[7,8] have been demonstrated as powerful applications of fluorescence image reconstruction by deep learning. In their seminal paper, using a custom-built multimodal reflectance microscope, Cheng et al. also showed that fluorescent stain predictions from reflected light information could be used to achieve 2D segmentation of monolayer cells in culture. Notably, however, *ex vivo* tissue microscopy offers a very different challenge to cell microscopy. Tissue is made up of multiple different cell types and extracellular features in dense, layer-upon-layer arrangements in a way that is not present *in vitro.* The image information is also fundamentally different due to the histological preparation steps (fixation, embedding, and sectioning) that are different or not required for cultured cells. Here, we recognized that during confocal imaging with entirely standard equipment, there is always freely available "byproduct" reflected light. Our work now shows that this carries sufficient information, with sufficient penetration, to establish accurate 2D and 3D segmentations of cells in tissues. To do this, we use classification and probability mapping—as opposed to regression-based fluorescent stain predictions—as intensity uniformity across the outputted probability maps is advantageous to the cell segmentation task. Moreover, we provide the software, data, and video tutorials necessary to remove the programming barrier to access, making this accessible for everyone. Because generalizability is essential to the practical utility of any method, we carefully demonstrate our approach using data from two different confocal microscopes across 40× and 63× objective lenses in three different tissues using reflectance from three different laser lines across four image resolutions (from 3.5 to 8.3 pixels per μm). We also demonstrate the compatibility of the approach with both frozen and FFPE tissue section types.

An important aspect of the presented method is that it allows the assembly of large amounts of sample-matched training data without the need of cell annotation. This enables conventional U-Net models to be trained with bespoke exemplifications of the task,[13,18] maximizing performance while providing data at a scale sufficient to avoid memorization and enable rigorous cross-validation testing.[19] These training data are prepared using antibody-independent affinity staining, enabling easy transfer across species[2] while minimizing pixel labeling errors through avoidance of non-specific binding.[7]

In this way, the presented work enables a move beyond disaggregated flow cytometry measurements and qualitative microscopy reporting by harnessing label-free information from every cell in a tissue section such that cell content and *in situ* location can be reported together. Detailed cell-cell interactions (nearest-neighbor-type relationships) in complex tissue environments are achievable and will provide the bridge between deep immunological knowledge of single cell types and macroscopic tissue function. All data, code, and methodological steps are available for download alongside detailed video tutorials demonstrating deployment in Python, MATLAB, or provided standalone software for Windows.

Limitations of the study

This technique relies on the existence of a relationship between the reflectance signal and the fluorescence information used to determine the ground truth.[18,20] Moreover, this relationship must describe the cellular structure in a manner that enables accurate cell-object segmentations. Here, we demonstrate that label-free predictions of cytoskeletal or cell membrane structure enable this from frozen and paraffin-embedded tissue sections for diverse lymphoid tissues (spleen, Peyer's patch, and mesenteric lymph node), where cell relationships are so important in establishing fundamental biology including responses to infection, vaccination, and carcinogenesis. However, this approach may not work in every tissue type as it is dependent upon the specific structural morphology of cell and tissue and the resultant optical scattering coefficients.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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  - Data and code availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS

(C–F) Label-free probability maps outputted from a 3D U-Net neural network (C and D) with insets demonstrating the (E) label-free probability map representation for the cytoskeleton compared with (F) fluorescent cytoskeletal F-actin staining.
(G and H) 3D label-free cell segmentation results where (repeated) filled colors represent individual cell objects.
(I–N) Validation of the 3D label-free approach using tissue sections immunolabelled for FOXP3 or matched isotype control with no nuclei or actin staining present.
(J and K) Flow cytometry-type gating using cell-object fluorescence distributions to establish $FOXP3^+$ events from cell intensity and fluorescence volume information.
(L) 3D projection of the label-free cell segmentation results. On the left, gated $FOXP3^+$ cells are identified using red surface overlays. On the right, both $FOXP3^+$ events (red) and their touching nearest-cell neighbors (cyan) are shown *in situ*. (M), 3D projections of individual $FOXP3^+$ cell objects cut out and montaged from the label-free segmentation. An intranuclear core of FOXP3 staining is visible surrounded by the label-free probability map for the cytoskeleton classification.
(N) 3D projections of individual $FOXP3^+$ cell objects and their touching nearest-cell neighbors cut out and montaged using the label-free cell segmentation.
(A, C, and G) Scale bars: 50 μm.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.crmeth.2023.100398.

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AUTHOR CONTRIBUTIONS

J.W.W., J.J.P., and P.R. conceived the concept. J.W.W., J.J.P., R.E.H., C.E.B., and P.T. designed the biological experiments. J.W.W., J.R., R.E.H., and M.M. carried out the immunofluorescence labeling and collected the image data. J.W.W., P.R., C.M.C.G., and H.D.S. designed and optimized the deep-learning method. J.W.W. and P.R. wrote the MATLAB implementation. C.M.B. and J.W.W. wrote the Python implementation. P.R. and J.W.W. wrote the standalone software. J.W.W., J.J.P., H.D.S., and P.R. wrote the manuscript in close collaboration with all authors. All authors reviewed, contributed in full, and approved the final version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rat anti-mouse CD3-EF450	Thermo Fisher	Cat #48-0032-82
Rat anti-mouse CD4-PE	Thermo Fisher	Cat #12-0041-83
Hamster anti-mouse CD11c-EF660	Thermo Fisher	Cat #50-0114-82
Rat anti-mouse FOXP3-EF660	Thermo Fisher	Cat #50-5773-82
Rabbit anti-mouse CD3	Abcam	Cat #AB5690
Hamster anti-mouse CD11c	Abcam	Cat #AB33483
Goat anti-Rabbit IgG (H + L) Alexa Fluor 568	Thermo Fisher	Cat #A-11011
Goat anti-Hamster IgG (H + L) Alexa Fluor 488	Thermo Fisher	Cat #A-11008
Biological samples		
Frozen C57BL/6J mouse spleen sections	This paper	N/A
Frozen C57BL/6J mouse Peyer's patch sections	This paper	N/A
Frozen C57BL/6J mouse mesenteric lymph node sections	This paper	N/A
Formalin-fixed paraffin embedded C57BL/6J mouse Peyer's patch sections	This paper	N/A
Chemicals, peptides, and recombinant proteins		
Hoescht 33,342	Thermo Fisher	Cat #H3570
Phalloidin-AlexaFluor 647	Thermo Fisher	Cat #A22287
Wheat germ agglutinin-AlexaFluor 555	Thermo Fisher	Cat #W32464
Deposited data		
Raw and analyzed microscopy data	This paper	https://www.ebi.ac.uk/biostudies/studies/S-BSST742
Experimental models: Organisms/strains		
Mouse: C57BL/6J	Charles River	Cat #027
Software and algorithms		
MATLAB code	This paper	Method S1 or https://www.ebi.ac.uk/biostudies/studies/S-BSST742
Python code	This paper	Method S1 or https://www.ebi.ac.uk/biostudies/studies/S-BSST742
Standalone label free prediction software	This paper	https://www.ebi.ac.uk/biostudies/studies/S-BSST742
MATLAB R2021a (or later)	MathWorks	https://uk.mathworks.com/products/new_products/release2021a.html
Deep Learning Toolbox 14.3	MathWorks	https://uk.mathworks.com/help/deeplearning/
Image Processing Toolbox 11.4	MathWorks	https://uk.mathworks.com/products/image.html
Computer Vision Toolbox 10.1	MathWorks	https://uk.mathworks.com/products/computer-vision.html
Python 3.6	python.org	https://www.python.org/downloads/release/python-360/
tensorflowgpu 1.9.0	tensorflow.org	https://pypi.org/project/tensorflow-gpu/1.9.0/
Keras 2.1.5	keras.io	https://pypi.org/project/keras/2.1.5/
numpy 1.18.1	numpy.org	https://pypi.org/project/numpy/1.18.1/
scipy 1.4.1	scipy.org	https://pypi.org/project/scipy/1.4.1/
pandas 1.0.3	pandas.pydata.org	https://pypi.org/project/pandas/1.0.3/

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
scikit-learn 0.22.1	scikit-learn.org	https://pypi.org/project/scikit-learn/0.21.1/
scikit-image 0.16.2	scikit-image.org	https://pypi.org/project/scikit-image/0.16.2/
pillow 8.4.0	pillow.readthedocs.io	https://pypi.org/project/Pillow/8.4.0/
ipython 7.13.0	ipython.org	https://pypi.org/project/ipython/7.13.0/
opencv 4.2.0.34	opencv.org	https://pypi.org/project/opencv-python/4.2.0.34/
javabridge 1.0.19	pythonhosted.org	https://pypi.org/project/javabridge/
bioformats 1.5.2	openmicroscopy.org	https://pypi.org/project/python-bioformats/1.5.2/
matplotlib 3.3.4	matplotlib.org	https://pypi.org/project/matplotlib/3.3.4/
H5py 2.10.0	h5py.org	https://pypi.org/project/h5py/2.10.0/
Imageio 2.11.0	imageio.readthedocs.io	https://pypi.org/project/imageio/2.11.0/
Java SE Development kit 11.0	oracle.com	https://www.oracle.com/uk/java/technologies/javase/jdk11-archive-downloads.html
CUDA Toolkit 9.0	developer.nvidia.com	https://developer.nvidia.com/cuda-90-download-archive
cuDNN 7.6.4	developer.nvidia.com	https://developer.nvidia.com/cudnn
Cell Profiler 4.1.3 (or later)	cellprofiler.org	https://cellprofiler.org/releases
Vaa3D	alleninstitute.org	https://github.com/Vaa3D/release/releases/
Other		
Zeiss LSM 780 confocal microscope	Zeiss	https://www.zeiss.com/microscopy/en/products/light-microscopes/confocal-microscopes.html
Leica SP8 confocal microscope	Leica Microsystems	https://www.leica-microsystems.com/products/confocal-microscopes/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, John W. Wills (jw2020@cam.ac.uk).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- Microscopy data are publically available as of the date of publication from the BioStudies database (https://www.ebi.ac.uk/biostudies/) under accession number S-BSST742.
- All original code (in MATLAB and Python languages) is available in the Method S1 file. All code as well as the precompiled, Windows software has also been deposited at the BioStudies database (https://www.ebi.ac.uk/biostudies/) under accession number S-BSST742 alongside screencast tutorial videos demonstrating deployment. These files are publicly available as of the date of publication.
- Any additional information required to reanalyse the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Murine tissues

Spleen, ileum (containing Peyer's patches) and mesenteric lymph node tissues were collected from healthy, male C57BL/6 mice (n = 4) (8-12 week-old) sacrificed by carbon dioxide asphyxiation. Tissues for cryosection analysis were snap-frozen in isopentane cooled on dry ice before storage in liquid nitrogen until use. Tissues for FFPE processing were fixed in neutral buffered formalin (4 h) prior to transfer to tissue cassettes. Samples were embedded in paraffin by dehydrating through an aqueous ethanol series (5 min each 20%, 50% 70% (100% x2) v/v) followed by three changes of 100% xylene (30°C) then three changes of paraffin wax (62°C). All animal work complied with the University of Cambridge Ethics Committee regulations and was performed under the Home Office Project License numbers 80/2572 and P48B8DA35.

METHOD DETAILS

Tissue sectioning

Frozen tissues were transferred into the cryostat chamber (- 15°C) and acclimatised for 30 min. Samples were trimmed to remove any excess fat, and transferred to cryomolds containing pre-chilled optimal cutting temperature compound (OCT) (#00411243, VWR). Sections were cut at 25 or 100 micron thicknesses (for 2D or 3D imaging, respectively) and collected on Super-Frost Plus adhesion treated slides (#J1800AMNT, Thermo) before resting at room temperature for 2 h prior to immunofluorescence labeling. Formalin-fixed, paraffin embedded (FFPE) sections were cut at 5 μm thickness. FFPE sections were dewaxed by baking at 60°C for 1 h prior to changing twice through xylene. Prior to fluorescence counterstaining, FFPE sections were rehydrated using a reverse ethanol series (100%, 70%, 50%, 10%; 5 min each) followed by immersion in water (1 min).

Immunofluorescence labeling

Tissue sections were ringed with hydrophobic barrier pen (Vector, #H-4000). Frozen sections were fixed using fresh 4% paraformaldehyde in 0.1 M PBS (pH 7.4) at room temperature for 10 or 20 min (25 μm or 100 μm sections, respectively). All subsequent steps were carried out under gentle agitation on a rotating shaker. To facilitate antibody penetration, the frozen sections were permeabilised for 2 or 4 h using 0.3% (v/v) Triton X-100 in 0.1 M PBS (pH 7.4) (25 μm or 100 μm sections, respectively). Sections were then blocked using 25 mM TBS (pH 7.4) supplemented with 10% (v/v) goat serum (ThermoFisher, #16210064), 2% (w/v) BSA (BioSera, #PM-T1726) and 20 mM glycine for 2 h. Primary antibodies or isotype controls were prepared in block buffer and added at 150 μL per section for 18–24 h at 4°C. All subsequent steps took place at room temperature. Sections were washed (3 × 3 min, TBS) prior to incubation with secondary antibodies (when needed) diluted in block buffer for 4 or 8 h (see Table S1 for antibody concentrations, fluorophores conjugations and manufacturer information) (25 μm or 100 μm sections, respectively). After washing (3 × 3 min, TBS) frozen sections destined for the provision of training data underwent nuclear and f-actin counterstaining using 2 μg/mL Hoechst 33,342 (#H3570, Thermo) and 500 nM phalloidin-AlexaFluor 647 (#A22287, Thermo) in TBS for 1 h. The cellular structure of the FFPE sections was counterstained by labeling cell membranes using 20 μg/mL wheat-germ agglutinin (WGA) conjugated with Alexa Fluor 555 (#W32464, Thermo). After counterstaining, all sections were washed for a final time (1 × 3 min, TBS) before mounting with #1.5 coverslips in Prolong Glass mountant (#P36980, Thermo).

Antibody controls

Three antibody controls commonly used by the flow cytometry community were measured in tissue-matched serial sections. Secondary-only controls received just the secondary antibody in absence of any primary antibody. Any signal in the collection channel for this control thus represented endogenous tissue autofluorescence or contributions from the fluorophore-conjugated secondary antibody binding non-specifically in the tissue section. Fluorescence-minus-one (FMO) controls contained all of the fluorescent stains – bar the one under quantification. Here, any signal in the collection channel was typically from 'spill over' from the other fluorophores into this empty channel. Finally, 'isotype controls' switched out the primary antibody for an irrelevant antibody (*i.e.,* raised against an antigen not present in the sample) but otherwise identical (*i.e.* same isoclass) to the primary antibody. Here, non-specific binding of this irrelevant primary antibody or capture by, *e.g.,* Fc receptors led to signal in the collection channel, informing on the level of non-specific binding.

Confocal microscopy

The label-free strategy was developed using image data collected from two commonplace (Leica SP8/Zeiss LSM 780) laser scanning confocal microscopy platforms. The SP8 was inverted configuration, whilst LSM780 was upright. No modifications from standard were necessary to enable the presented approach. Detailed instructions for setting up reflected light collection are provided in Method S1. Image data for the presented 2D analyses were collected using the SP8 via 40X/1.3 or 63X/1.4 oil immersion objectives. Reflectance was collected from the 488 nm laser via detector placement +/− 3 nm either side of the excitation line (*i.e.,* detection in the range 485–491 nm). 3D data were collected using the LSM 780 via the 40X/1.3 oil immersion objective. Reflectance was collected from the 561 nm laser via detector placement +/− 9 nm either side of the excitation line (*i.e.,* 552-600 nm). Tilescans were conducted with 10% edge overlap to facilitate registration. Details of the tissue specimen, image dimensions and pixel/voxel densities are provided for all image data in Table S1.

QUANTIFICATION AND STATISTICAL ANALYSIS

Label-free cell segmentation workflow

Neural network training data was collected from parallel tissue sections to those undergoing immunofluorescence labeling. Training data was achieved by collecting fluorescence images for nuclei and cytoskeletal f-actin (frozen sections) or nuclei and the cell membrane (FFPE sections) (fluorescence staining described above) alongside the 'paired' reflectance signal. Binary pixel classification labels were assembled by thresholding the fluorescence information to create pixel label classes representing 'nuclei', 'cytoskeleton' and 'background' classifications. Because of the paired nature of the training and test image-data (*i.e.,* collected using the same microscope settings) input reflectance data were rescaled in the zero-one interval with no contrast

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adjustment prior to inputting into the 2D or 3D U-Nets (architectures shown, Method S1). Using cross-entropy loss, the networks were then trained to output the probability that pixels in the reflectance image belonged to each of the classifications with the probability image from the loss function serving as the direct input for cell segmentation. Network training, optimisation and validation testing was conducted using MATLAB R2021a and the Deep Learning, Image Processing and Computer Vision toolboxes (described, Method S1). Scripts for running the 2D and 3D U-Nets were also written for Python 3 using keras/TensorFlow-gpu 1.9 (TensorFlow install guide and U-Net scripts described, Method S1). The probability map images outputted by the U-Net networks were segmented into 2D or 3D cell objects using marker-controlled watershed algorithms deployed in CellProfiler[12] (version 4.1.3) (instructions for installing CellProfiler and running the 2D and 3D image analysis pipelines are provided in Method S1). In all instances, neural networks were trained on data from one tissue section before validation testing using data collected from an entirely different tissue section.

2D U-NET

Reflectance data were passed to the network as patches with dimensions 256 × 256 × 1 (x, y, channels) with augmentation by x/y reflection and rotation. The three-class U-Net architecture used an encoder depth of 4 with 64 filters in the first layer (shown, Method S1). Complete up-convolutional expansion was used to provide probability maps of the same size as the input images. Training lasted for 50 epochs (frozen sections) or 150 epochs (FFPE sections) using a batch size of 12 with zero-center normalisation (demonstrated, Video S1). Training was optimised using stochastic gradient descent using cross-entropy loss. The initial learning rate was 0.05, dropping every 10 epochs by 0.1 under momentum 0.9 and L2 regularisation $1x10^{-4}$. Patches were shuffled every epoch.

3D U-NET

Reflectance data were patched through the network with input dimensions 64 × 64 × 64x1 (x, y, z, channels) and augmentation by x/y reflection and rotation. The three-class U-NET architecture used an encoder depth of 4 with 64 filters in the first layer (shown, Method S1). Complete up-convolutional expansion was used to output probability maps of the same dimensions as the source microscopy data. Training lasted for 150 epochs using a batch size of 8 with zero-center normalisation. Training was optimised under ADAM using cross-entropy loss. The initial learning rate was $5x10^{-4}$, dropping every 5 epochs by 0.95 under L2 regularisation $1x10^{-4}$. Patches were shuffled every epoch.

2D/3D U-NET: Standalone windows software

The label-free prediction software (described, Method S1) for Windows was built in MATLAB R2021a using the MATLAB App Designer and MATLAB Compiler. This enables 2D/3-D U-NET training and deployment via a simple graphical user interface removing the need for programming expertise.

Segmentation accuracy

Using the Jaccard index (intersection over union) approach, 2D and 3D label-free cell segmentation accuracies were assessed by comparing pixel positions within automatically segmented cell objects against those inside manually-drawn cell outline annotations. To assess the 3D segmentations, annotations of XY as well as ZY and XY dimensions were used to fully explore the validity of the segmented cell objects along all three dimensions. The Jaccard index was calculated as:

$$J(A,M) = \frac{|A \cap M|}{|A \cup M|} = \frac{|A \cap M|}{|A| + |M| - |A \cap M|} \quad \text{(Equation 1)}$$

Where, J is the Jaccard distance for two sets containing pixel positions for the automated segmentation (A) and the manual annotation (M) respectively. A score of 0 represents no overlap (*i.e.,* false negative) whereas 1 represents exact pixel-for-pixel overlap. It is acknowledged that this approach is a relatively harsh success measure and that a score of ~0.7 indicates a good segmentation result.[13] This is due in-part to the inaccuracies that are inevitably present even in the human annotated data (*e.g.,* due to outline smoothing, ambiguity in determining the precise position of each cell's boundary from the fluorescent staining information and available image resolution *etc.*).

Single-cell data extraction

After cell segmentation, subsequent CellProfiler modules enabled image preprocessing and cell feature extraction. 2D and 3D CellProfiler workflows are demonstrated in the Method S1. Immunofluorescence channels were thresholded at the level required to remove ~95% of fluorescence in tissue-matched, secondary antibody-only control images.[2] Fluorescence intensity values per cell, alongside cell size and shape features were then measured for all channels. Integration of binarized immunofluorescence images was used to measure the fluorescence area/volume.

Image analysis and data visulisation

Following recommended best practice,[21] cell-objects lying outside of the fifth or 95th percentiles by area (2D analyses) or volume (3D analyses) were discarded. Nearest-cell neighbors to gated startpoint cells were identified using a spherical structuring element to

dilate the startpoint cell's boundary by 3 pixels before identifying neighboring objects subsequently eroded. Immunofluorescence visualisations and gated cell-object surface overlays were created using the freely available Vaa3D software.[22]

ADDITIONAL RESOURCES

Image-data, code and screencast tutorials demonstrating deployment of the described label-free cell segmentation method using MATLAB (R2021a, using Deep Learning, Image Processing and Computer Vision toolboxes), Python 3 (using keras/Tensorflow-gpu 1.9) or via precompiled, standalone software for Windows are downloadable from the BioStudies database (https://www.ebi.ac.uk/biostudies/) under accession number S-BSST742.

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Supplemental information

Label-free cell segmentation of diverse lymphoid tissues in 2D and 3D

John W. Wills, Jack Robertson, Pani Tourlomousis, Clare M.C. Gillis, Claire M. Barnes, Michelle Miniter, Rachel E. Hewitt, Clare E. Bryant, Huw D. Summers, Jonathan J. Powell, and Paul Rees

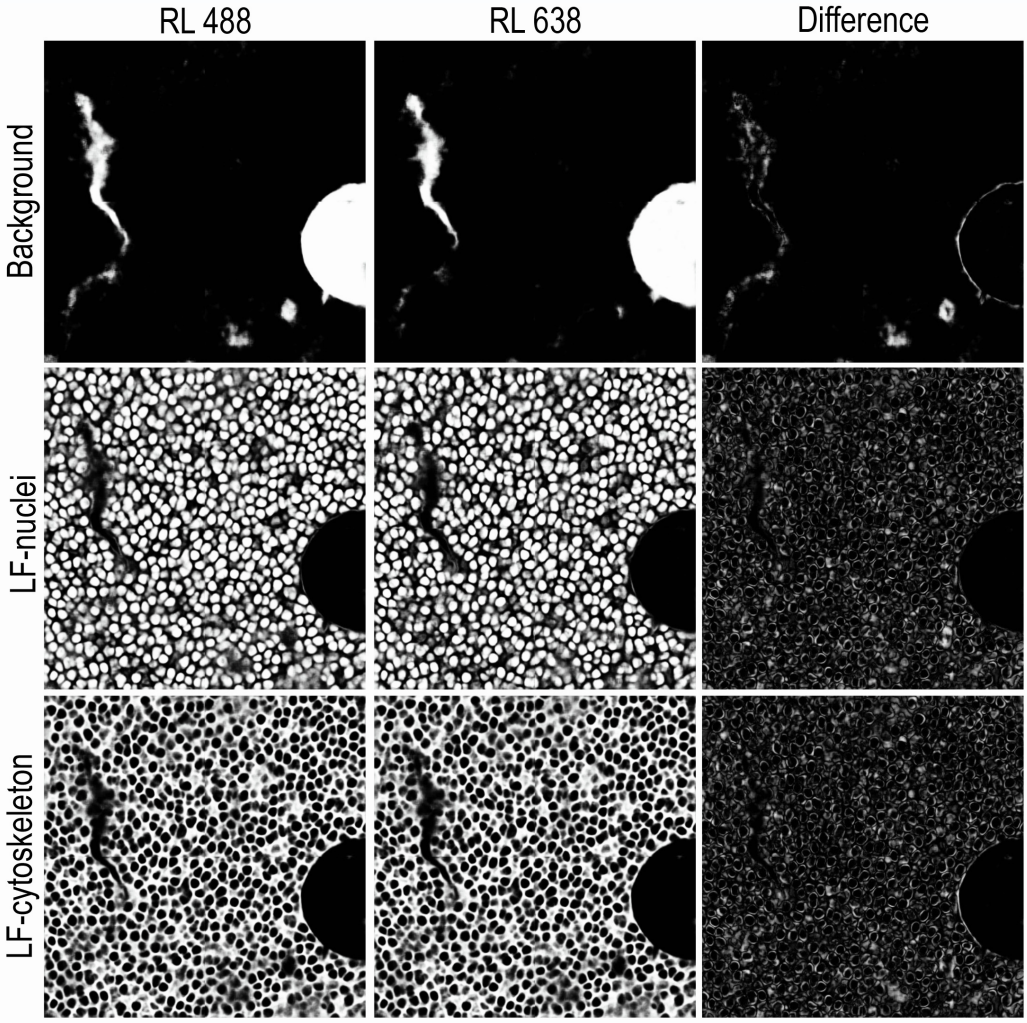


Figure S1 related to Figure 1 – Comparing label-free probability maps using reflectance data obtained using 488 nm or 638 nm laser excitation. The choice of excitation wavelength for generating the reflectance signal has minimal influence on the probability maps obtained from the network (left versus middle; difference shown right). The user can therefore reasonably use whatever is available on their individual microscope. For some applications, choosing a longer excitation wavelength may reduce fluorophore photobleaching / improve tissue penetrance and reflectance recovery - for instance, during 3-D Z-stack imaging in thicker tissue specimens.

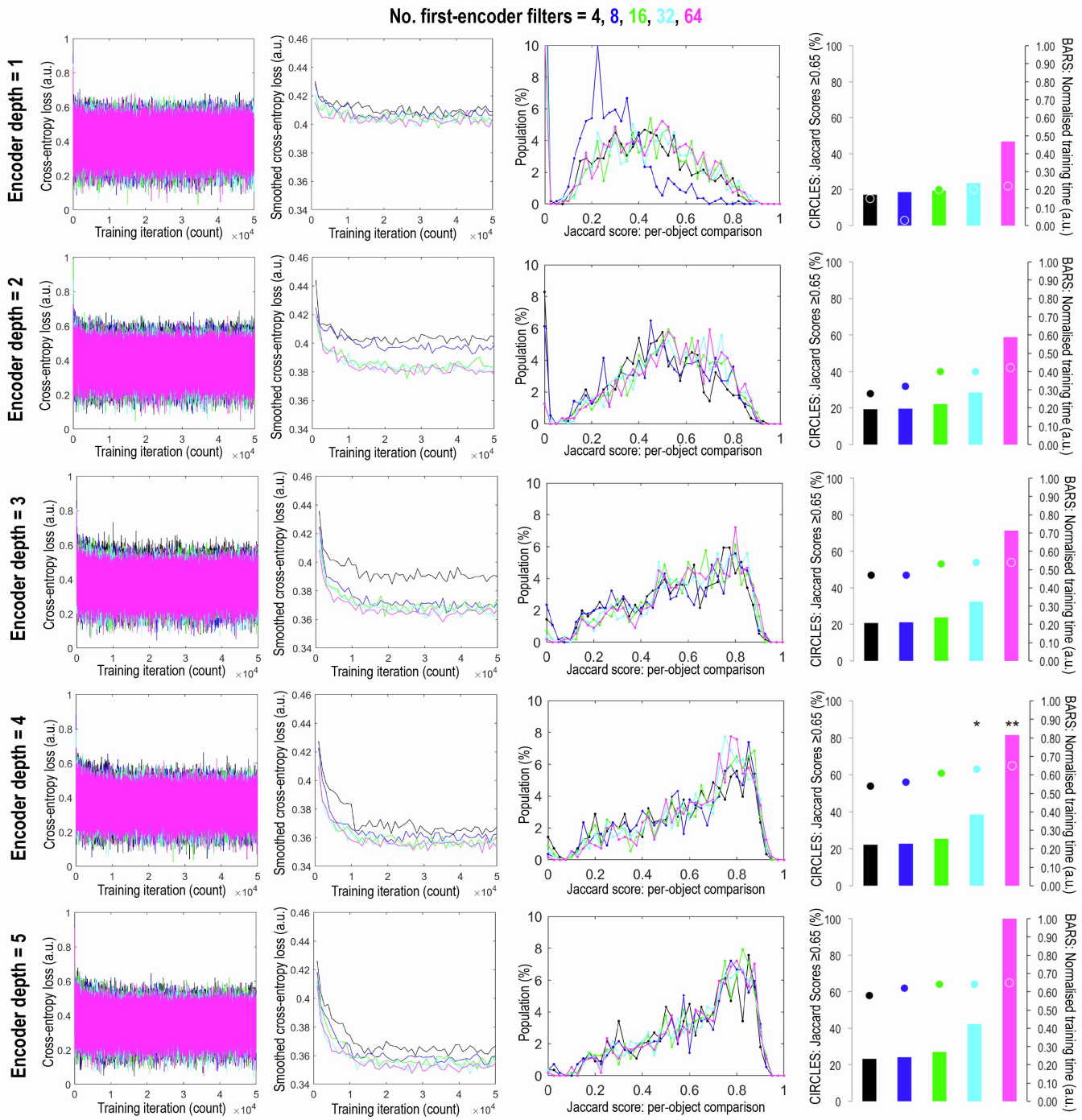


Figure S2 related to Figure 1 – Number of first-encoder filters and encoder depth optimisation to define the best performing 2-D Unet model. Bars represent normalised training time whilst circles indicate label-free cell segmentation accuracies (assessed by Jaccard index). The best performing model used an encoder depth of 4 with 64 filters at the level of the first encoder (indicated, **). Of note, using 32 filters instead of 64 can achieve a training speed up of ~50% for a negligible (~1%) decrease in segmentation accuracy (indicated, *). Increasing the encoder depth to 5 did not further improve cell segmentation accuracies (bottom row).

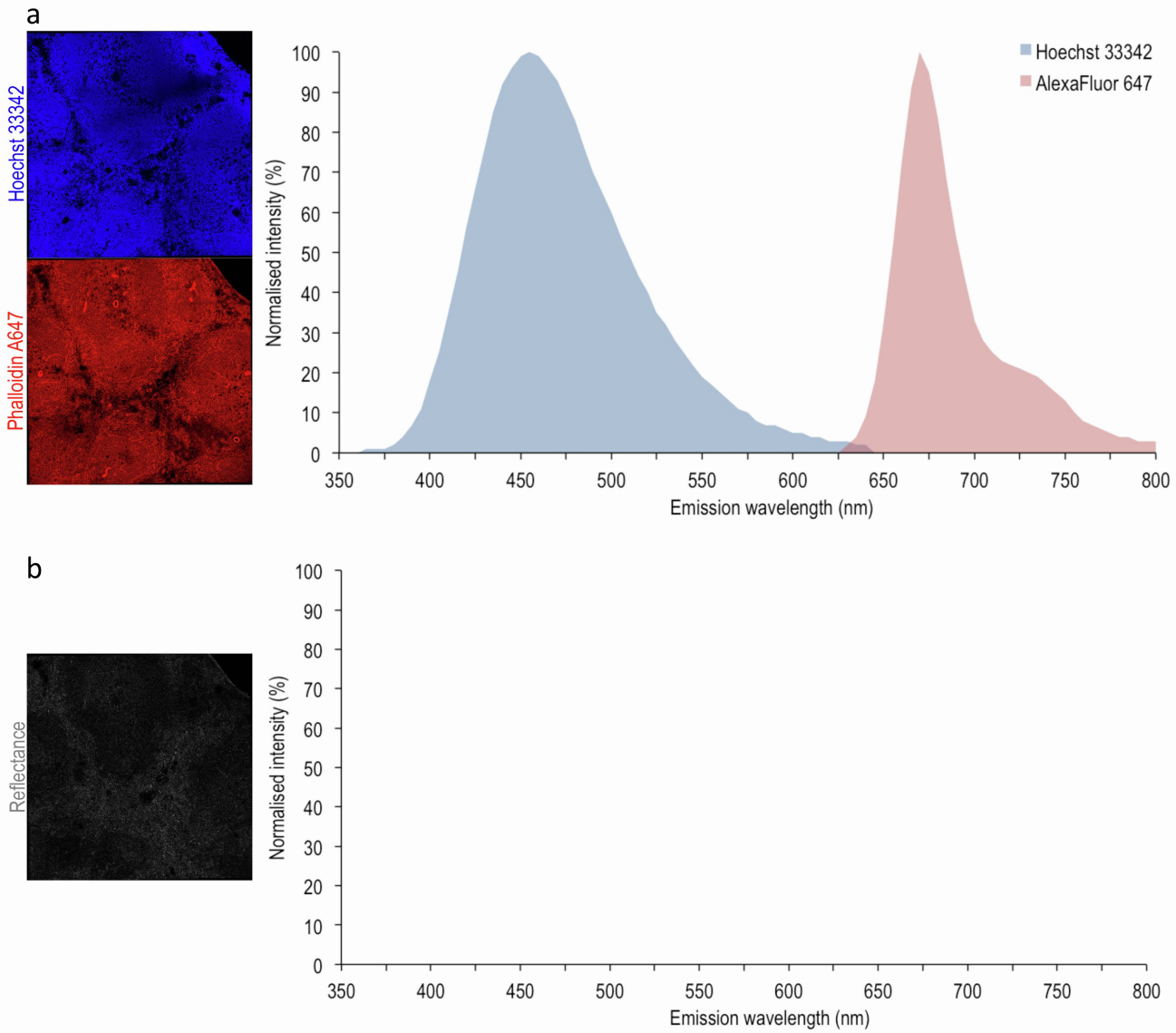


Figure S3 related to Figure 1 – Spectral bandwidth saving achieved by the label-free cell segmentation strategy. a, Emission spectra for Hoechst 33342 and AlexaFluor 647 as might typically be used to delineate cell nuclei and cell cytoskeletons when carrying out fluorescence-based cell segmentation. **b**, Harnessing reflectance information, the label-free cell segmentation method described here removes the need for these fluorescence stains leaving the spectrum entirely open for sensitive experimental measurements with single-cell quantification.

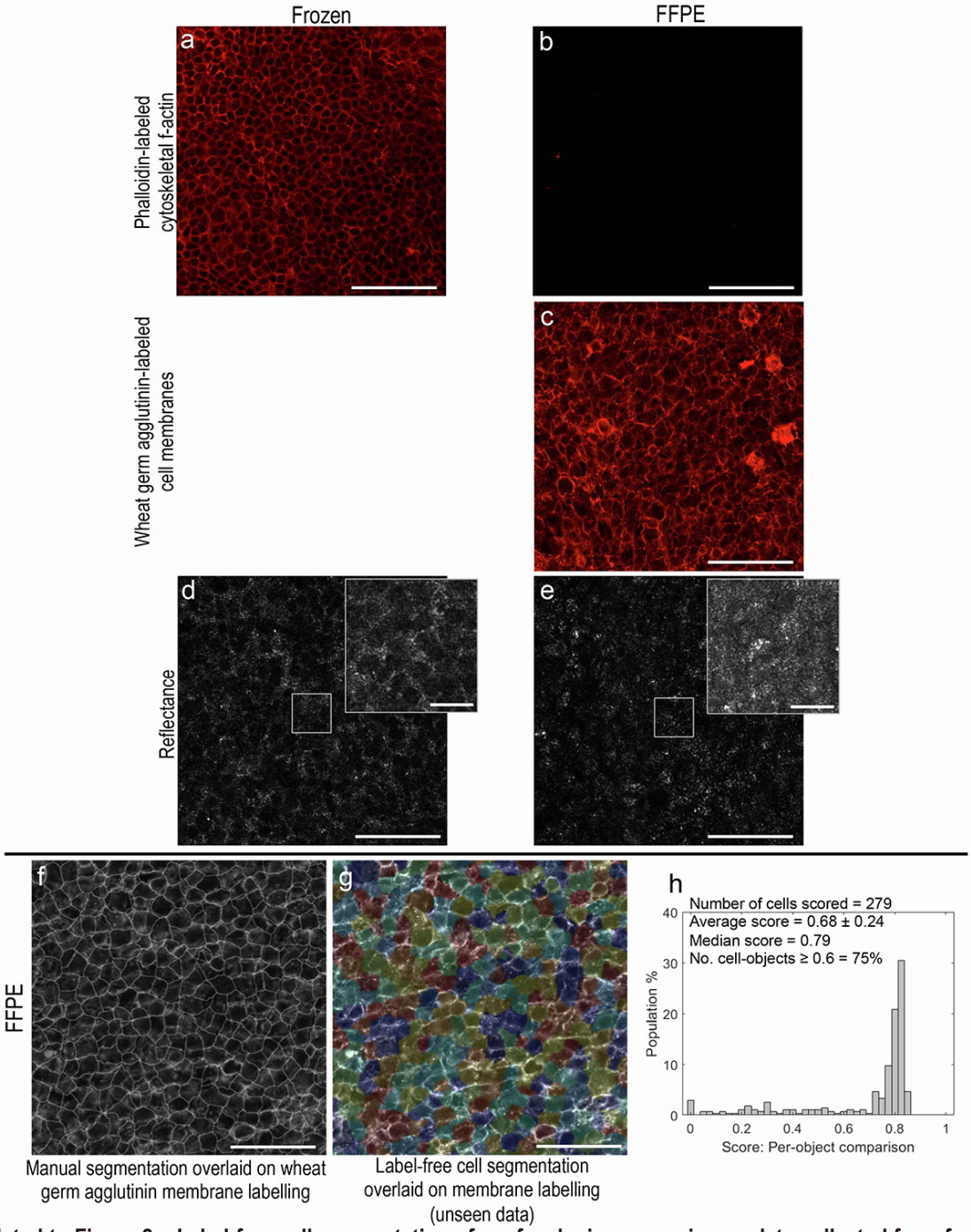


Figure S4 related to Figure 2 – Label-free cell segmentation of confocal microscopy image-data collected from formalin-fixed, paraffin embedded tissue sections. a, In frozen cryostat sections, f-actin staining using phalloidin conjugates clearly delineates cell outlines providing ground truth to enable the presented label-free cell segmentation approach. **b**, In contrast, in formalin-fixed paraffin embedded (FFPE) tissue sections, phalloidin staining fails because solvent exposure during the fixation and paraffin embedding process degrades the actin cytoskeleton. **c**, Demonstrated here using murine Peyer's patch tissue sections, successful ground truth labelling can be restored in the FFPE section-type by switching to cell membrane (*i.e.,* phospholipid) staining using wheat germ agglutinin (WGA) fluorescence conjugates. **d/e** Comparison of the reflectance signal from the frozen and FFPE section-types. Cytoskeletal degradation appears to change the reflectance images observed from the FFPE tissue: the faint trace of the cell outlines visible in the frozen sections is no longer apparent and instead the intracellular regions appear to exhibit the highest reflectance signal. **f-h** Despite this, a relationship between the reflectance signal and a WGA-delineated ground truth is still determinable by the neural network allowing (**g/h**) successful label-free cell segmentation direct from the reflectance signal. **h**, Intersection over union (IOU) score distribution comparing a (**f**) hand-drawn segmentation and the (**g**) automated, label-free cell segmentation outcome. An IOU score of 1 represents a perfect, per-pixel overlap between the hand-drawn and automated cell segmentations. Within the comparison presented here, scores ≥ 0.6 are seen to represent a good match, approaching the limits of hand-drawing accuracy. By harnessing ground truth from other fluorescence labels, the label-free strategy can operate in *both* FFPE and frozen tissue-types. Given that tissue archiving in FFPE format is commonplace worldwide, this finding dramatically increases the application domain of the presented label-free cell segmentation strategy. *Scale bars: a/b = 20 microns; c/d = 100 microns; e/f = 75 microns; g/h = 50 microns.*

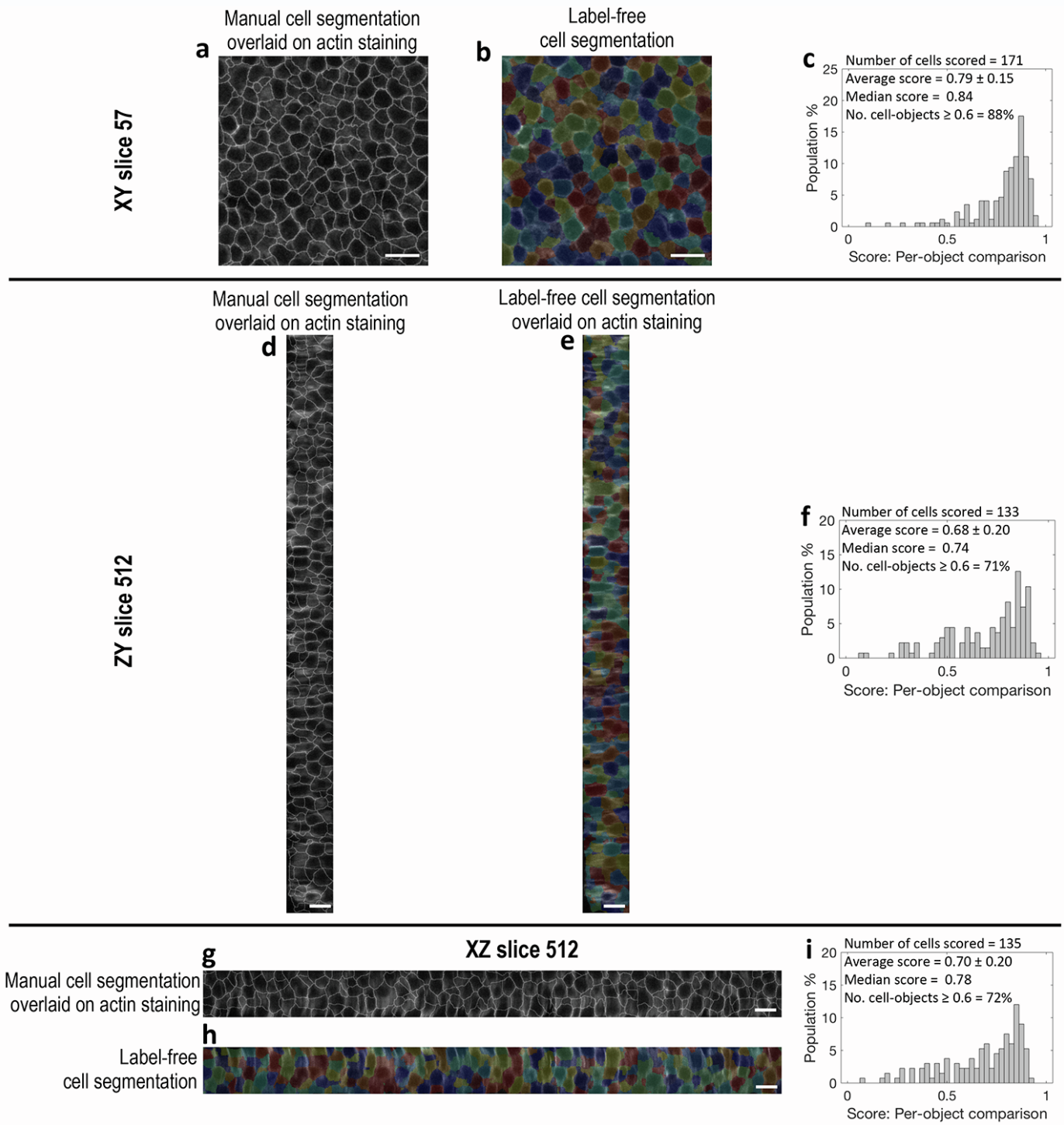


Figure S5 related to Figure 4 – Assessing 3-D label-free cell segmentation accuracies using mouse Peyer's patch tissue. a, Hand-drawn cell segmentations performed using the nuclei/actin fluorescence information for Z-planes (**a**) 57 in the XY dimension (**d**) 512 in ZY dimension and (**g**) 512 in the XZ dimension (unseen test image-data is 512x512x114 (X,Y,Z)). **b/e/h**, Automated cell segmentations for the same image-regions as (**a/d/g)** but achieved label-free direct from the reflectance signal. **c/f/i,** Cell-object intersection-over-union score distributions comparing – cell-object by cell-object – the (**a/d/g**) hand-drawn segmentations against the (**b/e/h**) automated, label-free cell segmentations. An IOU score of 1 represents perfect, per-pixel overlap between the hand-drawn and automated cell segmentations. Within the comparison presented here, scores ≥ 0.6 are seen to represent a good match, approaching the limits of hand-drawing accuracy. Encouragingly, the 3-D approach outperformed the segmentation accuracies achieved in 2-D (shown, **Figure 2**). *Scale bars equal 20 microns.*

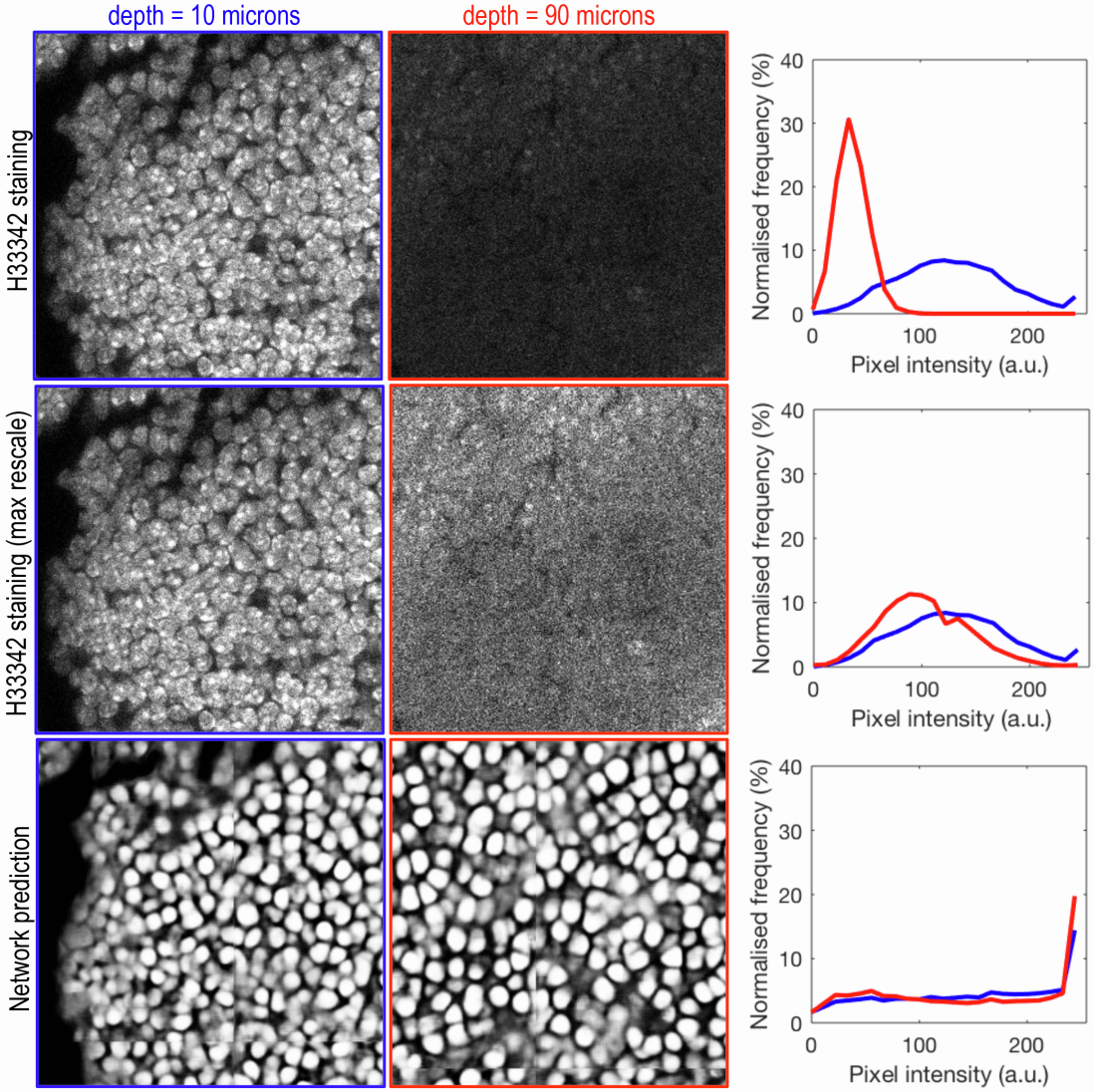


Figure S6 related to Figure 4 – Fluorescence versus label-free nuclei predictions at Z-depths of ~10 and ~90 microns. Using reflectance information from a 638 nm excitation laser, the 3-D network is able to consistently recover nuclear information long after the blue nuclear stain (Hoechst 33342) has decayed from multiple scattering effects (bottom right versus middle right). The resultant pixel intensity histograms from the probability map images are extremely stable (bottom right). This is advantageous for achieving consistent, depth-invariant 3-D cell segmentation in thick tissue specimens.

Table S1 related to Star Methods – Antibody and Image Information Table

PRIMARY ANTIBODIES	Product no	Supplier	Dilution primary	Stock concentration	Host	Secondary (detailed below)	Detection	Figure
Anti-mouse CD3-EF450	48-0032-82	Thermo Fisher	1:25	0.2 mg/mL	Rat	N/A	eFluor 450	Figure 1
Anti-mouse CD4-PE	12-0041-83	Thermo Fisher	1:25	0.2 mg/mL	Rat	N/A	R-phycoerythrin	Figure 1
Anti-mouse CD11c-EF660	50-0114-82	Thermo Fisher	1:25	0.2 mg/mL	Armenian Hamster	N/A	eFluor 660	Figure 1
Anti-mouse FOXP3-EF660	50-5773-82	Thermo Fisher	1:25	0.2 mg/mL	Rat	N/A	eFluor 660	Figure 2
Anti-mouse CD3	AB5690	Abcam	1:150	0.2 mg/mL	Rabbit	Anti-Rabbit AF568	AlexaFluor 568	Figure 3
Anti-mouse CD11c	AB33483	Abcam	1:400	0.5 mg/mL	Armenian Hamster	Anti-Hamster A488	AlexaFluor 488	Figure 3

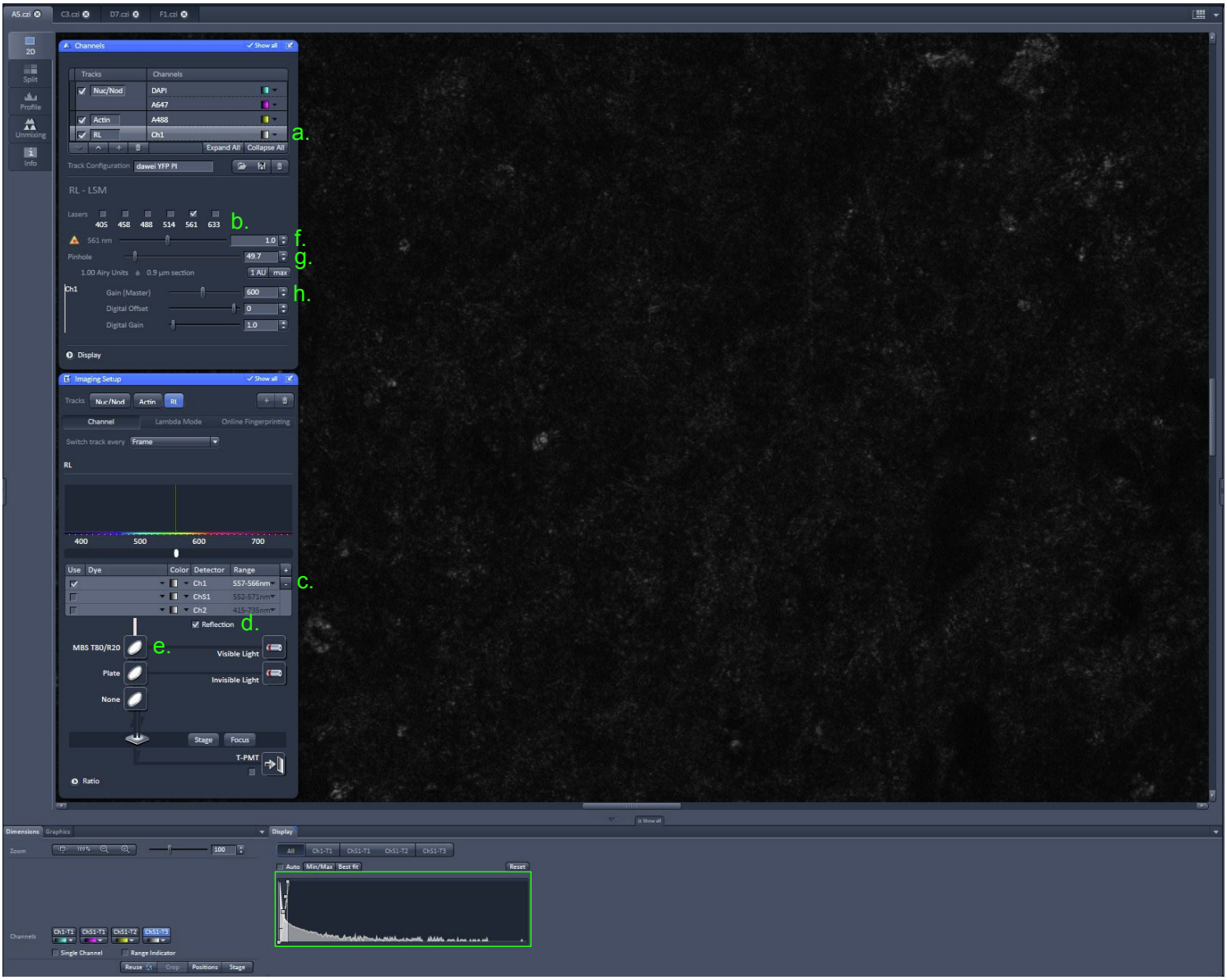
SECONDARY ANTIBODIES	Product no	Supplier	Stock concentration	Host	Secondary dilution	Fluorophore
Goat anti-Rabbit IgG (H+L)	A-11011	Thermo Fisher	2 mg/mL	Goat	1:400	AlexaFluor568
Goat anti-Hamster IgG (H+L)	A-11008	Thermo Fisher	2 mg/mL	Goat	1:400	AlexaFluor488

Figure ID	Section type	Tissue type (Sectioning orientation)	Objective lens Magnification / Numerical aperture (Microscope)	Pixel density Pixels per micron	Voxel size x,y,z µm	Reflectance Excitation laser (Detector placement) Wavelength, nm	Train / Test image(s) (number) x/y/z dimensions Pixel dimensions	Patch size / (patches per epoch)	Approx. Training time (single NVIDIA 1080 Ti GPU)
Figure 1	Cryostat (frozen)	Mouse spleen (transverse)	40X/1.3 (Leica SP8 inverted)	5.2842	0.1892 x 0.1892	488 (485-491)	Train: (1) 7617 x 7661 Test: (1) 5766 x 5787	256x256 (1000)	268 min (50 epochs)
Figure 4	Cryostat (frozen)	Mouse mesenteric lymph node (transverse)	40X/1.3 (Zeiss LSM780 upright)	4.8177	0.2076 x 0.2076 x 0.1500	561 (558-564)	Train: (2) 1024 x 1024 x 107 Test: (1) 1024 x 1024 x 111	64x64x64 (1000)	750 min (150 epochs)
Figure S1 Figure S6	Cryostat (frozen)	Mouse ileal Peyer's patch	40X/1.3 (Leica SP8 inverted)	3.5200	0.2840 x 0.2840 x 0.346	488 (485-491) 638 (635-641)	Train: (3) 512 x 512 x 103 Test: (1) 512 x 512 x 103	64x64x64 (500)	320 min (150 epochs)
Figure 3 Video S1	Cryostat (frozen)	Mouse ileal Peyer's patch (transverse)	63X/1.4 (Leica SP8 inverted)	8.324	0.1201 x 0.1201	488 (485-491)	Train: (1) 7607 x 11253 Test: (1) 7610 x 11247	256x256 (1500)	360 min (50 epochs)
Figure S4	FFPE	Mouse ileal Peyer's patch (transverse)	40X/1.3 (Leica SP8 inverted)	7.0463	0.1419 x 0.1419	638 (635-641)	Train: (1) 11364 x 11421 Test: (1) 7641 x 7660	256x256 (2000)	502 min (50 epochs)

Methods S1 – Related to Star Methods

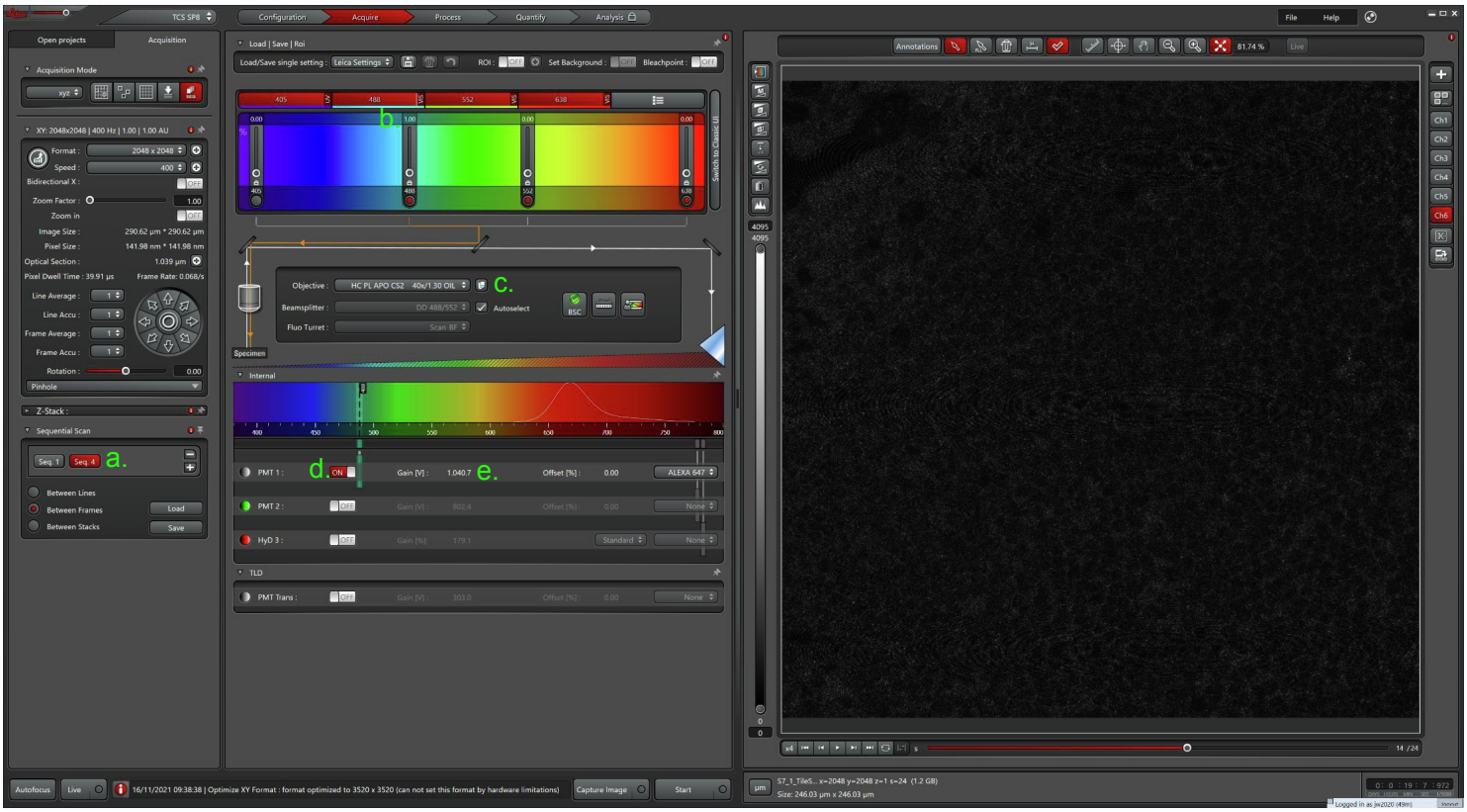
Table of contents –

Setting up sequential reflectance imaging using a standard Zeiss LSM780 confocal microscope.



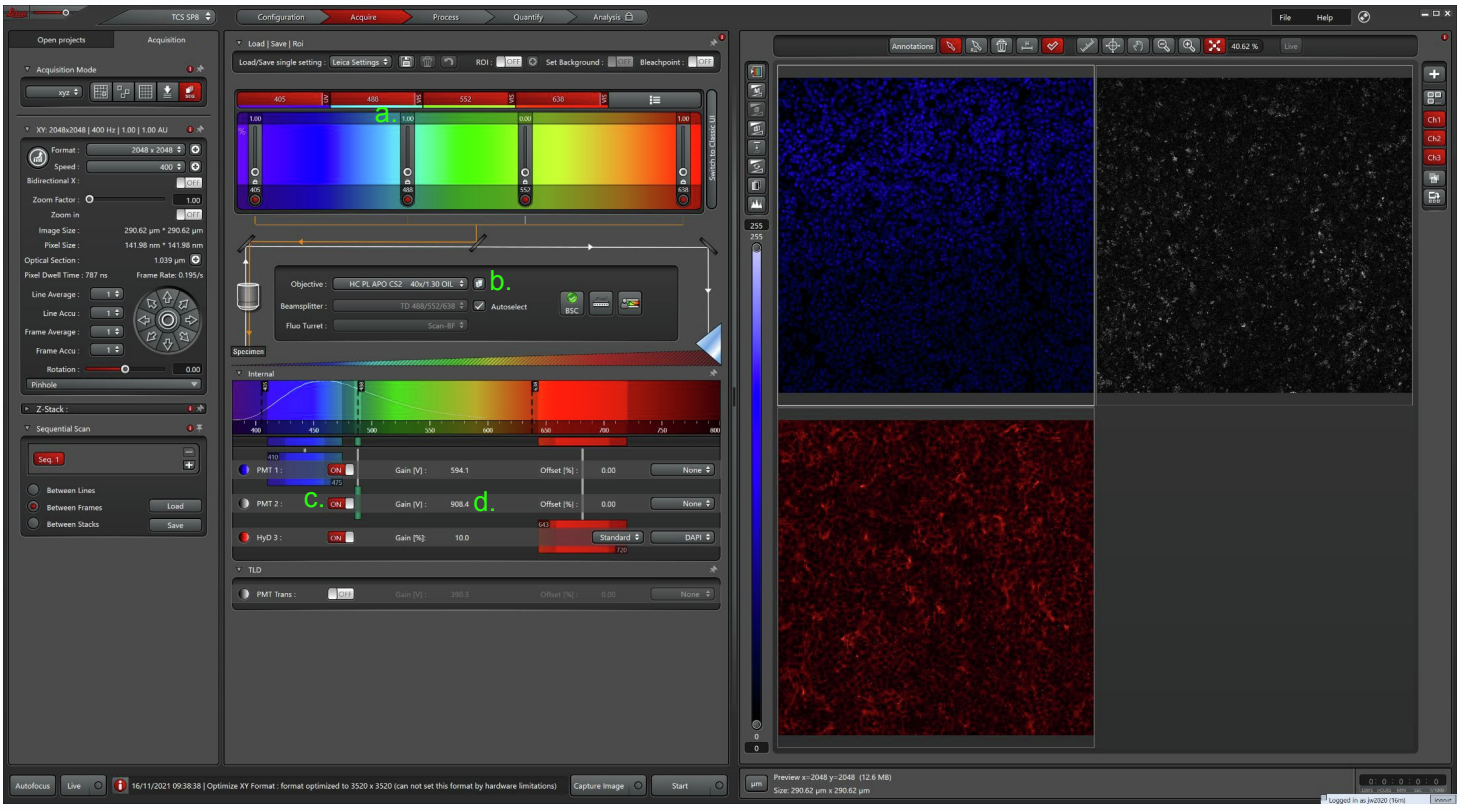
a, Once the fluorescence imaging sequences are set up, a new track for reflectance is added to the sequential scan. **b**, The desired excitation laser for reflectance imaging is selected (here, 561 nm). The choice of laser is not particularly important, but use of longer wavelengths may reduce fluorophore photobleaching and improve penetrance / recovery in thicker specimens (shown, **Figures S1/S6**). **c**, A photomultiplier detector (here Ch1) is turned on and the range set to approximately +/- 3 nm either side of the excitation wavelength (here 558 – 564 nm was entered, but the software rounds to display ~ 557 – 566 nm). **d**, The tick-box allowing reflected light to pass to the detector is turned on. **e**, The T80/R20 beam splitter is chosen (this indicates a transmission/reflection ratio of 80:20). **f**, A low laser excitation power (here, 1%) is entered. *N.B.,* use of a reflectance light path with high laser excitation power may damage the camera, so care should be taken here. The pinhole is set to ~ 1 airy unit, yielding an optical section of around ~ 1 micron with a high numerical aperture 40X or 63X objective. **g**, Running in 'live mode', the gain is slowly increased until the reflectance signal occupies approximately 80% of the range histogram (indicated, green box). Compressing the histogram in the range indicated yields a typical 'view' of the reflectance signal from a lymphoid tissue specimen (on display in the main image window).

Setting up sequential reflectance imaging using a standard Leica SP8 confocal microscope.



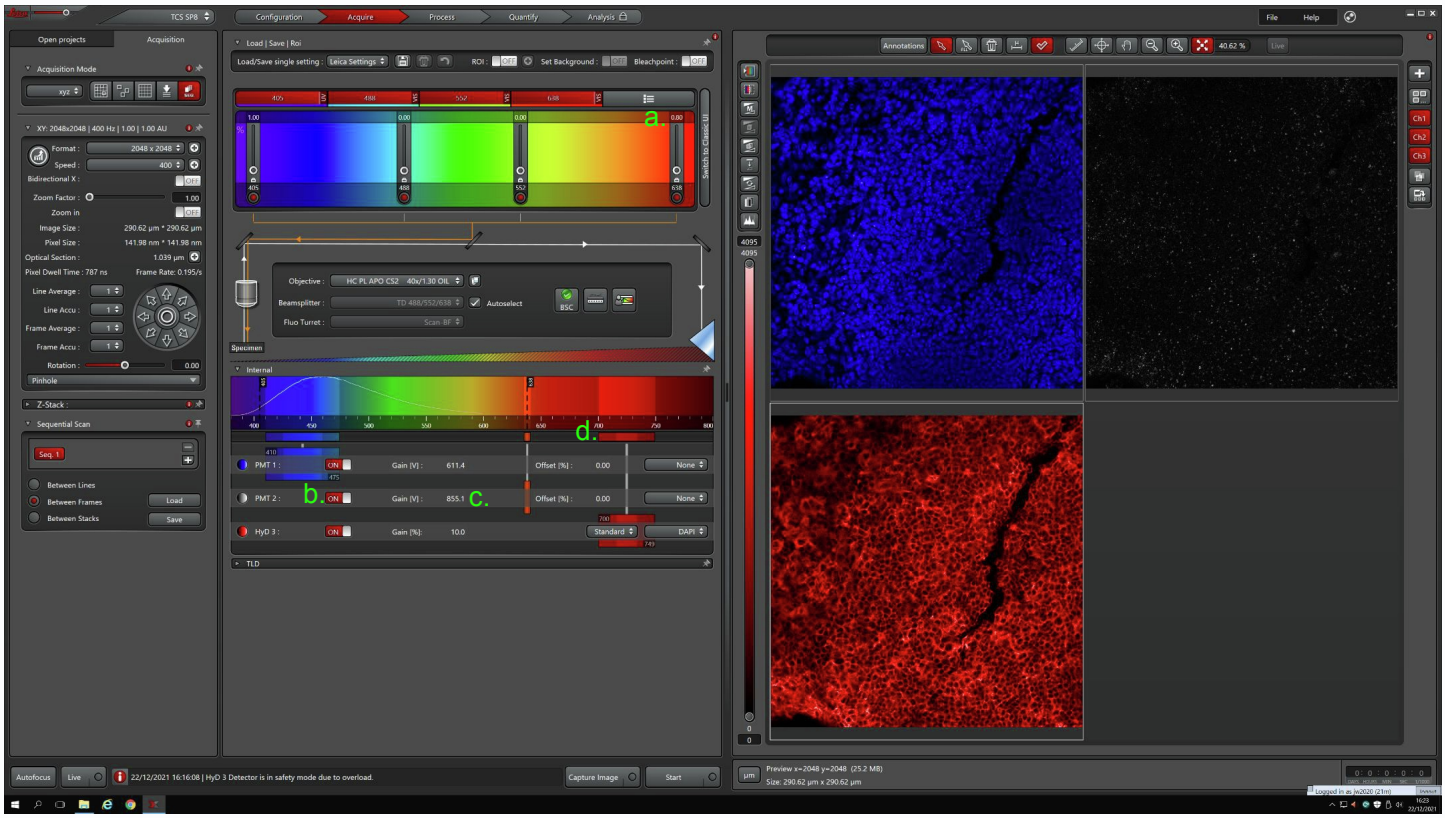
a, Once the fluorescence imaging sequence(s) are set up, a new track for reflectance is added to the sequential scan (here, 'Seq 4'). **b**, The desired excitation laser for reflectance imaging is turned on (here, 488 nm). The choice of laser is not particularly important, but use of longer wavelengths may reduce fluorophore photobleaching and improve penetrance / recovery in thicker specimens (shown, **Figures S1/S6**). A low laser excitation power (*e.g.,* 1%) is also specified at this step. *N.B.,* Use of a reflectance light path with high laser excitation powers could damage the camera, so care should be taken at this step. **c**, An appropriate beam splitter is chosen for the excitation line, or, the 'Autoselect' checkbox can be ticked to set this automatically. **d**, A photomultiplier detector (here PMT1) is turned on and the range set to approximately +/- 3 nm either side of the excitation wavelength (*i.e.,* here, 485-491 nm). **e**, Running in 'live mode', the gain is slowly increased until the reflectance signal occupies approximately 80% of the available intensity range. The main window shows a typical 'view' of the reflectance signal from a lymphoid tissue specimen under this setup.

Setting up simultaneous reflectance and fluorescence imaging using a standard Leica SP8 confocal microscope.



Once fluorescence excitation and collection are configured, (**a**) any remaining laser line can be used for reflectance imaging (*e.g.,* here, the 488 nm line is used). The choice of laser is not particularly important, but use of longer wavelengths may reduce fluorophore photobleaching and improve penetrance / recovery in thicker specimens (shown, **Figures S1/S6**). A low laser excitation power (*e.g.,* 1%) should also be specified here. Reflectance imaging with high laser excitation powers could damage the camera, so care should be taken at this step. **b**, An appropriate beam splitter for the excitation lasers is chosen, or the 'Autoselect' checkbox ticked to enable automatic setting. **c**, A free detector (here PMT2) is turned on and the range set to approximately +/- 3 nm either side of the excitation wavelength (*i.e.,* 485-491 nm). **d**, Running in 'live mode', the gain is slowly increased until the reflectance signal occupies approximately 80% of the available intensity range. The top-right image in the main window shows a typical 'view' of the reflectance signal from a lymphoid tissue specimen under this setup. This approach allows reflectance data to be concomitantly collected alongside fluorescence without adding the additional run-time of further sequences. *N.B.,* It is worth noting that in a similar way, a laser that is already being used for fluorescence excitation may also be used to obtain reflectance data (exemplified **page below**). For example, here, PMT2 could be moved up to collect reflectance from the 638 nm laser in the range 635-641 nm. Doing this has the advantage of reducing the photon budget for the sample. However, it also necessitates that enough excitation power is being used to obtain a good reflectance signal, and that a free detector can be moved within the necessary detection range. This is not always compatible with optimal fluorescence imaging – hence the setup shown here.

Setting up 'free' reflectance imaging alongside fluorescence collection using a standard Leica SP8 confocal microscope.



Once fluorescence excitation and collection are configured, (**a**) any remaining detector can be used to simultaneously collect the reflectance signal from one of the excitation lasers being used to stimulate fluorescence (*e.g.,* here, 'PMT2' is used to collect reflectance from the 638 nm laser line (**b**) – which is also being used to excite AlexaFluor 647). This is achieved by placing the detector approximately +/- 3 nm either side of the excitation wavelength (*i.e.,* 635-641 nm). **c**, Running in 'live mode', the gain is slowly increased until the reflectance signal occupies approximately 80% of the available intensity range. The top-right image in the main window shows a typical 'view' of the reflectance signal from a lymphoid tissue specimen under this setup. Simultaneous reflectance imaging has the advantage of reducing the photon budget for the sample, as the reflectance information is effectively recovered for 'free' by harnessing scatter from a laser that is already in use. However, this setup also necessitates that enough excitation power is available to obtain a good reflectance signal, and that a free detector can be moved into the necessary detection range. To achieve this here without saturating the AlexaFluor 647 signal (shown bottom-left in the main image window) the AlexaFluor647 detection range was narrowed (**d**) to ~ 700-750 nm. Where this setup cannot be accommodated one of the other options that instead use a dedicated laser for reflectance imaging should be utilised (shown, **three above pages).**

Windows 10: Running the label-free cell segmentation pipeline using standalone software

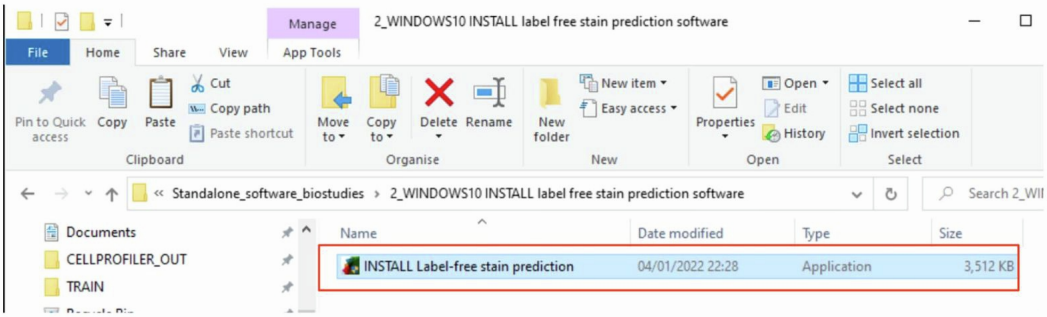
Running this software requires:

- Windows 10 machine (NVIDIA GPU desirable for model training)
- Label-free stain prediction standalone software (BioStudies download)
- MATLAB runtime 9.11 (installs automatically alongside software – see below).

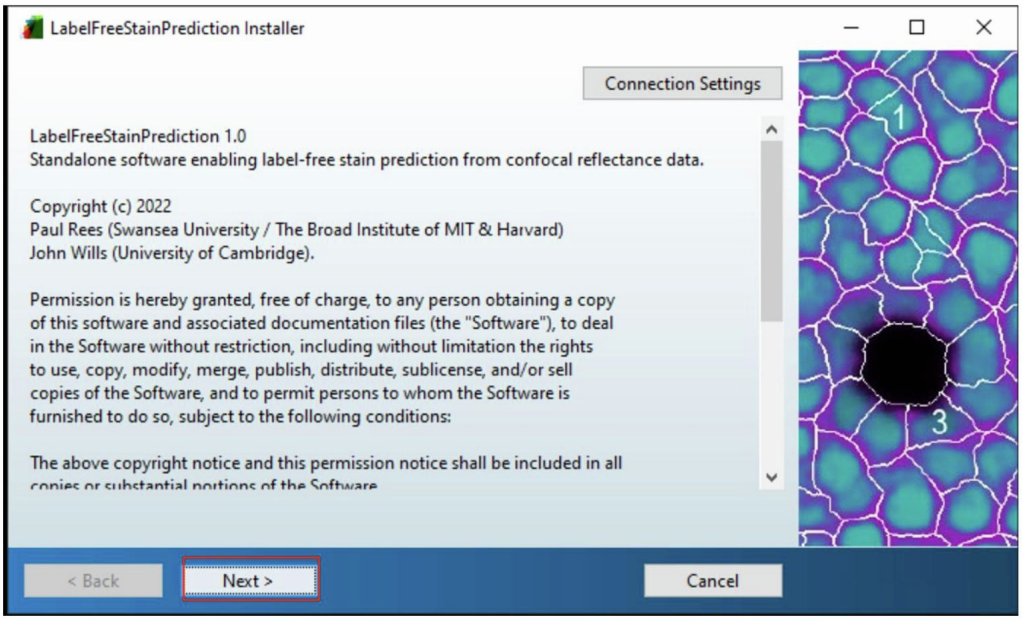
Note: This software is free to install. No MATLAB license is required to run this standalone software.

Installation Steps:

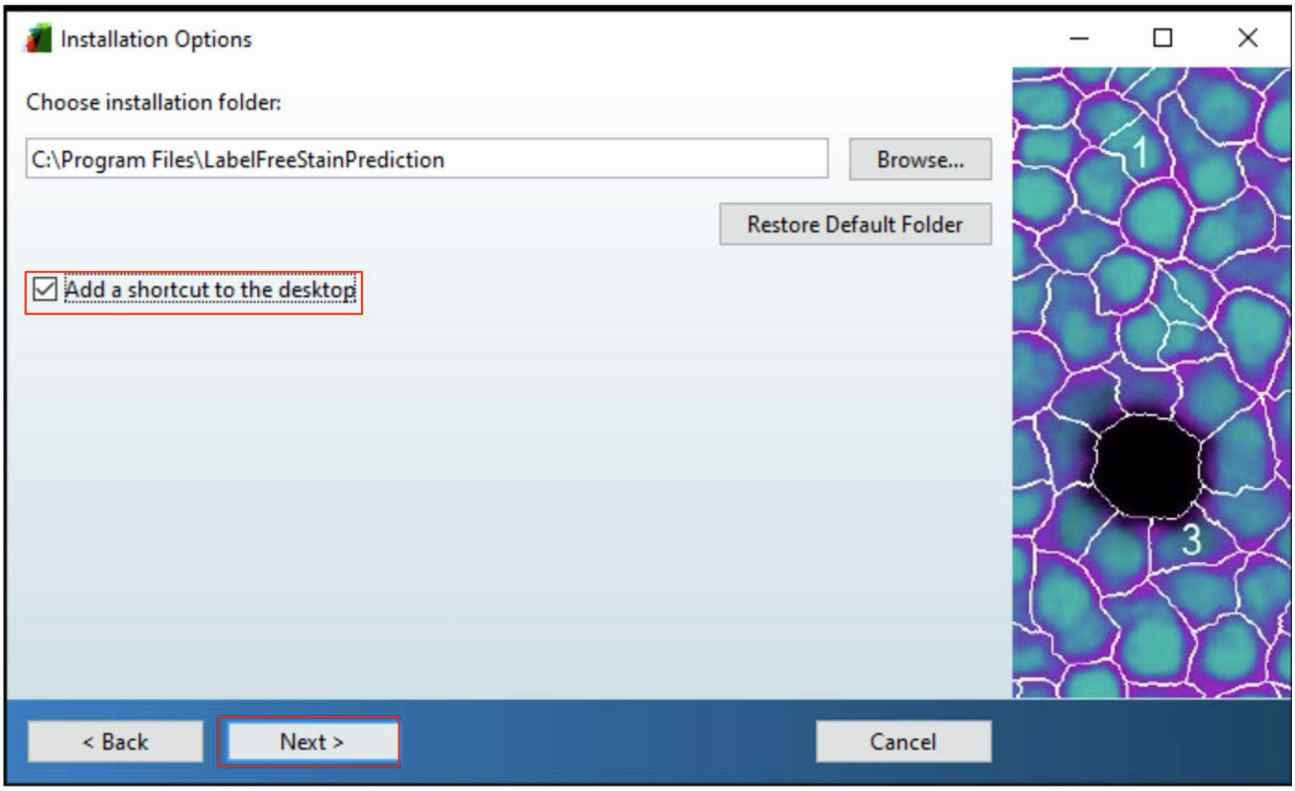
1. **Install the Label Free Stain Prediction software** by running the Windows 10 installer from the BioStudies download



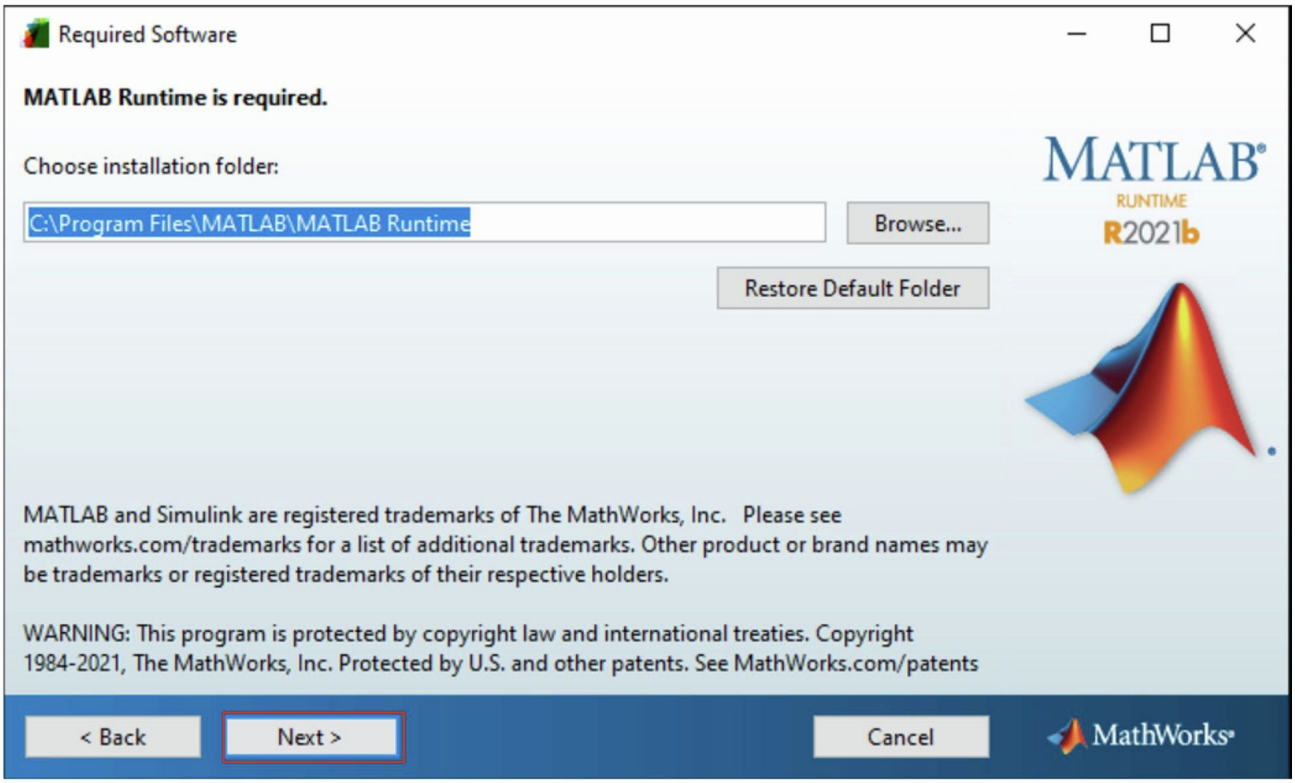
2. Follow through the setup procedure to install the software:



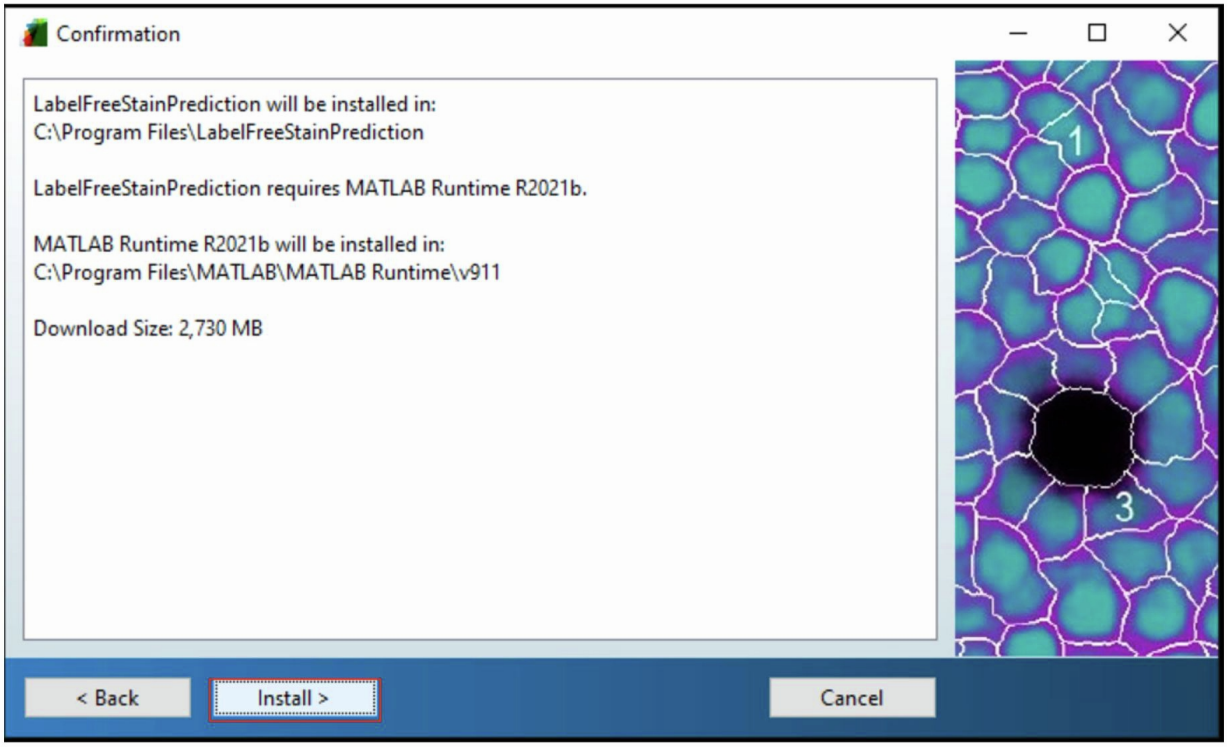
- Check 'add shortcut to the desktop':



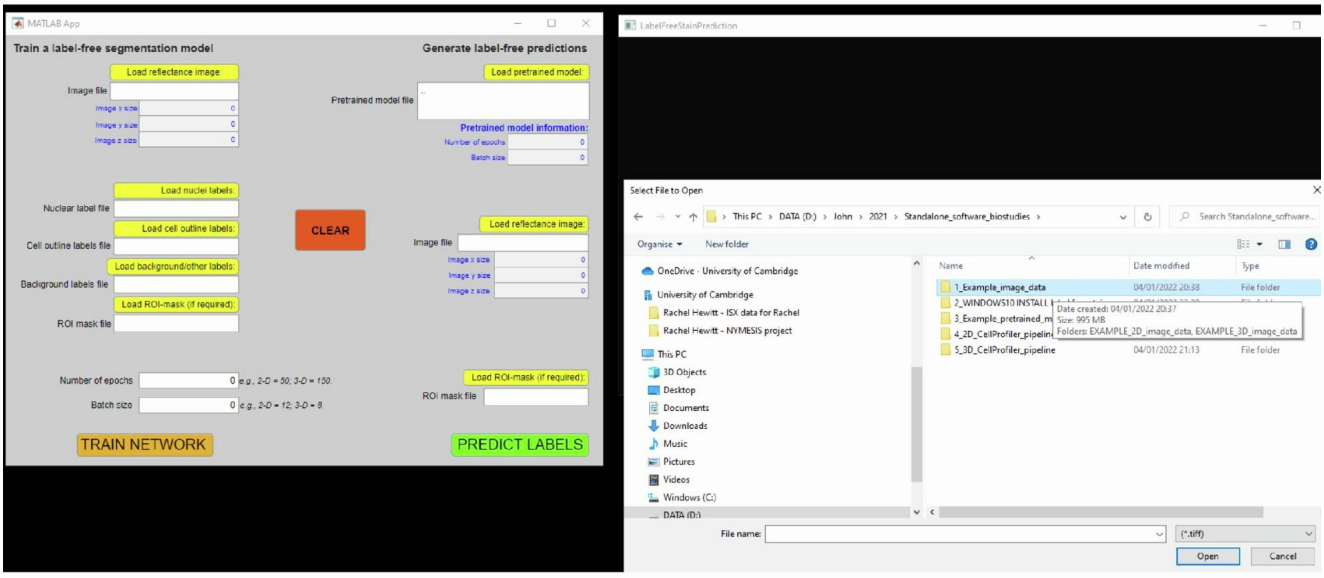
- When prompted, install MATLAB runtime (free; no license is required):



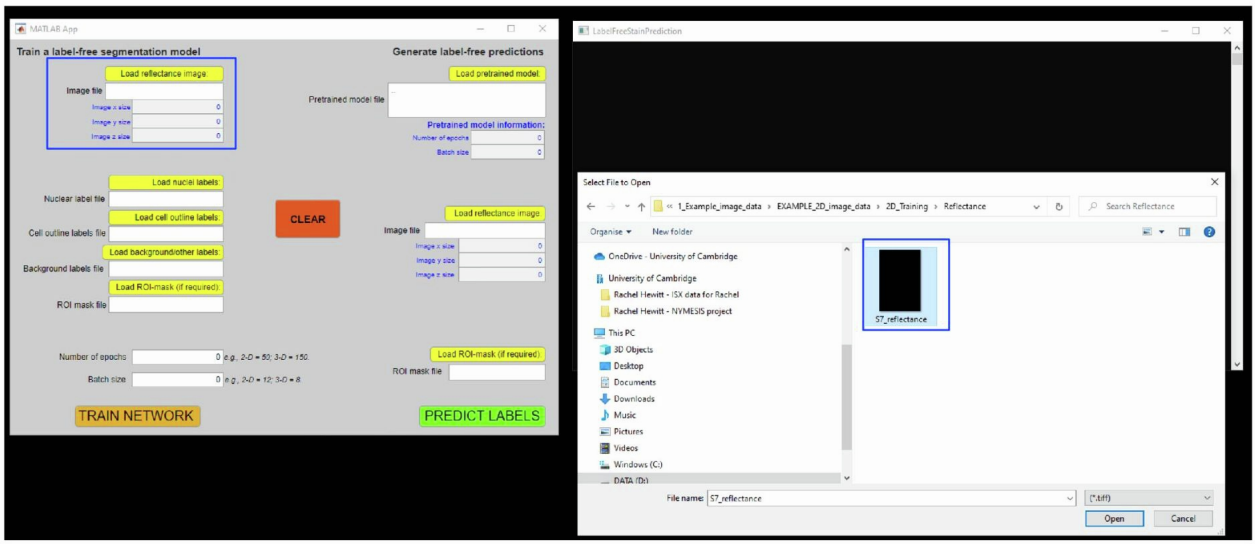
- click ‘install’ to install the label free stain prediction software and MATLAB runtime 9.11

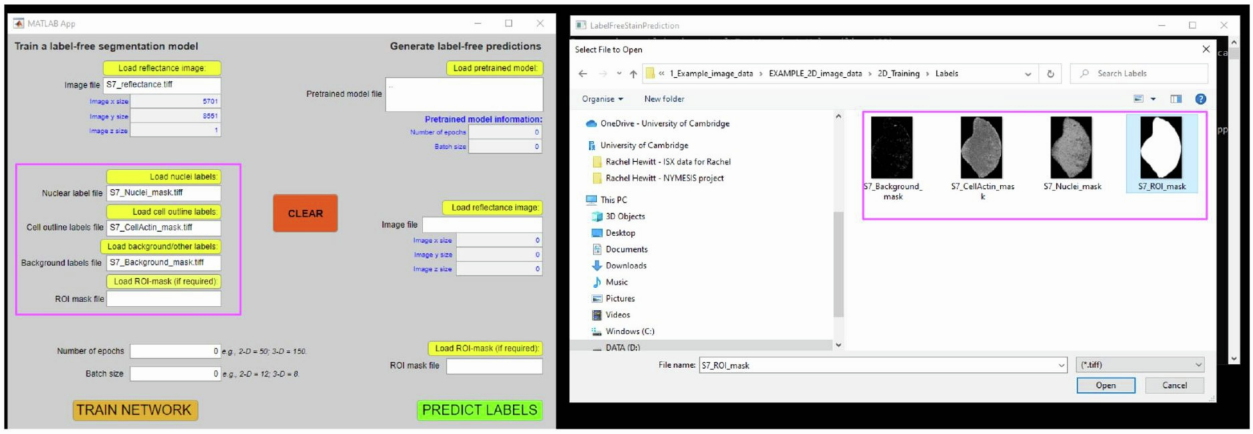


3. Training 2-D or 3-D UNET model using the standalone software
-A screencast video is included with the BioStudies archive.
-Launch the Label Free Stain Prediction software from the desktop icon
-Example 2-D and 3-D image data and training labels are included in the BioStudies download.



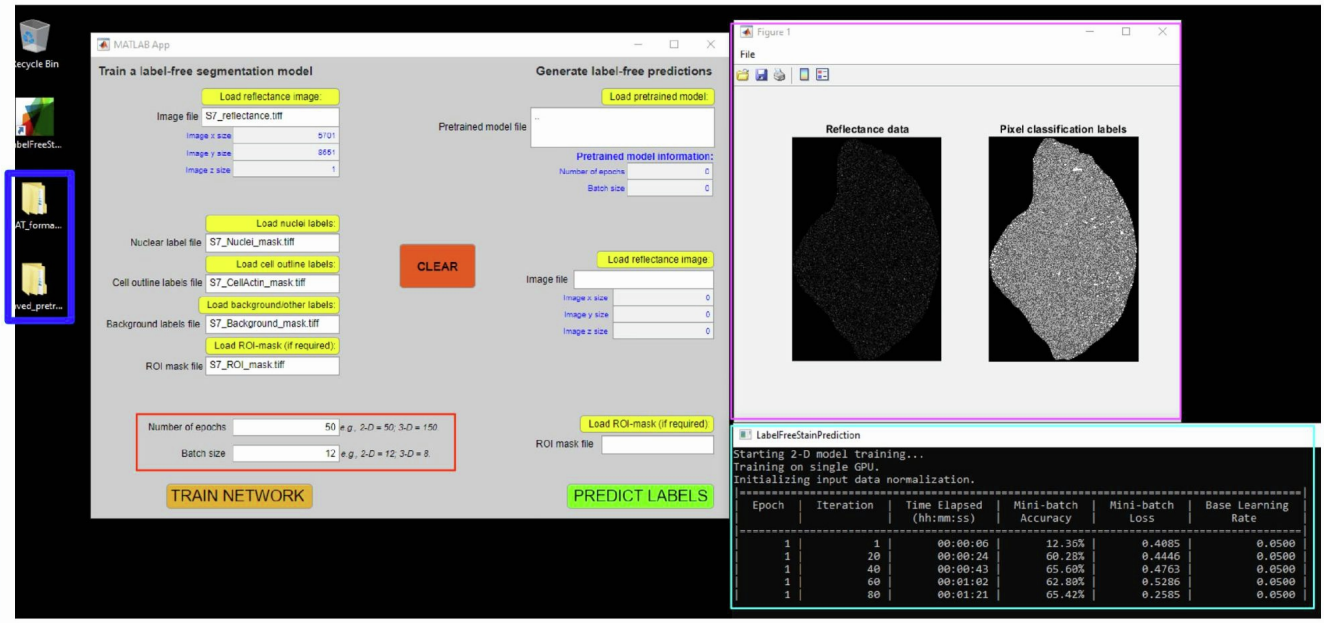
- To Train a 2-D or 3-D UNET model to predict the probability images needed to enable the label-free segmentation, load reflectance data and matching labels for 'Nuclei', 'Cell Outlines' (e.g. actin) and 'background/other' classes.
-The label images should be in .tiff format with the stains represented as foreground (i.e., white).
-2-D or 3-D data will be automatically detected.





- If desired, a ROI-mask can also be loaded to mask the outputted probability maps to the desired tissue region.
- If this isn't needed – just leave this box blank.

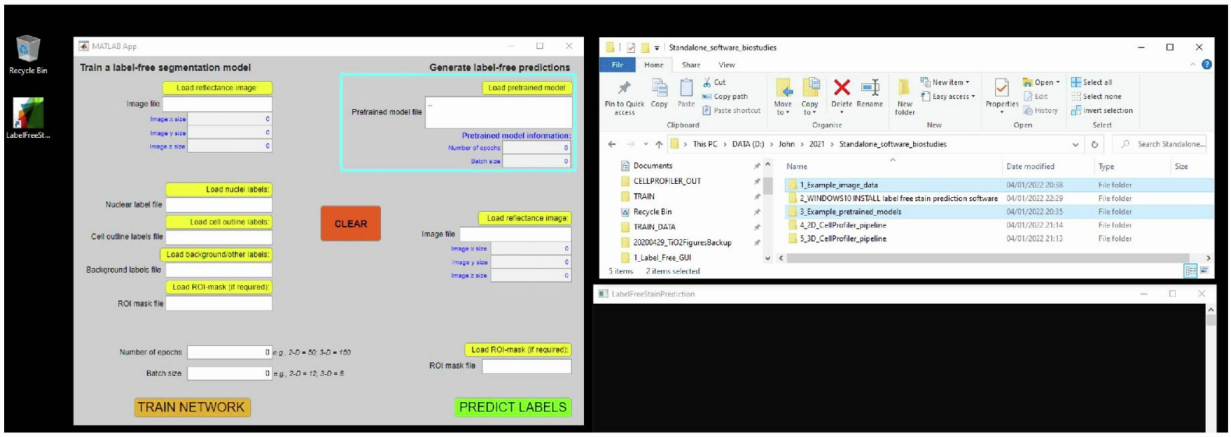
- Set the desired batch-size and number of training epochs and click 'Train Network'.
- After a few seconds, the loaded reflectance data and labels will be indicated in "Figure 1"
- Progress can be tracked in the Command Prompt console
- MAT formatted data and the trained model will be saved to folders on the desktop once training completes:
- Training with the example data takes ~6h on an NVIDIA 1080 GTX GPU. (Previously trained models are provided).



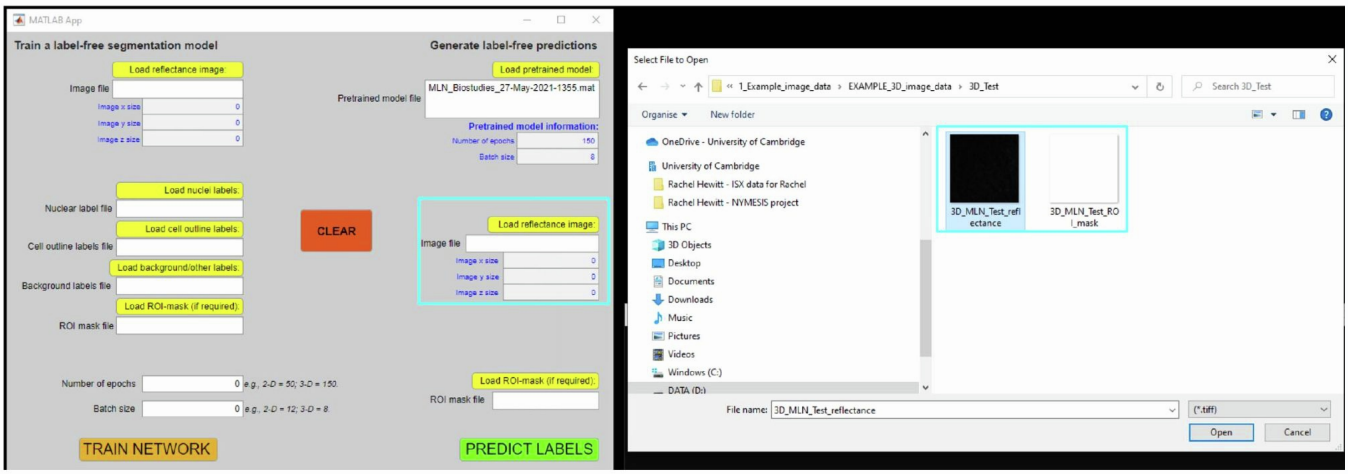
4. Using a pretrained 2-D or 3-D UNET model to generate probability images for label-free segmentation using unseen reflectance data.

-On the right-side of the software, load a pretrained model.

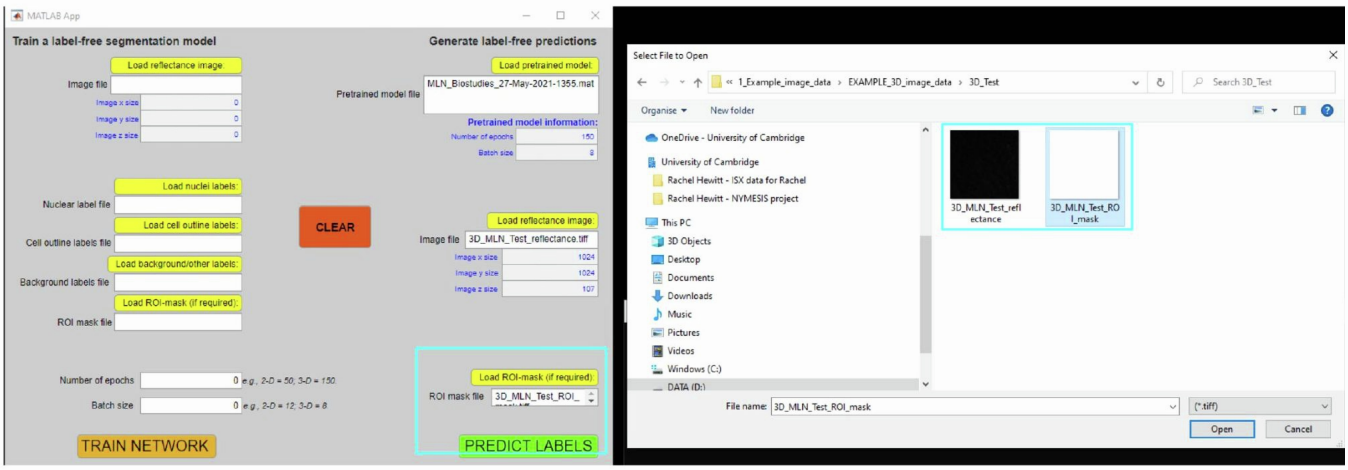
-This can be from Step 3 (above), or, example pretrained networks are provided in 'Folder 3' of the BioStudies download. Example unseen 'test' image-data is also provided in 'Folder 1' in 2-D and 3-D.



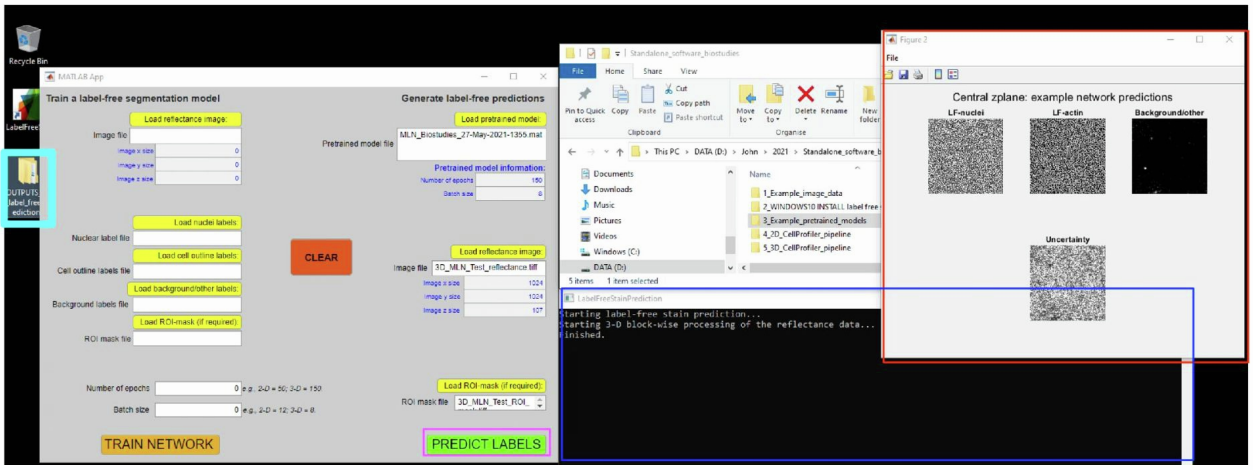
- **Load** 2-D or 3-D reflectance data:



- If desired, **load a ROI-mask** to limit the probability maps to the desired tissue region:
- (Leave blank if not required).



- Click ‘Predict Labels’ to generate the label-free probability images
- Progress can be tracked in the Command Prompt console
- Figure 1 demonstrates probability images for each class (central z-plane for 3-D data)
- Outputs are saved to the Desktop ready for loading into the CellProfiler pipelines enabling cell feature extraction (described below).



Windows 10: Running the label-free cell segmentation deep learning scripts on an NVIDIA GPU using MATLAB R2021b and the Deep Learning Toolbox

Running these deep learning scripts in MATLAB requires:

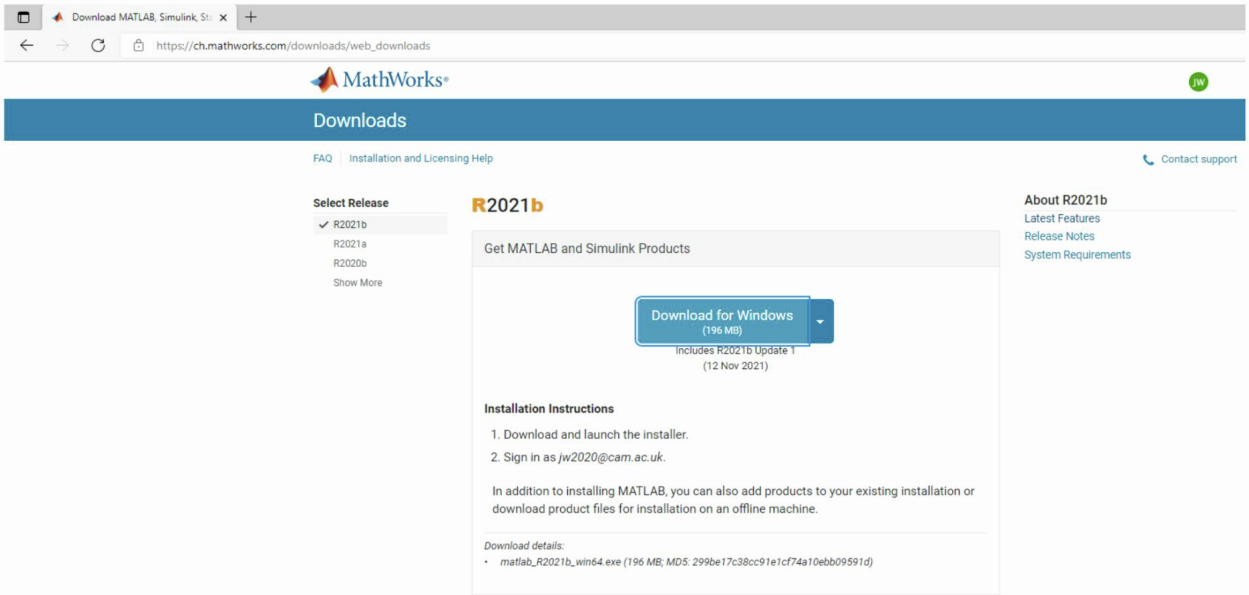
- MATLAB (version R2019a or later)
- Deep Learning Toolbox
- Image Processing Toolbox
- Computer Vision Toolbox

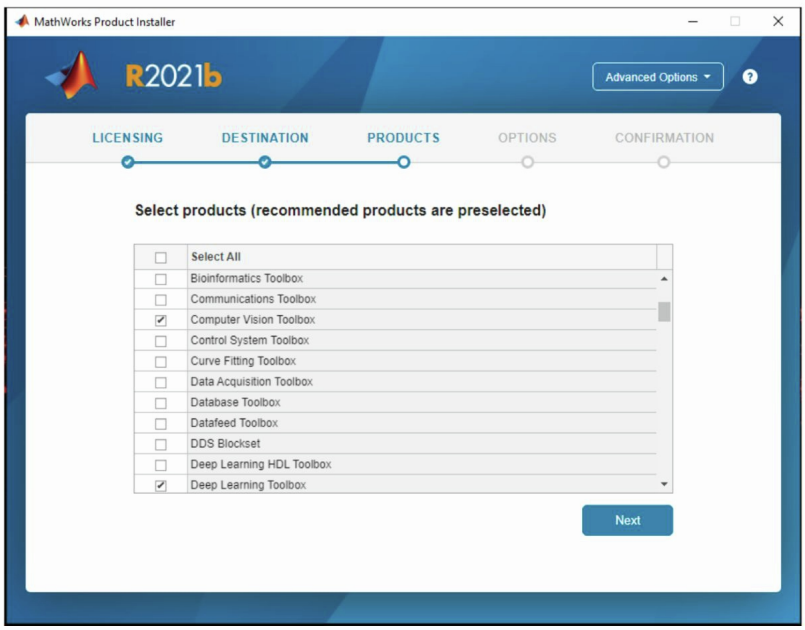
Installation Steps:

1. Install MATLAB

https://ch.mathworks.com/downloads/web_downloads

- Login to your MathWorks account to access the downloads page at the link above.
- Download and run the installer for MATLAB R2021b.

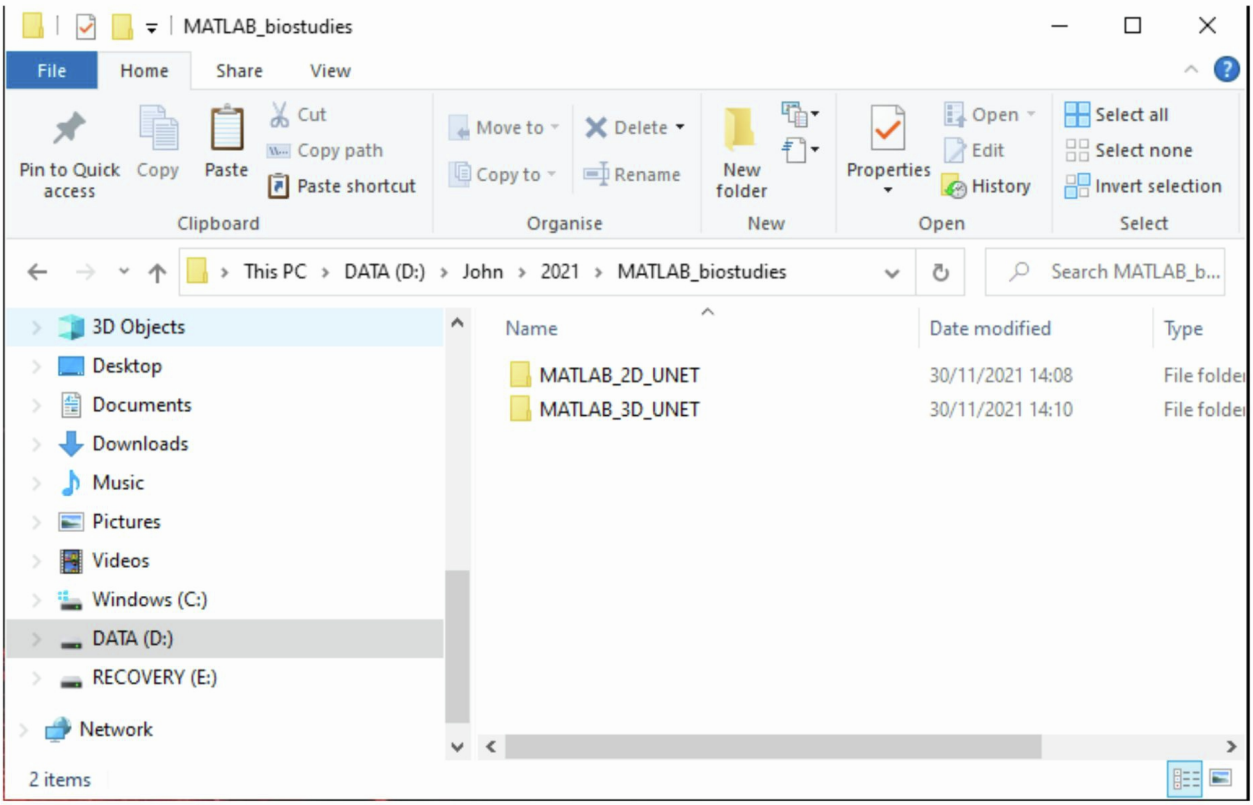




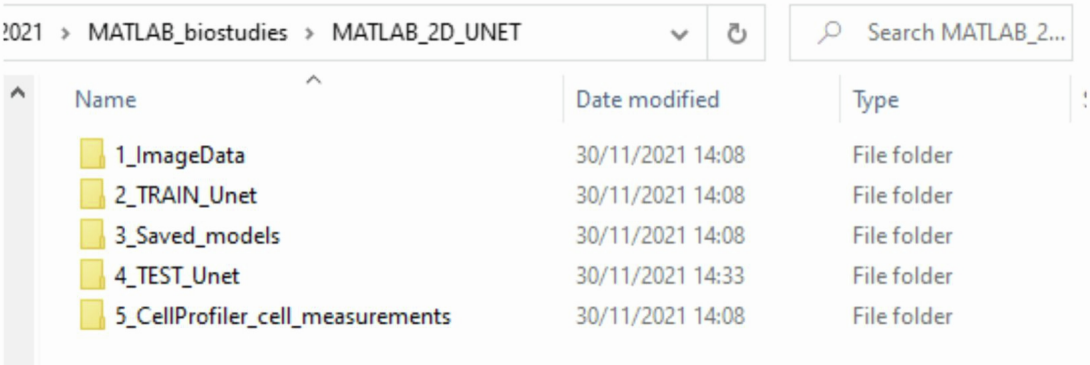
- During installation, install the Computer Vision, Deep Learning and Image Processing Toolboxes by selecting them at the 'Products' step of the installation.

- Once the installation is complete, you can proceed to running the scripts.

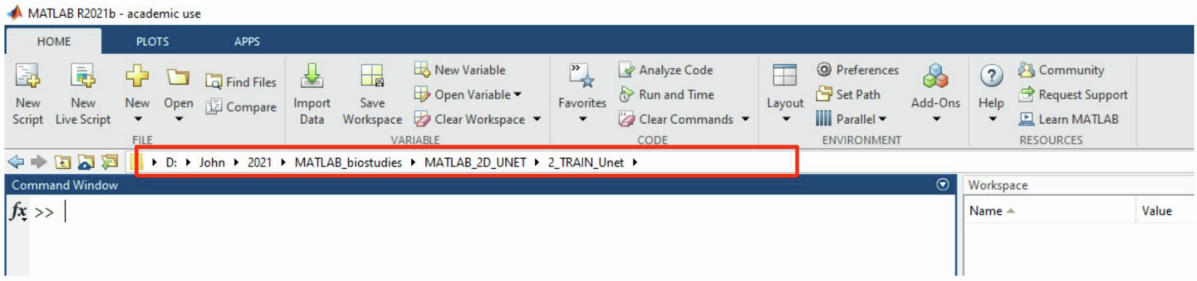
2. Training a 2-D UNET Model
-A screencast video is included with the BioStudies archive.
- Download and unzip the MATLAB BioStudies project archive at a suitable location on your computer.



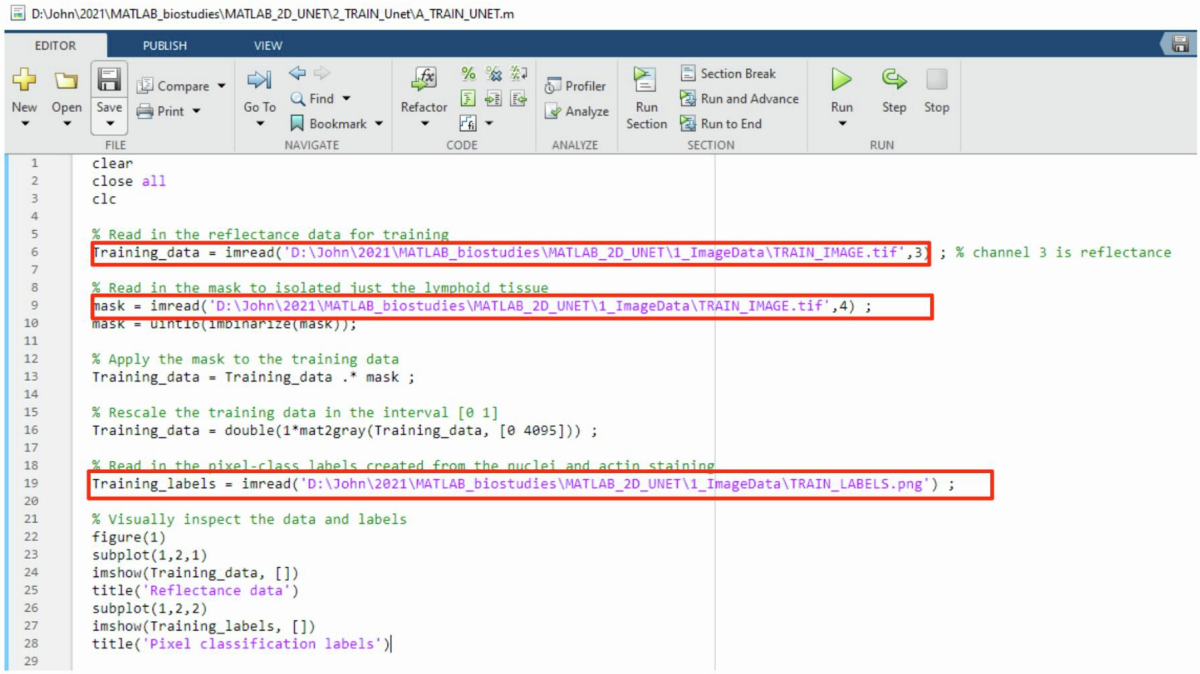
- Inside the MATLAB_2D_UNET folder, there is a sequentially-organised workflow starting with raw data and ending with a CellProfiler pipeline that obtains cell features from the label-free cell segmentation:



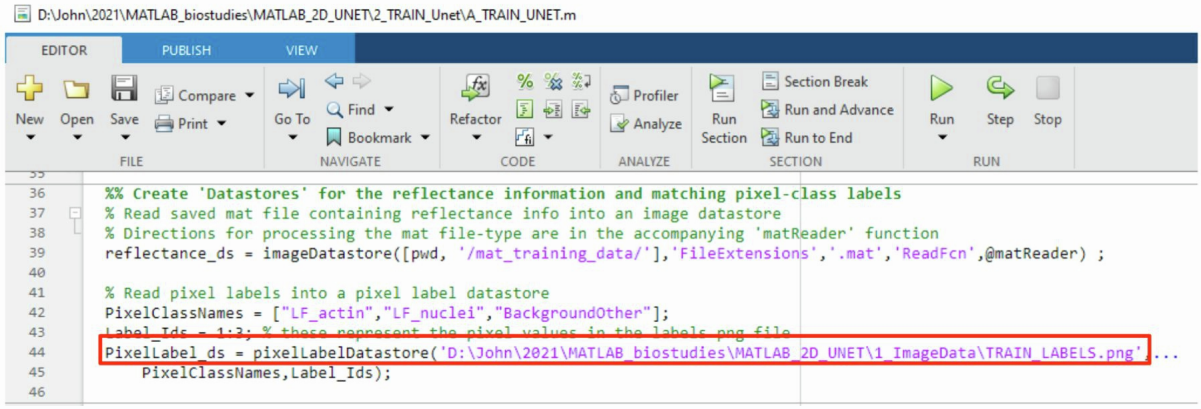
- To train a 2-D UNET model, open MATLAB and set the working directory to the "2_TRAIN_Unet" directory. Open the "A_TRAIN_UNET" script.



- Update the path to the training data (located in the "1_ImageData" folder).
- Update the path to tissue ROI mask which isolates the lymphoid follicle from the surrounding tissue (located in the "1_ImageData" folder).
- Update the path to the pixel classification labels (located in the "1_ImageData" folder)



- On Line 46, update the path to the pixel classification labels (located in the "1_ImageData" folder) used by the "pixelLabelDatastore" function.

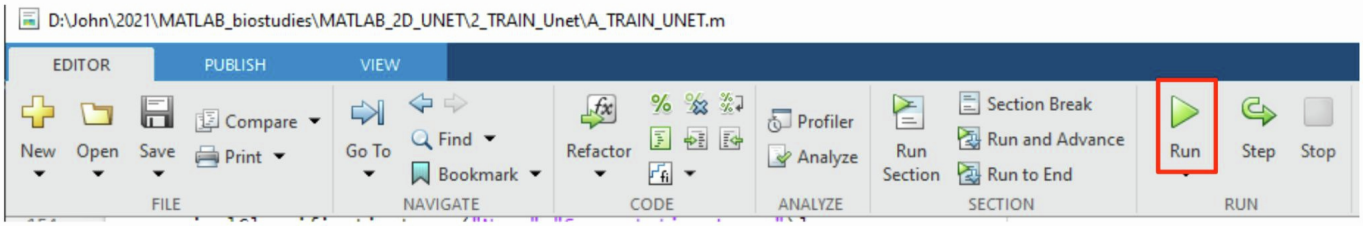


- At the bottom of the script on Line 195, update the path to the saved_network_directory.
- This should be folder 3 of the workflow ("3_Saved_models").

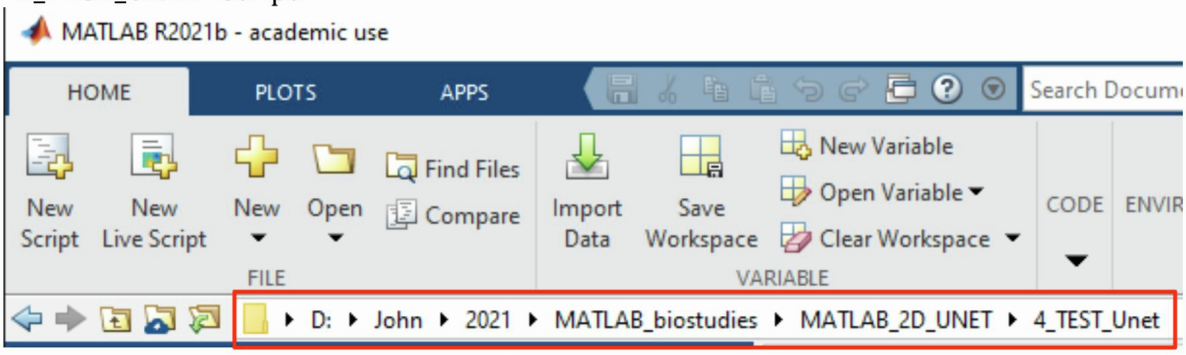
```
191  %% Train and save the network
192  close all
193
194  % Specify location to save the network
195  saved_network_directory = 'D:\John\2021\MATLAB_biostudies\MATLAB_2D_UNET\3_Saved_models\';
196
197  % Train the network
198  modelDateTime = datestr(now,'dd-mmm-yyyy-HH-MM-SS');
199  [net,info] = trainNetwork(training_ds,lgraph,options);
200
201  % Timestamp and save the network after training
202  save([saved_network_directory,'PeyersPatchBiostudies_',modelDateTime,'.mat'],...
203      'net','options','augmenter','info');
204
```

Zoom: 90%

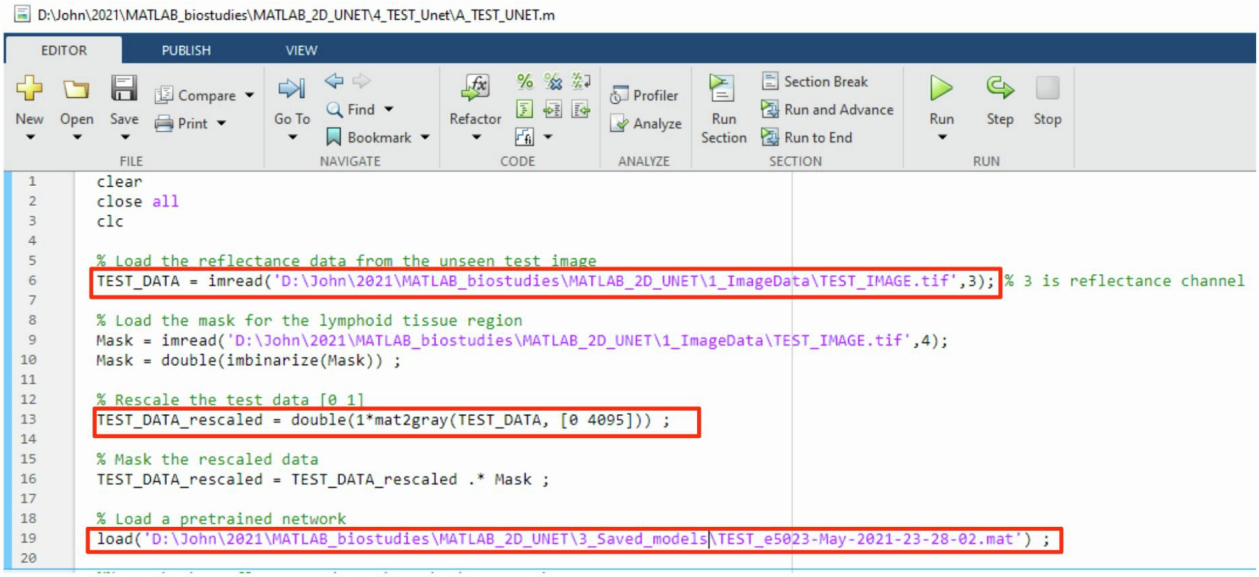
- Run the script by clicking the green arrow at the top.
- Model training takes several hours (~ 4h on a NVIDIA 1080 Ti GPU)
- The newly-trained model will be saved in the "3_Saved_models" directory with a new time/date stamp.



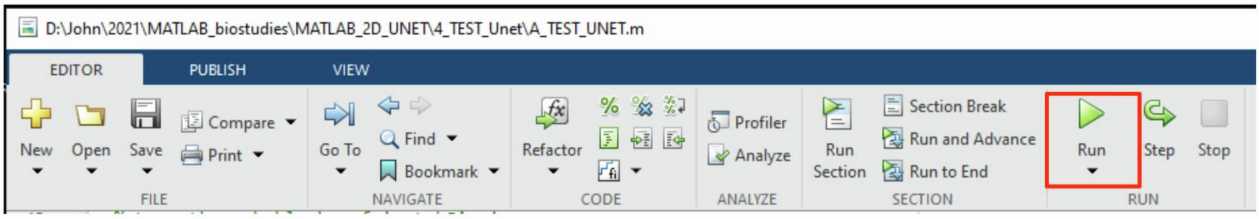
3. Testing a pretrained 2-D model using unseen data
-A screencast video is included with the BioStudies archive.
- Change the MATLAB working directory to the "4_Test_Unet" directory. Open the "A_TEST_UNET" script.



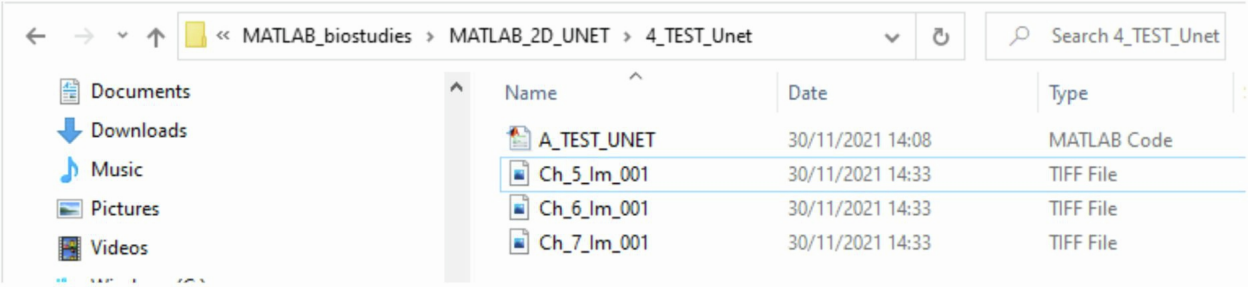
- Change the path to the unseen test image (located in the "1_ImageData" folder).
- Specify the ROI mask which identifies the lymphoid tissue (located in the "1_ImageData" folder).
- Specify which pretrained network to use (pretrained networks are located in the "3_Saved_models" folder.



- Click the "Run" button to process the unseen reflectance data with the selected pretrained network:



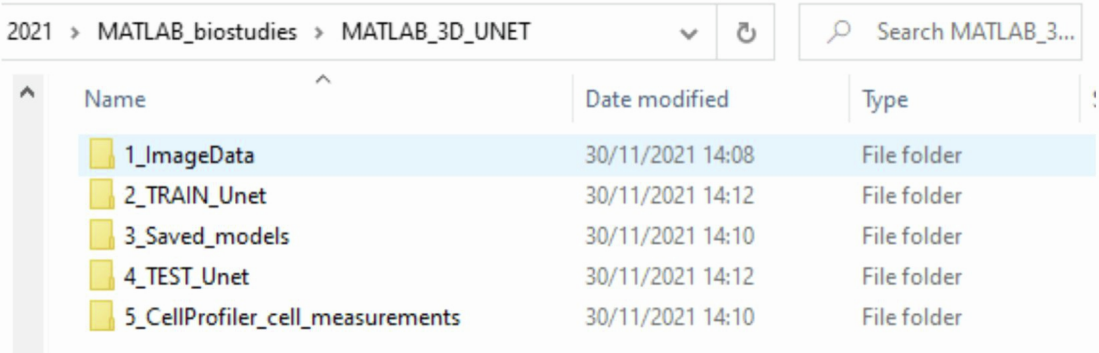
- Probability maps for the LF-nuclei, LF-actin and Background/Other classes will be saved in the working directory named ready for input into the CellProfiler pipeline (filenames Ch_5_lm_001, Ch_6_lm_001, Ch_7_lm_001, respectively).



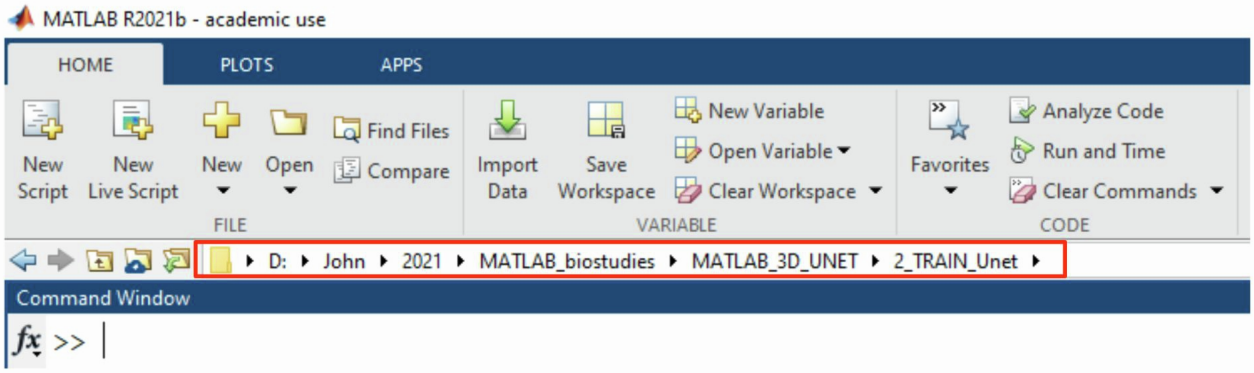
4. Training a 3-D UNET Model

-A screencast video is included with the BioStudies archive.

- Inside the MATLAB_3D_UNET folder, there is a sequentially-organised workflow starting with raw data and ending with a CellProfiler pipeline that obtains 3-D cell features from the label-free cell segmentation:



- To train a 3-D UNET model, open MATLAB and set the working directory to the "2_TRAIN_Unet" directory. Open the "A_TRAIN_3D_UNET" script.



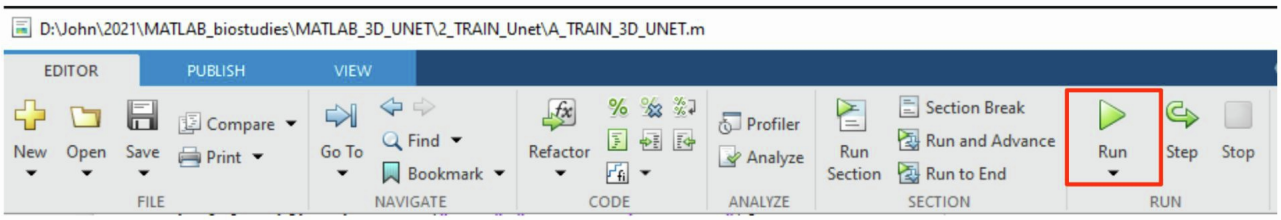
- Specify the path to the bioformats library. This is included inside the “2_TRAIN_Unet” folder (“bfmatlab”).
- Update the path to the training data directory (located in the “1_ImageData/TRAIN/DATA” folder).
- Specify the channel number in the training data that contains the reflectance information (here, ‘3’).
- Specify the location of the pixel classification training labels (located at “1_ImageData/TRAIN/LABELS” folder.

```
clear
close all
clc

% Specify path to bioformats library
addpath('D:\John\2021\MATLAB_biostudies\MATLAB_3D_UNET\2_TRAIN_Unet\bfmatlab\')

% Specify directory containing training data
Training_data_directory = 'D:\John\2021\MATLAB_biostudies\MATLAB_3D_UNET\1_ImageData\TRAIN\DATA\' ;

% Specify channel number in training data that contains reflectance information
RL_training_directory = 3;

% Specify directory containing training labels
Training_labels_path = 'D:\John\2021\MATLAB_biostudies\MATLAB_3D_UNET\1_ImageData\TRAIN\LABELS\' ;

```

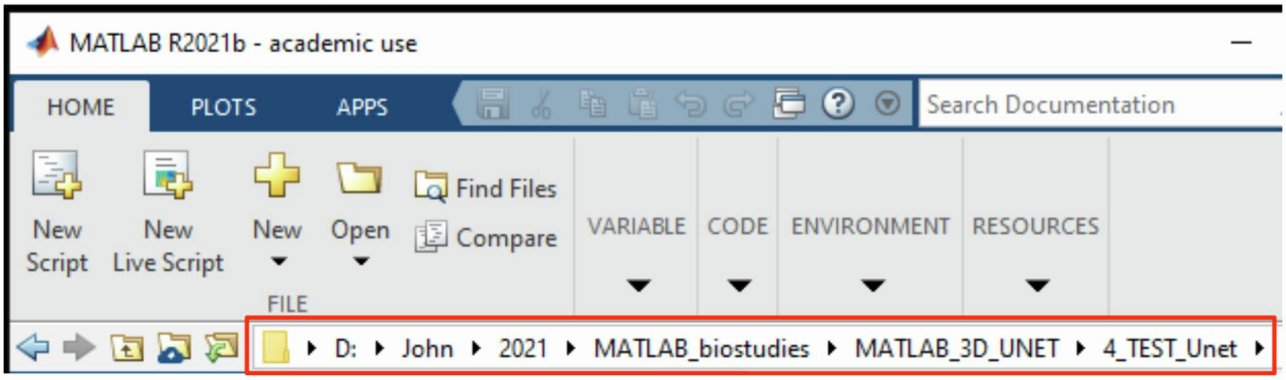
- Specify the location to save the model when training completes. This should be the “3_Saved_models” directory.

```
%% Train and save the network
close all

% Specify location to save the network
saved_network_directory = 'D:\John\2021\MATLAB_biostudies\MATLAB_3D_UNET\3_Saved_models\';

% Train the network
modelDateTime = datestr(now,'dd-mmm-yyyy-HHMM');
[net,info] = trainNetwork(Training_ds,lgraph,options);

% Timestamp and save the network after training
save([saved_network_directory,'MLN_Biostudies_',modelDateTime,'.mat'],'net','options','info');

```

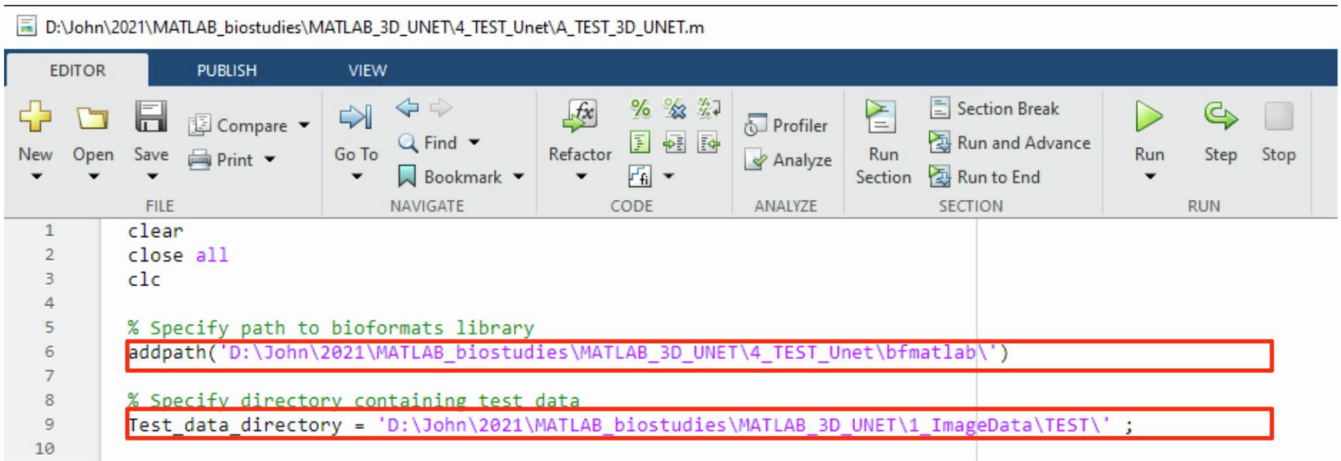
- Click “Run” to commence model training.
- This takes considerable time (approximately 6h on a NVIDIA 1080Ti GPU card).



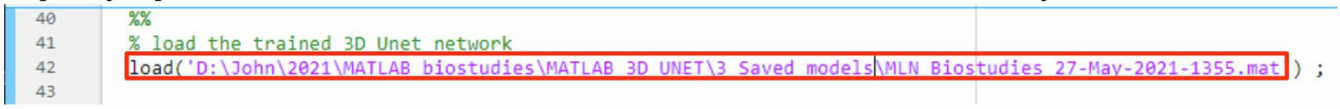
6. Testing a pretrained 3-D model using unseen data
-A screencast video is included with the BioStudies archive.
- Change the MATLAB working directory to the "4_Test_Unet" directory. Open the "A_TEST_3D_UNET" script.



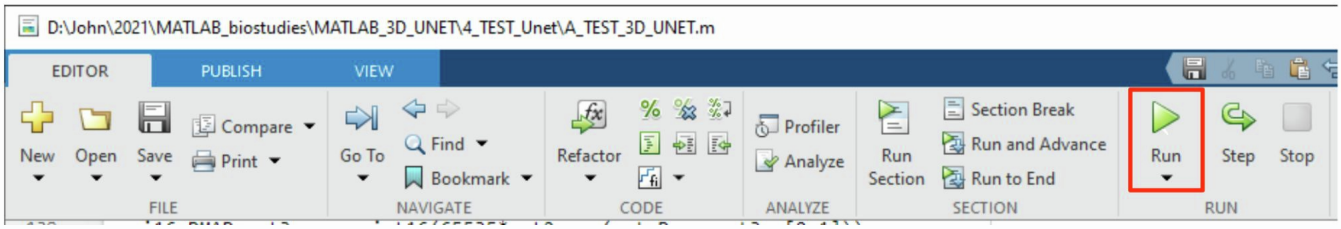
- Specify the path to the Bioformats library. (This is included inside the "4_TEST_Unet" folder ("bfmatlab").
- Specify the location of the unseen test image-data. This is located inside the "1_ImageData" folder at "1_ImageData/TEST/".



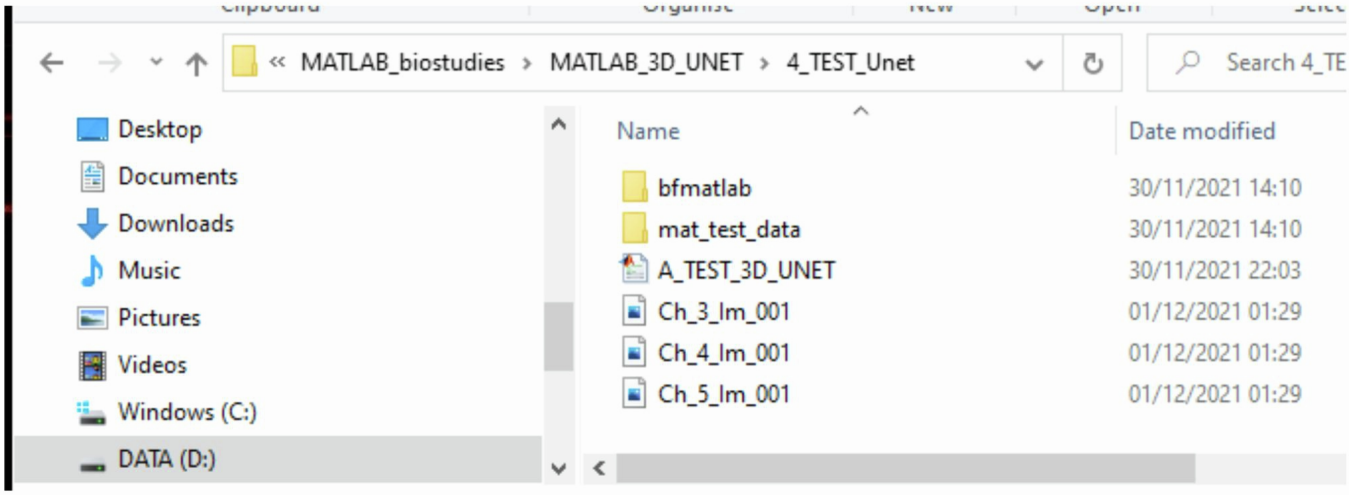
- Specify a pretrained 3-D Unet model from the "3_Saved_models" directory.



- Click "Run" to process the unseen reflectance data with the 3-D Unet model.



- Probability maps for the LF-nuclei, LF-actin and Background/Other classes will be saved as multipage .TIFF files in the working directory named ready for input into the CellProfiler pipeline (filenames Ch_3_lm_001, Ch_4_lm_001, Ch_5_lm_001, respectively).



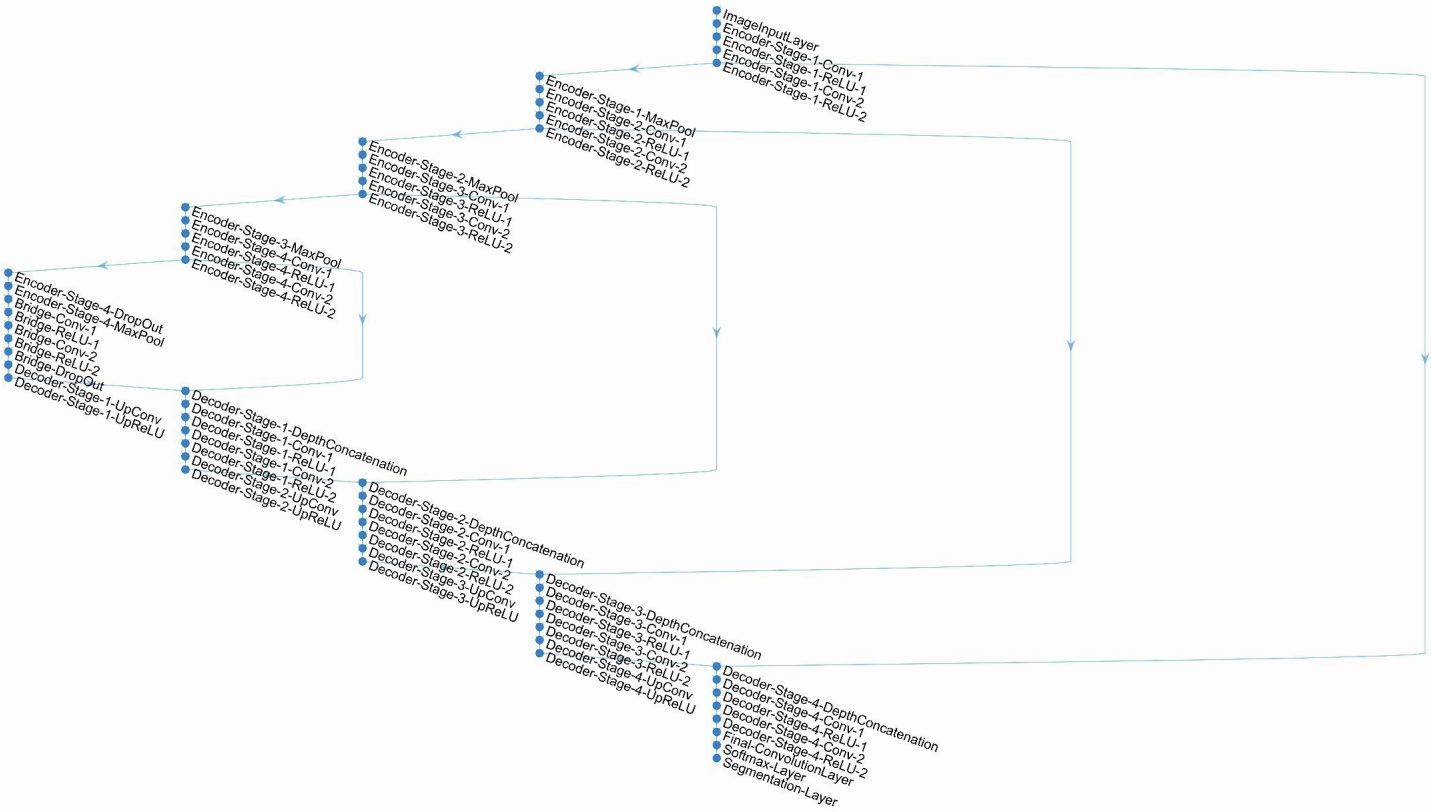
```
% MATLAB SCRIPT: TRAIN 2D UNET
% All code, image-data and screen-cast tutorial videos available for download at
% the Biostudies database under accession number S-BSST742

clear
close all
clc

% Read in the reflectance data for training
Training_data =
imread('D:\John\2021\MATLAB_biostudies\MATLAB_2D_UNET\1_ImageData\TRAIN_IMAGE.ti
f',3) ; % channel 3 is reflectance

% Read in the mask to isolated just the lymphoid tissue
mask =
imread('D:\John\2021\MATLAB_biostudies\MATLAB_2D_UNET\1_ImageData\TRAIN_IMAGE.ti
f',4) ;
mask = uint16(imbinarize(mask));

% Apply the mask to the training data
Training_data = Training_data .* mask ;

% Rescale the training data in the interval [0 1]
Training_data = double(1*mat2gray(Training_data, [0 4095])) ;

% Read in the pixel-class labels created from the nuclei and actin staining
Training_labels =
imread('D:\John\2021\MATLAB_biostudies\MATLAB_2D_UNET\1_ImageData\TRAIN_LABELS.p
ng') ;

% Visually inspect the data and labels
figure(1)
subplot(1,2,1)
imshow(Training_data, [])
title('Reflectance data')
subplot(1,2,2)
imshow(Training_labels, [])
title('Pixel classification labels')

%% Once happy with labels and data, commit the reflectance information data to
sub-directory in .mat format
if ~exist('mat_training_data', 'dir')
    mkdir('mat_training_data');
end
save([pwd,'/mat_training_data/','Training_data.mat'],'Training_data');

%% Create 'Datastores' for the reflectance information and matching pixel-class
labels
% Read saved mat file containing reflectance info into an image datastore
% Directions for processing the mat file-type are in the accompanying
'matReader' function
reflectance_ds = imageDatastore([pwd,
'/mat_training_data/'],'FileExtensions','.mat','ReadFcn',@matReader) ;

% Read pixel labels into a pixel label datastore
PixelClassNames = ["LF_actin","LF_nuclei","BackgroundOther"];
Label_Ids = 1:3; % these represent the pixel values in the labels png file
PixelLabel_ds =
pixelLabelDatastore('D:\John\2021\MATLAB_biostudies\MATLAB_2D_UNET\1_ImageData\T
RAIN_LABELS.png',...
```

```
    PixelClassNames,Label_Ids);

%% Define augmentation options
augmenter = imageDataAugmenter(...       %%% description and defaults %%%
    'FillValue',0,...              % define out-of-bounds points when resampling
0
    'RandXReflection',true,...     % Random reflection in the left-right
direction false
    'RandYReflection',true,...     % Random reflection in the top-bottom
direction false
    'RandRotation',[0 360],...     % Range of rotation, in degrees [0 0]
    'RandScale',[1 1],...          % Range of uniform (isotropic) scaling [1 1]
    'RandXScale',[1 1],...         % Range of horizontal scaling [1 1]
    'RandYScale',[1 1],...         % Range of vertical scaling [1 1]
    'RandXShear',[0 0],...         % Range of horizontal shear [0 0]
    'RandYShear',[0 0],...         % Range of vertical shear [0 0]
    'RandXTranslation',[0 0],...   % Range of horizontal translation [0 0]
    'RandYTranslation',[0 0]...    % Range of vertical translation [0 0]
    ) ;

%% Create a training datastore comprising matching patches (256x256 pixels) of
image-data and training labels
training_ds =
randomPatchExtractionDatastore(reflectance_ds,PixelLabel_ds,[256,256],...
    'PatchesPerImage',12000,'DataAugmentation',augmenter); % 743 draws == 1
epoch

%% CREATE THE UNET
% input layer 256x256x1
% encoder depth 4
% 64 filters at the level of the first encoder

lgraph = layerGraph();
tempLayers = [
    imageInputLayer([256 256 1],"Name","ImageInputLayer")
    convolution2dLayer([3 3],64,"Name","Encoder-Stage-1-Conv-
1","Padding","same","WeightsInitializer","he")
    reluLayer("Name","Encoder-Stage-1-ReLU-1")
    convolution2dLayer([3 3],64,"Name","Encoder-Stage-1-Conv-
2","Padding","same","WeightsInitializer","he")
    reluLayer("Name","Encoder-Stage-1-ReLU-2")];
lgraph = addLayers(lgraph,tempLayers);

tempLayers = [
    maxPooling2dLayer([2 2],"Name","Encoder-Stage-1-MaxPool","Stride",[2 2])
    convolution2dLayer([3 3],128,"Name","Encoder-Stage-2-Conv-
1","Padding","same","WeightsInitializer","he")
    reluLayer("Name","Encoder-Stage-2-ReLU-1")
    convolution2dLayer([3 3],128,"Name","Encoder-Stage-2-Conv-
2","Padding","same","WeightsInitializer","he")
    reluLayer("Name","Encoder-Stage-2-ReLU-2")];
lgraph = addLayers(lgraph,tempLayers);

tempLayers = [
    maxPooling2dLayer([2 2],"Name","Encoder-Stage-2-MaxPool","Stride",[2 2])
    convolution2dLayer([3 3],256,"Name","Encoder-Stage-3-Conv-
1","Padding","same","WeightsInitializer","he")
    reluLayer("Name","Encoder-Stage-3-ReLU-1")
    convolution2dLayer([3 3],256,"Name","Encoder-Stage-3-Conv-
2","Padding","same","WeightsInitializer","he")
```

```
    reluLayer("Name","Encoder-Stage-3-ReLU-2")];
lgraph = addLayers(lgraph,tempLayers);

tempLayers = [
    maxPooling2dLayer([2 2],"Name","Encoder-Stage-3-MaxPool","Stride",[2 2])
    convolution2dLayer([3 3],512,"Name","Encoder-Stage-4-Conv-
1","Padding","same","WeightsInitializer","he")
    reluLayer("Name","Encoder-Stage-4-ReLU-1")
    convolution2dLayer([3 3],512,"Name","Encoder-Stage-4-Conv-
2","Padding","same","WeightsInitializer","he")
    reluLayer("Name","Encoder-Stage-4-ReLU-2")];
lgraph = addLayers(lgraph,tempLayers);

tempLayers = [
    dropoutLayer(0.5,"Name","Encoder-Stage-4-DropOut")
    maxPooling2dLayer([2 2],"Name","Encoder-Stage-4-MaxPool","Stride",[2 2])
    convolution2dLayer([3 3],1024,"Name","Bridge-Conv-
1","Padding","same","WeightsInitializer","he")
    reluLayer("Name","Bridge-ReLU-1")
    convolution2dLayer([3 3],1024,"Name","Bridge-Conv-
2","Padding","same","WeightsInitializer","he")
    reluLayer("Name","Bridge-ReLU-2")
    dropoutLayer(0.5,"Name","Bridge-DropOut")
    transposedConv2dLayer([2 2],512,"Name","Decoder-Stage-1-
UpConv","BiasLearnRateFactor",2,"Stride",[2 2],"WeightsInitializer","he")
    reluLayer("Name","Decoder-Stage-1-UpReLU")];
lgraph = addLayers(lgraph,tempLayers);

tempLayers = [
    depthConcatenationLayer(2,"Name","Decoder-Stage-1-DepthConcatenation")
    convolution2dLayer([3 3],512,"Name","Decoder-Stage-1-Conv-
1","Padding","same","WeightsInitializer","he")
    reluLayer("Name","Decoder-Stage-1-ReLU-1")
    convolution2dLayer([3 3],512,"Name","Decoder-Stage-1-Conv-
2","Padding","same","WeightsInitializer","he")
    reluLayer("Name","Decoder-Stage-1-ReLU-2")
    transposedConv2dLayer([2 2],256,"Name","Decoder-Stage-2-
UpConv","BiasLearnRateFactor",2,"Stride",[2 2],"WeightsInitializer","he")
    reluLayer("Name","Decoder-Stage-2-UpReLU")];
lgraph = addLayers(lgraph,tempLayers);

tempLayers = [
    depthConcatenationLayer(2,"Name","Decoder-Stage-2-DepthConcatenation")
    convolution2dLayer([3 3],256,"Name","Decoder-Stage-2-Conv-
1","Padding","same","WeightsInitializer","he")
    reluLayer("Name","Decoder-Stage-2-ReLU-1")
    convolution2dLayer([3 3],256,"Name","Decoder-Stage-2-Conv-
2","Padding","same","WeightsInitializer","he")
    reluLayer("Name","Decoder-Stage-2-ReLU-2")
    transposedConv2dLayer([2 2],128,"Name","Decoder-Stage-3-
UpConv","BiasLearnRateFactor",2,"Stride",[2 2],"WeightsInitializer","he")
    reluLayer("Name","Decoder-Stage-3-UpReLU")];
lgraph = addLayers(lgraph,tempLayers);

tempLayers = [
    depthConcatenationLayer(2,"Name","Decoder-Stage-3-DepthConcatenation")
    convolution2dLayer([3 3],128,"Name","Decoder-Stage-3-Conv-
1","Padding","same","WeightsInitializer","he")
    reluLayer("Name","Decoder-Stage-3-ReLU-1")
```

```
    convolution2dLayer([3 3],128,"Name","Decoder-Stage-3-Conv-
2","Padding","same","WeightsInitializer","he")
    reluLayer("Name","Decoder-Stage-3-ReLU-2")
    transposedConv2dLayer([2 2],64,"Name","Decoder-Stage-4-
UpConv","BiasLearnRateFactor",2,"Stride",[2 2],"WeightsInitializer","he")
    reluLayer("Name","Decoder-Stage-4-UpReLU")];
lgraph = addLayers(lgraph,tempLayers);

tempLayers = [
    depthConcatenationLayer(2,"Name","Decoder-Stage-4-DepthConcatenation")
    convolution2dLayer([3 3],64,"Name","Decoder-Stage-4-Conv-
1","Padding","same","WeightsInitializer","he")
    reluLayer("Name","Decoder-Stage-4-ReLU-1")
    convolution2dLayer([3 3],64,"Name","Decoder-Stage-4-Conv-
2","Padding","same","WeightsInitializer","he")
    reluLayer("Name","Decoder-Stage-4-ReLU-2")
    convolution2dLayer([1 1],3,"Name","Final-
ConvolutionLayer","Padding","same","WeightsInitializer","he")
    softmaxLayer("Name","Softmax-Layer")
    pixelClassificationLayer("Name","Segmentation-Layer")];
lgraph = addLayers(lgraph,tempLayers);

clear tempLayers;

% encoder / decoder connections
lgraph = connectLayers(lgraph,"Encoder-Stage-1-ReLU-2","Encoder-Stage-1-
MaxPool");
lgraph = connectLayers(lgraph,"Encoder-Stage-1-ReLU-2","Decoder-Stage-4-
DepthConcatenation/in2");
lgraph = connectLayers(lgraph,"Encoder-Stage-2-ReLU-2","Encoder-Stage-2-
MaxPool");
lgraph = connectLayers(lgraph,"Encoder-Stage-2-ReLU-2","Decoder-Stage-3-
DepthConcatenation/in2");
lgraph = connectLayers(lgraph,"Encoder-Stage-3-ReLU-2","Encoder-Stage-3-
MaxPool");
lgraph = connectLayers(lgraph,"Encoder-Stage-3-ReLU-2","Decoder-Stage-2-
DepthConcatenation/in2");
lgraph = connectLayers(lgraph,"Encoder-Stage-4-ReLU-2","Encoder-Stage-4-
DropOut");
lgraph = connectLayers(lgraph,"Encoder-Stage-4-ReLU-2","Decoder-Stage-1-
DepthConcatenation/in2");
lgraph = connectLayers(lgraph,"Decoder-Stage-1-UpReLU","Decoder-Stage-1-
DepthConcatenation/in1");
lgraph = connectLayers(lgraph,"Decoder-Stage-2-UpReLU","Decoder-Stage-2-
DepthConcatenation/in1");
lgraph = connectLayers(lgraph,"Decoder-Stage-3-UpReLU","Decoder-Stage-3-
DepthConcatenation/in1");
lgraph = connectLayers(lgraph,"Decoder-Stage-4-UpReLU","Decoder-Stage-4-
DepthConcatenation/in1");

% analyzeNetwork(lgraph) % comment in to check structure and errors
plot(lgraph) % comment in to show network structure

%% Specify training options
options = trainingOptions('sgdm',...
    'InitialLearnRate',0.05, ...
    'Momentum',0.9,...
    'L2Regularization',0.0001,...
    'MaxEpochs',50,...
    'MiniBatchSize',12,...
```

```
    'LearnRateSchedule','piecewise',...
    'Shuffle','every-epoch',...
    'GradientThresholdMethod','l2norm',...
    'GradientThreshold',0.05, ...
    'Plots','training-progress', ...
    'VerboseFrequency',20,...
    'ExecutionEnvironment','auto');

%% Train and save the network
close all

% Specify location to save the network
saved_network_directory =
'D:\John\2021\MATLAB_biostudies\MATLAB_2D_UNET\3_Saved_models\';

% Train the network
modelDateTime = datestr(now,'dd-mmm-yyyy-HH-MM-SS');
[net,info] = trainNetwork(training_ds,lgraph,options);

% Timestamp and save the network after training
save([saved_network_directory,'PeyersPatchBiostudies_',modelDateTime,'.mat'],...
    'net','options','augmenter','info');
```



2-D Unet architecture schematic. The network uses an input layer for the reflectance data of 256x256x1 (x, y, channels). The best performing three-class Unet architecture uses an encoder depth of 4 with 64 filters at the level of the first encoder (shown, **Figure S2**). The network uses complete up-convolutional expansion to yield outputted probability maps that are identically sized to the input layer.

```
% MATLAB SCRIPT: TEST 2D UNET
% All code, image-data and screen-cast tutorial videos available for download at
% the Biostudies database under accession number S-BSST742

clear
close all
clc

% Load the reflectance data from the unseen test image
TEST_DATA =
imread('D:\John\2021\MATLAB_biostudies\MATLAB_2D_UNET\1_ImageData\TEST_IMAGE.tif
',3); % 3 is reflectance channel

% Load the mask for the lymphoid tissue region
Mask =
imread('D:\John\2021\MATLAB_biostudies\MATLAB_2D_UNET\1_ImageData\TEST_IMAGE.tif
',4);
Mask = double(imbinarize(Mask)) ;

% Rescale the test data [0 1]
TEST_DATA_rescaled = double(1*mat2gray(TEST_DATA, [0 4095])) ;

% Mask the rescaled data
TEST_DATA_rescaled = TEST_DATA_rescaled .* Mask ;

% Load a pretrained network
load('D:\John\2021\MATLAB_biostudies\MATLAB_2D_UNET\3_Saved_models\Rescaled0-
1_2DUNET_S7-RL-SA_enc-4_filt-64_e50.mat') ;

%% Patch the reflectance data through the network
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
% Set the patch sizes to be passed to the net
patchSize = [2048 2048]; % decrease if insufficient GPU memory eg., [1024 1024]

% Segment blockwise then reassemble in full
% Define image dimensions
[height, width, nChannel] = size(TEST_DATA_rescaled);
patch = zeros([patchSize, nChannel], 'like', TEST_DATA_rescaled);

% Pad image to have dimensions as multiples of patchSize
padSize(1) = patchSize(1) - mod(height, patchSize(1));
padSize(2) = patchSize(2) - mod(width, patchSize(2));
im_pad = padarray (TEST_DATA_rescaled, padSize, 0, 'post');
[height_pad, width_pad, nChannel_pad] = size(im_pad);

% Preallocate some matrices to receive the network outputs
out_Uncertainty_Scores = zeros([size(im_pad,1), size(im_pad,2)], 'double');
out_Pmap_cat1 = out_Uncertainty_Scores;
out_Pmap_cat2 = out_Uncertainty_Scores;
out_Pmap_cat3 = out_Uncertainty_Scores;

% Loop through blocks of 'patchSize'
    for loop = 1:patchSize(1):height_pad
        for j =1:patchSize(2):width_pad
            for p = 1:nChannel
                patch(:,:,p) = squeeze( im_pad( loop:loop+patchSize(1)-1,...
                                                j:j+patchSize(2)-1,p));
            end
            % deploy net
```

```
            [patch_seg, Scores, allScores] = semanticseg(patch, net,
'OutputType', 'double',...
                'ExecutionEnvironment','auto');
            % catch what comes out
            out_Uncertainty_Scores(loop:loop+patchSize(1)-1, j:j+patchSize(2)-1)
= Scores;
            out_Pmap_cat1(loop:loop+patchSize(1)-1, j:j+patchSize(2)-1) =
allScores(:,:,1);
            out_Pmap_cat2(loop:loop+patchSize(1)-1, j:j+patchSize(2)-1) =
allScores(:,:,2);
            out_Pmap_cat3(loop:loop+patchSize(1)-1, j:j+patchSize(2)-1) =
allScores(:,:,3);
        end
    end

% Remove padding from the network outputs
out_Uncertainty_Scores = out_Uncertainty_Scores(1:height, 1:width);
out_Pmap_cat1 = out_Pmap_cat1(1:height, 1:width);
out_Pmap_cat2 = out_Pmap_cat2(1:height, 1:width);
out_Pmap_cat3 = out_Pmap_cat3(1:height, 1:width);

%% visualise network outputs
figure(2)
clf(figure(2))
ax1 = subplot(2,3,1);
imshow(out_Pmap_cat1,[])
title('LF-actin')
ax2 = subplot(2,3,2) ;
imshow(out_Pmap_cat2,[])
title('LF-nuclei')
ax3 = subplot(2,3,3);
imshow(out_Pmap_cat3,[])
title('Background/other')
ax4 = subplot(2,3,5);
imshow(out_Uncertainty_Scores,[])
title('Uncertainty')
linkaxes([ax1 ax2 ax3 ax4], 'xy')

%% Map to 16-bit and save for loading into CellProfiler
ui16_PMAP_cat1_xy = uint16(65535*mat2gray(out_Pmap_cat1, [0 1])) ;
imwrite(ui16_PMAP_cat1_xy, ['Ch_6_Im_001','.tiff']) ;

ui16_PMAP_cat2_xy = uint16(65535*mat2gray(out_Pmap_cat2, [0 1])) ;
imwrite(ui16_PMAP_cat2_xy, ['Ch_5_Im_001','.tiff']) ;

ui16_PMAP_cat3_xy = uint16(65535*mat2gray(out_Pmap_cat3, [0 1])) ;
imwrite(ui16_PMAP_cat3_xy, ['Ch_7_Im_001','.tiff']) ;
```

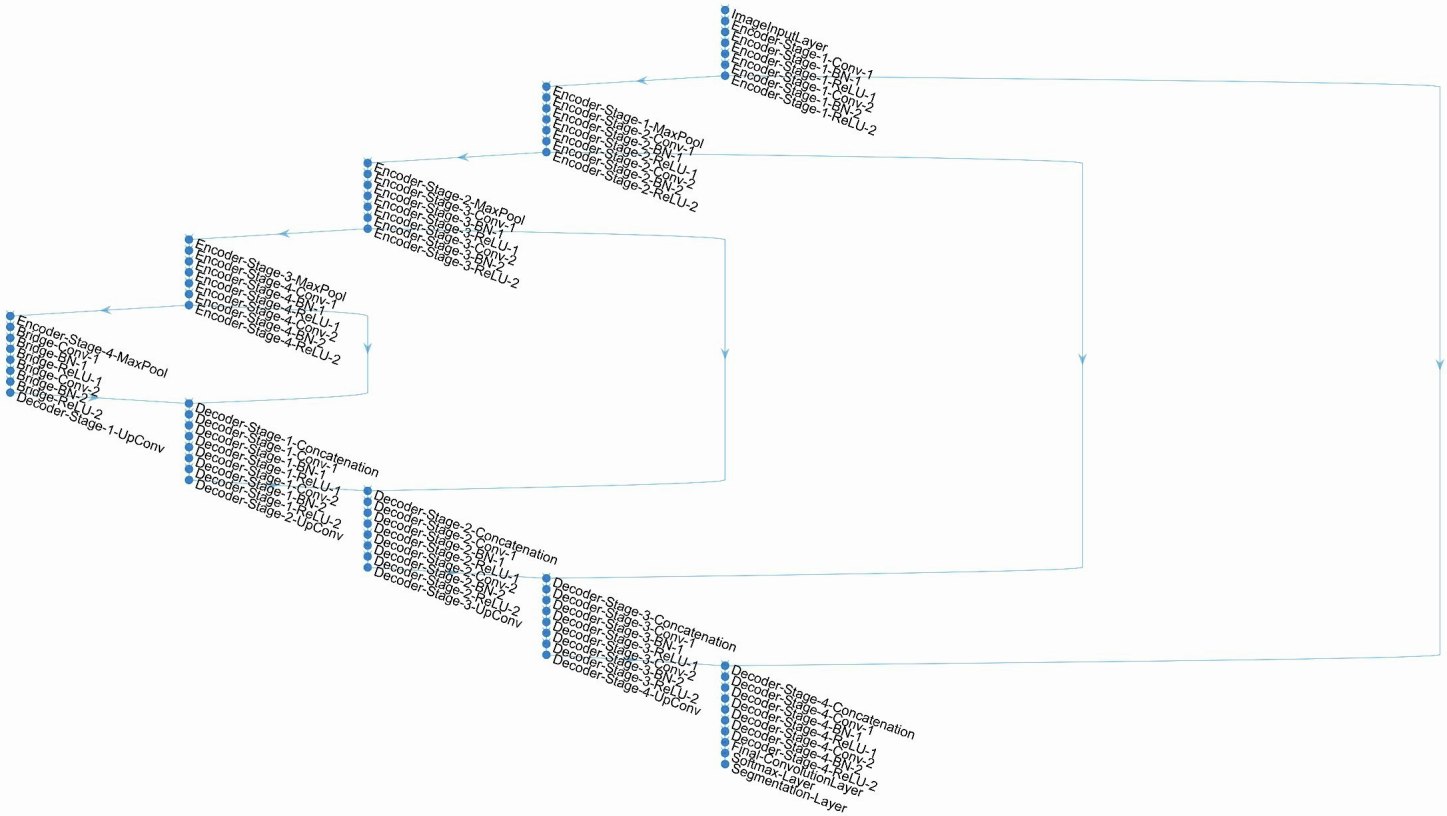
```
% MATLAB SCRIPT: Train 3D UNET
% All code, image-data and screen-cast tutorial videos available for download at
% the Biostudies database under accession number S-BSST742

clear
close all
clc

% Specify path to bioformats library
addpath('D:\John\2021\MATLAB_biostudies\MATLAB_3D_UNET\2_TRAIN_Unet\bfmatlab\')

% Specify directory containing training data
Training_data_directory =
'D:\John\2021\MATLAB_biostudies\MATLAB_3D_UNET\1_ImageData\TRAIN\DATA\' ;

% Specify channel number in training data that contains reflectance information
RL_training_directory = 3;

% Specify directory containing training labels
Training_labels_path =
'D:\John\2021\MATLAB_biostudies\MATLAB_3D_UNET\1_ImageData\TRAIN\LABELS\' ;

%% COMMIT TRAINING DATA TO DIRECTORY rescaled [0 1] IN MAT FORMAT
if ~exist('mat_training_data', 'dir')
    mkdir('mat_training_data');
end

for loop = 1:2
    counter = sprintf('%03d',loop) ;

    % find metadata describing file
    reader = bfGetReader([Training_data_directory,'TRAIN_',counter,'.tif']);
    omeMeta = reader.getMetadataStore();
    number_of_channels = omeMeta.getChannelCount(0);
    stackSizeZ = omeMeta.getPixelsSizeZ(0).getValue(); % number of Z slices

    % Load reflectance information
    for zplane = 1:stackSizeZ
        iPlane = reader.getIndex(zplane -1, RL_training_directory -1, 0) + 1; %
because zplanes and channels are numbered from zero
        channel_zimage{zplane} = bfGetPlane(reader, iPlane);
    end

    IM_DATA = cat(3,channel_zimage{:}) ;
    IM_DATA = double(1*mat2gray(IM_DATA, [0 65535])) ;
    save([pwd,'/mat_training_data/','TRAIN_DATA_',counter,'.mat'], 'IM_DATA') ;
end

%% COMMIT TRAINING LABELS TO DIRECTORY IN MAT FORMAT
if ~exist('mat_training_labels', 'dir')
    mkdir('mat_training_labels');
end

for loop = 1:2
    counter = sprintf('%03d',loop) ;

        % load label information
        for zplane = 1:stackSizeZ
            iplane =
imread([Training_labels_path,'LABELS_',counter,'.tif'],zplane);
```

```
            label_zimage{zplane} = iplane ;
        end

        IM_LABELS = cat(3,label_zimage{:});
        IM_LABELS = double(IM_LABELS) ;
        save([pwd,'/mat_training_labels/','TRAIN_LABELS_',counter,'.mat'],
'IM_LABELS') ;
end

% Once data is prepared for training, clear workspace
clear ;

%% Create 'Datastores' for the reflectance information and matching pixel-class
labels
% Read saved mat file containing reflectance info into an image datastore
% Directions for processing the mat file-type are in the accompanying 'matRead'
function
data_location = [pwd, '\mat_training_data\'] ;
volReader = @(x) JWmatRead(x) ;
reflectance_ds =
imageDatastore(data_location,'FileExtensions','.mat','ReadFcn',volReader);

% Read pixel labels into a pixel label datastore
label_location = [pwd, '\mat_training_labels\'] ;
labelReader = @(x) JWLabelRead(x);
classNames = ["LFNuclei","LFActin","BackgroundOther"];
pixelLabelID = 1:3; % these represent the pixel values in the labels file
PixelLabel_ds = pixelLabelDatastore(label_location,classNames,pixelLabelID,...
    'FileExtensions','.mat','ReadFcn',labelReader);

%% Set up patch extraction from reflectance datastore
patchSize = [64 64 32];
patchPerImage = 375;
MiniBatchSize = 8; % set the batch size
reflectance_patches_ds =
randomPatchExtractionDatastore(reflectance_ds,PixelLabel_ds,patchSize,'PatchesPe
rImage',patchPerImage);
reflectance_patches_ds.MiniBatchSize = MiniBatchSize;

% Augment the patches using 'augment3dPatch' function
Training_ds = transform(reflectance_patches_ds,@JWaugment3dPatch);

%% CREATE THE 3D UNET
% input layer 64x64x32x1
% encoder depth 4
% 32 filters at the level of the first encoder

lgraph = layerGraph();

tempLayers = [
    image3dInputLayer([64 64 32 1],"Name","ImageInputLayer")
    convolution3dLayer([3 3 3],32,"Name","Encoder-Stage-1-Conv-
1","Padding","same","WeightsInitializer","he")
    batchNormalizationLayer("Name","Encoder-Stage-1-BN-1")
    reluLayer("Name","Encoder-Stage-1-ReLU-1")
    convolution3dLayer([3 3 3],64,"Name","Encoder-Stage-1-Conv-
2","Padding","same","WeightsInitializer","he")
    batchNormalizationLayer("Name","Encoder-Stage-1-BN-2")
    reluLayer("Name","Encoder-Stage-1-ReLU-2")];
lgraph = addLayers(lgraph,tempLayers);
```

```
tempLayers = [
    maxPooling3dLayer([2 2 2],"Name","Encoder-Stage-1-MaxPool","Stride",[2 2 2])
    convolution3dLayer([3 3 3],64,"Name","Encoder-Stage-2-Conv-
1","Padding","same","WeightsInitializer","he")
    batchNormalizationLayer("Name","Encoder-Stage-2-BN-1")
    reluLayer("Name","Encoder-Stage-2-ReLU-1")
    convolution3dLayer([3 3 3],128,"Name","Encoder-Stage-2-Conv-
2","Padding","same","WeightsInitializer","he")
    batchNormalizationLayer("Name","Encoder-Stage-2-BN-2")
    reluLayer("Name","Encoder-Stage-2-ReLU-2")];
lgraph = addLayers(lgraph,tempLayers);

tempLayers = [
    maxPooling3dLayer([2 2 2],"Name","Encoder-Stage-2-MaxPool","Stride",[2 2 2])
    convolution3dLayer([3 3 3],128,"Name","Encoder-Stage-3-Conv-
1","Padding","same","WeightsInitializer","he")
    batchNormalizationLayer("Name","Encoder-Stage-3-BN-1")
    reluLayer("Name","Encoder-Stage-3-ReLU-1")
    convolution3dLayer([3 3 3],256,"Name","Encoder-Stage-3-Conv-
2","Padding","same","WeightsInitializer","he")
    batchNormalizationLayer("Name","Encoder-Stage-3-BN-2")
    reluLayer("Name","Encoder-Stage-3-ReLU-2")];
lgraph = addLayers(lgraph,tempLayers);

tempLayers = [
    maxPooling3dLayer([2 2 2],"Name","Encoder-Stage-3-MaxPool","Stride",[2 2 2])
    convolution3dLayer([3 3 3],256,"Name","Encoder-Stage-4-Conv-
1","Padding","same","WeightsInitializer","he")
    batchNormalizationLayer("Name","Encoder-Stage-4-BN-1")
    reluLayer("Name","Encoder-Stage-4-ReLU-1")
    convolution3dLayer([3 3 3],512,"Name","Encoder-Stage-4-Conv-
2","Padding","same","WeightsInitializer","he")
    batchNormalizationLayer("Name","Encoder-Stage-4-BN-2")
    reluLayer("Name","Encoder-Stage-4-ReLU-2")];
lgraph = addLayers(lgraph,tempLayers);

tempLayers = [
    maxPooling3dLayer([2 2 2],"Name","Encoder-Stage-4-MaxPool","Stride",[2 2 2])
    convolution3dLayer([3 3 3],512,"Name","Bridge-Conv-
1","Padding","same","WeightsInitializer","he")
    batchNormalizationLayer("Name","Bridge-BN-1")
    reluLayer("Name","Bridge-ReLU-1")
    convolution3dLayer([3 3 3],1024,"Name","Bridge-Conv-
2","Padding","same","WeightsInitializer","he")
    batchNormalizationLayer("Name","Bridge-BN-2")
    reluLayer("Name","Bridge-ReLU-2")
    transposedConv3dLayer([2 2 2],1024,"Name","Decoder-Stage-1-
UpConv","BiasLearnRateFactor",2,"Stride",[2 2 2],"WeightsInitializer","he")];
lgraph = addLayers(lgraph,tempLayers);

tempLayers = [
    concatenationLayer(4,2,"Name","Decoder-Stage-1-Concatenation")
    convolution3dLayer([3 3 3],512,"Name","Decoder-Stage-1-Conv-
1","Padding","same","WeightsInitializer","he")
    batchNormalizationLayer("Name","Decoder-Stage-1-BN-1")
    reluLayer("Name","Decoder-Stage-1-ReLU-1")
    convolution3dLayer([3 3 3],512,"Name","Decoder-Stage-1-Conv-
2","Padding","same","WeightsInitializer","he")
    batchNormalizationLayer("Name","Decoder-Stage-1-BN-2")
```

```
    reluLayer("Name","Decoder-Stage-1-ReLU-2")
    transposedConv3dLayer([2 2 2],512,"Name","Decoder-Stage-2-
UpConv","BiasLearnRateFactor",2,"Stride",[2 2 2],"WeightsInitializer","he")];
lgraph = addLayers(lgraph,tempLayers);

tempLayers = [
    concatenationLayer(4,2,"Name","Decoder-Stage-2-Concatenation")
    convolution3dLayer([3 3 3],256,"Name","Decoder-Stage-2-Conv-
1","Padding","same","WeightsInitializer","he")
    batchNormalizationLayer("Name","Decoder-Stage-2-BN-1")
    reluLayer("Name","Decoder-Stage-2-ReLU-1")
    convolution3dLayer([3 3 3],256,"Name","Decoder-Stage-2-Conv-
2","Padding","same","WeightsInitializer","he")
    batchNormalizationLayer("Name","Decoder-Stage-2-BN-2")
    reluLayer("Name","Decoder-Stage-2-ReLU-2")
    transposedConv3dLayer([2 2 2],256,"Name","Decoder-Stage-3-
UpConv","BiasLearnRateFactor",2,"Stride",[2 2 2],"WeightsInitializer","he")];
lgraph = addLayers(lgraph,tempLayers);

tempLayers = [
    concatenationLayer(4,2,"Name","Decoder-Stage-3-Concatenation")
    convolution3dLayer([3 3 3],128,"Name","Decoder-Stage-3-Conv-
1","Padding","same","WeightsInitializer","he")
    batchNormalizationLayer("Name","Decoder-Stage-3-BN-1")
    reluLayer("Name","Decoder-Stage-3-ReLU-1")
    convolution3dLayer([3 3 3],128,"Name","Decoder-Stage-3-Conv-
2","Padding","same","WeightsInitializer","he")
    batchNormalizationLayer("Name","Decoder-Stage-3-BN-2")
    reluLayer("Name","Decoder-Stage-3-ReLU-2")
    transposedConv3dLayer([2 2 2],128,"Name","Decoder-Stage-4-
UpConv","BiasLearnRateFactor",2,"Stride",[2 2 2],"WeightsInitializer","he")];
lgraph = addLayers(lgraph,tempLayers);

tempLayers = [
    concatenationLayer(4,2,"Name","Decoder-Stage-4-Concatenation")
    convolution3dLayer([3 3 3],64,"Name","Decoder-Stage-4-Conv-
1","Padding","same","WeightsInitializer","he")
    batchNormalizationLayer("Name","Decoder-Stage-4-BN-1")
    reluLayer("Name","Decoder-Stage-4-ReLU-1")
    convolution3dLayer([3 3 3],64,"Name","Decoder-Stage-4-Conv-
2","Padding","same","WeightsInitializer","he")
    batchNormalizationLayer("Name","Decoder-Stage-4-BN-2")
    reluLayer("Name","Decoder-Stage-4-ReLU-2")
    convolution3dLayer([1 1 1],3,"Name","Final-
ConvolutionLayer","Padding","same","WeightsInitializer","he")
    softmaxLayer("Name","Softmax-Layer")
    pixelClassificationLayer("Name","Segmentation-Layer")];
lgraph = addLayers(lgraph,tempLayers);

clear tempLayers;

% encoder / decoder connections
lgraph = connectLayers(lgraph,"Encoder-Stage-1-ReLU-2","Encoder-Stage-1-
MaxPool");
lgraph = connectLayers(lgraph,"Encoder-Stage-1-ReLU-2","Decoder-Stage-4-
Concatenation/in2");
lgraph = connectLayers(lgraph,"Encoder-Stage-2-ReLU-2","Encoder-Stage-2-
MaxPool");
lgraph = connectLayers(lgraph,"Encoder-Stage-2-ReLU-2","Decoder-Stage-3-
Concatenation/in2");
```

```
lgraph = connectLayers(lgraph,"Encoder-Stage-3-ReLU-2","Encoder-Stage-3-
MaxPool");
lgraph = connectLayers(lgraph,"Encoder-Stage-3-ReLU-2","Decoder-Stage-2-
Concatenation/in2");
lgraph = connectLayers(lgraph,"Encoder-Stage-4-ReLU-2","Encoder-Stage-4-
MaxPool");
lgraph = connectLayers(lgraph,"Encoder-Stage-4-ReLU-2","Decoder-Stage-1-
Concatenation/in2");
lgraph = connectLayers(lgraph,"Decoder-Stage-1-UpConv","Decoder-Stage-1-
Concatenation/in1");
lgraph = connectLayers(lgraph,"Decoder-Stage-2-UpConv","Decoder-Stage-2-
Concatenation/in1");
lgraph = connectLayers(lgraph,"Decoder-Stage-3-UpConv","Decoder-Stage-3-
Concatenation/in1");
lgraph = connectLayers(lgraph,"Decoder-Stage-4-UpConv","Decoder-Stage-4-
Concatenation/in1");

% analyzeNetwork(lgraph) % comment in to check structure and errors
plot(lgraph) % comment in to show network structure

%% Specify training options
options = trainingOptions('adam', ...
    'MaxEpochs',150, ...
    'InitialLearnRate',5e-4, ...
    'L2Regularization',1e-4,...
    'LearnRateSchedule','piecewise', ...
    'LearnRateDropPeriod',5,...
    'LearnRateDropFactor',0.95, ...
    'Plots','training-progress', ...
    'Verbose',true, ...
    'VerboseFrequency',20,...
    'Shuffle','every-epoch',...%
    'ExecutionEnvironment','auto',...
    'MiniBatchSize',MiniBatchSize);

%% Train and save the network
close all

% Specify location to save the network
saved_network_directory =
'D:\John\2021\MATLAB_biostudies\MATLAB_3D_UNET\3_Saved_models\';

% Train the network
modelDateTime = datestr(now,'dd-mmm-yyyy-HHMM');
[net,info] = trainNetwork(Training_ds,lgraph,options);

% Timestamp and save the network after training
save([saved_network_directory,'MLN_Biostudies_',modelDateTime,'.mat'],'net','opt
ions','info');
```



3-D Unet architecture schematic. The network uses an input layer for the reflectance data of 64x64x64x1 (x, y, z, channels). The three-class Unet architecture uses an encoder depth of 4 with 64 filters at the level of the first encoder. The network uses complete up-convolutional expansion to yield outputted probability maps that are identically sized to the input layer.

```
% MATLAB SCRIPT: TEST 3D UNET
% All code, image-data and screen-cast tutorial videos available for download at
% the Biostudies database under accession number S-BSST742

clear
close all
clc

% Specify path to bioformats library
addpath('D:\John\2021\MATLAB_biostudies\MATLAB_3D_UNET\4_TEST_Unet\bfmatlab\')

% Specify directory containing test data
Test_data_directory =
'D:\John\2021\MATLAB_biostudies\MATLAB_3D_UNET\1_ImageData\TEST\' ;

% Specify channel number in test data that contains reflectance information
RL_channel_number = 2;

% COMMIT REFLECTANCE TEST DATA TO DIRECTORY RESCALED [0 1] IN MAT FORMAT
if ~exist('mat_test_data', 'dir')
    mkdir('mat_test_data');
end

for loop = 1:1
    counter = sprintf('%03d',loop) ;

    % find metadata describing file
    reader = bfGetReader([Test_data_directory,'TEST_',counter,'.tif']);
    omeMeta = reader.getMetadataStore();
    number_of_channels = omeMeta.getChannelCount(0);
    stackSizeZ = omeMeta.getPixelsSizeZ(0).getValue(); % number of Z slices

    % Load reflectance information
    for zplane = 1:stackSizeZ
        iPlane = reader.getIndex(zplane -1, RL_channel_number -1, 0) + 1; %
because zplanes and channels are numbered from zero
        channel_zimage{zplane} = bfGetPlane(reader, iPlane);
    end

    IM_DATA = cat(3,channel_zimage{:}) ;
    IM_DATA = double(1*mat2gray(IM_DATA, [0 65535])) ; % rescale zero-one
    save([pwd,'/mat_test_data/','TEST_DATA_',counter,'.mat'], 'IM_DATA') ;
end
clear

%%
% load the trained 3D Unet network
load('D:\John\2021\MATLAB_biostudies\MATLAB_3D_UNET\3_Saved_models\MLN_Biostudie
s_27-May-2021-1355.mat') ;

% load the rescaled reflectance test data
load([pwd,'/mat_test_data/','TEST_DATA_001.mat']);

%% Patch the reflectance data through the network
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
% Set the patch sizes to be passed to the net
patchSize = [256 256 64];

% Segment blockwise then reassemble in full
% Define image dimensions
```

```
[height, width, depth, nChannel] = size(IM_DATA);
patch = zeros([patchSize, nChannel], 'like', IM_DATA);
number_of_height_patches =ceil(height/patchSize(1));
number_of_width_patches  =ceil(width/patchSize(2));
number_of_depth_patches  =ceil(depth/patchSize(3));

% Pad image to have dimensions as multiples of patchSize
height_pad = number_of_height_patches*patchSize(1);
width_pad  = number_of_width_patches*patchSize(2);
depth_pad  = number_of_depth_patches*patchSize(3);

% Amount to pad different dimensions of image
padSize(1) = height_pad - height;
padSize(2) = width_pad - width;
padSize(3) = depth_pad - depth;

% Pad the image by correct amounts
im_pad = padarray (IM_DATA, padSize, 0, 'post');

% Preallocate some matrices to catch the probability maps
out_Uncertainty_Scores = zeros([size(im_pad,1), size(im_pad,2), size(im_pad,3)],
'double'); % needs to match OutputType in semanticseg below
out_scores_from_network_all_classes=zeros([size(im_pad,1), size(im_pad,2),
size(im_pad,3), 3], 'double');

% Loop through blocks of 'patchSize'
for loop_height = 1:number_of_height_patches
    for loop_width = 1:number_of_width_patches
        for loop_depth = 1:number_of_depth_patches

            start_height_position=(loop_height-1)*patchSize(1)+1;
            end_height_position=loop_height*patchSize(1);

            start_width_position=(loop_width-1)*patchSize(2)+1;
            end_width_position=loop_width*patchSize(2);

            start_depth_position=(loop_depth-1)*patchSize(3)+1;
            end_depth_position=loop_depth*patchSize(3);

            patch_to_deploy=...

im_pad(start_height_position:end_height_position,start_width_position:end_width_
position,start_depth_position:end_depth_position,:);

            % deploy net
            [patch_seg, Scores, allScores] = semanticseg(patch_to_deploy, net,
'OutputType', 'double',...
             'ExecutionEnvironment','auto');


out_Uncertainty_Scores(start_height_position:end_height_position,start_width_pos
ition:end_width_position,start_depth_position:end_depth_position)...
            =Scores;

out_scores_from_network_all_classes(start_height_position:end_height_position,st
art_width_position:end_width_position,start_depth_position:end_depth_position,:)
...
            =allScores;

        end
```

```
    end
end

% Script is general for n classes to this point
out_Pmap_cat1 = out_scores_from_network_all_classes(:,:,:,1);
out_Pmap_cat2 = out_scores_from_network_all_classes(:,:,:,2);
out_Pmap_cat3 = out_scores_from_network_all_classes(:,:,:,3);

% Remove padding from probability maps
out_Uncertainty_Scores = out_Uncertainty_Scores(1:height, 1:width, 1:depth);
out_Pmap_cat1 = out_Pmap_cat1(1:height, 1:width, 1:depth);
out_Pmap_cat2 = out_Pmap_cat2(1:height, 1:width, 1:depth);
out_Pmap_cat3 = out_Pmap_cat3(1:height, 1:width, 1:depth);

%% visualise network outputs
figure(2)
clf(figure(2))
ax1 = subplot(2,3,1);
imshow(out_Pmap_cat1(:,:,60),[])
title('LF-nuclei')
ax2 = subplot(2,3,2) ;
imshow(out_Pmap_cat2(:,:,60),[])
title('LF-actin')
ax3 = subplot(2,3,3);
imshow(out_Pmap_cat3(:,:,60),[])
title('Background/other')
ax4 = subplot(2,3,5);
imshow(out_Uncertainty_Scores(:,:,60),[])
title('Uncertainty')
linkaxes([ax1 ax2 ax3 ax4], 'xy')

%% Map to 16-bit and save for loading into CellProfiler
ui16_PMAP_cat1_xy = uint16(65535*mat2gray(out_Pmap_cat1, [0 1])) ;
ui16_PMAP_cat2_xy = uint16(65535*mat2gray(out_Pmap_cat2, [0 1])) ;
ui16_PMAP_cat3_xy = uint16(65535*mat2gray(out_Pmap_cat3, [0 1])) ;
data_to_save = cat(4,ui16_PMAP_cat1_xy,ui16_PMAP_cat2_xy,ui16_PMAP_cat3_xy);

% Map to 16-bit and save for loading into CellProfiler
for loop = 1:3
    CP_channel_number = num2str(loop +2) ;

    MultiChImgTile = data_to_save(:,:,:,loop) ;

    % spec a fiji description
    fiji_descr = ['ImageJ=1.52p' newline ...
                'images=' num2str(size(MultiChImgTile,3)*...
                                  size(MultiChImgTile,4)*...
                                  size(MultiChImgTile,5)) newline...
                'channels=' num2str(size(MultiChImgTile,4)) newline...
                'slices=' num2str(size(MultiChImgTile,3)) newline...
                'frames=' num2str(size(MultiChImgTile,5)) newline...
                'hyperstack=true' newline...
                'mode=grayscale' newline...
                'loop=false' newline...
                'min=0.0' newline...
                'max=65535.0'];  % bitdepth spec

    %%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
    t = Tiff(['Ch_',CP_channel_number,'_Im_','001','.tif'],'w') ;
    %%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
```

```
    tagstruct.ImageLength = size(MultiChImgTile,1);
    tagstruct.ImageWidth = size(MultiChImgTile,2);
    tagstruct.Photometric = Tiff.Photometric.MinIsBlack;
    tagstruct.BitsPerSample = 16;
    tagstruct.SamplesPerPixel = 1;
    tagstruct.Compression = Tiff.Compression.None; %% lzw is not compatible w
CP4 out-of-box
    tagstruct.PlanarConfiguration = Tiff.PlanarConfiguration.Chunky;
    tagstruct.SampleFormat = Tiff.SampleFormat.UInt;
    tagstruct.ImageDescription = fiji_descr;

        for frame = 1:size(MultiChImgTile,5)
            for slice = 1:size(MultiChImgTile,3)
                for channel = 1:size(MultiChImgTile,4)
                    t.setTag(tagstruct)
                    t.write(im2uint16(MultiChImgTile(:,:,slice,channel,frame)));
                    t.writeDirectory(); % saves a new page in the tiff file
                end
            end
        end
        t.close()
end
```

Windows 10: Running the label-free cell segmentation deep learning scripts on an NVIDIA GPU using Python 3.6 and Tensorflow-gpu 1.9.0

In brief, it is recommended to use these deep learning files with:

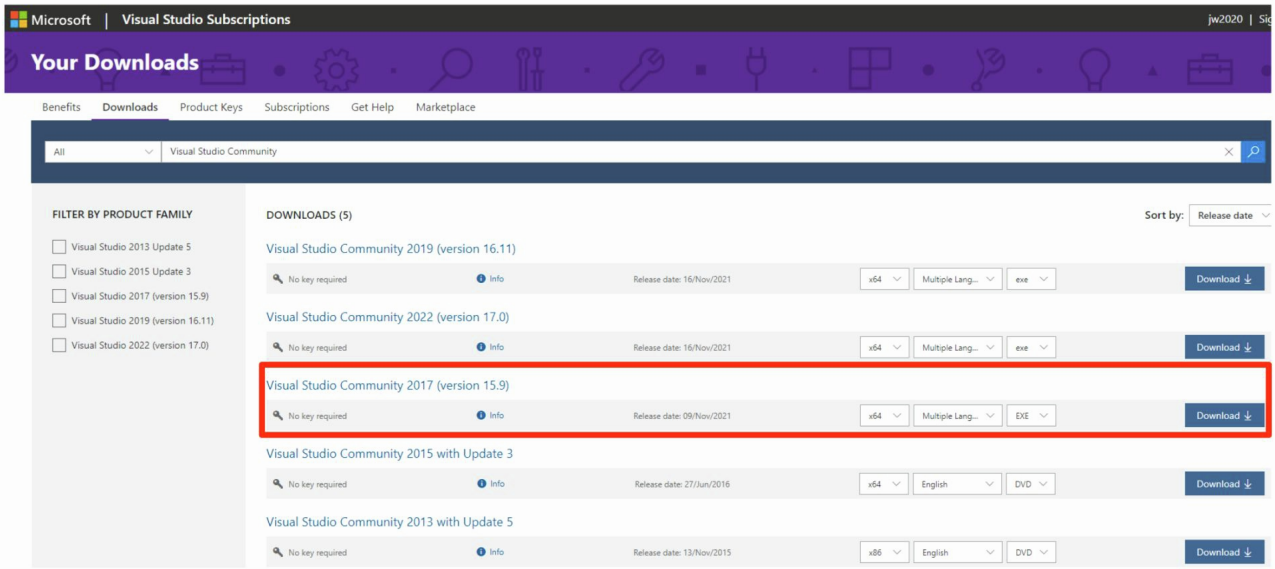
- Python 3.6
- Tensorflow-gpu 1.9.0
- Keras 2.1.5
- Numpy 1.18.1
- Scipy 1.4.1
- Java SE Development Kit 11.0
- Python-bioformats 1.5.2
- CUDA Toolkit 9.0 / cuDNN v7.6.4

Installation Steps:

1. Install Visual Studio Express Community 2017

https://visualstudio.microsoft.com/dev-essentials/#software

- This is necessary to enable the install of the CUDA toolkit.
- At the link above, join Visual Studio Development Essentials (free sign-up).
- Use the search tool to find, download and install Visual Studio Express Community 2017.
- Using the recommended configurations at every step of the installation works fine.



- Restarting your PC after installing Visual Studio is a probably a good idea.

2. Install CUDA Toolkit 9.0 and accompanying patches for Windows 10.

- Tensorflow-GPU 1.9.0 requires CUDA 9.0 - not whatever the latest version of the toolkit is.
- This is available at the Nvidia website "CUDA Toolkit Archive" --> select CUDA Toolkit 9.0

https://developer.nvidia.com/cuda-toolkit-archive

NVIDIA DEVELOPER JOIN

CUDA Toolkit Archive

Home

Previous releases of the CUDA Toolkit, GPU Computing SDK, documentation and developer drivers can be found using the links below. Please select the release you want from the list below, and be sure to check www.nvidia.com/drivers for more recent production drivers appropriate for your hardware configuration.

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CUDA Toolkit 11.5.1 (November 2021), Versioned Online Documentation

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CUDA Toolkit 11.4.2 (September 2021), Versioned Online Documentation
CUDA Toolkit 11.4.1 (August 2021), Versioned Online Documentation
CUDA Toolkit 11.4.0 (June 2021), Versioned Online Documentation
CUDA Toolkit 11.3.1 (May 2021), Versioned Online Documentation
CUDA Toolkit 11.3.0 (April 2021), Versioned Online Documentation
CUDA Toolkit 11.2.2 (March 2021), Versioned Online Documentation
CUDA Toolkit 11.2.1 (Feb 2021), Versioned Online Documentation
CUDA Toolkit 11.2.0 (Dec 2020), Versioned Online Documentation
CUDA Toolkit 11.1.1 (Oct 2020), Versioned Online Documentation
CUDA Toolkit 11.1.0 (Sept 2020), Versioned Online Documentation
CUDA Toolkit 11.0 Update1 (Aug 2020), Versioned Online Documentation
CUDA Toolkit 11.0 (May 2020), Versioned Online Documentation
CUDA Toolkit 10.2 (Nov 2019), Versioned Online Documentation
CUDA Toolkit 10.1 update2 (Aug 2019), Versioned Online Documentation
CUDA Toolkit 10.1 update1 (May 2019), Versioned Online Documentation
CUDA Toolkit 10.1 (Feb 2019), Online Documentation
CUDA Toolkit 10.0 (Sept 2018), Online Documentation
CUDA Toolkit 9.2 (May 2018),Online Documentation
CUDA Toolkit 9.1 (Dec 2017), Online Documentation
CUDA Toolkit 9.0 (Sept 2017), Online Documentation

- Select the target platform as Windows, X86_64, version 10 and the installer type as exe(local)

Select Target Platform

Click on the green buttons that describe your target platform. Only supported platforms will be shown.

Operating System

Windows | Linux | Mac OSX

Architecture

x86_64

Version

10 | 8.1 | 7 | Server 2016 | Server 2012 R2

Installer Type

exe (network) | exe (local)

Download Installers for Windows 10 x86_64

The base installer is available for download below.
There are 4 patches available. These patches require the base installer to be installed first.

Base Installer — Download (1.4 GB)

Installation Instructions:

1. Double click cuda_9.0.176_win10.exe
2. Follow on-screen prompts

Patch 1 (Released Jan 25, 2018) — Download (54.1 MB)

cuBLAS Patch Update: This update to CUDA 9.0 includes new GEMM kernels optimized for the Volta architecture and improved heuristics to select GEMM kernels for given input sizes.

Patch 2 (Released Mar 5, 2018) — Download (54.7 MB)

cuBLAS Patch Update: This update to CUDA 9 includes GEMM heuristics improvements to selects the most optimized algorithms for input sizes commonly used in Deep Learning RNNs. The update also includes other bug-fixes and performance enhancements.

Patch 3 (Released Jun 7, 2018) — Download (82.3 MB)

cuBLAS Patch Update: This update to cuBLAS addresses issues with Convolutional Seq2Seq and RNN inference performance.

Patch 4 (Released Aug 6, 2018) — Download (56.2 MB)

cuBLAS Patch Update: This update to cuBLAS includes optimized implementations of GEMV operations for mixed precision input and output types and important fixes to address performance issues.

The checksums for the installer and patches can be found in Installer Checksums.
For further information, see the Installation Guide for Microsoft Windows and the CUDA Quick Start Guide.

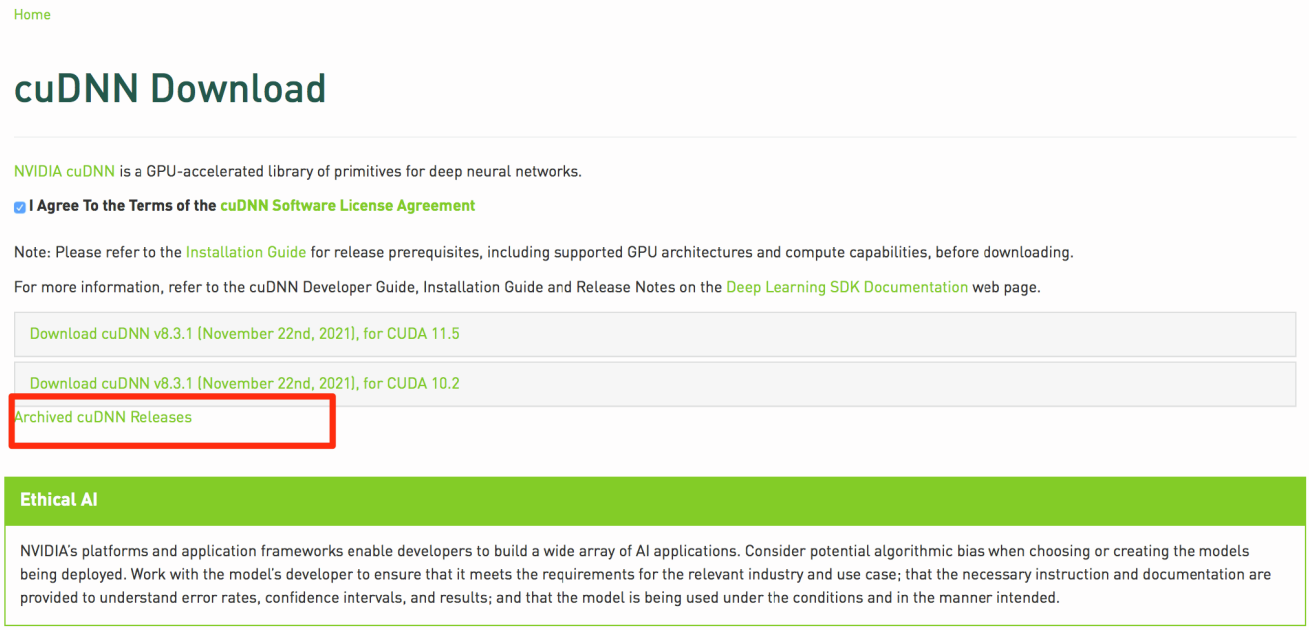
- First install the “base installer” following the standard, “express configurations” options.

- Then install Patches 1-4 sequentially in order by just following the on-screen prompts after each download.

3. Install cuDNN v7.6.4 for CUDA 9.0

- To download and install cuDNN, first join the NVIDIA Developer Program – which is free. https://developer.nvidia.com/cudnn-download-survey

- Once signed in, proceed the cuDNN download page and click archived cuDNN releases: https://developer.nvidia.com/rdp/cudnn-archive



- Scroll down to the option to download cuDNN v7.6.4 for CUDA 9.0 and download the library for Windows 10.

- Unzip the downloaded cuDNN .zip file.

- Inside are three files which need to be copied to the correct folder subdirectories of your Window 10 installation of the CUDA 9.0 toolkit:

- These files are **cudnn64_7.dll, cudnn.h** and **cudnn.lib**:

1. cudnn64_7.dll
Copy the file from the unzipped cuDNN download folder at e.g.,
<unzipped_download>/cudnn-9.0-windows10-x64-v7.6.2.24\cuda\bin\cudnn64_7.dll

and paste it into the NVIDIA GPU computing toolkit v9.0 subdirectory 'bin' located at e.g.,
C:\Program Files\NVIDIA GPU Computing Toolkit\CUDA\v9.0\bin\

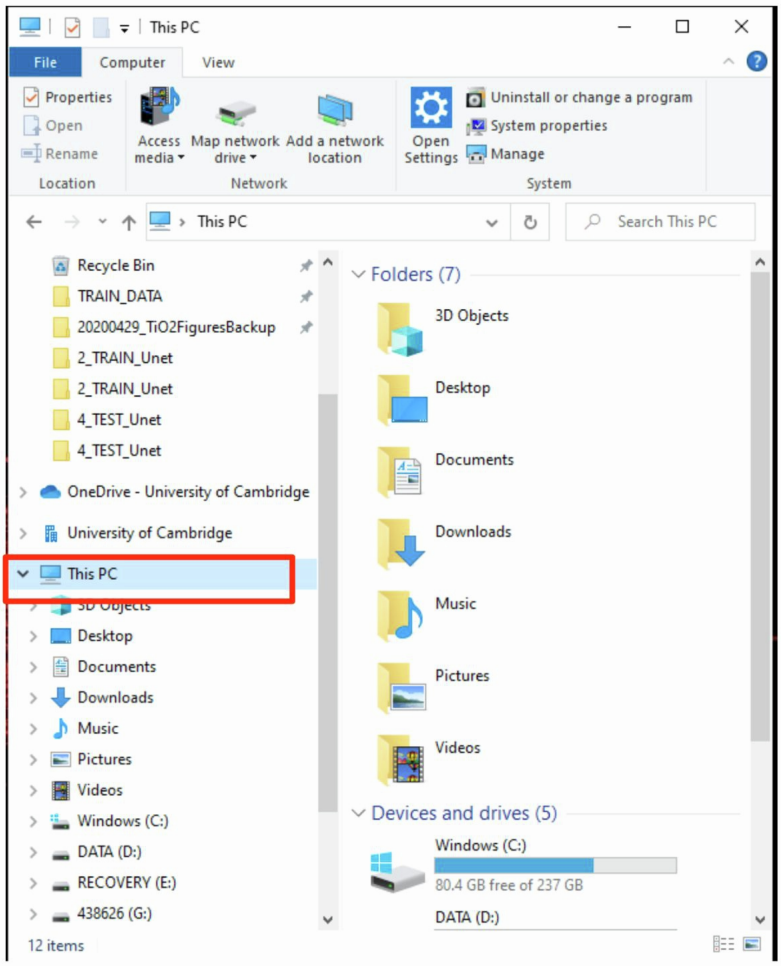
2. cudnn.h
Copy the file from the unzipped cuDNN download folder at e.g.,
<unzipped_download>/cudnn-9.0-windows10-x64-v7.6.2.24\cuda\ include\cudnn.h

and paste it into the NVIDIA GPU computing toolkit v9.0 subdirectory 'include' located at e.g.,
C:\Program Files\NVIDIA GPU Computing Toolkit\CUDA\v9.0\include\

3. cudnn.lib
Copy the file from the unzipped cuDNN download folder at e.g.,
<unzipped_download>/cudnn-9.0-windows10-x64-v7.6.2.24\cuda\lib\x64\cudnn.lib

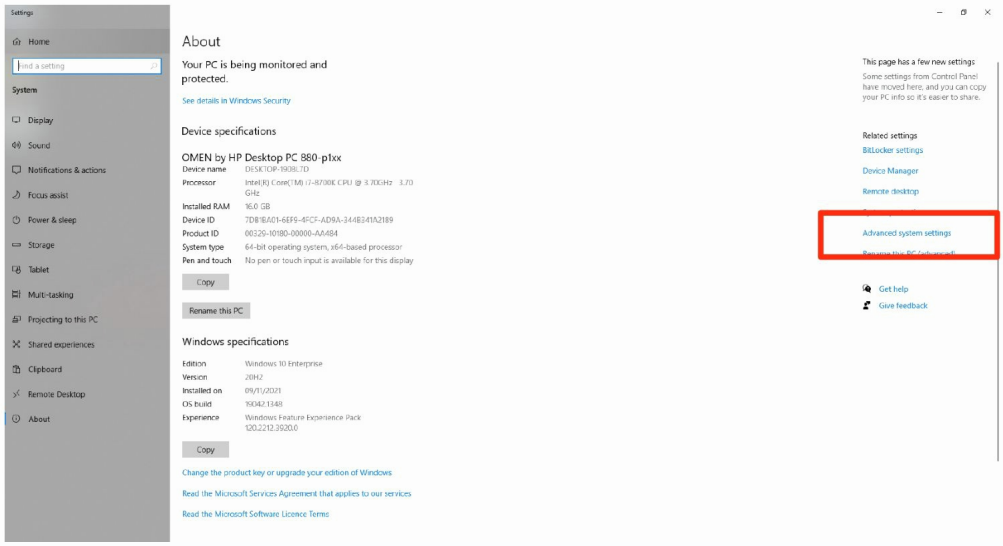
and paste it into the NVIDIA GPU computing toolkit v9.0 subdirectory 'include' located at e.g.,
C:\Program Files\NVIDIA GPU Computing Toolkit\CUDA\v9.0\lib\x64\

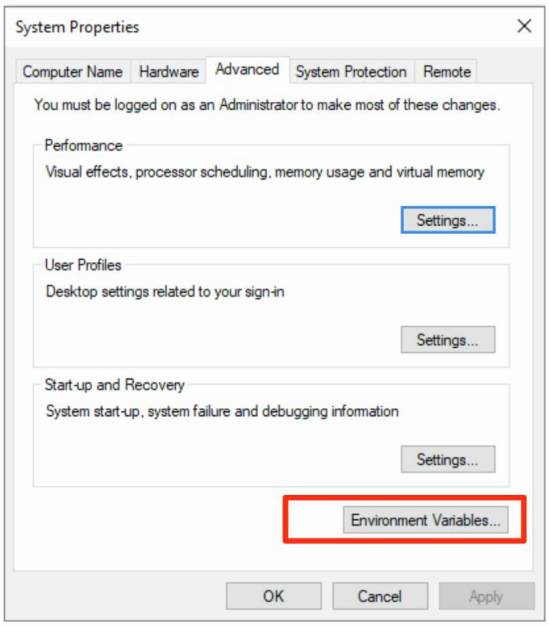
4. Check that the CUDA environment variables are set in Windows 10



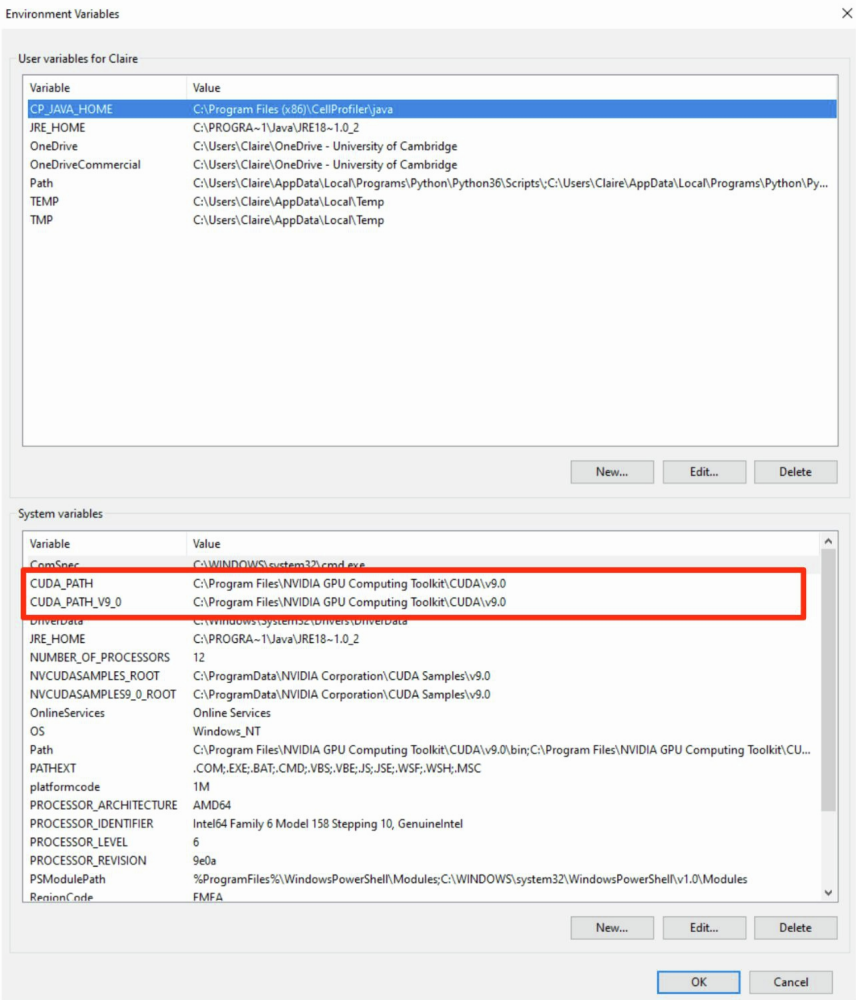
- From File Explorer, right-click on “This PC” on the left-side and select “Properties”

- On the right-side, of the dialogue that opens, click “Advanced system settings”





- In the “System Properties” dialogue box, click “Environment Variables” at the bottom



- Make sure in the bottom window (labelled "System Variables") that variables named CUDA_PATH and CUDA PATH V9.0 exist and point to the correct locations e.g.,

C:\Program Files\NVIDIA GPU Computing Toolkit\CUDA\v9.0\bin

and
C:\Program Files\NVIDIA GPU Computing Toolkit\CUDA\v9.0\libnvvp

respectively.

- These paths should be auto-installed. If they are missing on your system, they can be added by clicking the "New" button below the system variables bottom panel and entering the name and path into the dialogue box for your system.

5. Install Python 3.6.8

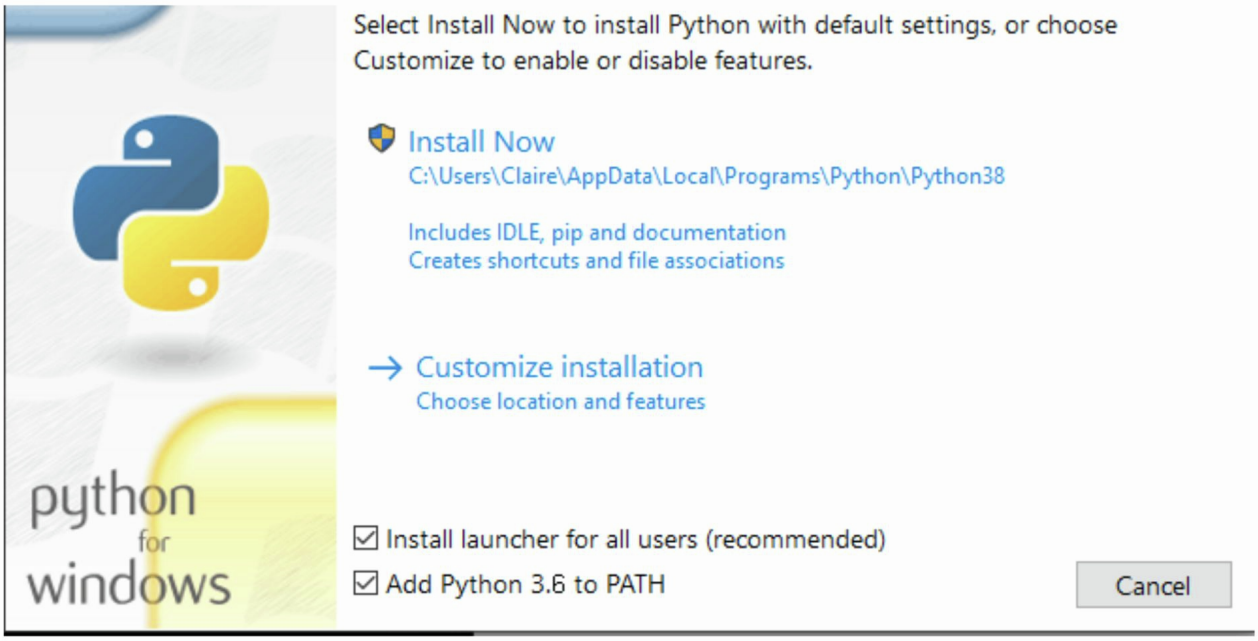
- To install Python version 3.6.8 navigate to the previous release at:
https://www.python.org/downloads/release/python-368/

Files

Version	Operating System	Description	MD5 Sum	File Size	GPG
Gzipped source tarball	Source release		48f393a04c2e66c77bfc114e589ec630	23010188	SIG
XZ compressed source tarball	Source release		51aac91bdf8be95ec0a62d174890821a	17212420	SIG
macOS 64-bit/32-bit installer	macOS	for Mac OS X 10.6 and later	eb1a23d762946329c2aa3448d256d421	33258809	SIG
macOS 64-bit installer	macOS	for OS X 10.9 and later	786c4d9183c754f58751d52f509bc971	27073838	SIG
Windows help file	Windows		0b04278f5bdb8ee85ae5ae66af0430b2	7868305	SIG
Windows x86-64 embeddable zip file	Windows	for AMD64/EM64T/x64	73df7cb2f1500ff36d7dbeeac3968711	7276004	SIG
Windows x86-64 executable installer	Windows	for AMD64/EM64T/x64	72f37686b7ab240ef70fdb931bdf3cb5	31830944	SIG
Windows x86-64 web-based installer	Windows	for AMD64/EM64T/x64	39dde5f535c16d642e84fc7a69f43e05	1331744	SIG
Windows x86 embeddable zip file	Windows		60470b4cceba52094121d43cd3f6ce3a	6560373	SIG
Windows x86 executable installer	Windows		9c7b1ebdd3a8df0eebfda2f107f1742c	30807656	SIG
Windows x86 web-based installer	Windows		80de96338691698e10a935ecd0bdaacb	1296064	SIG

- Scroll to the bottom of the page and download the "Windows x86-64 executable installer" with description "AMD64/EM64T/x64".

- Once downloaded, follow the on-screen prompts to install Python 3.6.8.

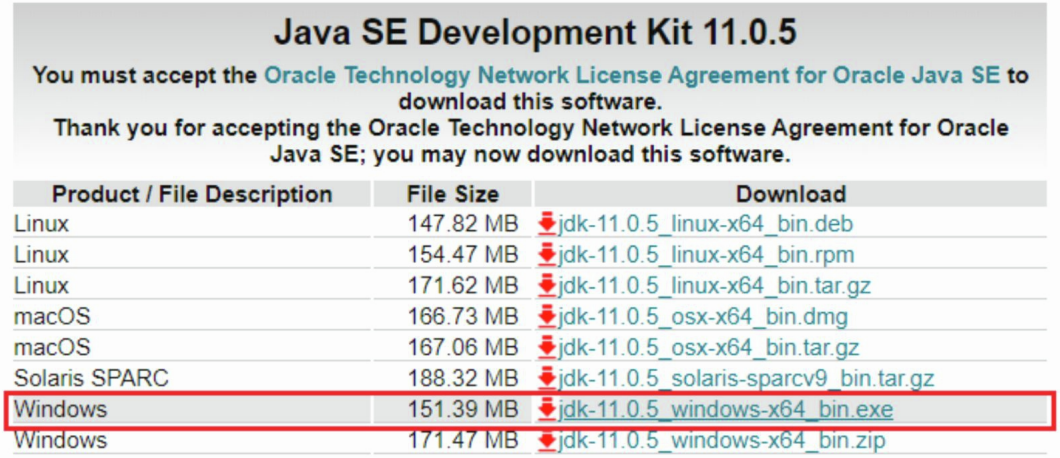


- Check the box to add Python to the Windows path.

6. Install Java Development Kit 11

- This is used by Python-bioformats to enable read/write of image-data.
- Download and install Java Development kit 11

https://www.oracle.com/java/technologies/downloads/#java11

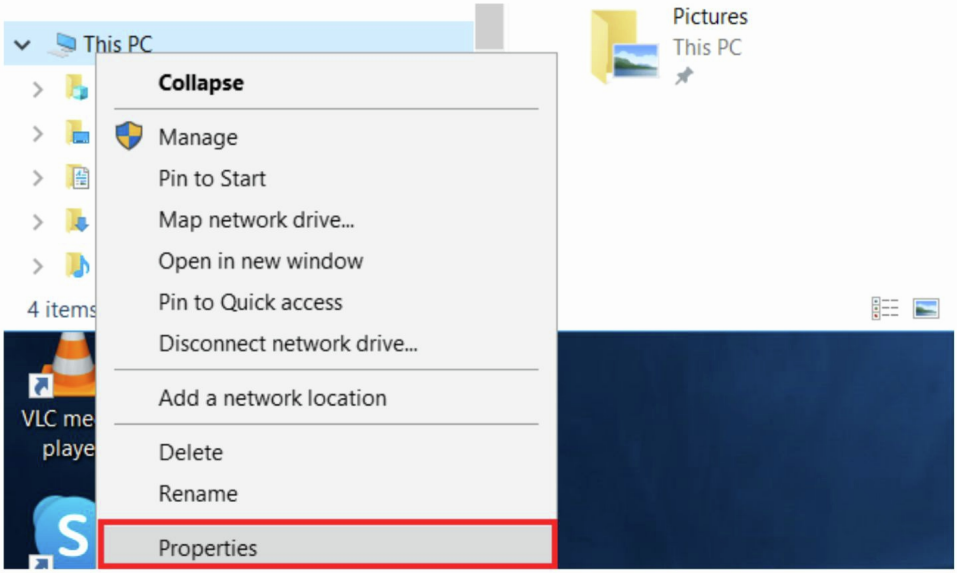


Java SE Development Kit 11.0.5

You must accept the Oracle Technology Network License Agreement for Oracle Java SE to download this software.
Thank you for accepting the Oracle Technology Network License Agreement for Oracle Java SE; you may now download this software.

Product / File Description	File Size	Download
Linux	147.82 MB	jdk-11.0.5_linux-x64_bin.deb
Linux	154.47 MB	jdk-11.0.5_linux-x64_bin.rpm
Linux	171.62 MB	jdk-11.0.5_linux-x64_bin.tar.gz
macOS	166.73 MB	jdk-11.0.5_osx-x64_bin.dmg
macOS	167.06 MB	jdk-11.0.5_osx-x64_bin.tar.gz
Solaris SPARC	188.32 MB	jdk-11.0.5_solaris-sparcv9_bin.tar.gz
Windows	151.39 MB	jdk-11.0.5_windows-x64_bin.exe
Windows	171.47 MB	jdk-11.0.5_windows-x64_bin.zip

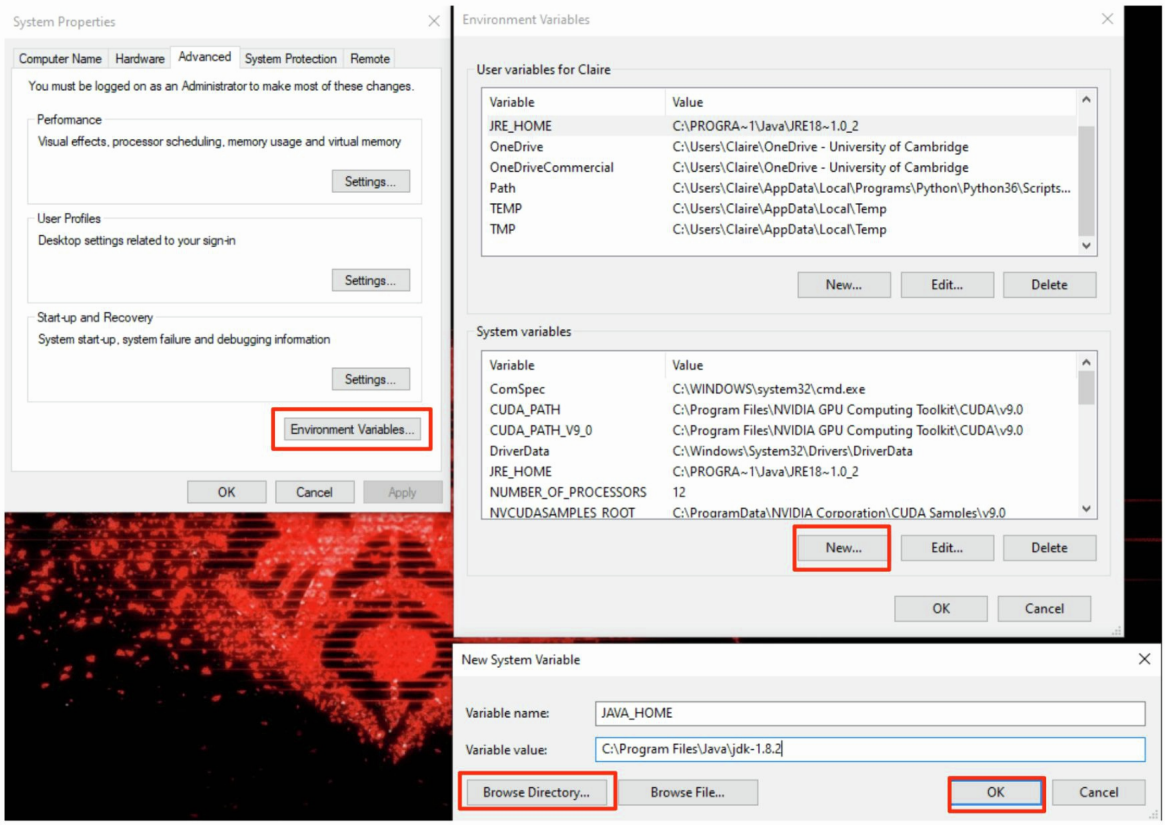
- Once Java SE Development Kit 11 is installed, set the **JAVA_HOME** environment variable and add the Java development kit to the Windows path. To do this, as above, open a File Explorer window, right-click on the '**This PC**' option and select '**Properties**' from the drop-down menu.



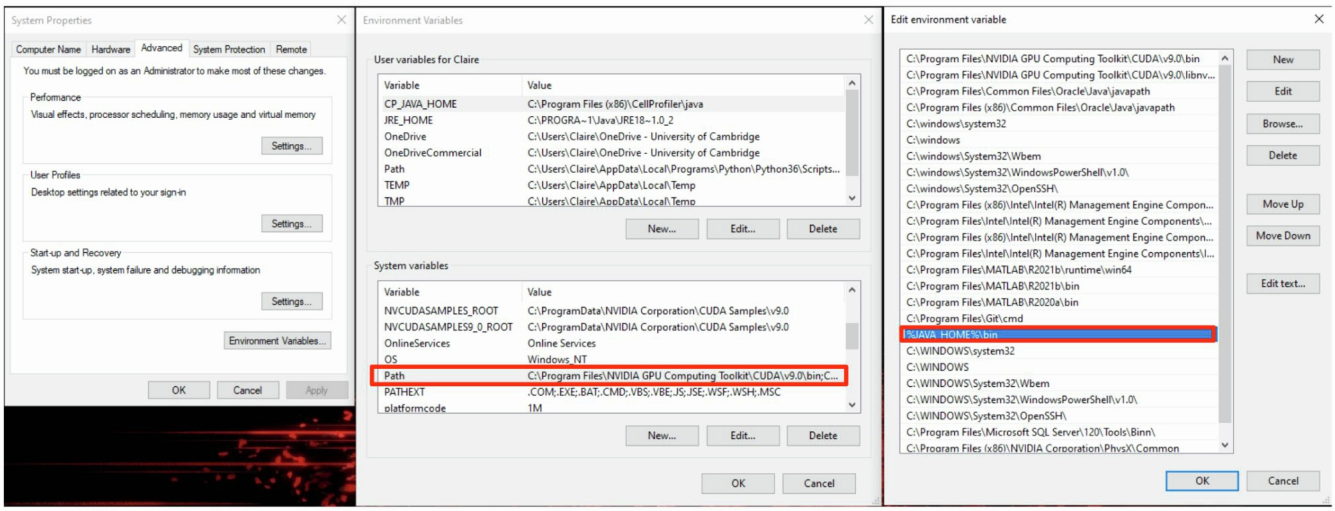
The control panel will pop up as a separate window. Select '**Advanced system settings'** from the list appearing at the right of the window.

In the 'system properties' dialogue that opens, select '**Environment Variables**'. This will cause the environment variables window to appear. To set the 'JAVA_HOME' variable, Click the '**New**' button option at the bottom of this window in the 'System variables' section.

Name the new variable 'JAVA_HOME' and use the '**Browse Directory**' option to specify the path to JDK 11. Select '**OK**' to create this new variable:



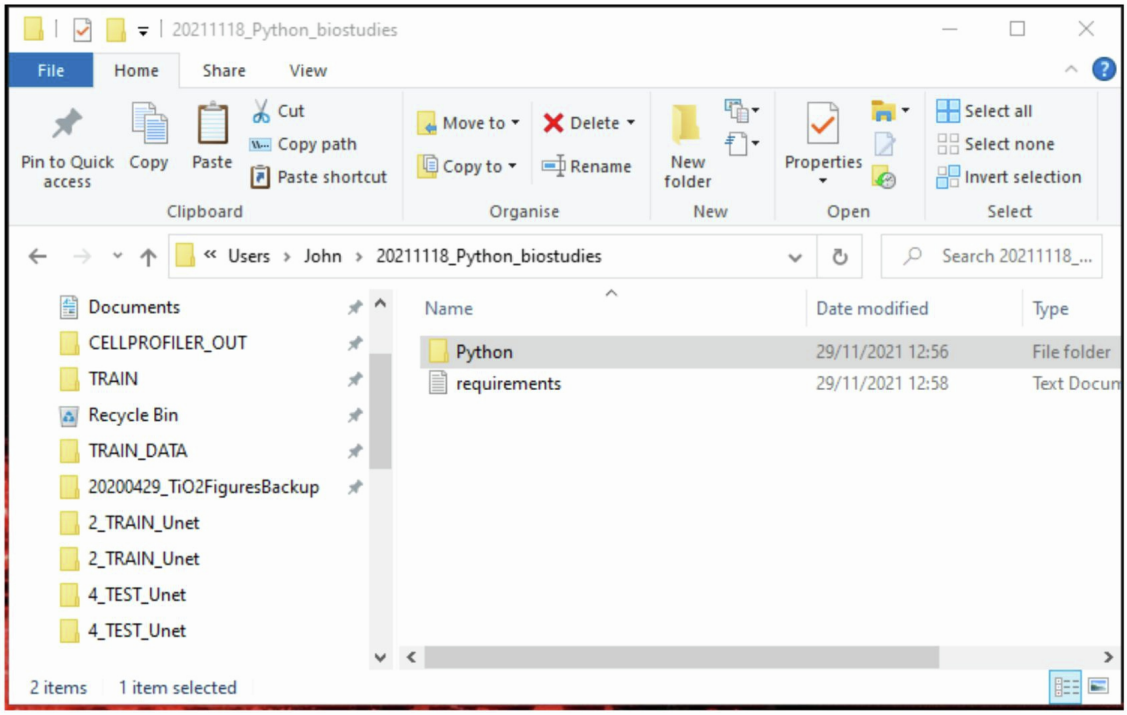
To add JDK11 to the Windows path, Click on the exisiting 'Path' system variable and click the '**Edit**' button. A new window will appear. Click new and type '**%JAVA_HOME%\bin**'.



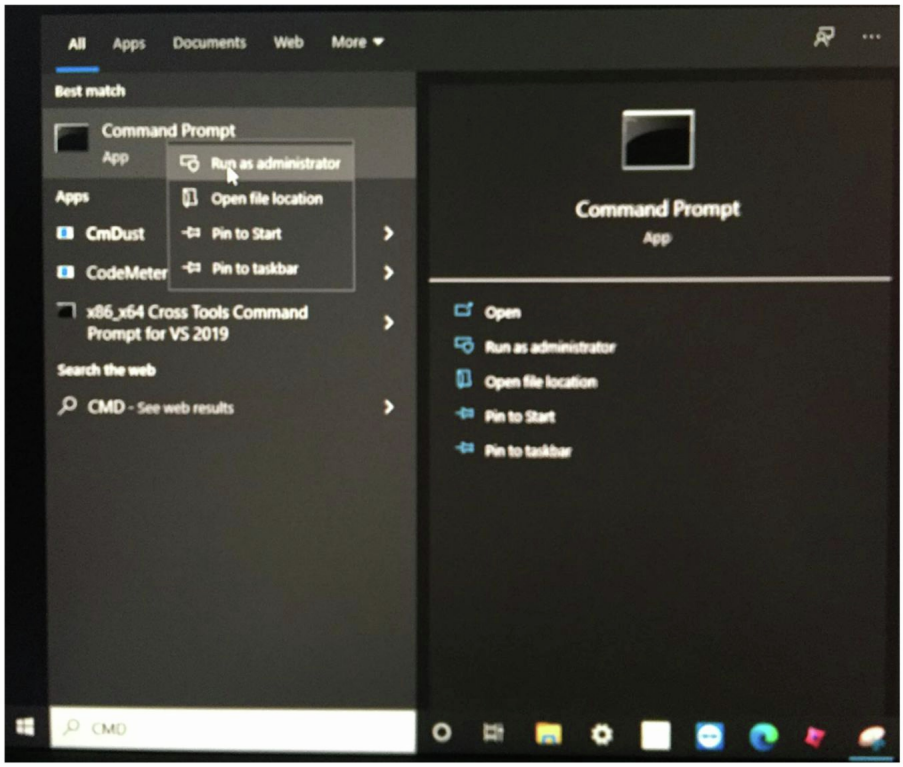
- Click '**OK**' to apply changes and close all opened windows.

7. Setup a virtual environment and install the dependencies necessary to run the code

- Download and unzip the "Python_biostudies" folder from the BioStudies project archive to a suitable location on your computer.



- Run command prompt as administrator:
- To do this, Search "CMD", right-click on "Command Prompt" from the search results dialogue and click "Run as administrator":



- Using the ‘cd’ command, change the current directory to the unzipped python scripts folder downloaded from Biostudies e.g.,

```
Administrator: Command Prompt
Microsoft Windows [Version 10.0.19042.1348]
(c) Microsoft Corporation. All rights reserved.

C:\WINDOWS\system32>cd C:\Users\John\20211118_Python_biostudies
```

- Make a new subdirectory ‘venv’ and initialise an instance of Python inside it e.g.,

```
C:\Users\John\20211118_Python_biostudies>python -m venv ./venv
```

- Activate the newly-created virtual environment e.g.,

```
C:\Users\John\20211118_Python_biostudies>venv\Scripts\activate.bat
```

- Update pip to the latest version (N.B., this is critical to the successful install of javabridge with Python 3.6)

```
(venv) C:\Users\John\20211118_Python_biostudies>python -m pip install --upgrade pip
```

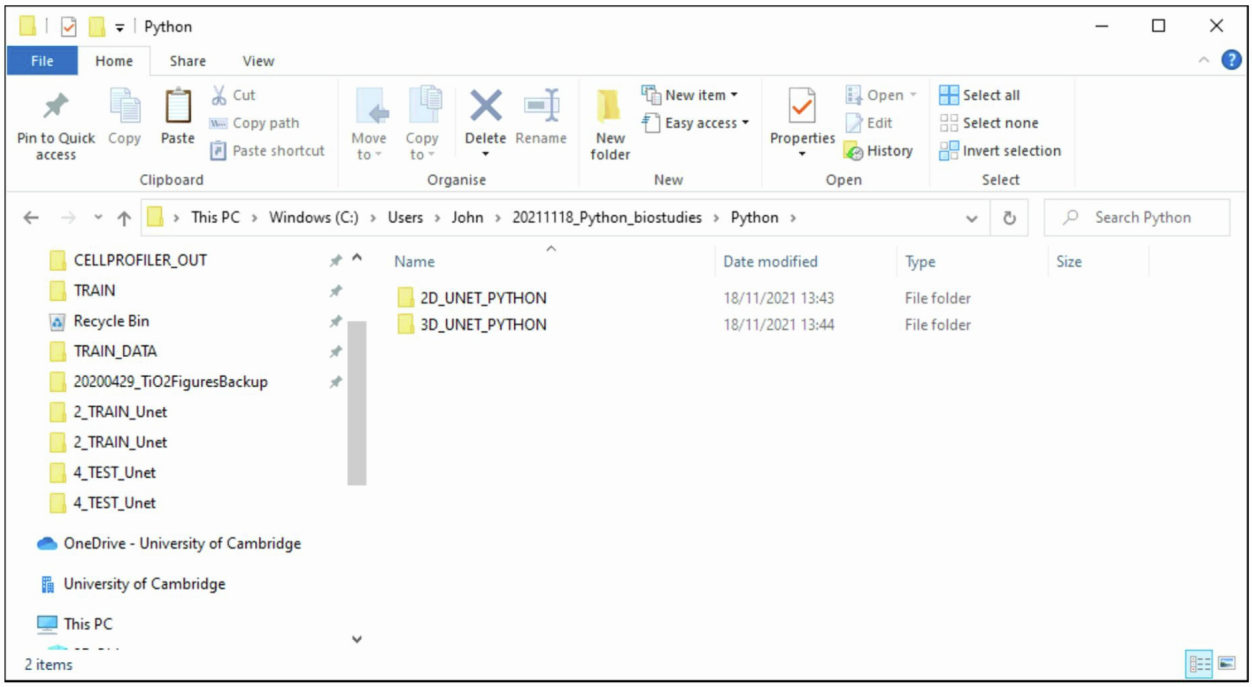
- Using the “requirements.txt” file included in the BioStudies download, install the required dependencies and the Spyder 4.0 Integrated Development Environment (IDE) to the newly-created virtual environment e.g.,

```
(venv) C:\Users\John\20211118_Python_biostudies>pip install -r requirements.txt
```

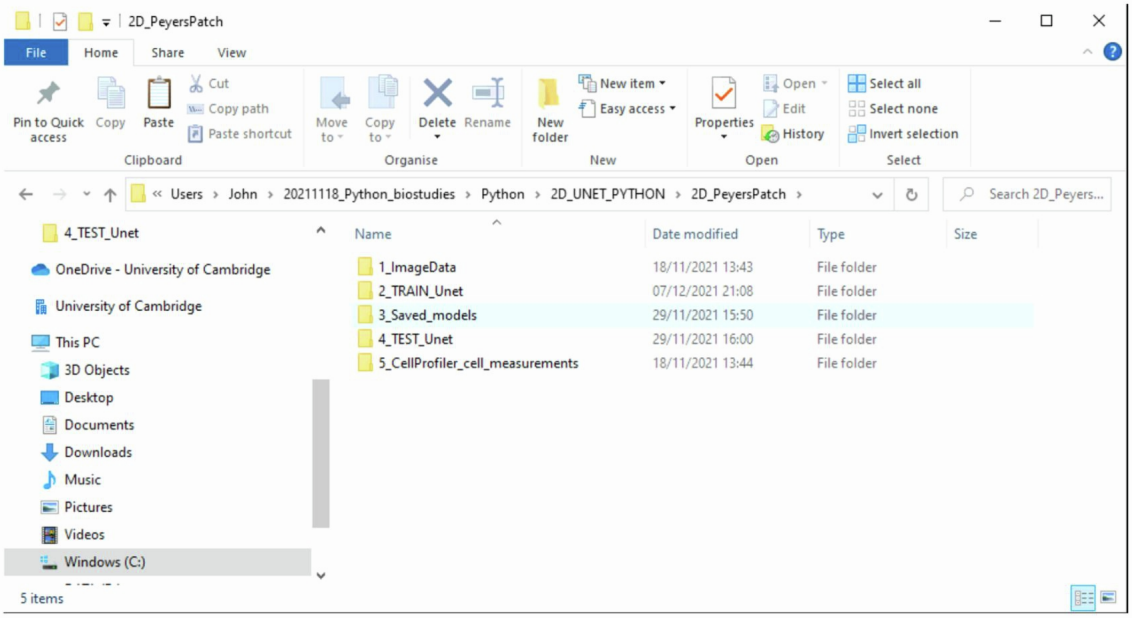
8. Training a 2-D UNET model

- A screencast video is included with the BioStudies project archive.

- Download and unzip the Python BioStudies project archive at a suitable location on your computer.



- Inside the 2D_UNET_Python folder, there is a sequentially-organised workflow starting with raw data and ending with a CellProfiler pipeline that obtains cell features from the label-free cell segmentation:



- In command prompt with the virtual environment active, type 'Spyder' to start the Spyder4 IDE.

```
(venv) C:\Users\John\20211118_Python_biostudies>spyder
```

- To train a 2-D UNET model, set the Spyder working directory to the “2_TRAIN_Unet” directory. Open the “A_TRAIN_UNET” script.

- Update the path to the training data (located in the “1_ImageData” folder).
- Update the path to the pixel classification labels (located in the “1_ImageData” folder)

```
# Read in the reflectance data for training
Training_data_holder = skimage.io.imread('C:/Users/John/20211118_Python_biostudies/Python/2D_UNET_PYTHON/2D_PeyersPatch/1_ImageData/TRAIN_IMAGE.tif')
Training_data = Training_data_holder[0,0,:,:,2]

#Read in the mask to isolated just the lymphoid tissue
mask = Training_data_holder[0,0,:,:,3]
thresh = threshold_otsu(Training_data)
mask = mask > thresh
mask = mask*1
mask = np.uint16(mask)

# Apply the mask to the training data
Training_data = np.multiply(Training_data,mask);
Training_data = np.double(Training_data);

# Rescale the training data in the interval [0 1]
Training_data_norm_holder = np.zeros((8551, 5701),np.double)
Training_data_norm = cv2.normalize(Training_data, Training_data_norm_holder , 1.0, 0.0, cv2.NORM_MINMAX)

#Read in the pixel-class labels created from the nuclei and actin staining
Training_labels_holder = skimage.io.imread('C:/Users/John/20211118_Python_biostudies/Python/2D_UNET_PYTHON/2D_PeyersPatch/1_ImageData/TRAIN_LABELS.png');
Training_labels = np.where(Training_labels_holder == 3, 0, Training_labels_holder)
```

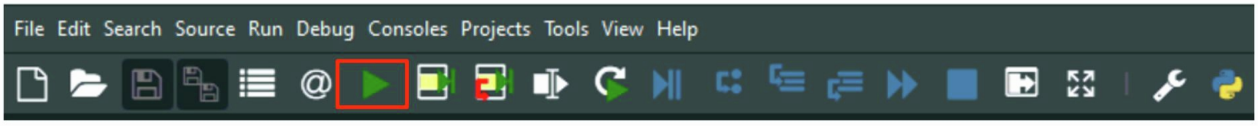
- Update the path saving the training data and pixel classification labels in numpy format.
- This should be inside the”2_TRAIN_Unet” directory.

```
#%% Once happy with labels and data, commit the reflectance information data to sub-directory in .npy format

MYDIR = C:/Users/John/20211118_Python_biostudies/Python/2D_UNET_PYTHON/2D_PeyersPatch/2_TRAIN_Unet/npy_training_data/'
CHECK_FOLDER = os.path.isdir(MYDIR)

# If folder doesn't exist, then create it.
if not CHECK_FOLDER:
    os.makedirs(MYDIR)

np.save(os.path.join(MYDIR, 'Training_data'), Training_data_norm)
```

- The script will save the model along with checkpoint values taken during training. You should specify where these files are to be saved. This should be folder 3 of the workflow: (“3_Saved_models”).

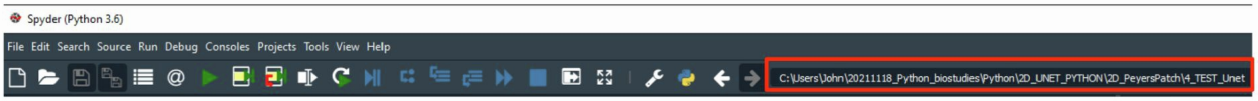
```
#%% Define path to safe network, time stamp network
checkpoint_filepath = 'C:/Users/John/20211118_Python_biostudies/Python/2D_UNET_PYTHON/2D_PeyersPatch/3_Saved_models/'
now = datetime.now()
dt_string = now.strftime("%d/%m/%Y%H:%M:%S")
NAME =dt_string
Name_file = 'PeyersPatchBiostudies_'
checkpoint_filepath_name = checkpoint_filepath + Name_file+dt_string


checkpoint_dir =  os.path.dirname(checkpoint_filepath_name)

#Specify that weights are to be saved
model_checkpoint_callback = tensorflow.keras.callbacks.ModelCheckpoint(
    filepath=checkpoint_dir,
    save_weights_only=True,
    monitor='val_accuracy',
    mode='max',
    save_best_only=True)

# Specify training options
BATCH_SIZE = 12
NUM_EPOCHS = 50

#Augment training data
train_generator = get_train_augmented(trainX=trainX, trainY=trainY, BATCH_SIZE=BATCH_SIZE)

#Train and save the network
history = model.fit_generator(train_generator, steps_per_epoch=len(trainX)/(BATCH_SIZE*2), epochs=NUM_EPOCHS, callbacks=[model_checkpoint_callback])

#Plot progress # this plots over the view of the data
plt.figure(2)
plt.plot(history.history['sparse_categorical_accuracy'])
plt.title('Model accuracy')
plt.ylabel('accuracy')
plt.xlabel('epoch')
plt.legend(['train', 'test'], loc='upper left')
plt.savefig('Training_plot.png')
plt.show()

#Save final fully trained model as a .h5 file
model.save("C:/Users/John/20211118_Python_biostudies/Python/2D_UNET_PYTHON/2D_PeyersPatch/3_Saved_models/my_h5_model.h5")
checkpoint_filepath_name_h5 = checkpoint_filepath + Name_file+dt_string+'.h5'
model.save(checkpoint_filepath_name_h5)
```

- To start model training, click the green arrow button at the top of the Spyder dialogue or type the script name at the command line.



- Model training takes several hours (~ 4h on a NVIDIA GTX 1080 Ti GPU)
- The newly-trained model will be saved in the "3_Saved_models" directory.

9. Testing a pretrained 2-D model using unseen data
- A screencast video is included with the BioStudies project archive.
- Change the Spyder working directory to the "4_Test_Unet" directory. Open the "A_TEST_UNET" script.



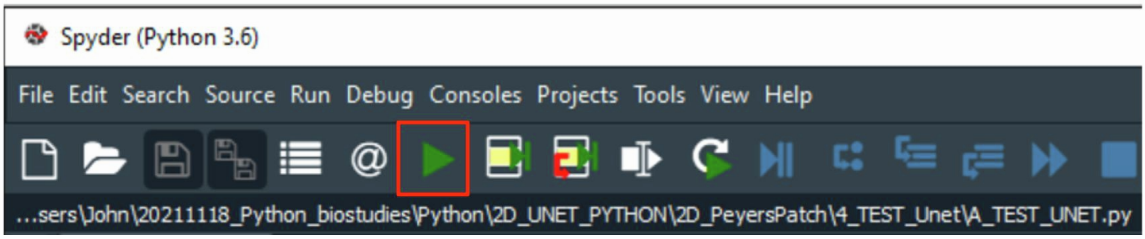
- Change the path to the unseen test image (located in the "1_ImageData" folder).

```
#%% Load the reflectance data from the unseen test image
TEST_DATA = skimage.io.imread('C:/Users/John/20211118_Python_biostudies/Python/2D_UNET_PYTHON/2D_PeyersPatch/1_ImageData/TEST_IMAGE.tif');
Test_data = TEST_DATA[0,0,:,:,2]

# Load the mask for the lymphoid tissue region
Mask = TEST_DATA[0,0,:,:,3]
thresh = threshold_otsu(Test_data)
Mask = Mask > thresh
Mask = Mask*1
Mask = np.uint16(Mask)


# Rescale the test data [0 1]
# Mask the rescaled data
Test_data = np.multiply(Test_data,Mask);
Test_data = np.double(Test_data);
Test_data_norm_holder = np.zeros((11247, 7610),np.double)
Test_data_rescaled = cv2.normalize(Test_data, Test_data_norm_holder , 1.0, 0.0, cv2.NORM_MINMAX)
```

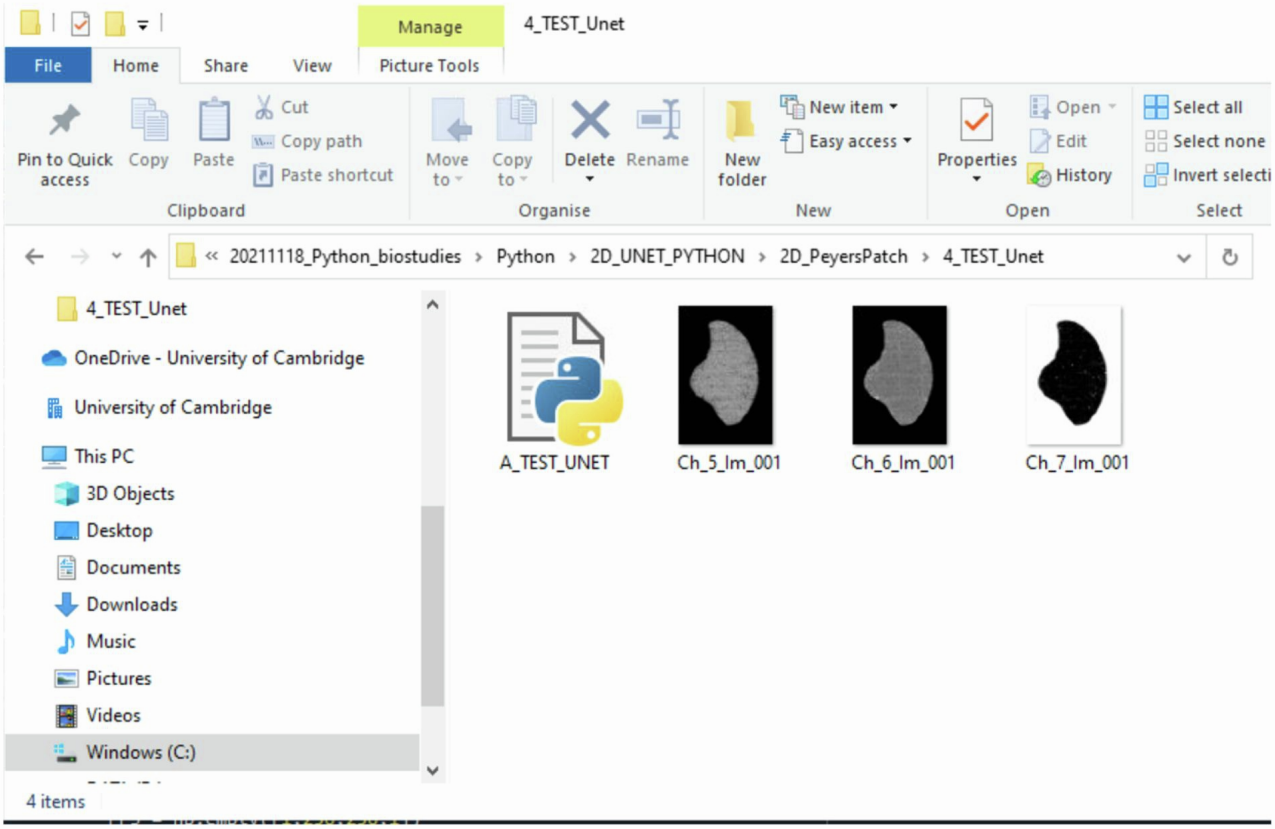
- Specify which pretrained network to use (pretrained networks are located in the "3_Saved_models" folder

```
# Load a pretrained network
pretrained_model = unet()
pretrained_model.load_weights('C:/Users/John/20211118_Python_biostudies/Python/2D_UNET_PYTHON/2D_PeyersPatch/3_Saved_models/my_h5_model_fully_trained.h5')
```

- Clicking the green arrow button or type the file name at the command line to process the unseen reflectance data with the selected pretrained network:



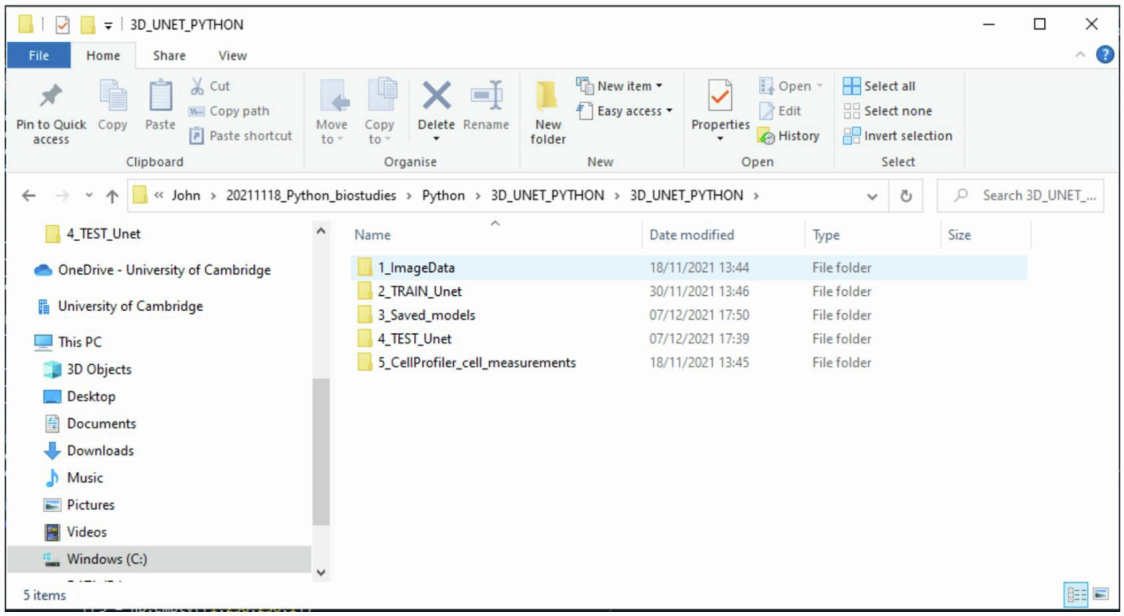
- Probability maps for the LF-nuclei, LF-actin and Background/Other classes will be saved in the working directory named ready for input into the CellProfiler pipeline (filenames Ch_5_lm_001, Ch_6_lm_001, Ch_7_lm_001, respectively).



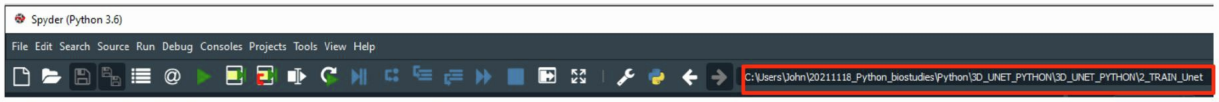
10. Training a 3-D UNET Model

- A screencast video is included with the BioStudies project archive.

- Inside the 3D_UNET_Python folder, there is a sequentially-organised workflow starting with raw data and ending with a CellProfiler pipeline that obtains 3-D cell features from the label-free cell segmentation:



- To train a 3-D UNET model, open Spyder and set the working directory to the “2_TRAIN_Unet” directory. Open the “A_TRAIN_UNET_3D” script.



- Specify the path to the training data (located in the “1_ImageData/TRAIN/DATA” folder).
- Specify the channel number in the training data that contains the reflectance information (here ‘3’).
- Specify the location of the pixel-classification training labels (located at “1_ImageData/TRAIN/LABELS” folder).
- Specify the directory where the training data should be saved in Numpy format.

```
#%% Specify directory containing training data
Training_data_directory = 'C:/Users/John/20211118_Python_biostudies/Python/3D_UNET_PYTHON/3D_UNET_PYTHON/1_ImageData/TRAIN/DATA/'

# Specify channel number in training data that contains reflectance information
RL_training_directory = 3;

# Specify directory containing training labels
Training_labels_path = 'C:/Users/John/20211118_Python_biostudies/Python/3D_UNET_PYTHON/3D_UNET_PYTHON/1_ImageData/TRAIN/LABELS/' ;


# COMMIT TRAINING DATA TO DIRECTORY rescaled [0 1] IN MAT FORMAT
MYDIR ='C:/Users/John/20211118_Python_biostudies/Python/3D_UNET_PYTHON/3D_UNET_PYTHON/1_ImageData/TRAIN/LABELS/mat_training_data/'
CHECK_FOLDER = os.path.isdir(MYDIR)

# If folder doesn't exist, then create it.
if not CHECK_FOLDER:
    os.makedirs(MYDIR)
```

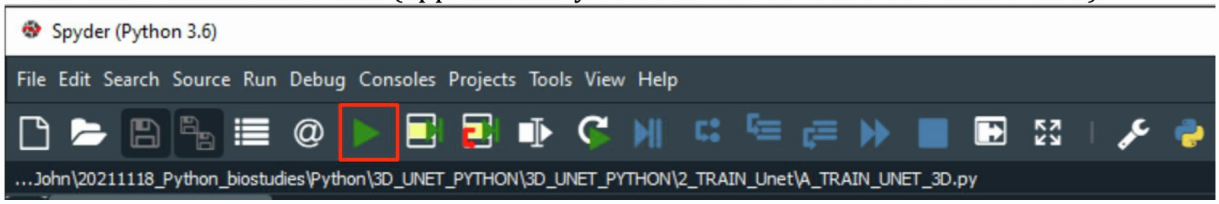
- Specify the checkpoint path during model training:

```
#%% Specify location to save the network
checkpoint_filepath = 'C:/Users/John/20211118_Python_biostudies/Python/3D_UNET_PYTHON/3D_UNET_PYTHON/3_Saved_models/'
now = datetime.now()
dt_string = now.strftime("%d/%m/%Y%H:%M:%S")
NAME =dt_string
Name_file = 'PeyersPatchBiostudies_'
checkpoint_filepath_name = checkpoint_filepath + Name_file+dt_string
```

- Specify the location to save the model when training completes. This should be the “3_Saved_models” directory.

```
#%% Train the model
history = model.fit(
    storeX,
    storeY,
    batch_size = BATCH_SIZE,
    epochs = NUM_EPOCHS,callbacks=[model_checkpoint_callback])

plt.plot(history.history['sparse_categorical_accuracy'])
plt.title('Model accuracy')
plt.ylabel('accuracy')
plt.xlabel('epoch')
plt.legend(['train', 'test'], loc='upper left')
plt.savefig('Training_plot.png')
plt.show()

model.save("C:/Users/John/20211118_Python_biostudies/Python/3D_UNET_PYTHON/3D_UNET_PYTHON/3_Saved_models/my_h53D_model.h5")
```

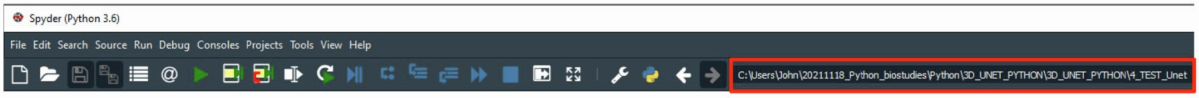
- Click “Run” to commence model training.
- This takes considerable time (approximately 6h on a NVIDIA GTX 1080 Ti GPU card).



11. Testing a pretrained 3-D UNET Model using unseen data

- A screencast video is included with the BioStudies project archive.

- Change the Python working directory to the "4_Test_Unet" directory. Open the "A_TEST_UNET_3D" script.



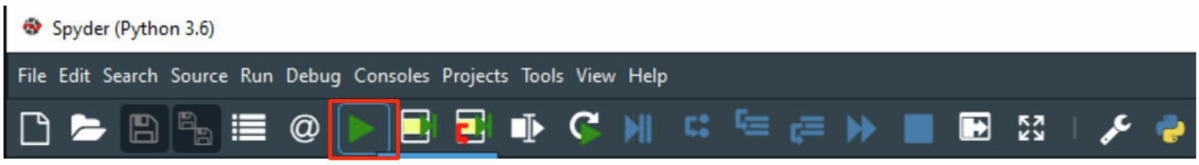
- Specify the location of the unseen test image-data. This is located inside the "1_ImageData" folder at "1_ImageData/TEST/".
- Specify the channel containing the reflectance information (here, '2').
- Specify where the unseen data should be stored upon conversion to Numpy format. This should be inside the "4_Test_Unet" folder.

```
#%% Specify directory containing test data
Test_data_directory = 'C:/Users/John/20211118_Python_biostudies/Python/3D_UNET_PYTHON/3D_UNET_PYTHON/1_ImageData/TEST/'

# Specify channel number in test data that contains reflectance information
RL_channel_number = 2;

#COMMIT REFLECTANCE TEST DATA TO DIRECTORY RESCALED [0 1] IN MAT FORMAT
MYDIR ='C:/Users/John/20211118_Python_biostudies/Python/3D_UNET_PYTHON/3D_UNET_PYTHON/4_TEST_Unet/npy_test_data/'
CHECK_FOLDER = os.path.isdir(MYDIR)
```

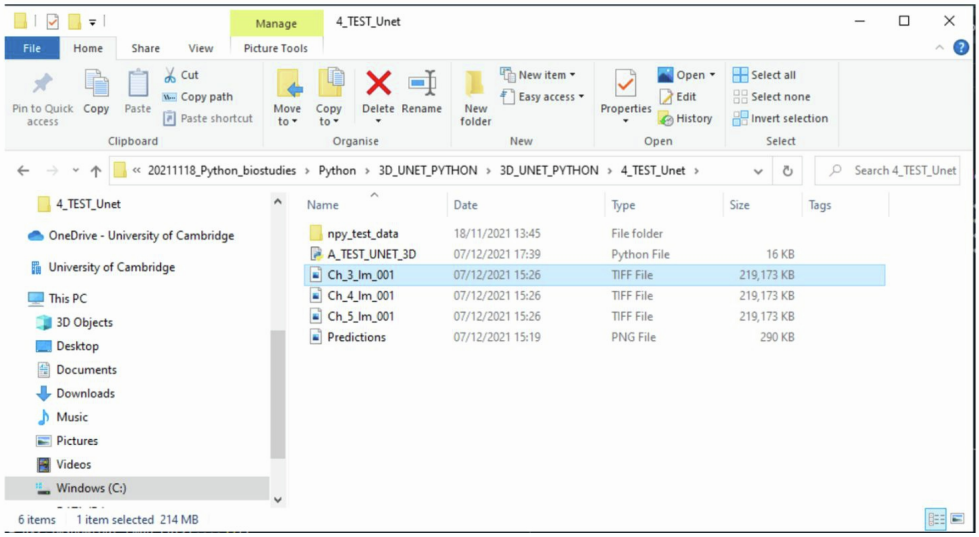
- Specify a pretrained 3-D Unet model from the "3_Saved_models" directory.

```
#%% load the trained 3D Unet network
#model.load_weights("C:/Users/John/20211118_Python_biostudies/Python/3D_UNET_PYTHON/3D_UNET_PYTHON/3_Saved_models/my_h53D_model.h5")
pretrained_model = unet3()
pretrained_model.load_weights("C:/Users/John/20211118_Python_biostudies/Python/3D_UNET_PYTHON/3D_UNET_PYTHON/3_Saved_models/my_h53D_model_updated.h5")
```

- Click the green arrow to process the unseen reflectance data with the 3-D Unet model.



- Probability maps for the LF-nuclei, LF-actin and Background/Other classes will be saved as multipage .TIFF files in the working directory named ready for input into the CellProfiler pipeline (filenames Ch_3_lm_001, Ch_4_lm_001, Ch_5_lm_001, respectively).



```
% PYTHON SCRIPT: TRAIN 2D UNET
% All code, image-data and screen-cast tutorial videos available for download at
% the Biostudies database under accession number S-BSST742


# -*- coding: utf-8 -*-
"""
Created on Mon Sep 20 23:58:04 2021

@author: Paul
"""
##############################################################################
#######################################################
#Import all required modules
##############################################################################
#######################################################

from PIL import Image
import cv2
import matplotlib.pyplot as plt
import matplotlib.pyplot as plt
import skimage
from skimage import data
from skimage.filters import threshold_otsu
import cv2
import os
import numpy as np
import skimage.transform as trans
import tensorflow
from tensorflow.keras.models import *
from tensorflow.keras.layers import *
from tensorflow.keras.optimizers import *
from tensorflow.keras.callbacks import ModelCheckpoint, LearningRateScheduler
from tensorflow.keras import backend as keras
from tensorflow.keras.preprocessing.image import ImageDataGenerator
import sklearn.feature_extraction
from sklearn.datasets import load_sample_image
from sklearn.feature_extraction import image
from datetime import datetime
from keras.utils import to_categorical
from tensorflow.python.keras.optimizers import *
import skimage.io
import h5py # this was missing

#%%

# Read in the reflectance data for training
Training_data_holder =
skimage.io.imread('C:/Users/John/20211118_Python_biostudies/Python/2D_UNET_PYTHO
N/2D_PeyersPatch/1_ImageData/TRAIN_IMAGE.tif')
Training_data = Training_data_holder[0,0,:,:,2]

#Read in the mask to isolated just the lymphoid tissue
mask = Training_data_holder[0,0,:,:,3]
thresh = threshold_otsu(Training_data)
mask = mask > thresh
mask = mask*1
mask = np.uint16(mask)

# Apply the mask to the training data
```

```
Training_data = np.multiply(Training_data,mask);
Training_data = np.double(Training_data);

# Rescale the training data in the interval [0 1]
Training_data_norm_holder = np.zeros((8551, 5701),np.double)
Training_data_norm = cv2.normalize(Training_data, Training_data_norm_holder ,
1.0, 0.0, cv2.NORM_MINMAX)

#Read in the pixel-class labels created from the nuclei and actin staining
Training_labels_holder =
skimage.io.imread('C:/Users/John/20211118_Python_biostudies/Python/2D_UNET_PYTHO
N/2D_PeyersPatch/1_ImageData/TRAIN_LABELS.png') ;
Training_labels = np.where(Training_labels_holder == 3, 0,
Training_labels_holder)


#% Visually inspect the data and labels
fig1, (ax1, ax2) = plt.subplots(1, 2) # figure1
ax1.imshow(Training_data_norm)
ax1.set_title('Reflectance data')
ax2.imshow(Training_labels)
ax2.set_title('Pixel classification labels')


#%% Once happy with labels and data, commit the reflectance information data to
sub-directory in .npy format

MYDIR =
'C:/Users/John/20211118_Python_biostudies/Python/2D_UNET_PYTHON/2D_PeyersPatch/2
_TRAIN_Unet/npy_training_data/'
CHECK_FOLDER = os.path.isdir(MYDIR)

# If folder doesn't exist, then create it.
if not CHECK_FOLDER:
    os.makedirs(MYDIR)

np.save(os.path.join(MYDIR, 'Training_data'), Training_data_norm)

# Create matrices to store training data
Patching_data = np.zeros((8551, 5701,2))
Patching_data[:,:,0] =  Training_data_norm
Patching_data[:,:,1] = Training_labels

#Create a training data comprising matching patches (256x256 pixels) of image-
data and training labels
Patched_images =
sklearn.feature_extraction.image.extract_patches_2d(Patching_data, patch_size =
[256, 256], max_patches=12000, random_state=None)

#%%
##############################################################################
#######################################################
# CREATE THE UNET
# input layer 256x256x1
# encoder depth 4
# 64 filters at the level of the first encoder
##############################################################################
#######################################################

def unet(pretrained_weights = None,input_size=(256,256,1), n_class=3):
```

```
    inputs = tensorflow.keras.Input(shape=input_size)
    conv1 = Conv2D(64, 3, activation = 'relu', dilation_rate=2,padding = 'same',
kernel_initializer = 'he_normal')(inputs)
    conv1 = BatchNormalization()(conv1)
    conv1 = Conv2D(64, 3, activation = 'relu', dilation_rate=2,padding = 'same',
kernel_initializer = 'he_normal')(conv1)
    conv1 = BatchNormalization()(conv1)
    pool1 = MaxPooling2D(pool_size=(2, 2))(conv1)
    conv2 = Conv2D(128, 3, activation = 'relu', dilation_rate=2,padding =
'same', kernel_initializer = 'he_normal')(pool1)
    conv2 = BatchNormalization()(conv2)
    conv2 = Conv2D(128, 3, activation = 'relu', dilation_rate=2, padding =
'same', kernel_initializer = 'he_normal')(conv2)
    conv2 = BatchNormalization()(conv2)
    pool2 = MaxPooling2D(pool_size=(2, 2))(conv2)
    conv3 = Conv2D(256, 3, activation = 'relu', padding = 'same',
kernel_initializer = 'he_normal')(pool2)
    conv3 = BatchNormalization()(conv3)
    conv3 = Conv2D(256, 3, activation = 'relu', padding = 'same',
kernel_initializer = 'he_normal')(conv3)
    conv3 = BatchNormalization()(conv3)
    pool3 = MaxPooling2D(pool_size=(2, 2))(conv3)
    conv4 = Conv2D(512, 3, activation = 'relu', padding = 'same',
kernel_initializer = 'he_normal')(pool3)
    conv4 = BatchNormalization()(conv4)
    conv4 = Conv2D(512, 3, activation = 'relu', padding = 'same',
kernel_initializer = 'he_normal')(conv4)
    conv4 = BatchNormalization()(conv4)
    drop4 = Dropout(0.5)(conv4, training=True)
    pool4 = MaxPooling2D(pool_size=(2, 2))(drop4)

    conv5 = Conv2D(1024, 3, activation = 'relu', padding = 'same',
kernel_initializer = 'he_normal')(pool4)
    conv5 = BatchNormalization()(conv5)
    conv5 = Conv2D(1024, 3, activation = 'relu', padding = 'same',
kernel_initializer = 'he_normal')(conv5)
    conv5 = BatchNormalization()(conv5)
    drop5 = Dropout(0.5)(conv5, training=True)

    up6 = Conv2D(512, 2, activation = 'relu', padding = 'same',
kernel_initializer = 'he_normal')(UpSampling2D(size = (2,2))(drop5))
    merge6 = concatenate([drop4,up6], axis = 3)
    conv6 = Conv2D(512, 3, activation = 'relu', padding = 'same',
kernel_initializer = 'he_normal')(merge6)
    conv6 = Conv2D(512, 3, activation = 'relu', padding = 'same',
kernel_initializer = 'he_normal')(conv6)


    up7 = Conv2D(256, 2, activation = 'relu', padding = 'same',
kernel_initializer = 'he_normal')(UpSampling2D(size = (2,2))(conv6))
    merge7 = concatenate([conv3,up7], axis = 3)
    conv7 = Conv2D(256, 3, activation = 'relu', padding = 'same',
kernel_initializer = 'he_normal')(merge7)
    conv7 = Conv2D(256, 3, activation = 'relu', padding = 'same',
kernel_initializer = 'he_normal')(conv7)


    up8 = Conv2D(128, 2, activation = 'relu', padding = 'same',
kernel_initializer = 'he_normal')(UpSampling2D(size = (2,2))(conv7))
```

```
    merge8 = concatenate([conv2,up8], axis = 3)
    conv8 = Conv2D(128, 3, activation = 'relu', padding = 'same',
kernel_initializer = 'he_normal')(merge8)
    conv8 = Conv2D(128, 3, activation = 'relu', padding = 'same',
kernel_initializer = 'he_normal')(conv8)


    up9 = Conv2D(64, 2, activation = 'relu', padding = 'same',
kernel_initializer = 'he_normal')(UpSampling2D(size = (2,2))(conv8))
    merge9 = concatenate([conv1,up9], axis = 3)
    conv9 = Conv2D(64, 3, activation = 'relu', padding = 'same',
kernel_initializer = 'he_normal')(merge9)
    conv9 = Conv2D(64, 3, activation = 'relu', padding = 'same',
kernel_initializer = 'he_normal')(conv9)


    conv10 = Conv2D(n_class, 1, activation = 'softmax')(conv9)

    model = tensorflow.keras.Model(inputs = inputs, outputs = conv10)

    model.compile(optimizer = Adam(lr = 0.0001),loss =
'sparse_categorical_crossentropy', metrics = ['sparse_categorical_accuracy'])


    if(pretrained_weights):
      model=keras.models.load_model(pretrained_weights)

    return model



#%% Split data into image and label data and reshape ready for training
trainX = Patched_images[:,:,:,0]
trainX = np.asarray(trainX).reshape((12000, 256, 256,1))
trainY = Patched_images[:,:,:,1]
trainY = np.asarray(trainY).reshape((12000, 256, 256, 1))



#Define model for data
model = unet(pretrained_weights = None,input_size = (256,256,1),n_class=3)


# Define augmentation options
def get_train_augmented(trainX=trainX, trainY=trainY, BATCH_SIZE=12):



    aug_X = ImageDataGenerator(rotation_range=360, zoom_range=[1,1],
width_shift_range=[0,0], height_shift_range=[0,0], horizontal_flip=True,
vertical_flip = True, shear_range = 0,fill_mode = "constant",cval=0.0)
    aug_Y = ImageDataGenerator(rotation_range=360, zoom_range=[1,1],
width_shift_range=[0,0], height_shift_range=[0,0], horizontal_flip=True,
vertical_flip = True, shear_range = 0,fill_mode = "constant",cval=0.0)

    aug_X.fit(trainX, augment=True, seed=1)
    aug_Y.fit(trainY, augment=True, seed=1)

    X_train_augmented = aug_X.flow(trainX, batch_size=BATCH_SIZE,shuffle=True,
seed=1)
```

```
    Y_train_augmented = aug_Y.flow(trainY, batch_size=BATCH_SIZE, shuffle=True,
seed=1)

    train_generator = zip(X_train_augmented, Y_train_augmented)

    for (X_train_augmented,Y_train_augmented) in train_generator:
        yield (X_train_augmented,Y_train_augmented)




#%% Define path to safe network, time stamp network
checkpoint_filepath =
'C:/Users/John/20211118_Python_biostudies/Python/2D_UNET_PYTHON/2D_PeyersPatch/3
_Saved_models/'
now = datetime.now()
dt_string = now.strftime("%d/%m/%Y%H:%M:%S")
NAME =dt_string
Name_file = 'PeyersPatchBiostudies_'
checkpoint_filepath_name = checkpoint_filepath + Name_file+dt_string


checkpoint_dir =  os.path.dirname(checkpoint_filepath_name)

#Specify that weights are to be saved
model_checkpoint_callback = tensorflow.keras.callbacks.ModelCheckpoint(
    filepath=checkpoint_dir,
    save_weights_only=True,
    monitor='val_accuracy',
    mode='max',
    save_best_only=True)

# Specify training options
BATCH_SIZE = 12
NUM_EPOCHS = 50

#Augment training data
train_generator = get_train_augmented(trainX=trainX, trainY=trainY,
BATCH_SIZE=BATCH_SIZE)

#Train and save the network
history = model.fit_generator(train_generator,
steps_per_epoch=len(trainX)/(BATCH_SIZE*2), epochs=NUM_EPOCHS,
callbacks=[model_checkpoint_callback])

#Plot progress # this plots over the view of the data
plt.figure(2)
plt.plot(history.history['sparse_categorical_accuracy'])
plt.title('Model accuracy')
plt.ylabel('accuracy')
plt.xlabel('epoch')
plt.legend(['train', 'test'], loc='upper left')
plt.savefig('Training_plot.png')
plt.show()

#Save final fully trained model as a .h5 file
model.save("C:/Users/John/20211118_Python_biostudies/Python/2D_UNET_PYTHON/2D_Pe
yersPatch/3_Saved_models/my_h5_model.h5")
checkpoint_filepath_name_h5 = checkpoint_filepath + Name_file+dt_string+'.h5'
model.save(checkpoint_filepath_name_h5)
```

```
% PYTHON SCRIPT: TEST 2D UNET
% All code, image-data and screen-cast tutorial videos available for download at
% the Biostudies database under accession number S-BSST742


# -*- coding: utf-8 -*-
"""
Created on Tue Sep 21 02:46:52 2021

@author: Paul
"""
#Import all required modules
import skimage
from skimage import data
from skimage.filters import threshold_otsu
from tensorflow.keras.preprocessing.image import ImageDataGenerator
import sklearn.feature_extraction
from sklearn.datasets import load_sample_image
from sklearn.feature_extraction import image
import matplotlib.cm as cm
import matplotlib.pyplot as plt
import matplotlib.cbook as cbook
from matplotlib.path import Path
from matplotlib.patches import PathPatch
import PIL
import numpy as np
import cv2
import tensorflow
from tensorflow.keras.models import *
from tensorflow.keras.layers import *
from tensorflow.keras.optimizers import *
from tensorflow.keras.callbacks import ModelCheckpoint, LearningRateScheduler
from tensorflow.keras import backend as keras
from tensorflow.keras.preprocessing.image import ImageDataGenerator
import sklearn.feature_extraction
from sklearn.datasets import load_sample_image
from sklearn.feature_extraction import image
from datetime import datetime
from keras.utils import to_categorical
from tensorflow.python.keras.optimizers import *
import skimage.io

#%% Load the reflectance data from the unseen test image
TEST_DATA =
skimage.io.imread('C:/Users/John/20211118_Python_biostudies/Python/2D_UNET_PYTHO
N/2D_PeyersPatch/1_ImageData/TEST_IMAGE.tif');
Test_data = TEST_DATA[0,0,:,:,2]

# Load the mask for the lymphoid tissue region
Mask = TEST_DATA[0,0,:,:,3]
thresh = threshold_otsu(Test_data)
Mask = Mask > thresh
Mask = Mask*1
Mask = np.uint16(Mask)


# Rescale the test data [0 1]
# Mask the rescaled data
Test_data = np.multiply(Test_data,Mask);
Test_data = np.double(Test_data);
```

```
Test_data_norm_holder = np.zeros((11247, 7610),np.double)
Test_data_rescaled = cv2.normalize(Test_data, Test_data_norm_holder , 1.0, 0.0,
cv2.NORM_MINMAX)

#%%
##############################################################################
#################################################
# Define model for testing
##############################################################################
#################################################
def unet(pretrained_weights = None,input_size=(256,256,1), n_class=3):

    inputs = tensorflow.keras.Input(shape=input_size)
    conv1 = Conv2D(64, 3, activation = 'relu', dilation_rate=2,padding = 'same',
kernel_initializer = 'he_normal')(inputs)
    conv1 = BatchNormalization()(conv1)
    conv1 = Conv2D(64, 3, activation = 'relu', dilation_rate=2,padding = 'same',
kernel_initializer = 'he_normal')(conv1)
    conv1 = BatchNormalization()(conv1)
    pool1 = MaxPooling2D(pool_size=(2, 2))(conv1)
    conv2 = Conv2D(128, 3, activation = 'relu', dilation_rate=2,padding =
'same', kernel_initializer = 'he_normal')(pool1)
    conv2 = BatchNormalization()(conv2)
    conv2 = Conv2D(128, 3, activation = 'relu', dilation_rate=2, padding =
'same', kernel_initializer = 'he_normal')(conv2)
    conv2 = BatchNormalization()(conv2)
    pool2 = MaxPooling2D(pool_size=(2, 2))(conv2)
    conv3 = Conv2D(256, 3, activation = 'relu', padding = 'same',
kernel_initializer = 'he_normal')(pool2)
    conv3 = BatchNormalization()(conv3)
    conv3 = Conv2D(256, 3, activation = 'relu', padding = 'same',
kernel_initializer = 'he_normal')(conv3)
    conv3 = BatchNormalization()(conv3)
    pool3 = MaxPooling2D(pool_size=(2, 2))(conv3)
    conv4 = Conv2D(512, 3, activation = 'relu', padding = 'same',
kernel_initializer = 'he_normal')(pool3)
    conv4 = BatchNormalization()(conv4)
    conv4 = Conv2D(512, 3, activation = 'relu', padding = 'same',
kernel_initializer = 'he_normal')(conv4)
    conv4 = BatchNormalization()(conv4)
    drop4 = Dropout(0.5)(conv4, training=True)
    pool4 = MaxPooling2D(pool_size=(2, 2))(drop4)

    conv5 = Conv2D(1024, 3, activation = 'relu', padding = 'same',
kernel_initializer = 'he_normal')(pool4)
    conv5 = BatchNormalization()(conv5)
    conv5 = Conv2D(1024, 3, activation = 'relu', padding = 'same',
kernel_initializer = 'he_normal')(conv5)
    conv5 = BatchNormalization()(conv5)
    drop5 = Dropout(0.5)(conv5, training=True)

    up6 = Conv2D(512, 2, activation = 'relu', padding = 'same',
kernel_initializer = 'he_normal')(UpSampling2D(size = (2,2))(drop5))
    merge6 = concatenate([drop4,up6], axis = 3)
    conv6 = Conv2D(512, 3, activation = 'relu', padding = 'same',
kernel_initializer = 'he_normal')(merge6)
    conv6 = Conv2D(512, 3, activation = 'relu', padding = 'same',
kernel_initializer = 'he_normal')(conv6)
```

```
    up7 = Conv2D(256, 2, activation = 'relu', padding = 'same',
kernel_initializer = 'he_normal')(UpSampling2D(size = (2,2))(conv6))
    merge7 = concatenate([conv3,up7], axis = 3)
    conv7 = Conv2D(256, 3, activation = 'relu', padding = 'same',
kernel_initializer = 'he_normal')(merge7)
    conv7 = Conv2D(256, 3, activation = 'relu', padding = 'same',
kernel_initializer = 'he_normal')(conv7)


    up8 = Conv2D(128, 2, activation = 'relu', padding = 'same',
kernel_initializer = 'he_normal')(UpSampling2D(size = (2,2))(conv7))
    merge8 = concatenate([conv2,up8], axis = 3)
    conv8 = Conv2D(128, 3, activation = 'relu', padding = 'same',
kernel_initializer = 'he_normal')(merge8)
    conv8 = Conv2D(128, 3, activation = 'relu', padding = 'same',
kernel_initializer = 'he_normal')(conv8)


    up9 = Conv2D(64, 2, activation = 'relu', padding = 'same',
kernel_initializer = 'he_normal')(UpSampling2D(size = (2,2))(conv8))
    merge9 = concatenate([conv1,up9], axis = 3)
    conv9 = Conv2D(64, 3, activation = 'relu', padding = 'same',
kernel_initializer = 'he_normal')(merge9)
    conv9 = Conv2D(64, 3, activation = 'relu', padding = 'same',
kernel_initializer = 'he_normal')(conv9)


    conv10 = Conv2D(n_class, 1, activation = 'softmax')(conv9)


    model = tensorflow.keras.Model(inputs = inputs, outputs = conv10)


    model.compile(optimizer = Adam(lr = 0.0001),loss =
'sparse_categorical_crossentropy', metrics = ['sparse_categorical_accuracy'])



    if(pretrained_weights):
      model=keras.models.load_model(pretrained_weights)

    return model


#%%
##############################################################################
#################################################
# Patch the reflectance data through the network
##############################################################################
#################################################
# Set the patch sizes to be passed to the net
patchSize = [256,256]
Test_data_rescaled =
np.asarray(Test_data_rescaled).reshape((Test_data_rescaled.shape[0],
Test_data_rescaled.shape[1],1))

# Segment blockwise then reassemble in full
# Define image dimensions
[height,width,nChannel] = np.shape(Test_data_rescaled)
```

```
patch = np.zeros([patchSize[0], patchSize[1],
nChannel],dtype=Test_data_rescaled.dtype);

# Pad image to have dimensions as multiples of patchSize
padSize = np.empty([1, 2])
padSize[0,0] = patchSize[0] - np.remainder(height, patchSize[0]);
padSize[0,1] = patchSize[1] - np.remainder(width, patchSize[1]);
Starting_shape = np.shape(Test_data_rescaled)
a1 = Starting_shape[0]+padSize[0,0]
a2 = Starting_shape[1]+padSize[0,1]

im_pad = np.zeros([int(a1),int(a2)])
im_pad[:Test_data_rescaled.shape[0],:Test_data_rescaled.shape[1]] =
Test_data_rescaled[:,:,0]
im_pad = np.asarray(im_pad).reshape((im_pad.shape[0], im_pad.shape[1],1))
[height_pad, width_pad, nChannel_pad] = np.shape(im_pad);

# Preallocate some matrices to receive the network outputs
out_Uncertainty_Scores = np.zeros([np.shape(im_pad)[0], np.shape(im_pad)[1]],
'double');

out_Pmap_cat1 = np.zeros([np.shape(im_pad)[0], np.shape(im_pad)[1]], 'double');
out_Pmap_cat2 = np.zeros([np.shape(im_pad)[0], np.shape(im_pad)[1]], 'double');
out_Pmap_cat3 = np.zeros([np.shape(im_pad)[0], np.shape(im_pad)[1]], 'double');

# Load a pretrained network
pretrained_model = unet()
pretrained_model.load_weights('C:/Users/John/20211118_Python_biostudies/Python/2
D_UNET_PYTHON/2D_PeyersPatch/3_Saved_models/my_h5_model_fully_trained.h5')

# Loop through blocks of 'patchSize'
for loop in range(1, int(height_pad),int(patchSize[0])):
    print(loop)
    for j in range(1,int(width_pad),int(patchSize[1])):

        for p in range(1,(nChannel+1)):
            PP3 = np.empty([1,256,256,1])

            im_pad_touse =im_pad[(loop-1):((loop-1)+patchSize[0]),(j-1):((j-
1)+(patchSize[1]))]
            patch[:,:,0] = np.squeeze(im_pad_touse,axis=None)

            PP3[0,:,:,:]=patch
            # deploy net
            predictions = pretrained_model.predict(PP3)

            scores = np.max(predictions,axis=3)
            predictions.argmax(axis=3)

            out_Uncertainty_Scores[(loop-1):(loop+patchSize[0]-1), (j-
1):(j+patchSize[1]-1)] = scores;
            out_Pmap_cat1[(loop-1):(loop+patchSize[0]-1), (j-1):(j+patchSize[1]-
1)] = predictions[0,:,:,0];
            out_Pmap_cat2[(loop-1):(loop+patchSize[0]-1), (j-1):(j+patchSize[1]-
1)] = predictions[0,:,:,1];
            out_Pmap_cat3[(loop-1):(loop+patchSize[0]-1), (j-1):(j+patchSize[1]-
1)] = predictions[0,:,:,2];


# Remove padding from the network outputs
```

```
out_Uncertainty_Scores = out_Uncertainty_Scores[0:height, 0:width];
out_Pmap_cat1 = out_Pmap_cat1[0:height, 0:width];
out_Pmap_cat2 = out_Pmap_cat2[0:height, 0:width];
out_Pmap_cat3 = out_Pmap_cat3[0:height, 0:width];

image_to_plot = np.zeros((11247, 7610,3))
image_to_plot[:,:,0] = out_Pmap_cat1
image_to_plot[:,:,1] = out_Pmap_cat2
image_to_plot[:,:,2] = out_Pmap_cat3

#%% visualise network outputs
fig, (ax1, ax2, ax3, ax4) = plt.subplots(1, 4, sharey=True)
fig.set_size_inches(14, 12)

im = ax1.imshow(out_Pmap_cat1)
ax1.title.set_text('Background/Other')

im = ax2.imshow(out_Pmap_cat2)
ax2.title.set_text('LF-Actin')

im = ax3.imshow(out_Pmap_cat3)
ax3.title.set_text('LF-Nuclei')

im = ax4.imshow(out_Uncertainty_Scores)
ax4.title.set_text('Uncertainty')

plt.savefig('Predictions.png')

#%% Map to 16-bit and save for loading into CellProfiler
# Background/other
data_norm_holder = np.zeros((11247, 7610),np.uint16)
ui16_PMAP_cat1_xy = cv2.normalize(out_Pmap_cat1, data_norm_holder , 65535.0,
0.0, cv2.NORM_MINMAX)
img_data2 = (ui16_PMAP_cat1_xy).astype(dtype=np.uint16)
rawtiff=PIL.Image.fromarray(img_data2)
rawtiff.save('Ch_7_Im_001'+'.tiff')

# LF-Actin
data_norm_holder = np.zeros((11247, 7610),np.uint16)
ui16_PMAP_cat2_xy = cv2.normalize(out_Pmap_cat2, data_norm_holder , 65535.0,
0.0, cv2.NORM_MINMAX)
img_data2 = (ui16_PMAP_cat2_xy).astype(dtype=np.uint16)
rawtiff=PIL.Image.fromarray(img_data2)
rawtiff.save('Ch_6_Im_001'+'.tiff')

# LF-Nuclei
data_norm_holder = np.zeros((11247, 7610),np.uint16)
ui16_PMAP_cat3_xy = cv2.normalize(out_Pmap_cat3, data_norm_holder , 65535.0,
0.0, cv2.NORM_MINMAX)
img_data2 = (ui16_PMAP_cat3_xy).astype(dtype=np.uint16)
rawtiff=PIL.Image.fromarray(img_data2)
rawtiff.save('Ch_5_Im_001'+'.tiff')
```

```
% PYTHON SCRIPT: TRAIN 3D UNET
% All code, image-data and screen-cast tutorial videos available for download at
% the Biostudies database under accession number S-BSST742


# -*- coding: utf-8 -*-
"""
Created on Mon Jun 14 15:21:44 2021

@author: Paul
"""

from PIL import Image
import javabridge
import bioformats
javabridge.start_vm(class_path=bioformats.JARS)
import cv2
import matplotlib.pyplot as plt
import matplotlib.pyplot as plt
import skimage
from skimage import data
from skimage.filters import threshold_otsu
import os
import numpy as np
import skimage.transform as trans
import numpy as np
import tensorflow
from tensorflow.keras.models import *
from tensorflow.keras.layers import *
from tensorflow.keras.optimizers import *
from tensorflow.keras.callbacks import ModelCheckpoint, LearningRateScheduler
from tensorflow.keras import backend as keras
from tensorflow.keras.preprocessing.image import ImageDataGenerator
import sklearn.feature_extraction
from sklearn.datasets import load_sample_image
from sklearn.feature_extraction import image
from datetime import datetime
from keras.utils import to_categorical
from tensorflow.python.keras.optimizers import *
import random
import matplotlib.pyplot as plt
from mpl_toolkits.mplot3d.art3d import Poly3DCollection
import numpy as np

from skimage import exposure, io, util


#%% Specify directory containing training data
Training_data_directory =
'C:/Users/John/20211118_Python_biostudies/Python/3D_UNET_PYTHON/3D_UNET_PYTHON/1
_ImageData/TRAIN/DATA/'

# Specify channel number in training data that contains reflectance information
RL_training_directory = 3;

# Specify directory containing training labels
Training_labels_path =
'C:/Users/John/20211118_Python_biostudies/Python/3D_UNET_PYTHON/3D_UNET_PYTHON/1
_ImageData/TRAIN/LABELS/' ;
```

```
# COMMIT TRAINING DATA TO DIRECTORY rescaled [0 1] IN MAT FORMAT
MYDIR
='C:/Users/John/20211118_Python_biostudies/Python/3D_UNET_PYTHON/3D_UNET_PYTHON/
1_ImageData/TRAIN/LABELS/mat_training_data/'
CHECK_FOLDER = os.path.isdir(MYDIR)

# If folder doesn't exist, then create it.
if not CHECK_FOLDER:
    os.makedirs(MYDIR)


Training_labels_holder =
skimage.io.imread('C:/Users/John/20211118_Python_biostudies/Python/3D_UNET_PYTHO
N/3D_UNET_PYTHON/1_ImageData/TRAIN/LABELS/LABELS_001.tif') ;
Training_labels = np.where(Training_labels_holder == 3, 0,
Training_labels_holder)


#%%
for i in range(0,2):

    loop = i+1

    counter = '00'+str(loop)

    # find metadata describing file
    TOTAL_path = Training_data_directory+'TRAIN_'+counter+'.tif'

    reader = bioformats.ImageReader(TOTAL_path)
    NUMBERIMAGES = reader.rdr.getSeriesCount(TOTAL_path)
    number_of_channels = reader.rdr.getSizeC(TOTAL_path)
    Xlength = reader.rdr.getSizeX(TOTAL_path)
    Ylength = reader.rdr.getSizeY(TOTAL_path)

    stackSizeZ  = reader.rdr.getSizeZ(TOTAL_path)# number of Z slices
    channel_zimage = np.zeros((Xlength,Ylength,stackSizeZ))


    # Load reflectance information
    for zplane in range(1,stackSizeZ+1):

        channel_zimage[:,:,(zplane-1)] = bioformats.load_image(TOTAL_path,z =
(zplane-1), c= (RL_training_directory-1))
    IM_DATA = channel_zimage


    IM_DATA = np.double(IM_DATA);
    DIM = IM_DATA.shape
    IM_DATA_norm_holder = np.zeros((DIM[0],DIM[1],DIM[2]),np.double)
    IM_DATA_norm = cv2.normalize(IM_DATA, IM_DATA_norm_holder, 1.0, 0.0,
cv2.NORM_MINMAX)
    pw = os.getcwd()
    FILENAME = pw+'\\npy_training_data\\'+'TRAIN_DATA_'+counter
    FOLDER_CHECK = pw+'\\npy_training_data\\'
    CHECK_FOLDER = os.path.isdir(FOLDER_CHECK)
    if not CHECK_FOLDER:
        os.makedirs(FOLDER_CHECK)

    np.save(FILENAME,IM_DATA_norm)
```

```
pw = os.getcwd()

#%% COMMIT TRAINING LABELS TO DIRECTORY IN MAT FORMAT
FOLDER_NAME = pw+'\\npy_training_labels\\'
CHECK_FOLDER2 = os.path.isdir(FOLDER_NAME)
if not CHECK_FOLDER2:
    os.makedirs(FOLDER_NAME)

for i in range(0,2):
    loop = i +1
    counter = '00'+str(loop)
    label_zimage = [];
    label_zimage = np.zeros((Xlength,Ylength,stackSizeZ))
    # load label information
    for zplane in range(1,stackSizeZ+1):

        TOTAL_path = Training_labels_path+'LABELS_'+counter+'.tif'
        iplane = bioformats.load_image(TOTAL_path,z=(zplane-1), c=0,rescale =
False )

        label_zimage[:,:,(zplane-1)] = iplane

    IM_LABELS = np.double(label_zimage);
    DIM = IM_LABELS.shape
    IM_LABELS_norm_holder = np.zeros((DIM[0],DIM[1],DIM[2]),np.double)
    IM_LABELS_norm_holder[:,:,:] = label_zimage[:,:,:]
    pw = os.getcwd()
    FILENAME = pw+'\\npy_training_labels\\'+'TRAIN_LABELS_'+counter
    FOLDER_CHECK = pw+'\\npy_training_labels\\'
    CHECK_FOLDER = os.path.isdir(FOLDER_CHECK)
    if not CHECK_FOLDER:
        os.makedirs(FOLDER_CHECK)

    np.save(FILENAME, IM_LABELS_norm_holder)

#%%
NUMBER_batch = 1

del  channel_zimage,  IM_LABELS, IM_DATA,   label_zimage

# Prepare to extract patches
patchPerImage = 375;
xrand = random.sample(range(0,959), patchPerImage)
yrand = random.sample(range(0,959), patchPerImage)
zrand = np.random.choice(78,patchPerImage, replace=True)


# Define random patch extraction and augmentarion of patches
def get_train_patched(xrand, yrand, zrand,BATCH_SIZE,batch_number =
NUMBER_batch, trainXm=IM_DATA_norm, trainYm=IM_LABELS_norm_holder):

    patched_data_image = np.zeros((BATCH_SIZE,64,64,32))
    patched_data_labels = np.zeros((BATCH_SIZE,64,64,32))
    for i in range(0,BATCH_SIZE):
        val = (i + batch_number)-1;
        patched_data_image[i,:,:,:] =
trainXm[(xrand[val]):(xrand[val]+64),(yrand[val]):(yrand[val]+64),(zrand[val]):(
zrand[val]+32)];
```

```
        patched_data_labels[i,:,:,:] =
trainYm[(xrand[val]):(xrand[val]+64),(yrand[val]):(yrand[val]+64),(zrand[val]):(
zrand[val]+32)];
    return patched_data_image, patched_data_labels


def get_train_augmented(trainXm,trainYm ,BATCH_SIZE,batch_number =
NUMBER_batch):
    for i in range(0,BATCH_SIZE):
        rand_number = random.sample(range(BATCH_SIZE), 1)
        my_arr = [np.rot90, np.flipud, np.fliplr]
        if int(rand_number[0])<3:
            trainXm[i,:,:,:] = my_arr[int(rand_number[0])](trainXm[i,:,:,:])
            trainYm[i,:,:,:] = my_arr[int(rand_number[0])](trainYm[i,:,:,:])


        elif int(rand_number[0]) ==3:

            trainXm[i,:,:,:] = np.fliplr(trainXm[i,:,:,:])
            trainYm[i,:,:,:] = np.fliplr(trainYm[i,:,:,:])
            trainXm[i,:,:,:] = np.rot90(trainXm[i,:,:,:])
            trainYm[i,:,:,:] = np.rot90(trainYm[i,:,:,:])

        else:
            trainXm[i,:,:,:] = trainXm[i,:,:,:]
            trainYm[i,:,:,:] = trainYm[i,:,:,:]
        return trainXm, trainYm

#%%
##############################################################################
#################################################
# CREATE THE 3D UNET
# input layer 64x64x32x1
# encoder depth 4
# 32 filters at the level of the first encoder
##############################################################################
#################################################

def unet3(pretrained_weights = None,input_size= (64,64,32,1), n_class=4):

    inputs = tensorflow.keras.Input(shape=input_size)
    conv1 = Conv3D(32, kernel_size=(3, 3, 3),dilation_rate=2,padding = 'same',
kernel_initializer = 'he_normal')(inputs)
    conv1 = BatchNormalization()(conv1)
    conv1 = Activation('relu')(conv1)
    conv1 = Conv3D(64, kernel_size=(3, 3, 3), dilation_rate=2,padding = 'same',
kernel_initializer = 'he_normal')(conv1)
    conv1 = BatchNormalization()(conv1)
    up1 = Activation('relu')(conv1)
    pool1 = MaxPooling3D(pool_size=(2,2,2),strides = (2,2,2))(up1)
    conv2 = Conv3D(64, kernel_size=(3, 3, 3), dilation_rate=2,padding = 'same',
kernel_initializer = 'he_normal')(pool1)
    conv2 = BatchNormalization()(conv2)
    conv2 = Activation('relu')(conv2)
    conv2 = Conv3D(128, kernel_size=(3, 3, 3), dilation_rate=2,padding = 'same',
kernel_initializer = 'he_normal')(up1)
    conv2 = BatchNormalization()(conv2)
    up2 = Activation('relu')(conv2)
    pool2 = MaxPooling3D(pool_size=(2,2,2),strides = (2,2,2))(up2)
```

```
    conv3 = Conv3D(128, kernel_size=(3, 3, 3), dilation_rate=2,padding = 'same',
kernel_initializer = 'he_normal')(pool2)
    conv3 = BatchNormalization()(conv3)
    conv3 = Activation('relu')(conv3)
    conv3 = Conv3D(256, kernel_size=(3, 3, 3), dilation_rate=2,padding = 'same',
kernel_initializer = 'he_normal')(conv3)
    conv3 = BatchNormalization()(conv3)
    up3 = Activation('relu')(conv3)
    pool3 = MaxPooling3D(pool_size=(2,2,2),strides = (2,2,2))(up3)
    conv4 = Conv3D(256, kernel_size=(3, 3, 3),  dilation_rate=2,padding =
'same', kernel_initializer = 'he_normal')(pool3)
    conv4 = BatchNormalization()(conv4)
    conv4 = Activation('relu')(conv4)
    conv4 = Conv3D(512, kernel_size=(3, 3, 3),  dilation_rate=2,padding =
'same', kernel_initializer = 'he_normal')(conv4)
    conv4 = BatchNormalization()(conv4)
    up4 = Activation('relu')(conv4)
    pool4 = MaxPooling3D(pool_size=(2,2,2),strides = (2,2,2))(up4)
    conv5 = Conv3D(512, kernel_size=(3, 3, 3),  dilation_rate=2,padding =
'same', kernel_initializer = 'he_normal')(pool4)
    conv5 = BatchNormalization()(conv5)
    conv5 = Activation('relu')(conv5)
    conv5 = Conv3D(1024, kernel_size=(3, 3, 3),  dilation_rate=2,padding =
'same', kernel_initializer = 'he_normal')(conv5)
    conv5 = BatchNormalization()(conv5)
    conv5 = Activation('relu')(conv5)
    drop5 = Conv3DTranspose(1024, kernel_size=(2, 2, 2),strides=(2, 2, 2),
activation = 'relu', dilation_rate=2,padding = 'same', kernel_initializer =
'he_normal')(conv5)

    merge6 = concatenate([drop5,up4], axis = 4)
    conv6 = Conv3D(512, kernel_size=(3, 3, 3),  dilation_rate=2,padding =
'same', kernel_initializer = 'he_normal')(merge6)
    conv6 = BatchNormalization()(conv6)
    conv6 = Activation('relu')(conv6)
    conv6 = Conv3D(512, kernel_size=(3, 3, 3),  dilation_rate=2,padding =
'same', kernel_initializer = 'he_normal')(conv6)
    conv6 = BatchNormalization()(conv6)
    conv6 = Activation('relu')(conv6)
    drop6 = Conv3DTranspose(512, kernel_size=(2, 2, 2),strides=(2, 2, 2),
activation = 'relu', dilation_rate=2,padding = 'same', kernel_initializer =
'he_normal')(conv6)
    merge7 = concatenate([drop6,up3], axis = 4)

    conv7 = Conv3D(256, kernel_size=(3, 3, 3), dilation_rate=2,padding = 'same',
kernel_initializer = 'he_normal')(merge7)
    conv7 = BatchNormalization()(conv7)
    conv7 = Activation('relu')(conv7)
    conv7 = Conv3D(256, kernel_size=(3, 3, 3), dilation_rate=2,padding = 'same',
kernel_initializer = 'he_normal')(conv7)
    conv7 = BatchNormalization()(conv7)
    conv7 = Activation('relu')(conv7)
    drop7 = Conv3DTranspose(256, kernel_size=(2, 2, 2),strides=(2, 2, 2),
activation = 'relu', dilation_rate=2,padding = 'same', kernel_initializer =
'he_normal')(conv7)
    merge8 = concatenate([drop7,up2], axis = 4)


    conv8 = Conv3D(128, kernel_size=(3, 3, 3),  dilation_rate=2,padding =
'same', kernel_initializer = 'he_normal')(merge8)
```

```
    conv8 = BatchNormalization()(conv8)
    conv8 = Activation('relu')(conv8)
    conv8 = Conv3D(128, kernel_size=(3, 3, 3),  dilation_rate=2,padding =
'same', kernel_initializer = 'he_normal')(conv8)
    conv8 = BatchNormalization()(conv8)
    conv8 = Activation('relu')(conv8)
    drop8 = Conv3DTranspose(128, kernel_size=(2, 2, 2),strides=(2, 2, 2),
activation = 'relu', dilation_rate=2,padding = 'same', kernel_initializer =
'he_normal')(conv8)
    conv8 = BatchNormalization()(drop8)
    conv8 = Activation('relu')(conv8)
    drop8 = Conv3DTranspose(128, kernel_size=(2, 2, 2),strides=(2, 2, 2),
activation = 'relu', dilation_rate=2,padding = 'same', kernel_initializer =
'he_normal')(conv7)


    merge9 = concatenate([drop8,up1], axis = 4)
    conv9 = Conv3D(64, kernel_size=(3, 3, 3),  dilation_rate=2,padding = 'same',
kernel_initializer = 'he_normal')(merge9)
    conv9 = BatchNormalization()(conv9)
    conv9 = Activation('relu')(conv9)
    conv9 = Conv3D(64, kernel_size=(3, 3, 3),  dilation_rate=2,padding = 'same',
kernel_initializer = 'he_normal')(conv9)
    conv9 = BatchNormalization()(conv9)
    conv9 = Activation('relu')(conv9)

    conv9 = Conv3D(n_class, kernel_size=(1, 1, 1),activation = 'softmax')(conv9)


    model = tensorflow.keras.Model(inputs = inputs, outputs = conv9)
    model.compile(optimizer = Adam(lr = 0.0005),loss =
'sparse_categorical_crossentropy', metrics = ['sparse_categorical_accuracy'])

    if(pretrained_weights):
      model=keras.models.load_model(pretrained_weights)

    return model


#%% Specify location to save the network
checkpoint_filepath =
'C:/Users/John/20211118_Python_biostudies/Python/3D_UNET_PYTHON/3D_UNET_PYTHON/3
_Saved_models/'
now = datetime.now()
dt_string = now.strftime("%d/%m/%Y%H:%M:%S")
NAME =dt_string
Name_file = 'PeyersPatchBiostudies_'
checkpoint_filepath_name = checkpoint_filepath + Name_file+dt_string

checkpoint_dir =  os.path.dirname(checkpoint_filepath_name)


model_checkpoint_callback = tensorflow.keras.callbacks.ModelCheckpoint(
    filepath=checkpoint_dir,
    save_weights_only=True,
    monitor='val_accuracy',
    mode='max',
    save_best_only=True)

#Specify training options
```

```
BATCH_SIZE = 8
NUM_EPOCHS = 50
patchPerImage = 375;
steps = np.floor(patchPerImage/8);

NUMBER_batch = 1;



start = 0;
finish = 8;
storeX = np.zeros((375,64,64,32,1))
storeY = np.zeros((375,64,64,32,1))

#Extract patches
for i in range(1,int(steps)):


    [patched_data_image, patched_data_labels]=get_train_patched(xrand, yrand,
zrand, BATCH_SIZE, batch_number = NUMBER_batch, trainXm=IM_DATA_norm,
trainYm=IM_LABELS_norm_holder)



    [trainXmgg, trainYmgg] =
get_train_augmented(BATCH_SIZE=8,trainXm=patched_data_image,
trainYm=patched_data_labels, batch_number = NUMBER_batch)
    trainYmgg = trainYmgg.reshape((8,64,64,32,1))
    trainXmgg = trainXmgg.reshape((8,64,64,32,1))

    trainXmgg = trainXmgg.astype('float32')
    trainYmgg = trainYmgg.astype('float32')

    storeX[start:finish,:,:,:,:] = trainXmgg
    storeY[start:finish,:,:,:,:] = trainYmgg

    start = finish
    finish = start+8
    NUMBER_batch = NUMBER_batch+1


[patched_data_image, patched_data_labels]=get_train_patched(xrand, yrand, zrand,
BATCH_SIZE=(patchPerImage-finish), batch_number = NUMBER_batch,
trainXm=IM_DATA_norm, trainYm=IM_LABELS_norm_holder)


[trainXmgg, trainYmgg] = get_train_augmented(BATCH_SIZE=(patchPerImage-
finish),trainXm=patched_data_image, trainYm=patched_data_labels, batch_number =
NUMBER_batch)
trainYmgg = trainYmgg.reshape((patchPerImage-finish,64,64,32,1))
trainXmgg = trainXmgg.reshape((patchPerImage-finish,64,64,32,1))

trainXmgg = trainXmgg.astype('float32')
trainYmgg = trainYmgg.astype('float32')

storeX[finish:patchPerImage,:,:,:,:] = trainXmgg
storeY[finish:patchPerImage,:,:,:,:] = trainYmgg

model = unet3(pretrained_weights = None,input_size =
(64,64,32,1),n_class=4)#change to 3
```

```
#%% Train the model
history = model.fit(
    storeX,
    storeY,
    batch_size = BATCH_SIZE,
    epochs = NUM_EPOCHS,callbacks=[model_checkpoint_callback])

plt.plot(history.history['sparse_categorical_accuracy'])
plt.title('Model accuracy')
plt.ylabel('accuracy')
plt.xlabel('epoch')
plt.legend(['train', 'test'], loc='upper left')
plt.savefig('Training_plot.png')
plt.show()

model.save("C:/Users/John/20211118_Python_biostudies/Python/3D_UNET_PYTHON/3D_UN
ET_PYTHON/3_Saved_models/my_h53D_model.h5")
```

```
% PYTHON SCRIPT: TEST 3D UNET
% All code, image-data and screen-cast tutorial videos available for download at
% the Biostudies database under accession number S-BSST742


# -*- coding: utf-8 -*-
"""
Created on Mon Sep 20 16:37:00 2021

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"""
from PIL import Image
import javabridge
import bioformats
javabridge.start_vm(class_path=bioformats.JARS)
import cv2
import matplotlib.pyplot as plt
import matplotlib.pyplot as plt
import skimage
from skimage import data
from skimage.filters import threshold_otsu
import os
import numpy as np
import skimage.transform as trans
import numpy as np
import tensorflow
from tensorflow.keras.models import *
from tensorflow.keras.layers import *
from tensorflow.keras.optimizers import *
from tensorflow.keras.callbacks import ModelCheckpoint, LearningRateScheduler
from tensorflow.keras import backend as keras
from tensorflow.keras.preprocessing.image import ImageDataGenerator
import sklearn.feature_extraction
from sklearn.datasets import load_sample_image
from sklearn.feature_extraction import image
from datetime import datetime
from keras.utils import to_categorical
from tensorflow.python.keras.optimizers import *
import random
import matplotlib.pyplot as plt
from mpl_toolkits.mplot3d.art3d import Poly3DCollection
import numpy as np

from skimage import exposure, io, util
from skimage import data
from tensorflow.keras.preprocessing.image import ImageDataGenerator
import sklearn.feature_extraction
from sklearn.datasets import load_sample_image
from sklearn.feature_extraction import image
import matplotlib.cm as cm

import matplotlib.cbook as cbook
from matplotlib.path import Path
from matplotlib.patches import PathPatch
import math
import json
import numpy as np
import PIL

import imageio
```

```
#%% Specify directory containing test data
Test_data_directory =
'C:/Users/John/20211118_Python_biostudies/Python/3D_UNET_PYTHON/3D_UNET_PYTHON/1
_ImageData/TEST/'

# Specify channel number in test data that contains reflectance information
RL_channel_number = 2;

#COMMIT REFLECTANCE TEST DATA TO DIRECTORY RESCALED [0 1] IN MAT FORMAT
MYDIR
='C:/Users/John/20211118_Python_biostudies/Python/3D_UNET_PYTHON/3D_UNET_PYTHON/
4_TEST_Unet/npy_test_data/'
CHECK_FOLDER = os.path.isdir(MYDIR)


# If folder doesn't exist, then create it.
if not CHECK_FOLDER:
    os.makedirs(MYDIR)


for i in range(0,1):

    loop = i+1

    counter = '00'+str(loop)

    TOTAL_path = Test_data_directory+'TEST_'+counter+'.tif'
    # find metadata describing file
    reader = bioformats.ImageReader(TOTAL_path)

    NUMBERIMAGES = reader.rdr.getSeriesCount(TOTAL_path)
    number_of_channels = reader.rdr.getSizeC(TOTAL_path)
    Xlength = reader.rdr.getSizeX(TOTAL_path)
    Ylength = reader.rdr.getSizeY(TOTAL_path)
    stackSizeZ  = reader.rdr.getSizeZ(TOTAL_path)

    channel_zimage = np.zeros((Xlength,Ylength,stackSizeZ))
    # Load reflectance information
    for zplane in range(1,stackSizeZ+1):

        channel_zimage[:,:,(zplane-1)] = bioformats.load_image(TOTAL_path,z =
(zplane-1), c= (RL_channel_number-1))
    IM_DATA = channel_zimage


    IM_DATA = np.double(IM_DATA);
    DIM = IM_DATA.shape
    IM_DATA_norm_holder = np.zeros((DIM[0],DIM[1],DIM[2]),np.double)
    IM_DATA_norm = cv2.normalize(IM_DATA, IM_DATA_norm_holder, 1.0, 0.0,
cv2.NORM_MINMAX)
    pw = os.getcwd()
    FILENAME = pw+'\\npy_test_data\\'+'TEST_DATA_'+counter
    FOLDER_CHECK = pw+'\\npy_test_data\\'
    CHECK_FOLDER = os.path.isdir(FOLDER_CHECK)
    if not CHECK_FOLDER:
        os.makedirs(FOLDER_CHECK)

    np.save(FILENAME,IM_DATA_norm)
```

```
#%%
#Redefine network
def unet3(pretrained_weights = None,input_size= (64,64,32,1), n_class=4):

    inputs = tensorflow.keras.Input(shape=input_size)
    conv1 = Conv3D(32, kernel_size=(3, 3, 3),dilation_rate=2,padding = 'same',
kernel_initializer = 'he_normal')(inputs)
    conv1 = BatchNormalization()(conv1)
    conv1 = Activation('relu')(conv1)
    conv1 = Conv3D(64, kernel_size=(3, 3, 3), dilation_rate=2,padding = 'same',
kernel_initializer = 'he_normal')(conv1)
    conv1 = BatchNormalization()(conv1)
    up1 = Activation('relu')(conv1)
    pool1 = MaxPooling3D(pool_size=(2,2,2),strides = (2,2,2))(up1)
    conv2 = Conv3D(64, kernel_size=(3, 3, 3), dilation_rate=2,padding = 'same',
kernel_initializer = 'he_normal')(pool1)
    conv2 = BatchNormalization()(conv2)
    conv2 = Activation('relu')(conv2)
    conv2 = Conv3D(128, kernel_size=(3, 3, 3), dilation_rate=2,padding = 'same',
kernel_initializer = 'he_normal')(up1)
    conv2 = BatchNormalization()(conv2)
    up2 = Activation('relu')(conv2)
    pool2 = MaxPooling3D(pool_size=(2,2,2),strides = (2,2,2))(up2)
    conv3 = Conv3D(128, kernel_size=(3, 3, 3), dilation_rate=2,padding = 'same',
kernel_initializer = 'he_normal')(pool2)
    conv3 = BatchNormalization()(conv3)
    conv3 = Activation('relu')(conv3)
    conv3 = Conv3D(256, kernel_size=(3, 3, 3), dilation_rate=2,padding = 'same',
kernel_initializer = 'he_normal')(conv3)
    conv3 = BatchNormalization()(conv3)
    up3 = Activation('relu')(conv3)
    pool3 = MaxPooling3D(pool_size=(2,2,2),strides = (2,2,2))(up3)
    conv4 = Conv3D(256, kernel_size=(3, 3, 3),  dilation_rate=2,padding =
'same', kernel_initializer = 'he_normal')(pool3)
    conv4 = BatchNormalization()(conv4)
    conv4 = Activation('relu')(conv4)
    conv4 = Conv3D(512, kernel_size=(3, 3, 3),  dilation_rate=2,padding =
'same', kernel_initializer = 'he_normal')(conv4)
    conv4 = BatchNormalization()(conv4)
    up4 = Activation('relu')(conv4)
    pool4 = MaxPooling3D(pool_size=(2,2,2),strides = (2,2,2))(up4)
    conv5 = Conv3D(512, kernel_size=(3, 3, 3),  dilation_rate=2,padding =
'same', kernel_initializer = 'he_normal')(pool4)
    conv5 = BatchNormalization()(conv5)
    conv5 = Activation('relu')(conv5)
    conv5 = Conv3D(1024, kernel_size=(3, 3, 3),  dilation_rate=2,padding =
'same', kernel_initializer = 'he_normal')(conv5)
    conv5 = BatchNormalization()(conv5)
    conv5 = Activation('relu')(conv5)
    drop5 = Conv3DTranspose(1024, kernel_size=(2, 2, 2),strides=(2, 2, 2),
activation = 'relu', dilation_rate=2,padding = 'same', kernel_initializer =
'he_normal')(conv5)

    merge6 = concatenate([drop5,up4], axis = 4)
    conv6 = Conv3D(512, kernel_size=(3, 3, 3),  dilation_rate=2,padding =
'same', kernel_initializer = 'he_normal')(merge6)
    conv6 = BatchNormalization()(conv6)
    conv6 = Activation('relu')(conv6)
    conv6 = Conv3D(512, kernel_size=(3, 3, 3),  dilation_rate=2,padding =
'same', kernel_initializer = 'he_normal')(conv6)
```

```
    conv6 = BatchNormalization()(conv6)
    conv6 = Activation('relu')(conv6)
    drop6 = Conv3DTranspose(512, kernel_size=(2, 2, 2),strides=(2, 2, 2),
activation = 'relu', dilation_rate=2,padding = 'same', kernel_initializer =
'he_normal')(conv6)
    merge7 = concatenate([drop6,up3], axis = 4)

    conv7 = Conv3D(256, kernel_size=(3, 3, 3), dilation_rate=2,padding = 'same',
kernel_initializer = 'he_normal')(merge7)
    conv7 = BatchNormalization()(conv7)
    conv7 = Activation('relu')(conv7)
    conv7 = Conv3D(256, kernel_size=(3, 3, 3), dilation_rate=2,padding = 'same',
kernel_initializer = 'he_normal')(conv7)
    conv7 = BatchNormalization()(conv7)
    conv7 = Activation('relu')(conv7)
    drop7 = Conv3DTranspose(256, kernel_size=(2, 2, 2),strides=(2, 2, 2),
activation = 'relu', dilation_rate=2,padding = 'same', kernel_initializer =
'he_normal')(conv7)
    merge8 = concatenate([drop7,up2], axis = 4)


    conv8 = Conv3D(128, kernel_size=(3, 3, 3),  dilation_rate=2,padding =
'same', kernel_initializer = 'he_normal')(merge8)
    conv8 = BatchNormalization()(conv8)
    conv8 = Activation('relu')(conv8)
    conv8 = Conv3D(128, kernel_size=(3, 3, 3),  dilation_rate=2,padding =
'same', kernel_initializer = 'he_normal')(conv8)
    conv8 = BatchNormalization()(conv8)
    conv8 = Activation('relu')(conv8)
    drop8 = Conv3DTranspose(128, kernel_size=(2, 2, 2),strides=(2, 2, 2),
activation = 'relu', dilation_rate=2,padding = 'same', kernel_initializer =
'he_normal')(conv8)
    conv8 = BatchNormalization()(drop8)
    conv8 = Activation('relu')(conv8)
    drop8 = Conv3DTranspose(128, kernel_size=(2, 2, 2),strides=(2, 2, 2),
activation = 'relu', dilation_rate=2,padding = 'same', kernel_initializer =
'he_normal')(conv7)


    merge9 = concatenate([drop8,up1], axis = 4)
    conv9 = Conv3D(64, kernel_size=(3, 3, 3),  dilation_rate=2,padding = 'same',
kernel_initializer = 'he_normal')(merge9)
    conv9 = BatchNormalization()(conv9)
    conv9 = Activation('relu')(conv9)
    conv9 = Conv3D(64, kernel_size=(3, 3, 3),  dilation_rate=2,padding = 'same',
kernel_initializer = 'he_normal')(conv9)
    conv9 = BatchNormalization()(conv9)
    conv9 = Activation('relu')(conv9)

    conv9 = Conv3D(n_class, kernel_size=(1, 1, 1),activation = 'softmax')(conv9)




    model = tensorflow.keras.Model(inputs = inputs, outputs = conv9)
    model.compile(optimizer = Adam(lr = 0.0005),loss =
'sparse_categorical_crossentropy', metrics = ['sparse_categorical_accuracy'])
```

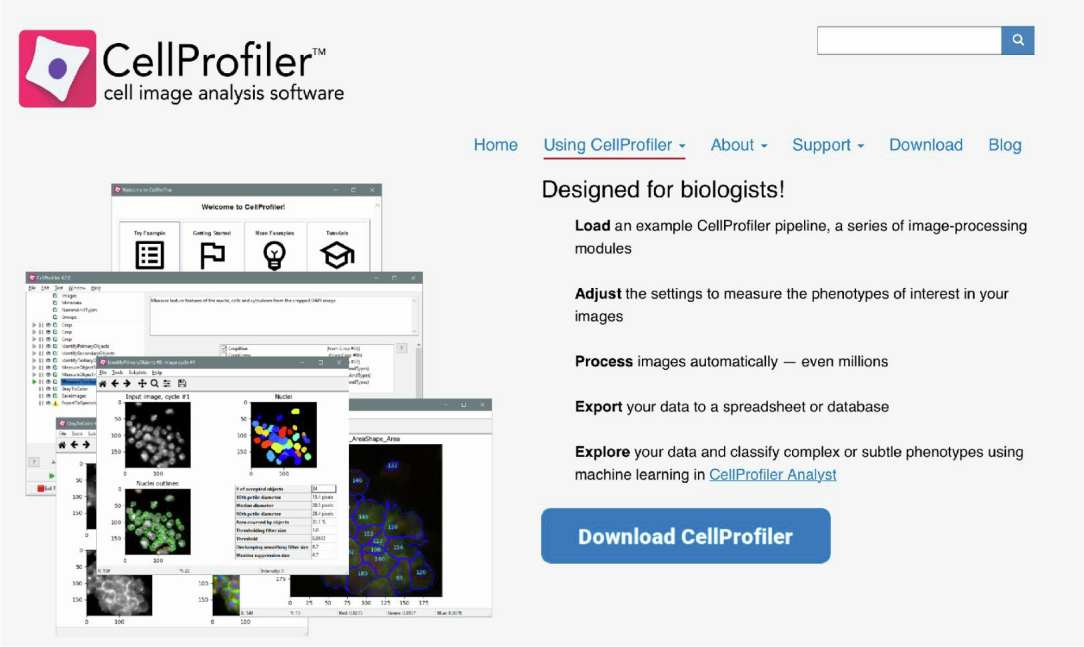
```
    if(pretrained_weights):
      model=keras.models.load_model(pretrained_weights)

    return model

#%% load the trained 3D Unet network
#model.load_weights("C:/Users/John/20211118_Python_biostudies/Python/3D_UNET_PYT
HON/3D_UNET_PYTHON/3_Saved_models/my_h53D_model.h5")
pretrained_model = unet3()
pretrained_model.load_weights("C:/Users/John/20211118_Python_biostudies/Python/3
D_UNET_PYTHON/3D_UNET_PYTHON/3_Saved_models/my_h53D_model_updated.h5")


#%% Patch the reflectance data through the network
##########################################################################
##############################################
# Set the patch sizes to be passed to the net

patchSize = [64, 64, 32]
# Segment blockwise then reassemble in full
# Define image dimensions
Shape_overall = np.shape(IM_DATA_norm)
height = Shape_overall[0]
width = Shape_overall[1]
depth = Shape_overall[2]

if len(Shape_overall) == 3:
    nChannel = 1
else:
    nChannel = Shape_overall[3]


patch = np.zeros([patchSize[0],patchSize[1],patchSize[2],
nChannel],dtype=IM_DATA_norm.dtype);
number_of_height_patches = math.ceil(height/patchSize[0]);
number_of_width_patches = math.ceil(width/patchSize[1]);
number_of_depth_patches = math.ceil(depth/patchSize[2]);

#Pad image to have dimensions as multiples of patchSize
height_pad = number_of_height_patches*patchSize[0];
width_pad  = number_of_width_patches*patchSize[1];
depth_pad  = number_of_depth_patches*patchSize[2];

# Amount to pad different dimensions of image
padSize = np.empty([1, 3])

# Pad the image by correct amounts
padSize[0,0] = height_pad;
padSize[0,1] = width_pad;
padSize[0,2] = depth_pad;

Starting_shape = np.shape(IM_DATA_norm)
a1 = padSize[0,0]
a2 = padSize[0,1]
a3 = padSize[0,2]
im_pad = np.zeros([int(a1),int(a2),int(a3)])

im_pad[:IM_DATA_norm.shape[0],:IM_DATA_norm.shape[1],:IM_DATA_norm.shape[2]] =
IM_DATA_norm[:,:,:]
```

```
im_pad = np.asarray(im_pad).reshape((im_pad.shape[0],
im_pad.shape[1],im_pad.shape[2],1))
[height_pad, width_pad, depth_pad, nChannel_pad] = np.shape(im_pad);

#Preallocate some matrices to catch the probability maps
out_Uncertainty_Scores = np.zeros([height_pad, width_pad, depth_pad],'double');
out_scores_from_network_all_classes = np.zeros([height_pad, width_pad,
depth_pad, 4], 'double');


#Loop through blocks of 'patchSize'
for loop_height in range(1,number_of_height_patches+1):

    for loop_width in range(1,number_of_width_patches+1):

        for loop_depth in range(1,number_of_depth_patches+1):
            PP3 = np.empty([1,64,64,32,1])
            start_height_position=(loop_height-1)*patchSize[0];
            end_height_position=loop_height*patchSize[0];

            start_width_position=(loop_width-1)*patchSize[1];
            end_width_position=loop_width*patchSize[1];

            start_depth_position=(loop_depth-1)*patchSize[2];
            end_depth_position=loop_depth*patchSize[2];


patch_to_deploy=im_pad[start_height_position:end_height_position,start_width_pos
ition:end_width_position,start_depth_position:end_depth_position,:];
            PP3[0,:,:,:]=patch_to_deploy
            # deploy net
            predictions = pretrained_model.predict(PP3)

            scores = np.max(predictions,axis=4)
            predictions.argmax(axis=3)




out_Uncertainty_Scores[start_height_position:end_height_position,start_width_pos
ition:end_width_position,start_depth_position:end_depth_position]=scores


out_scores_from_network_all_classes[start_height_position:end_height_position,st
art_width_position:end_width_position,start_depth_position:end_depth_position,0]
=predictions[0,:,:,:,0];

out_scores_from_network_all_classes[start_height_position:end_height_position,st
art_width_position:end_width_position,start_depth_position:end_depth_position,1]
=predictions[0,:,:,:,1];

out_scores_from_network_all_classes[start_height_position:end_height_position,st
art_width_position:end_width_position,start_depth_position:end_depth_position,2]
=predictions[0,:,:,:,2];

out_scores_from_network_all_classes[start_height_position:end_height_position,st
art_width_position:end_width_position,start_depth_position:end_depth_position,3]
=predictions[0,:,:,:,3];
```

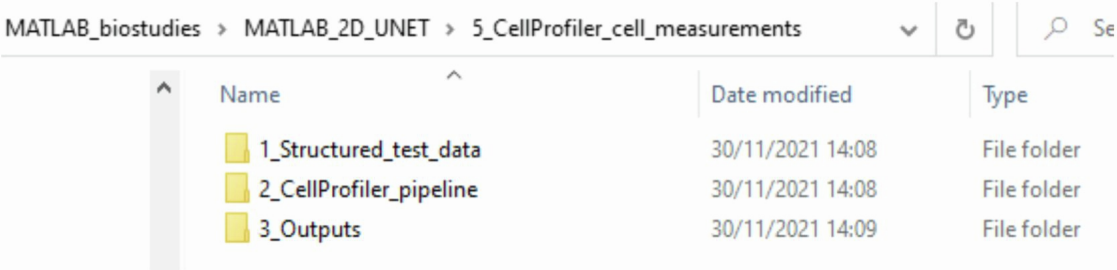
```
#Script is general for n classes to this point
out_Pmap_cat1 = out_scores_from_network_all_classes[:,:,:,0];
out_Pmap_cat2 = out_scores_from_network_all_classes[:,:,:,1];
out_Pmap_cat3 = out_scores_from_network_all_classes[:,:,:,2];

out_Uncertainty_Scores = out_Uncertainty_Scores[0:height, 0:width, 0:depth];

#Remove padding from probability maps
out_Pmap_cat1 = out_Pmap_cat1[0:height, 0:width, 0:depth];
out_Pmap_cat2 = out_Pmap_cat2[0:height, 0:width, 0:depth];
out_Pmap_cat3 = out_Pmap_cat3[0:height, 0:width, 0:depth];

#%%visualise network outputs
fig, (ax1, ax2, ax3, ax4) = plt.subplots(1, 4, sharey=True)
fig.set_size_inches(14, 12)
im = ax1.imshow(out_Pmap_cat1[:,:,59])
ax1.title.set_text('Background/Other')
im = ax2.imshow(out_Pmap_cat2[:,:,59])
ax2.title.set_text('LF-nuclei')
im = ax3.imshow(out_Pmap_cat3[:,:,59])
ax3.title.set_text('LF-actin')
im = ax4.imshow(out_Uncertainty_Scores[:,:,59])
ax4.title.set_text('Uncertainty')
plt.savefig('Predictions.png')

#%%Map to 16-bit and save for loading into CellProfiler
data_norm_holder = np.zeros((1024, 1024, 107),np.uint16)
ui16_PMAP_cat1_xy = cv2.normalize(out_Pmap_cat1, data_norm_holder , 65535.0,
0.0, cv2.NORM_MINMAX)
data_norm_holder = np.zeros((1024, 1024, 107),np.uint16)
ui16_PMAP_cat2_xy = cv2.normalize(out_Pmap_cat2, data_norm_holder , 65535.0,
0.0, cv2.NORM_MINMAX)
data_norm_holder = np.zeros((1024, 1024, 107),np.uint16)
ui16_PMAP_cat3_xy = cv2.normalize(out_Pmap_cat3, data_norm_holder , 65535.0,
0.0, cv2.NORM_MINMAX)
data_to_save = np.zeros((1024,1024,107,3))
data_to_save[:,:,:,0] = ui16_PMAP_cat1_xy
data_to_save[:,:,:,1] = ui16_PMAP_cat2_xy
data_to_save[:,:,:,2] = ui16_PMAP_cat3_xy

#%% write files out as multipage tiff
for channel in range(0,3):
    image_to_save = (data_to_save[:,:,:,channel]).astype(dtype=np.uint16)
    rearranged_image_to_save = np.transpose(image_to_save, axes=[2,0,1])

imageio.mimwrite('Ch_'+str(channel+3)+'_lm_001'+'.tiff',rearranged_image_to_sav
e)
```

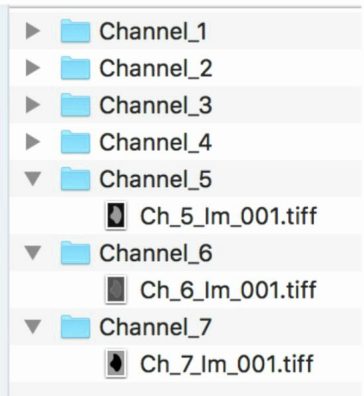
Extracting single-cell features using label-free cell segmentation and CellProfiler 4

- Download and install CellProfiler4 from the CellProfiler website.
https://cellprofiler.org



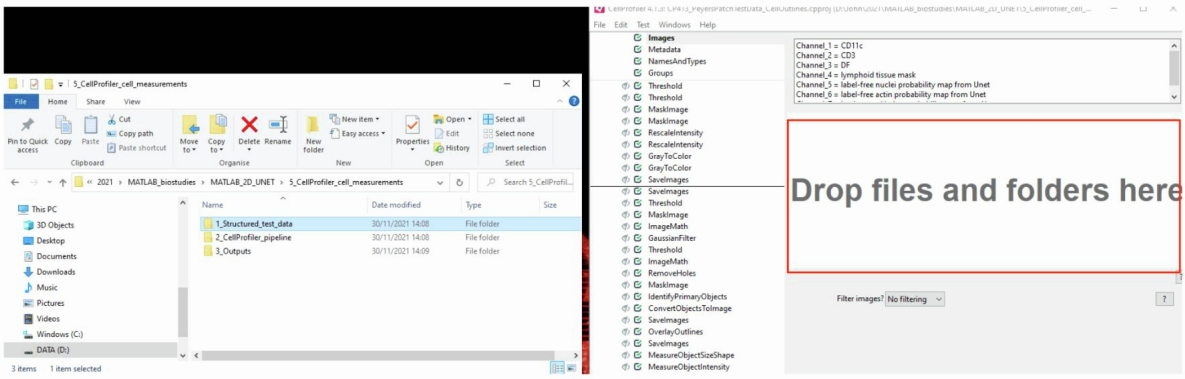
1. 2-D CellProfiler Pipeline
-A screencast video is included with the BioStudies archive.
- Folder 5 "5_CellProfiler_cell_measurements" in the 2-D BioStudies project archive contains example data, a CellProfiler pipeline and the subsequent CellProfiler single-cell outputs:



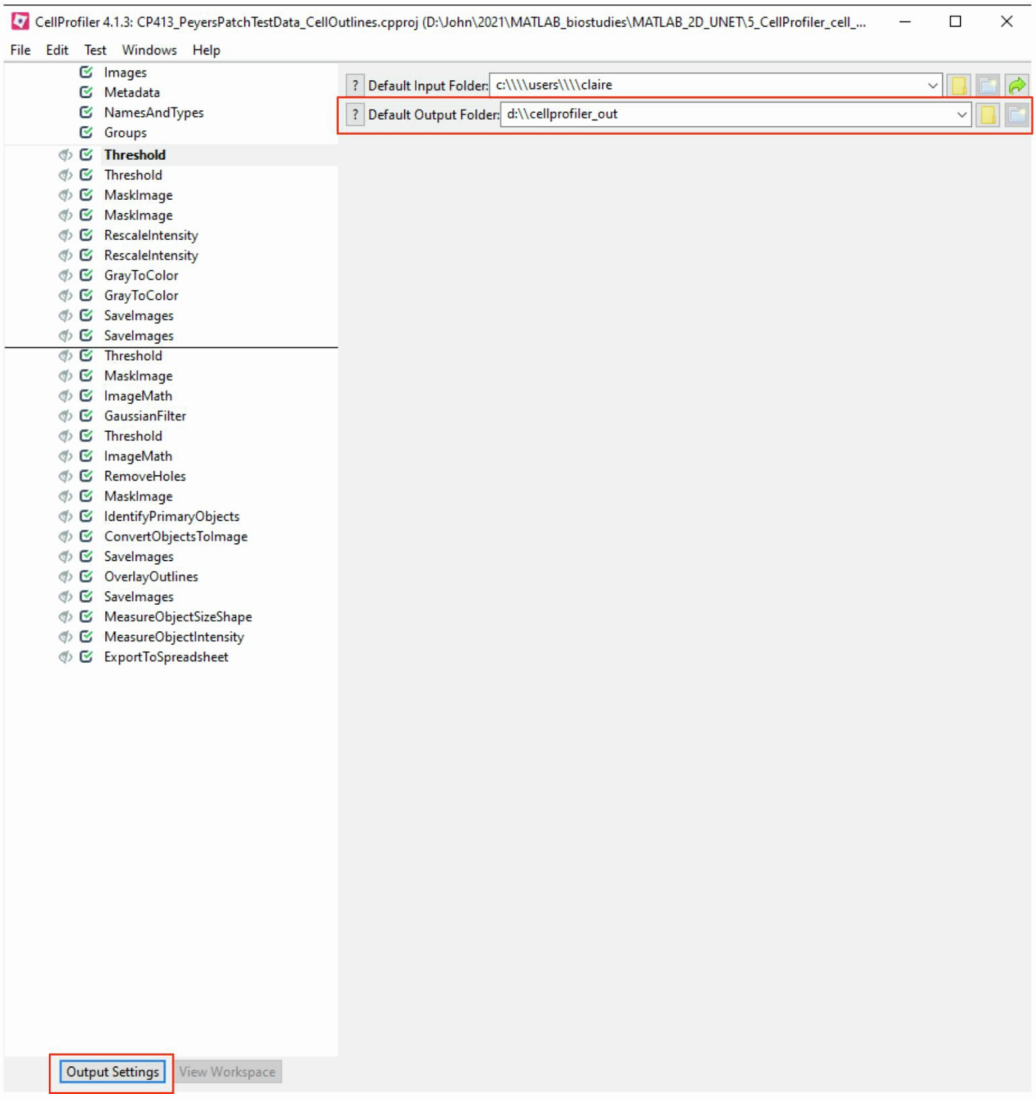


- The image-data (inside 1_Structured_test_data) contains four immunofluorescence channels followed by the probability maps obtained for the label-free nuclei, actin and background/other classes from either the Python or MATLAB deep learning scripts (channels 5, 6 and 7, respectively).

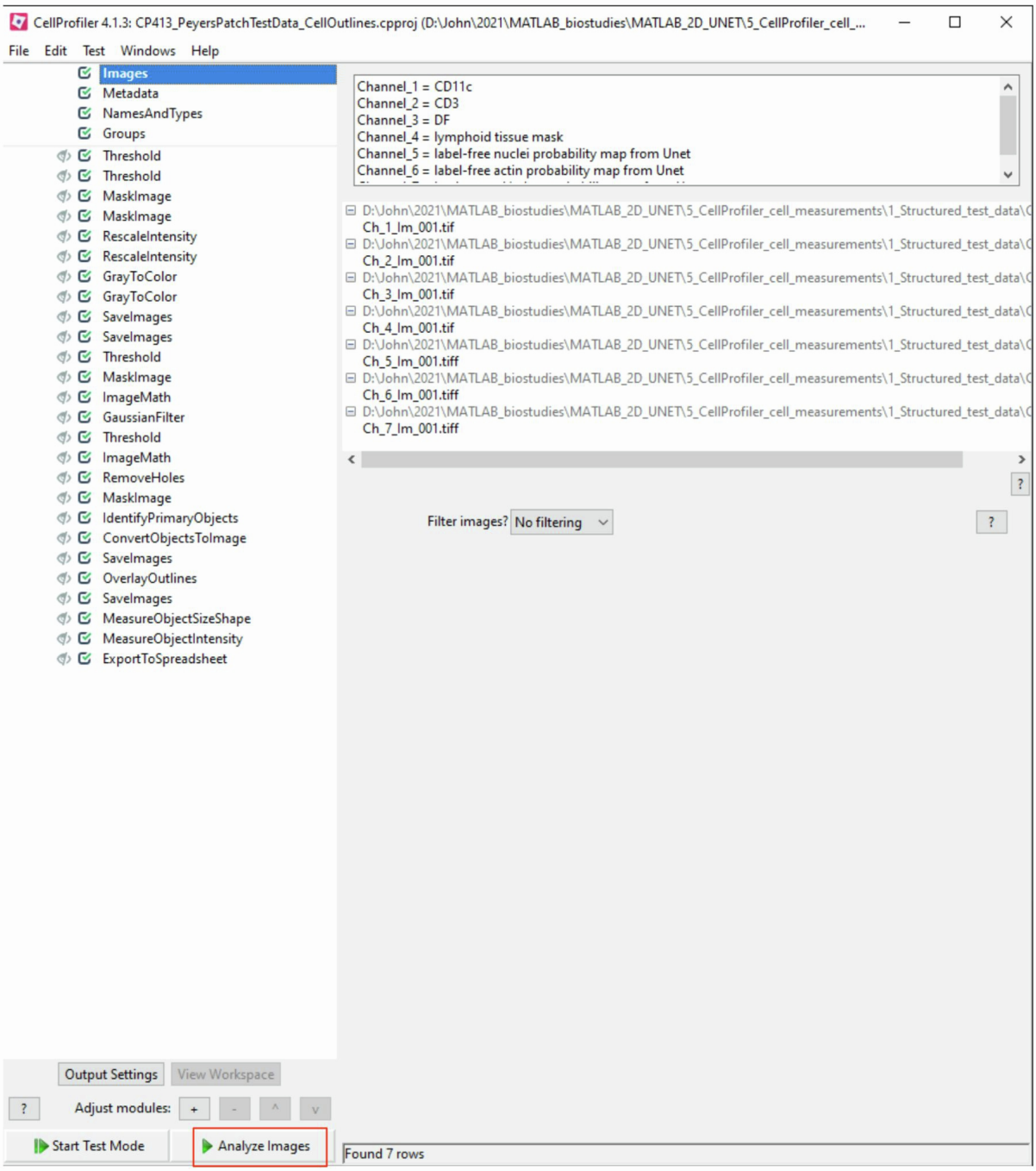
- Open CellProfiler 4 and load the image analysis pipeline from the “2_CellProfiler_pipeline” folder.
- Drag-and-drop the “1_structured_test_data” folder into the CellProfiler image-window to load the 2-D image-data:



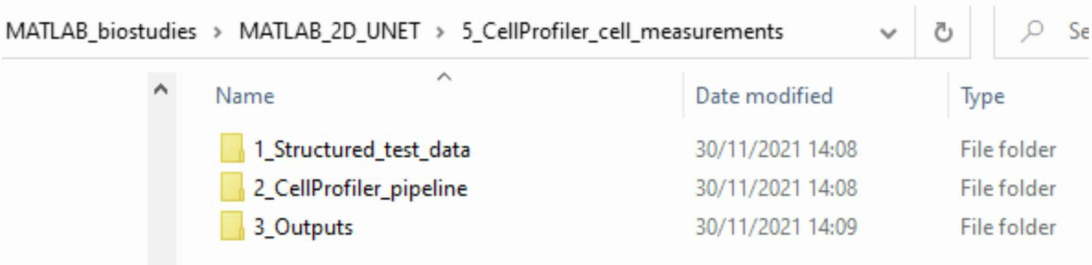
- Choose where to save the outputted cell features by clicking on the “Output Settings” tab at the bottom-left of the CellProfiler dialogue.



- To run the CellProfiler pipeline and save the cell features and other pipeline outputs at the specified location, click “Analyse Images” at the bottom-left of the CellProfiler screen.



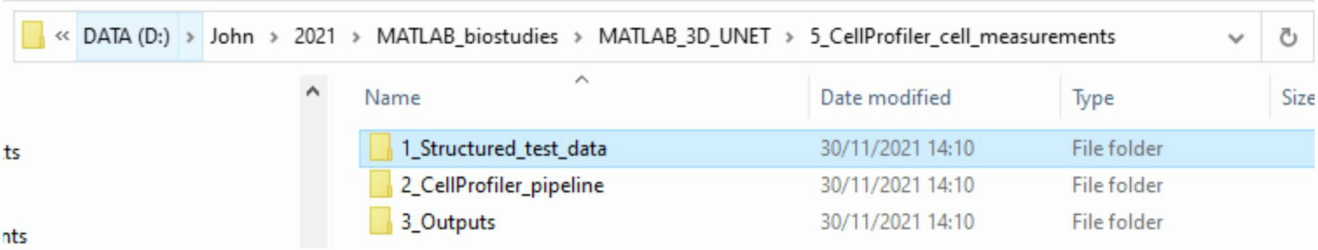
- Previously-saved CellProfiler outputs are also available inside the “3_Outputs” folder:

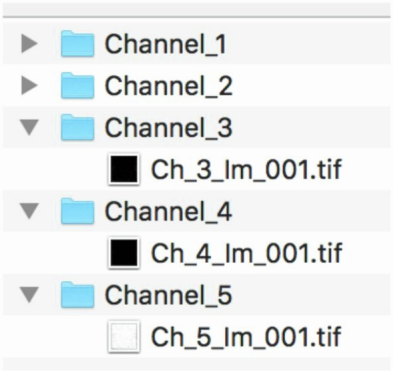


2. 3-D CellProfiler Pipeline

-A screencast video is included with the BioStudies archive.

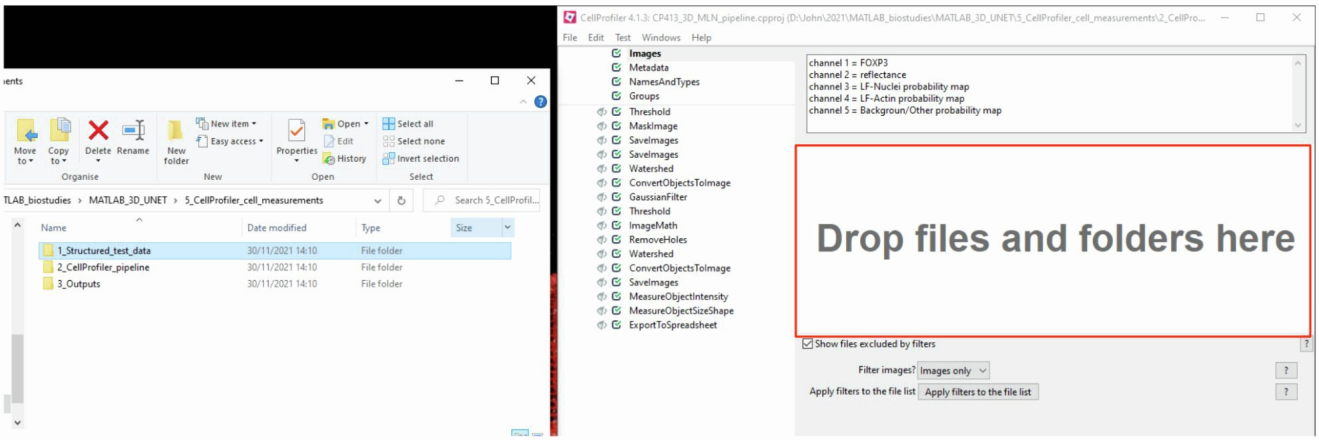
- Folder 5 “5_CellProfiler_cell_measurements” in the 3-D BioStudies project archive contains example data, a 3-D CellProfiler pipeline and the subsequent CellProfiler single-cell outputs:



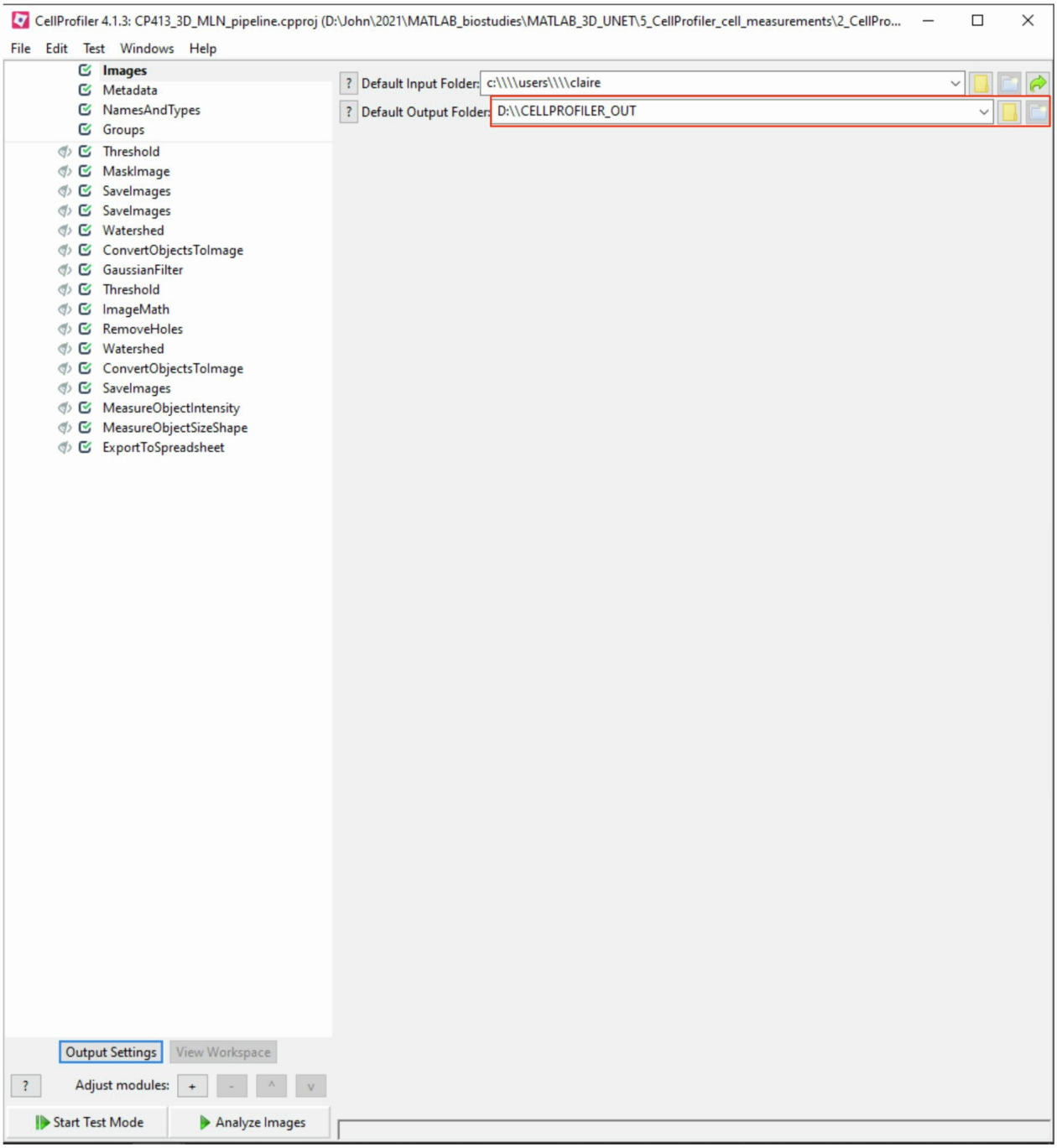


- The image-data (inside 1_Structured_test_data) contains two immunofluorescence channels followed by the 3-D probability maps obtained for the label-free nuclei, actin and background/other classes from either the Python or MATLAB deep learning scripts (channels 3, 4 and 5, respectively).

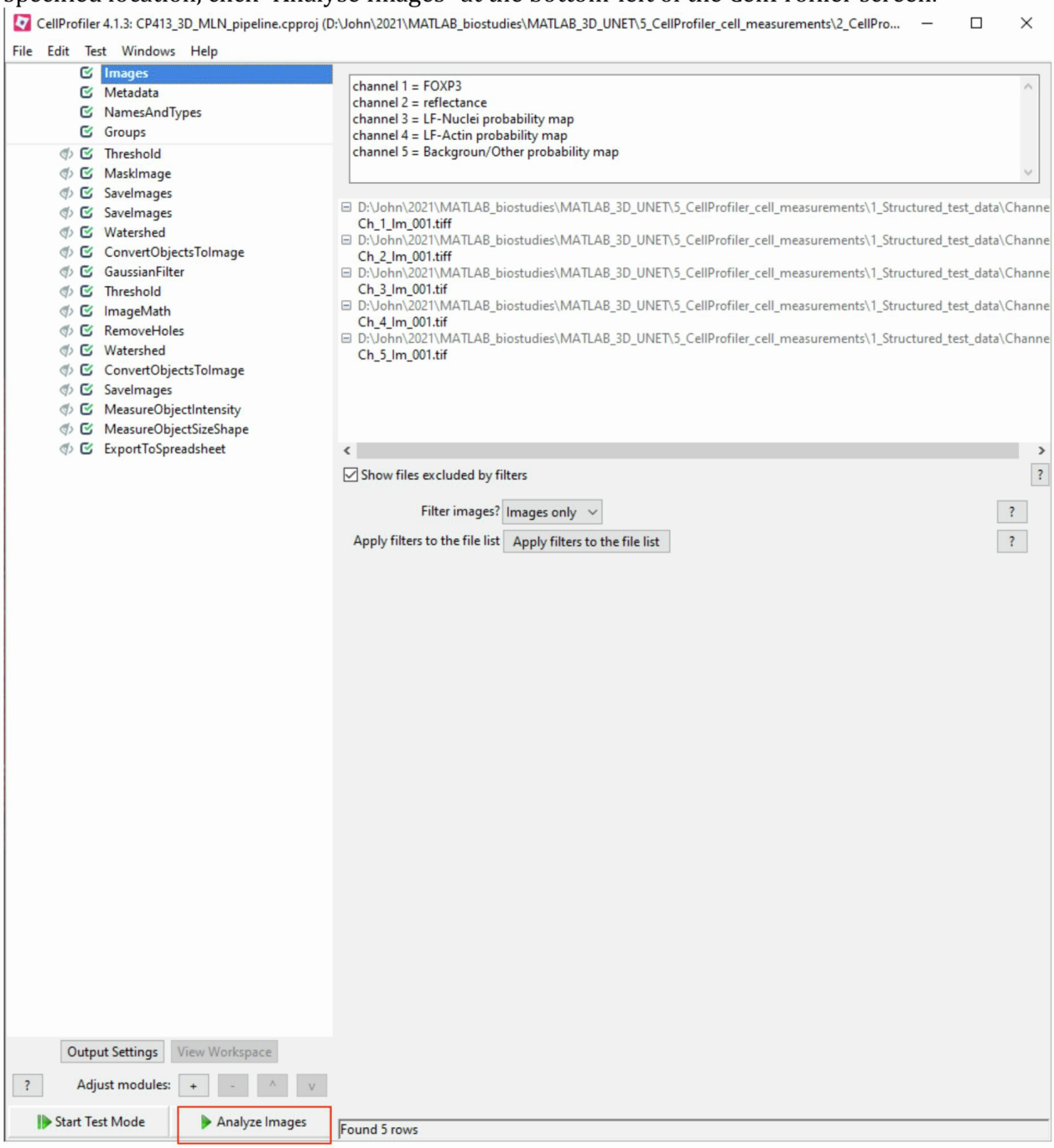
- Open CellProfiler 4 and load the image analysis pipeline from the “2_CellProfiler_pipeline” folder.
- Drag-and-drop the “1_structured_test_data” folder into the CellProfiler image-window to load the 2-D image-data:



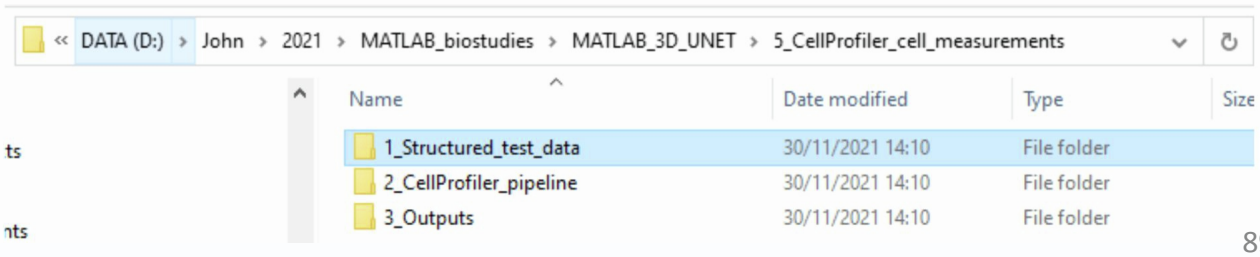
- Choose where to save the outputted cell features by clicking on the “Output Settings” tab at the bottom-left of the CellProfiler dialogue.



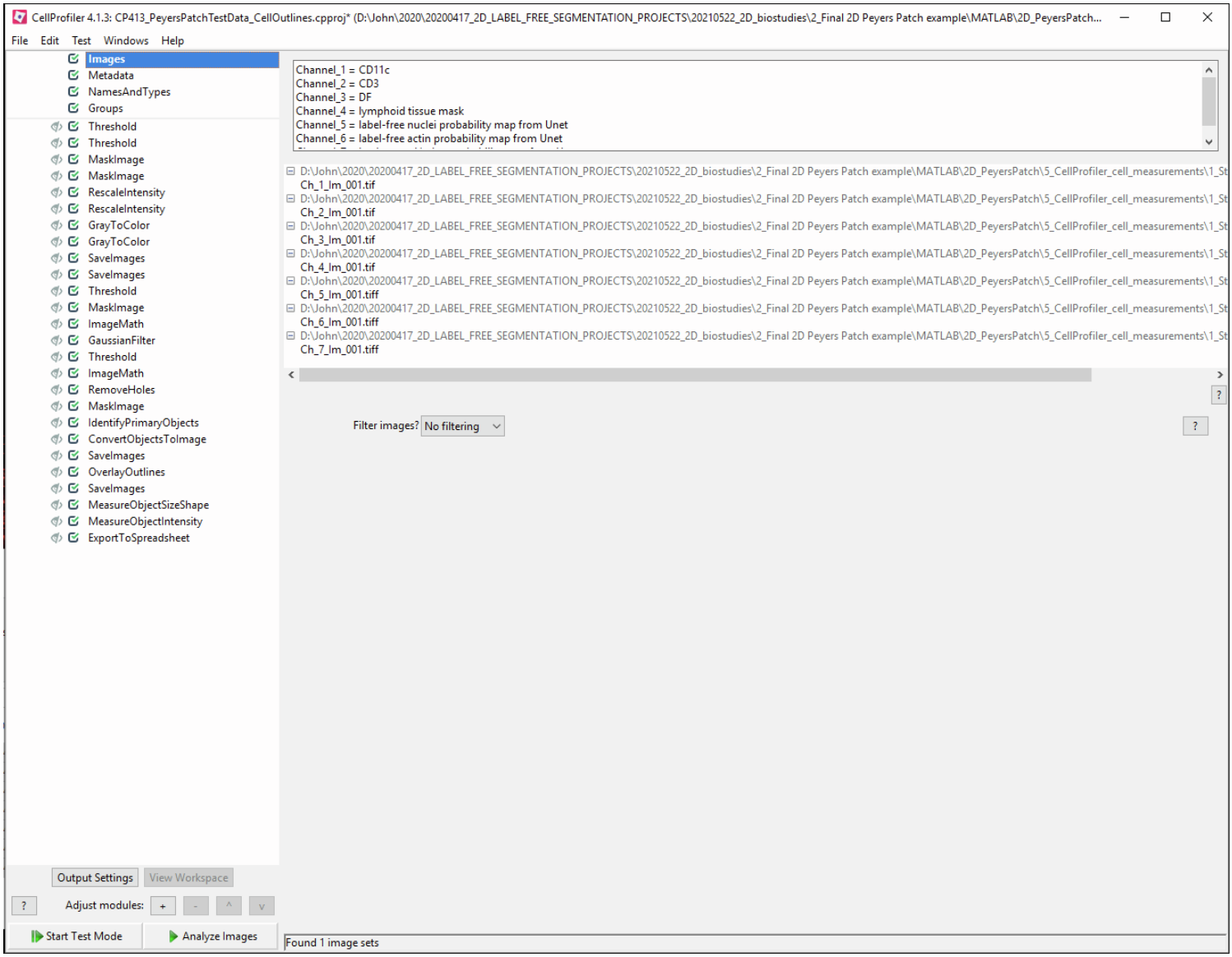
- To run the CellProfiler pipeline and save the cell features and other pipeline outputs at the specified location, click “Analyse Images” at the bottom-left of the CellProfiler screen.

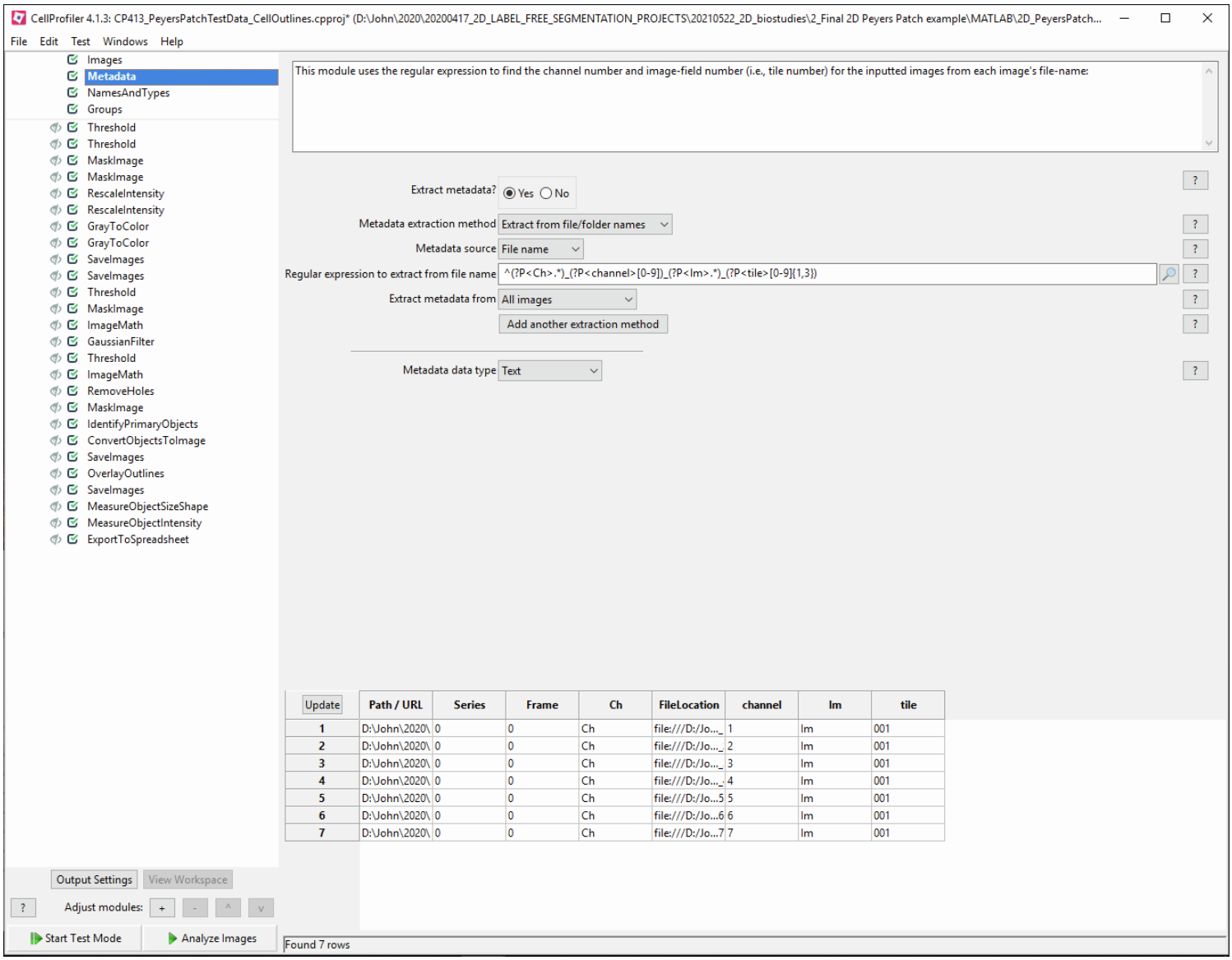


- Previously-saved CellProfiler outputs are also available inside the “3_Outputs” folder:



2-D Cell Profiler image analysis pipelines. This section presents screenshots of a CellProfiler image analysis pipeline used to achieve label-free cell segmentation in 2-D from the Unet network outputs, and to measure the intensity and size/shape features of identified cell-objects. To use the image analysis pipeline with new image data, the 'IdentifyPrimaryObjects' module simply needs adjusting so that the 'typical diameter of objects' size-range matches the pixel scaling of the new images. For newcomers to CellProfiler, we recommend downloading the image-data and pipeline from BioStudies database https://www.ebi.ac.uk/biostudies/ under accession number S-BSST742. This enables the pipeline to be run with the data described in the manuscript and allows the user to see how each module works.




CellProfiler 4.1.3: CP413_PeyersPatchTestData_CellOutlines.cpproj* (D:\John\2020\20200417_2D_LABEL_FREE_SEGMENTATION_PROJECTS\20210522_2D_biostudies\2_Final 2D Peyers Patch example\MATLAB\2D_PeyersPatch...
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ImageMath
GaussianFilter
Threshold
ImageMath
RemoveHoles
MaskImage
IdentifyPrimaryObjects
ConvertObjectsToImage
SaveImages
OverlayOutlines
SaveImages
MeasureObjectSizeShape
MeasureObjectIntensity
ExportToSpreadsheet
This module uses the regular expression to find the channel number and image-field number (i.e., tile number) for the inputted images from each image's file-name:
Extract metadata? Yes No
Metadata extraction method Extract from file/folder names
Metadata source File name
Regular expression to extract from file name ^(?P<Ch>.*)_(?P<channel>[0-9])_(?P<Im>.*)_(?P<tile>[0-9]{1,3})
Extract metadata from All images
Add another extraction method
Metadata data type Text
Update
Path / URL Series Frame Ch FileLocation channel Im tile
1 D:\John\2020\ 0 0 Ch file:///D:/Jo..._ 1 Im 001
2 D:\John\2020\ 0 0 Ch file:///D:/Jo..._ 2 Im 001
3 D:\John\2020\ 0 0 Ch file:///D:/Jo..._ 3 Im 001
4 D:\John\2020\ 0 0 Ch file:///D:/Jo..._ 4 Im 001
5 D:\John\2020\ 0 0 Ch file:///D:/Jo...5 5 Im 001
6 D:\John\2020\ 0 0 Ch file:///D:/Jo...6 6 Im 001
7 D:\John\2020\ 0 0 Ch file:///D:/Jo...7 7 Im 001
Output Settings
View Workspace
Adjust modules:
Start Test Mode
Analyze Images
Found 7 rows

CellProfiler 4.1.3: CP413_PeyersPatchTestData_CellOutlines.cpproj* (D:\John\2020\20200417_2D_LABEL_FREE_SEGMENTATION_PROJECTS\20210522_2D_biostudies\2_Final 2D Peyers Patch example\MATLAB\2D_PeyersPatch...

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ExportToSpreadsheet

Output Settings | View Workspace

? Adjust modules: + - ^ v

Start Test Mode | Analyze Images

Loads images and associates them together according the specified rule criteria

Assign a name to: Images matching rules

Process as 3D? ○ Yes ◉ No

Select the rule criteria: Match All of the following rules
Directory Does Contain Channel_1

Name to assign these images: c1

Select the image type: Grayscale image

Set intensity range from: Image bit-depth

Duplicate this image

Select the rule criteria: Match All of the following rules
Directory Does Contain Channel_2

Name to assign these images: c2

Select the image type: Grayscale image

Set intensity range from: Image bit-depth

Duplicate this image

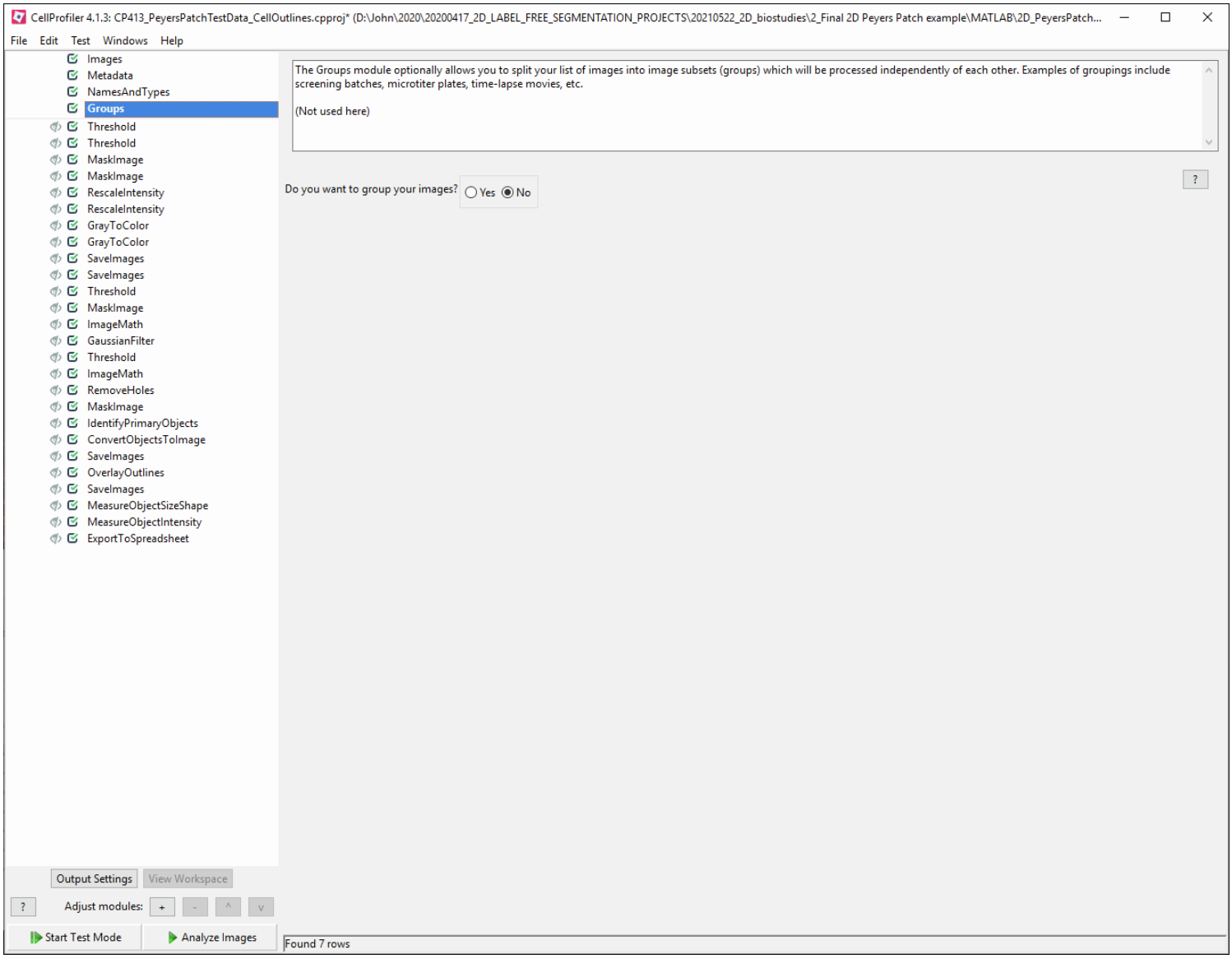
Remove this image

Select the rule criteria: Match All of the following rules
Directory Does Contain Channel_3

Name to assign these images: c3

Update	c1	c2	c3	c4	c5	c6	c7
1	Ch_1_Im_001.tif	Ch_2_Im_001.tif	Ch_3_Im_001.tif	Ch_4_Im_001.tif	Ch_5_Im_001.tiff	Ch_6_Im_001.tiff	Ch_7_Im_001.tiff

Found 7 rows


CellProfiler 4.1.3: CP413_PeyersPatchTestData_CellOutlines.cpproj* (D:\John\2020\20200417_2D_LABEL_FREE_SEGMENTATION_PROJECTS\20210522_2D_biostudies\2_Final 2D Peyers Patch example\MATLAB\2D_PeyersPatch...
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RemoveHoles
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IdentifyPrimaryObjects
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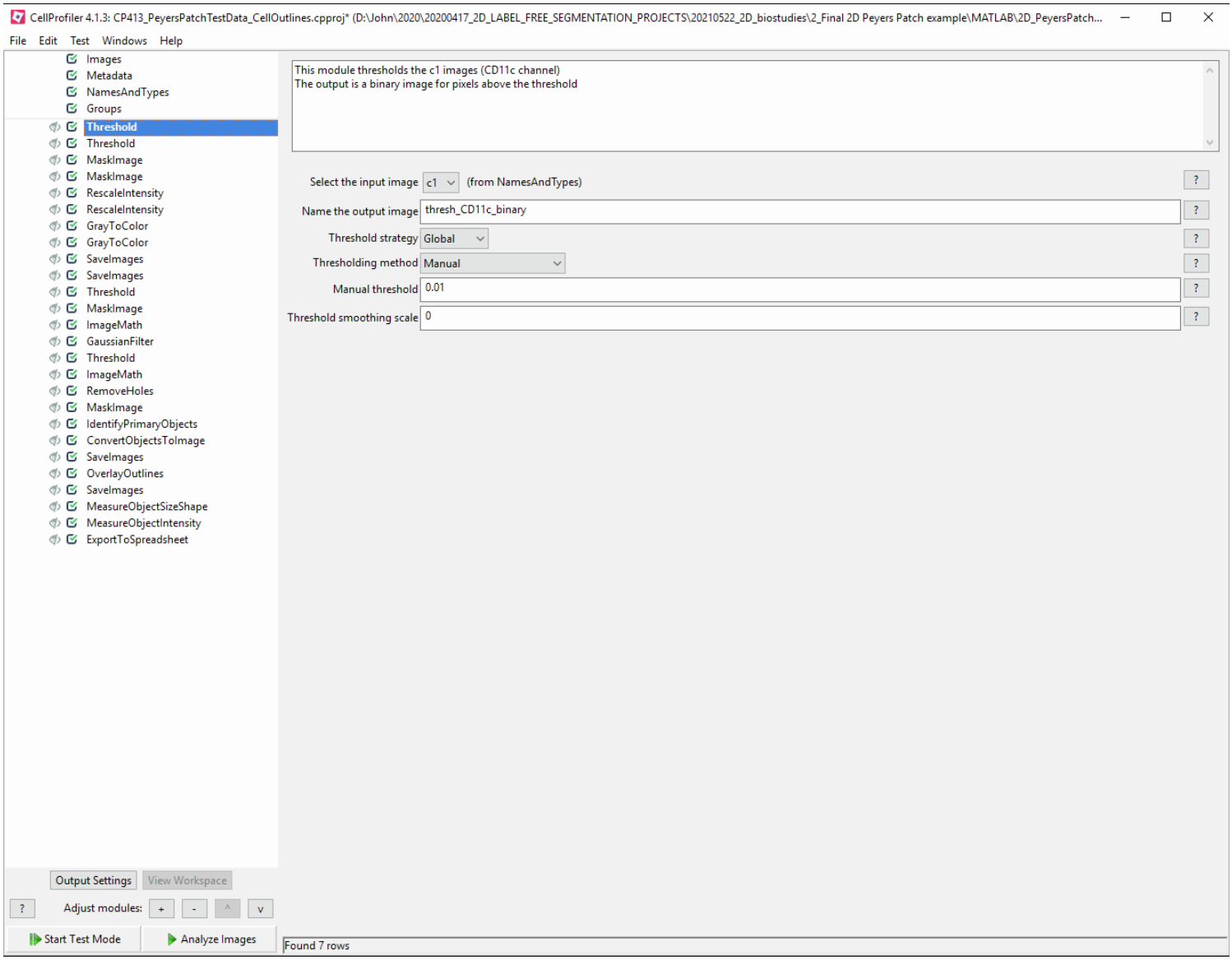
SaveImages
MeasureObjectSizeShape
MeasureObjectIntensity
ExportToSpreadsheet
The Groups module optionally allows you to split your list of images into image subsets (groups) which will be processed independently of each other. Examples of groupings include screening batches, microtiter plates, time-lapse movies, etc.
(Not used here)
Do you want to group your images?
Yes
No
?
Output Settings
View Workspace
?
Adjust modules:
+
-
^
v
Start Test Mode
Analyze Images
Found 7 rows


CellProfiler 4.1.3: CP413_PeyersPatchTestData_CellOutlines.cpproj* (D:\John\2020\20200417_2D_LABEL_FREE_SEGMENTATION_PROJECTS\20210522_2D_biostudies\2_Final 2D Peyers Patch example\MATLAB\2D_PeyersPatch...
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GaussianFilter
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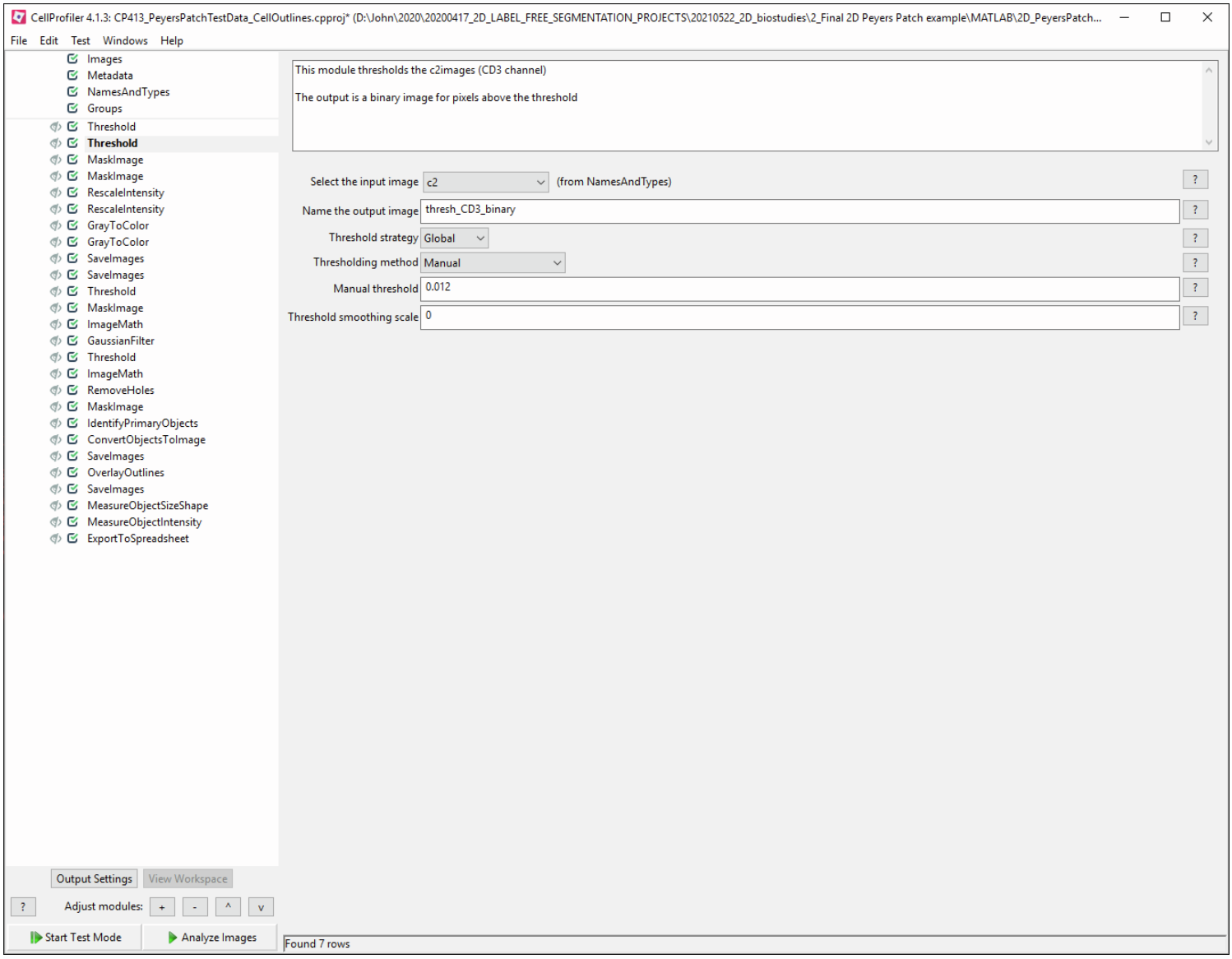
ImageMath
RemoveHoles
MaskImage
IdentifyPrimaryObjects
ConvertObjectsToImage
SaveImages
OverlayOutlines
SaveImages
MeasureObjectSizeShape
MeasureObjectIntensity
ExportToSpreadsheet
This module thresholds the c1 images (CD11c channel)
The output is a binary image for pixels above the threshold
Select the input image
c1
(from NamesAndTypes)
Name the output image
thresh_CD11c_binary
Threshold strategy
Global
Thresholding method
Manual
Manual threshold
0.01
Threshold smoothing scale
0
Output Settings
View Workspace
Adjust modules:
Start Test Mode
Analyze Images
Found 7 rows


CellProfiler 4.1.3: CP413_PeyersPatchTestData_CellOutlines.cpproj* (D:\John\2020\20200417_2D_LABEL_FREE_SEGMENTATION_PROJECTS\20210522_2D_biostudies\2_Final 2D Peyers Patch example\MATLAB\2D_PeyersPatch...
File Edit Test Windows Help
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GrayToColor
SaveImages
SaveImages
Threshold
MaskImage
ImageMath
GaussianFilter
Threshold
ImageMath
RemoveHoles
MaskImage
IdentifyPrimaryObjects
ConvertObjectsToImage
SaveImages
OverlayOutlines
SaveImages
MeasureObjectSizeShape
MeasureObjectIntensity
ExportToSpreadsheet
This module thresholds the c2images (CD3 channel)
The output is a binary image for pixels above the threshold
Select the input image
c2
(from NamesAndTypes)
Name the output image
thresh_CD3_binary
Threshold strategy
Global
Thresholding method
Manual
Manual threshold
0.012
Threshold smoothing scale
0
Output Settings
View Workspace
Adjust modules:
Start Test Mode
Analyze Images
Found 7 rows

CellProfiler 4.1.3: CP413_PeyersPatchTestData_CellOutlines.cpproj* (D:\John\2020\20200417_2D_LABEL_FREE_SEGMENTATION_PROJECTS\20210522_2D_biostudies\2_Final 2D Peyers Patch example\MATLAB\2D_PeyersPatch...

File Edit Test Windows Help

- Images
- Metadata
- NamesAndTypes
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- Threshold
- **MaskImage**
- MaskImage
- RescaleIntensity
- RescaleIntensity
- GrayToColor
- GrayToColor
- SaveImages
- SaveImages
- Threshold
- MaskImage
- ImageMath
- GaussianFilter
- Threshold
- ImageMath
- RemoveHoles
- MaskImage
- IdentifyPrimaryObjects
- ConvertObjectsToImage
- SaveImages
- OverlayOutlines
- SaveImages
- MeasureObjectSizeShape
- MeasureObjectIntensity
- ExportToSpreadsheet

This module creates a greyscale version of the c1 (CD11c) data - where the threshold calculated above is applied.

The outputted image from this module can be measured in each cell-object to calculate per-cell intensity values AFTER the background has been thresholded out. This is particulary important here, as integration of this image in cell objects allows for background correction according to the tissue-matched secondary-only and isotype controls.

Select the input image: c1 (from NamesAndTypes)

Name the output image: thresh_CD11c_greyscale

Use objects or an image as a mask?: Image

Select image for mask: thresh_CD11c_binary (from Threshold #05)

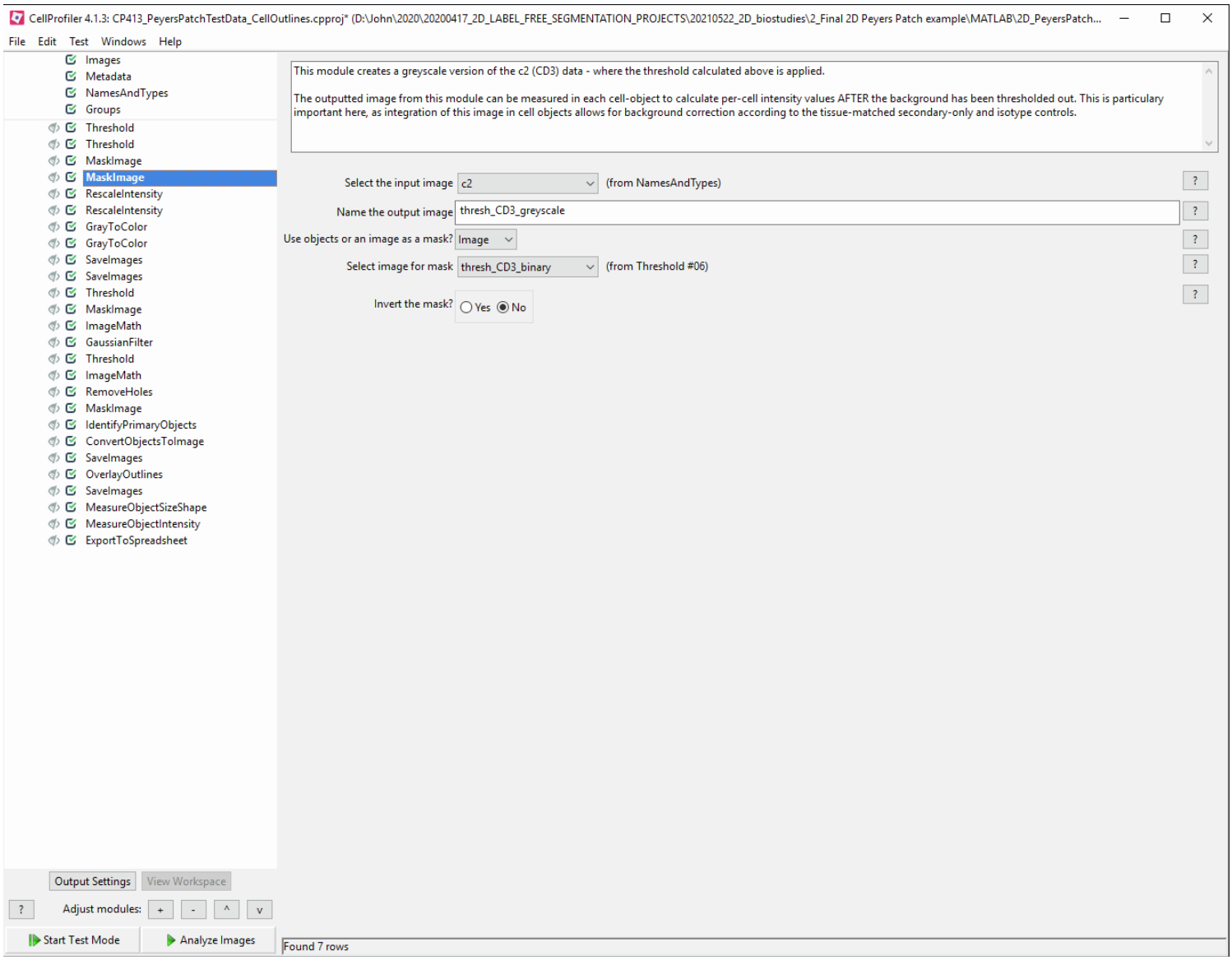
Invert the mask? ○ Yes ◉ No

Output Settings | View Workspace

? Adjust modules: + - ^ v

Start Test Mode | Analyze Images

Found 7 rows


CellProfiler 4.1.3: CP413_PeyersPatchTestData_CellOutlines.cpproj* (D:\John\2020\20200417_2D_LABEL_FREE_SEGMENTATION_PROJECTS\20210522_2D_biostudies\2_Final 2D Peyers Patch example\MATLAB\2D_PeyersPatch...
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ImageMath
GaussianFilter
Threshold
ImageMath
RemoveHoles
MaskImage
IdentifyPrimaryObjects
ConvertObjectsToImage
SaveImages
OverlayOutlines
SaveImages
MeasureObjectSizeShape
MeasureObjectIntensity
ExportToSpreadsheet
This module creates a greyscale version of the c2 (CD3) data - where the threshold calculated above is applied.
The outputted image from this module can be measured in each cell-object to calculate per-cell intensity values AFTER the background has been thresholded out. This is particulary important here, as integration of this image in cell objects allows for background correction according to the tissue-matched secondary-only and isotype controls.
Select the input image
c2
(from NamesAndTypes)
Name the output image
thresh_CD3_greyscale
Use objects or an image as a mask?
Image
Select image for mask
thresh_CD3_binary
(from Threshold #06)
Invert the mask?
Yes
No
Output Settings
View Workspace
Adjust modules:
Start Test Mode
Analyze Images
Found 7 rows

CellProfiler 4.1.3: CP413_PeyersPatchTestData_CellOutlines.cpproj* (D:\John\2020\20200417_2D_LABEL_FREE_SEGMENTATION_PROJECTS\20210522_2D_biostudies\2_Final 2D Peyers Patch example\MATLAB\2D_PeyersPatch...

File Edit Test Windows Help

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- Metadata
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- Threshold
- MaskImage
- MaskImage
- **RescaleIntensity**
- RescaleIntensity
- GrayToColor
- GrayToColor
- SaveImages
- SaveImages
- Threshold
- MaskImage
- ImageMath
- GaussianFilter
- Threshold
- ImageMath
- RemoveHoles
- MaskImage
- IdentifyPrimaryObjects
- ConvertObjectsToImage
- SaveImages
- OverlayOutlines
- SaveImages
- MeasureObjectSizeShape
- MeasureObjectIntensity
- ExportToSpreadsheet

This module rescales the 12-bit thresholded CD11c data for subsequent visulisation and saving.

Saturated pixels (i.e., 4095) in the source 12-bit image are rescaled to the maximum

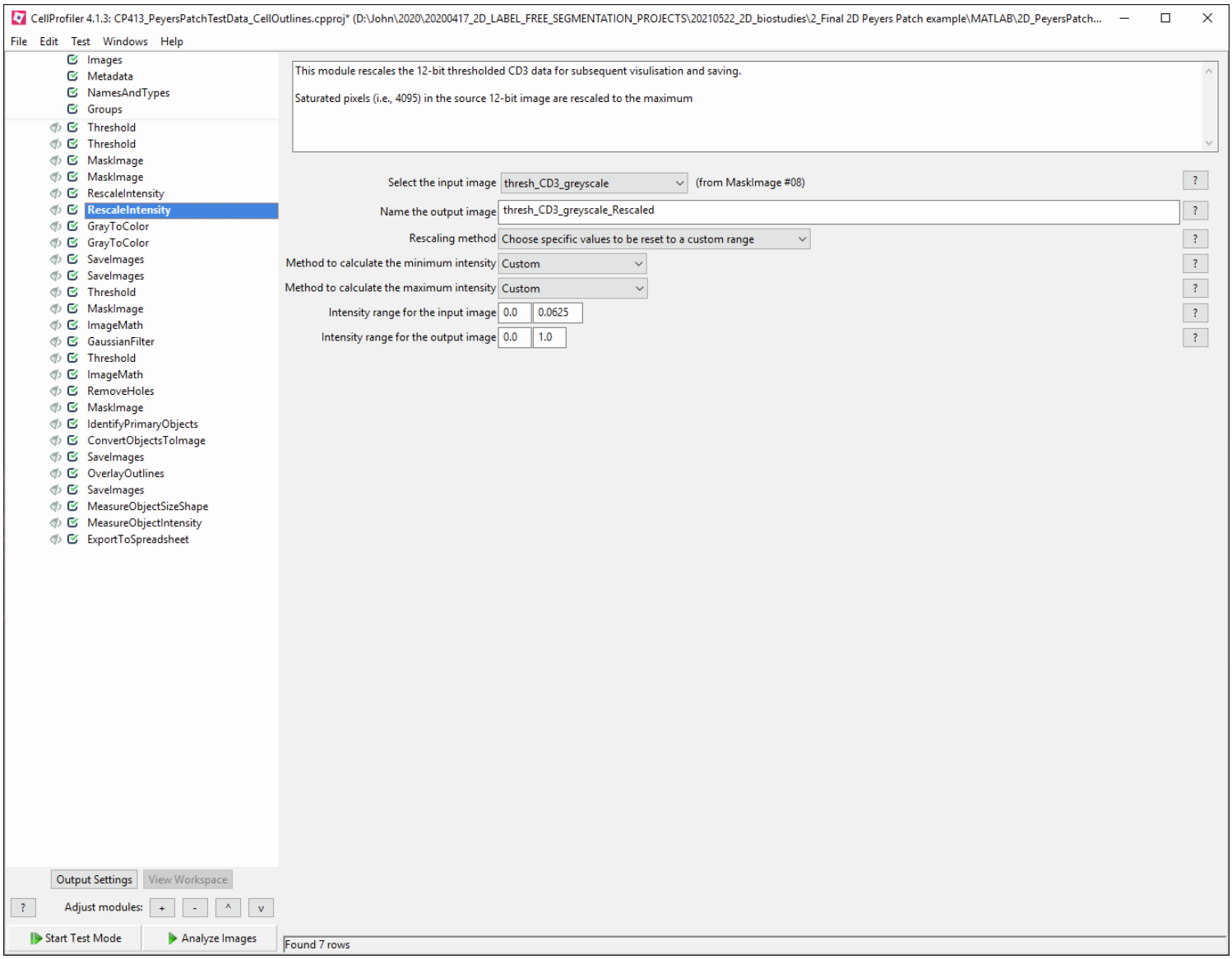
Select the input image	thresh_CD11c_greyscale	(from MaskImage #07)
Name the output image	thresh_CD11c_greyscale_Rescaled	
Rescaling method	Choose specific values to be reset to a custom range	
Method to calculate the minimum intensity	Custom	
Method to calculate the maximum intensity	Custom	
Intensity range for the input image	0.0	0.0625
Intensity range for the output image	0.0	1.0

Output Settings View Workspace

? Adjust modules: + - ^ v

Start Test Mode Analyze Images

Found 7 rows


CellProfiler 4.1.3: CP413_PeyersPatchTestData_CellOutlines.cpproj* (D:\John\2020\20200417_2D_LABEL_FREE_SEGMENTATION_PROJECTS\20210522_2D_biostudies\2_Final 2D Peyers Patch example\MATLAB\2D_PeyersPatch...
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MaskImage
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SaveImages
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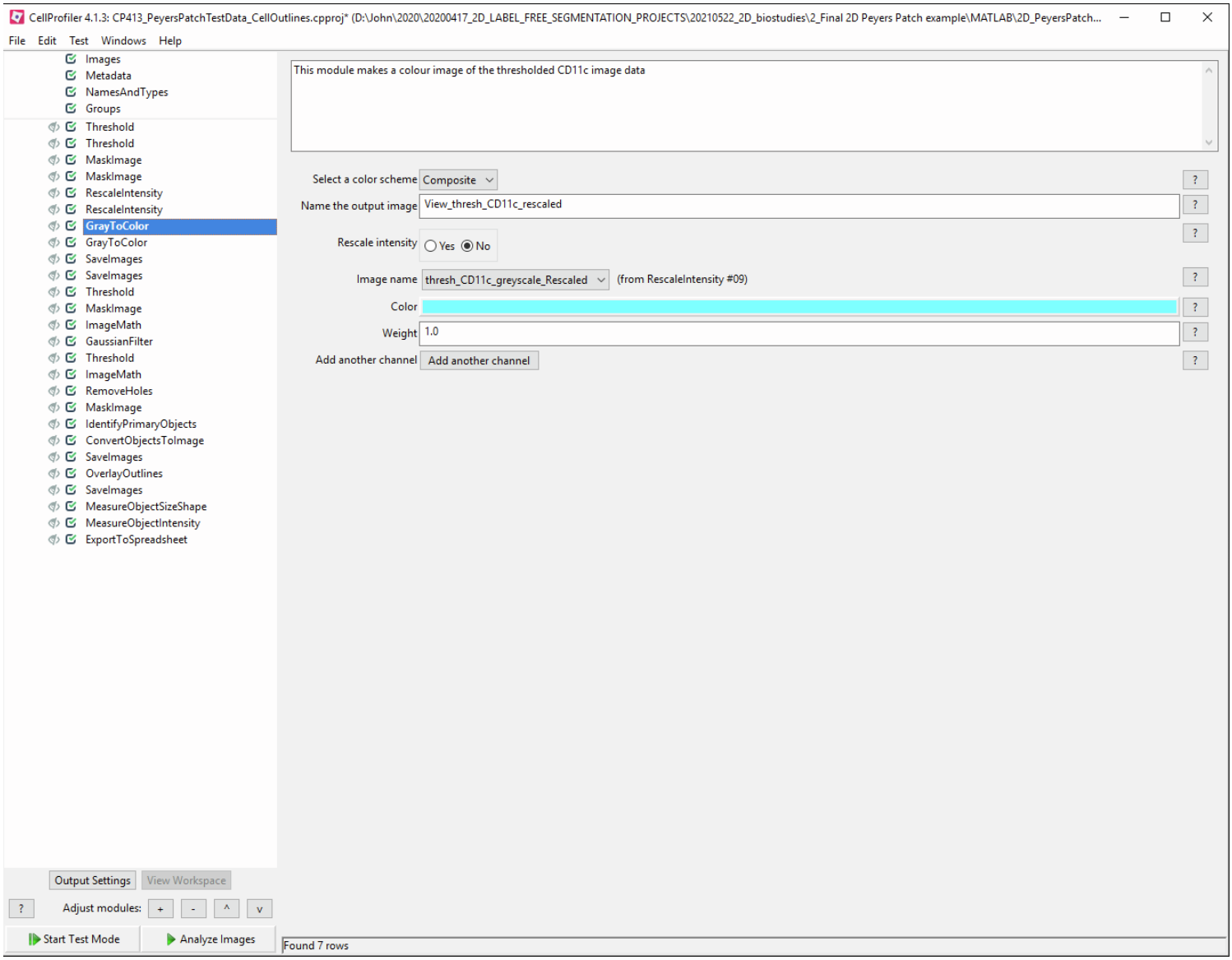
MaskImage
ImageMath
GaussianFilter
Threshold
ImageMath
RemoveHoles
MaskImage
IdentifyPrimaryObjects
ConvertObjectsToImage
SaveImages
OverlayOutlines
SaveImages
MeasureObjectSizeShape
MeasureObjectIntensity
ExportToSpreadsheet
This module rescales the 12-bit thresholded CD3 data for subsequent visulisation and saving.
Saturated pixels (i.e., 4095) in the source 12-bit image are rescaled to the maximum
Select the input image
thresh_CD3_greyscale
(from MaskImage #08)
Name the output image
thresh_CD3_greyscale_Rescaled
Rescaling method
Choose specific values to be reset to a custom range
Method to calculate the minimum intensity
Custom
Method to calculate the maximum intensity
Custom
Intensity range for the input image
0.0
0.0625
Intensity range for the output image
0.0
1.0
Output Settings
View Workspace
Adjust modules:
Start Test Mode
Analyze Images
Found 7 rows


CellProfiler 4.1.3: CP413_PeyersPatchTestData_CellOutlines.cpproj* (D:\John\2020\20200417_2D_LABEL_FREE_SEGMENTATION_PROJECTS\20210522_2D_biostudies\2_Final 2D Peyers Patch example\MATLAB\2D_PeyersPatch...
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MaskImage
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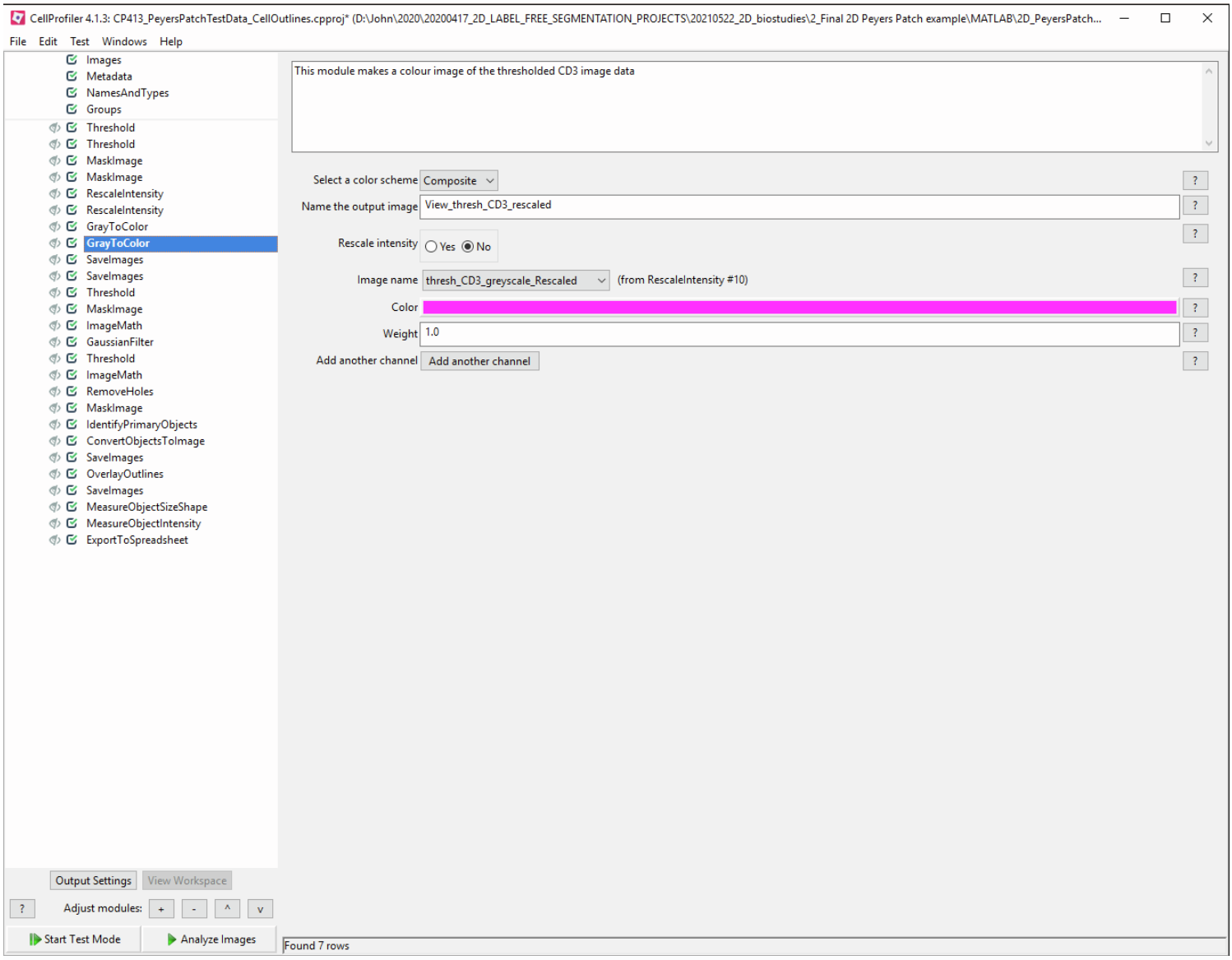
SaveImages
SaveImages
Threshold
MaskImage
ImageMath
GaussianFilter
Threshold
ImageMath
RemoveHoles
MaskImage
IdentifyPrimaryObjects
ConvertObjectsToImage
SaveImages
OverlayOutlines
SaveImages
MeasureObjectSizeShape
MeasureObjectIntensity
ExportToSpreadsheet
This module makes a colour image of the thresholded CD11c image data
Select a color scheme
Composite
Name the output image
View_thresh_CD11c_rescaled
Rescale intensity
Yes
No
Image name
thresh_CD11c_greyscale_Rescaled
(from RescaleIntensity #09)
Color
Weight
1.0
Add another channel
Add another channel
Output Settings
View Workspace
Adjust modules:
Start Test Mode
Analyze Images
Found 7 rows


CellProfiler 4.1.3: CP413_PeyersPatchTestData_CellOutlines.cpproj* (D:\John\2020\20200417_2D_LABEL_FREE_SEGMENTATION_PROJECTS\20210522_2D_biostudies\2_Final 2D Peyers Patch example\MATLAB\2D_PeyersPatch...
File Edit Test Windows Help
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ImageMath
GaussianFilter
Threshold
ImageMath
RemoveHoles
MaskImage
IdentifyPrimaryObjects
ConvertObjectsToImage
SaveImages
OverlayOutlines
SaveImages
MeasureObjectSizeShape
MeasureObjectIntensity
ExportToSpreadsheet
This module makes a colour image of the thresholded CD3 image data
Select a color scheme
Composite
Name the output image
View_thresh_CD3_rescaled
Rescale intensity
Yes
No
Image name
thresh_CD3_greyscale_Rescaled
(from RescaleIntensity #10)
Color
Weight
1.0
Add another channel
Add another channel
Output Settings
View Workspace
Adjust modules:
Start Test Mode
Analyze Images
Found 7 rows

CellProfiler 4.1.3: CP413_PeyersPatchTestData_CellOutlines.cpproj* (D:\John\2020\20200417_2D_LABEL_FREE_SEGMENTATION_PROJECTS\20210522_2D_biostudies\2_Final 2D Peyers Patch example\MATLAB\2D_PeyersPatch...

File Edit Test Windows Help

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- Threshold
- MaskImage
- MaskImage
- RescaleIntensity
- RescaleIntensity
- GrayToColor
- GrayToColor
- **SaveImages**
- SaveImages
- Threshold
- MaskImage
- ImageMath
- GaussianFilter
- Threshold
- ImageMath
- RemoveHoles
- MaskImage
- IdentifyPrimaryObjects
- ConvertObjectsToImage
- SaveImages
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- MeasureObjectSizeShape
- MeasureObjectIntensity
- ExportToSpreadsheet

Saves a view of the CD11c immunofluorescence after thresholding - as created in the above GrayToColor

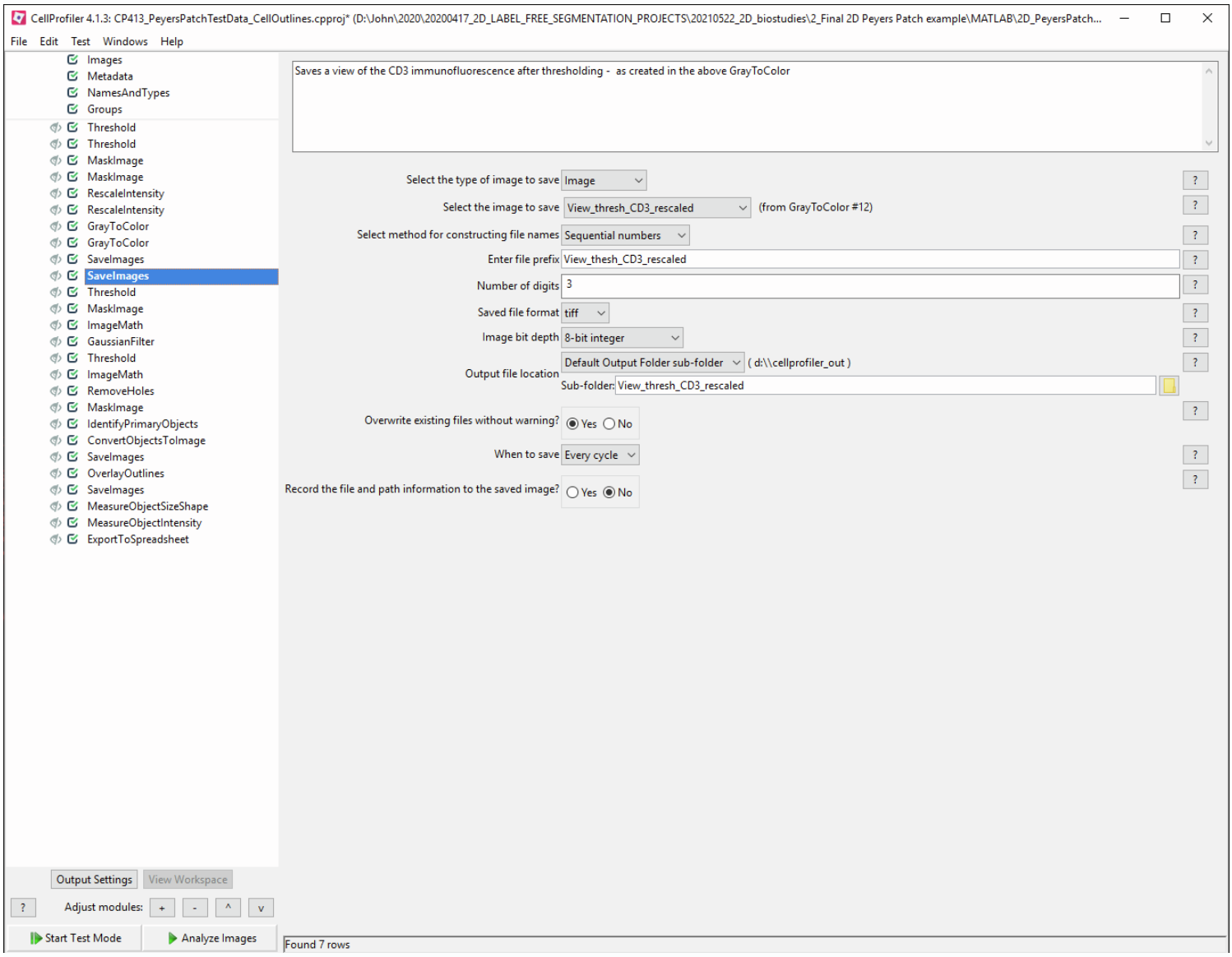
Select the type of image to save	Image
Select the image to save	View_thresh_CD11c_rescaled (from GrayToColor #11)
Select method for constructing file names	Sequential numbers
Enter file prefix	View_thesh_CD11c_rescaled
Number of digits	3
Saved file format	tiff
Image bit depth	8-bit integer
Output file location	Default Output Folder sub-folder (d:\\cellprofiler_out)
	Sub-folder: View_thresh_CD11c_rescaled
Overwrite existing files without warning?	(•) Yes ( ) No
When to save	Every cycle
Record the file and path information to the saved image?	( ) Yes (•) No

Output Settings | View Workspace

? Adjust modules: + - ^ v

Start Test Mode | Analyze Images

Found 7 rows


CellProfiler 4.1.3: CP413_PeyersPatchTestData_CellOutlines.cpproj* (D:\John\2020\20200417_2D_LABEL_FREE_SEGMENTATION_PROJECTS\20210522_2D_biostudies\2_Final 2D Peyers Patch example\MATLAB\2D_PeyersPatch...
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ImageMath
RemoveHoles
MaskImage
IdentifyPrimaryObjects
ConvertObjectsToImage
SaveImages
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SaveImages
MeasureObjectSizeShape
MeasureObjectIntensity
ExportToSpreadsheet
Saves a view of the CD3 immunofluorescence after thresholding - as created in the above GrayToColor
Select the type of image to save
Image
Select the image to save
View_thresh_CD3_rescaled
(from GrayToColor #12)
Select method for constructing file names
Sequential numbers
Enter file prefix
View_thesh_CD3_rescaled
Number of digits
3
Saved file format
tiff
Image bit depth
8-bit integer
Output file location
Default Output Folder sub-folder
(d:\\cellprofiler_out)
Sub-folder:
View_thresh_CD3_rescaled
Overwrite existing files without warning?
Yes
No
When to save
Every cycle
Record the file and path information to the saved image?
Yes
No
Output Settings
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Adjust modules:
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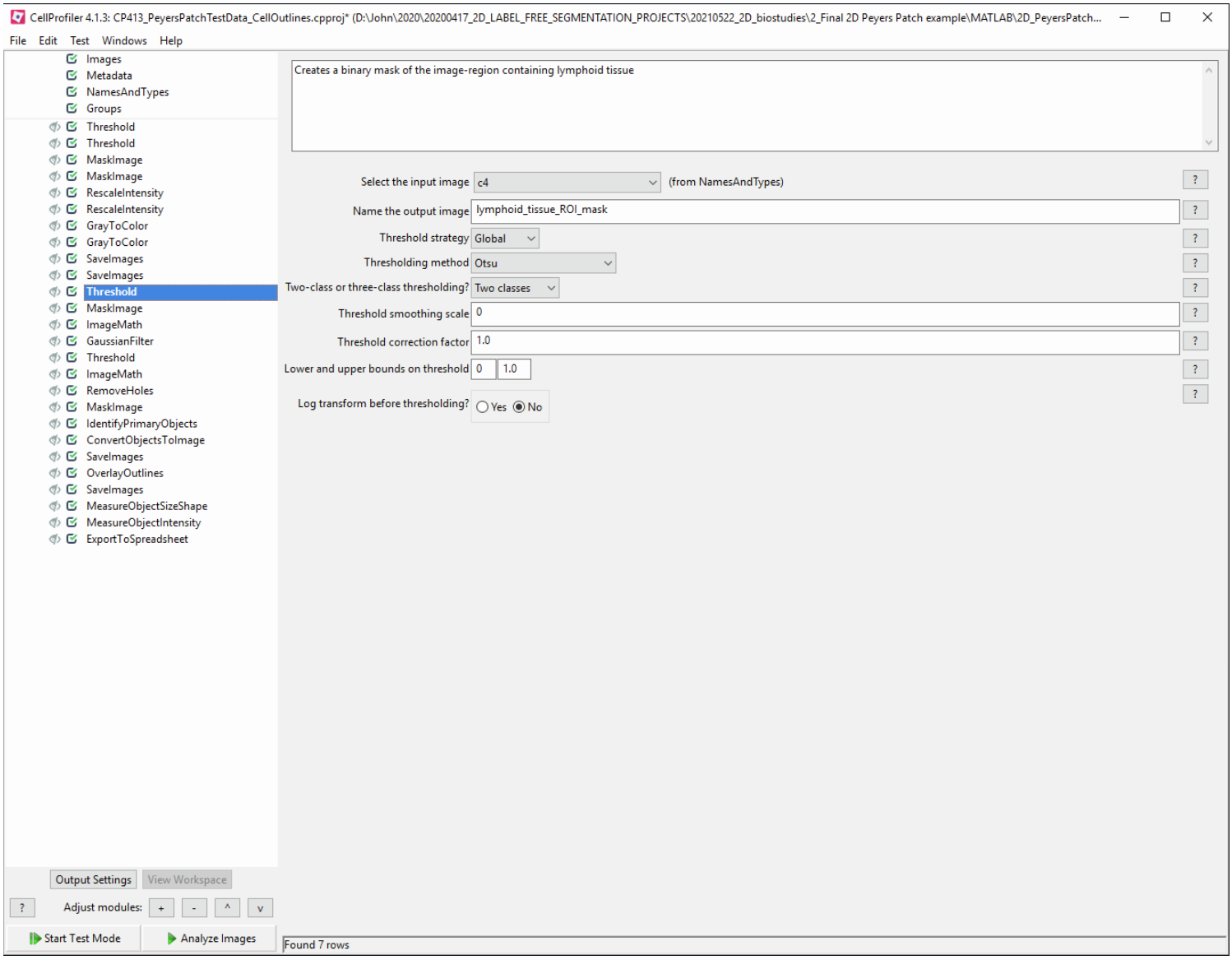
CellProfiler 4.1.3: CP413_PeyersPatchTestData_CellOutlines.cpproj* (D:\John\2020\20200417_2D_LABEL_FREE_SEGMENTATION_PROJECTS\20210522_2D_biostudies\2_Final 2D Peyers Patch example\MATLAB\2D_PeyersPatch...
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GaussianFilter
Threshold
ImageMath
RemoveHoles
MaskImage
IdentifyPrimaryObjects
ConvertObjectsToImage
SaveImages
OverlayOutlines
SaveImages
MeasureObjectSizeShape
MeasureObjectIntensity
ExportToSpreadsheet
Creates a binary mask of the image-region containing lymphoid tissue
Select the input image
c4
(from NamesAndTypes)
Name the output image
lymphoid_tissue_ROI_mask
Threshold strategy
Global
Thresholding method
Otsu
Two-class or three-class thresholding?
Two classes
Threshold smoothing scale
0
Threshold correction factor
1.0
Lower and upper bounds on threshold
0
1.0
Log transform before thresholding?
Yes
No
Output Settings
View Workspace
Adjust modules:
Start Test Mode
Analyze Images
Found 7 rows

CellProfiler 4.1.3: CP413_PeyersPatchTestData_CellOutlines.cpproj* (D:\John\2020\20200417_2D_LABEL_FREE_SEGMENTATION_PROJECTS\20210522_2D_biostudies\2_Final 2D Peyers Patch example\MATLAB\2D_PeyersPatch...

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- Threshold
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- MaskImage
- RescaleIntensity
- RescaleIntensity
- GrayToColor
- GrayToColor
- SaveImages
- SaveImages
- Threshold
- **MaskImage**
- ImageMath
- GaussianFilter
- Threshold
- ImageMath
- RemoveHoles
- MaskImage
- IdentifyPrimaryObjects
- ConvertObjectsToImage
- SaveImages
- OverlayOutlines
- SaveImages
- MeasureObjectSizeShape
- MeasureObjectIntensity
- ExportToSpreadsheet

Applies the lymphoid ROI mask to the LF-actin probability map used to enable cell-segmentation

Select the input image: c6 (from NamesAndTypes)

Name the output image: Masked_c6

Use objects or an image as a mask?: Image

Select image for mask: lymphoid_tissue_ROI_mask (from Threshold #15)

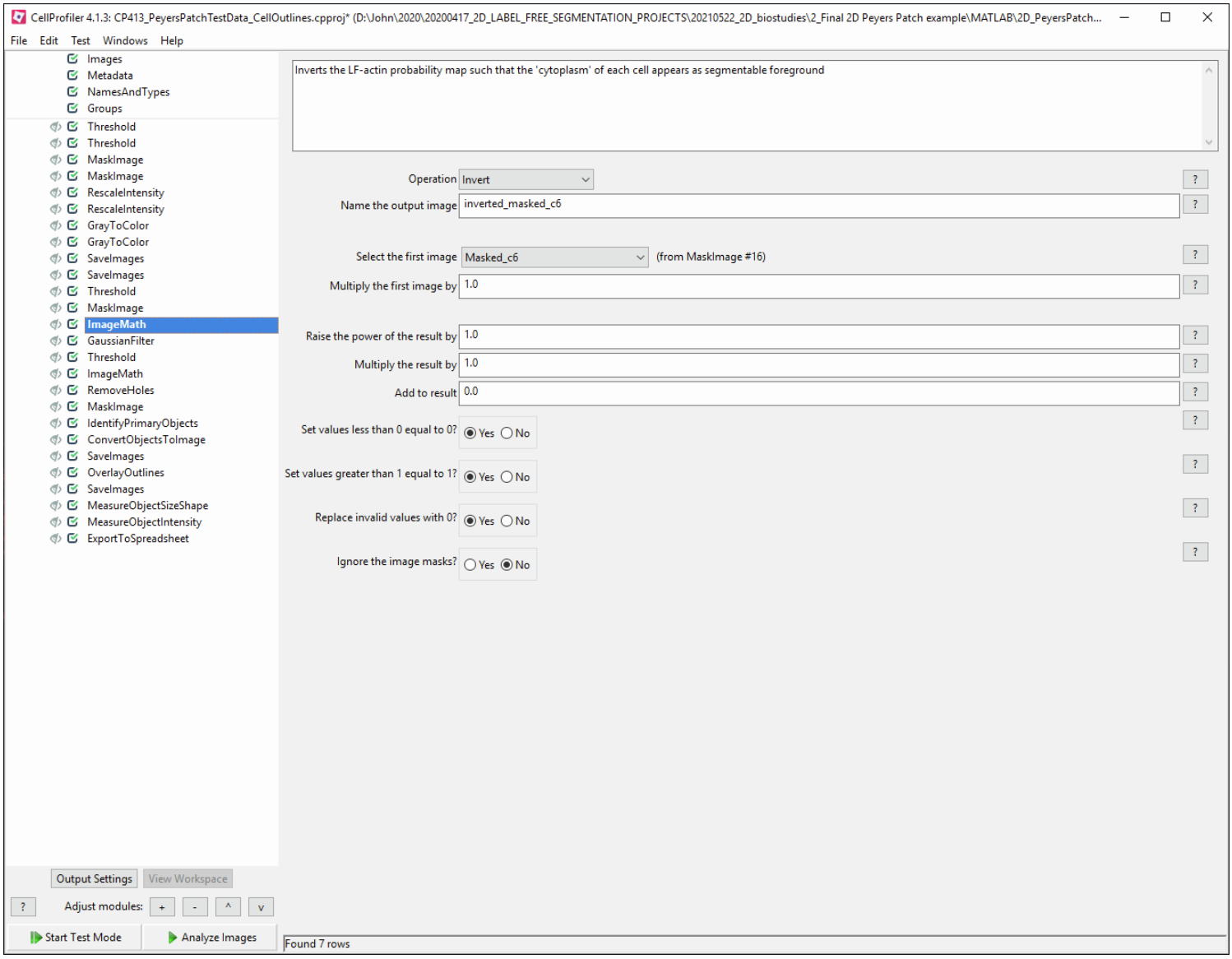
Invert the mask? ○ Yes ◉ No

Output Settings | View Workspace

? Adjust modules: + - ^ v

Start Test Mode | Analyze Images

Found 7 rows


CellProfiler 4.1.3: CP413_PeyersPatchTestData_CellOutlines.cpproj* (D:\John\2020\20200417_2D_LABEL_FREE_SEGMENTATION_PROJECTS\20210522_2D_biostudies\2_Final 2D Peyers Patch example\MATLAB\2D_PeyersPatch...
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Threshold
ImageMath
RemoveHoles
MaskImage
IdentifyPrimaryObjects
ConvertObjectsToImage
SaveImages
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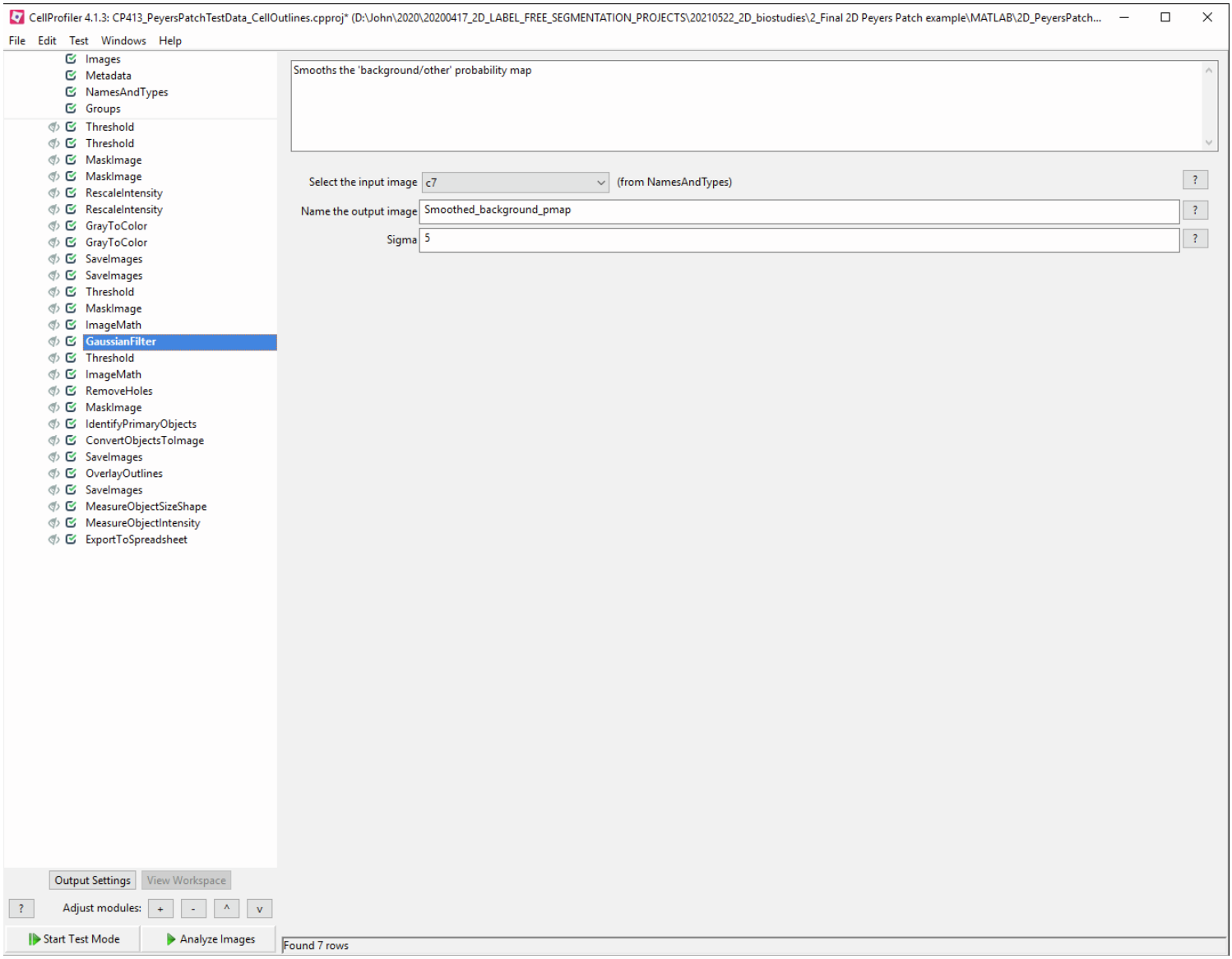
SaveImages
MeasureObjectSizeShape
MeasureObjectIntensity
ExportToSpreadsheet
Inverts the LF-actin probability map such that the 'cytoplasm' of each cell appears as segmentable foreground
Operation Invert
Name the output image inverted_masked_c6
Select the first image Masked_c6 (from MaskImage #16)
Multiply the first image by 1.0
Raise the power of the result by 1.0
Multiply the result by 1.0
Add to result 0.0
Set values less than 0 equal to 0? Yes No
Set values greater than 1 equal to 1? Yes No
Replace invalid values with 0? Yes No
Ignore the image masks? Yes No
Output Settings
View Workspace
Adjust modules:
Start Test Mode
Analyze Images
Found 7 rows


CellProfiler 4.1.3: CP413_PeyersPatchTestData_CellOutlines.cpproj* (D:\John\2020\20200417_2D_LABEL_FREE_SEGMENTATION_PROJECTS\20210522_2D_biostudies\2_Final 2D Peyers Patch example\MATLAB\2D_PeyersPatch...
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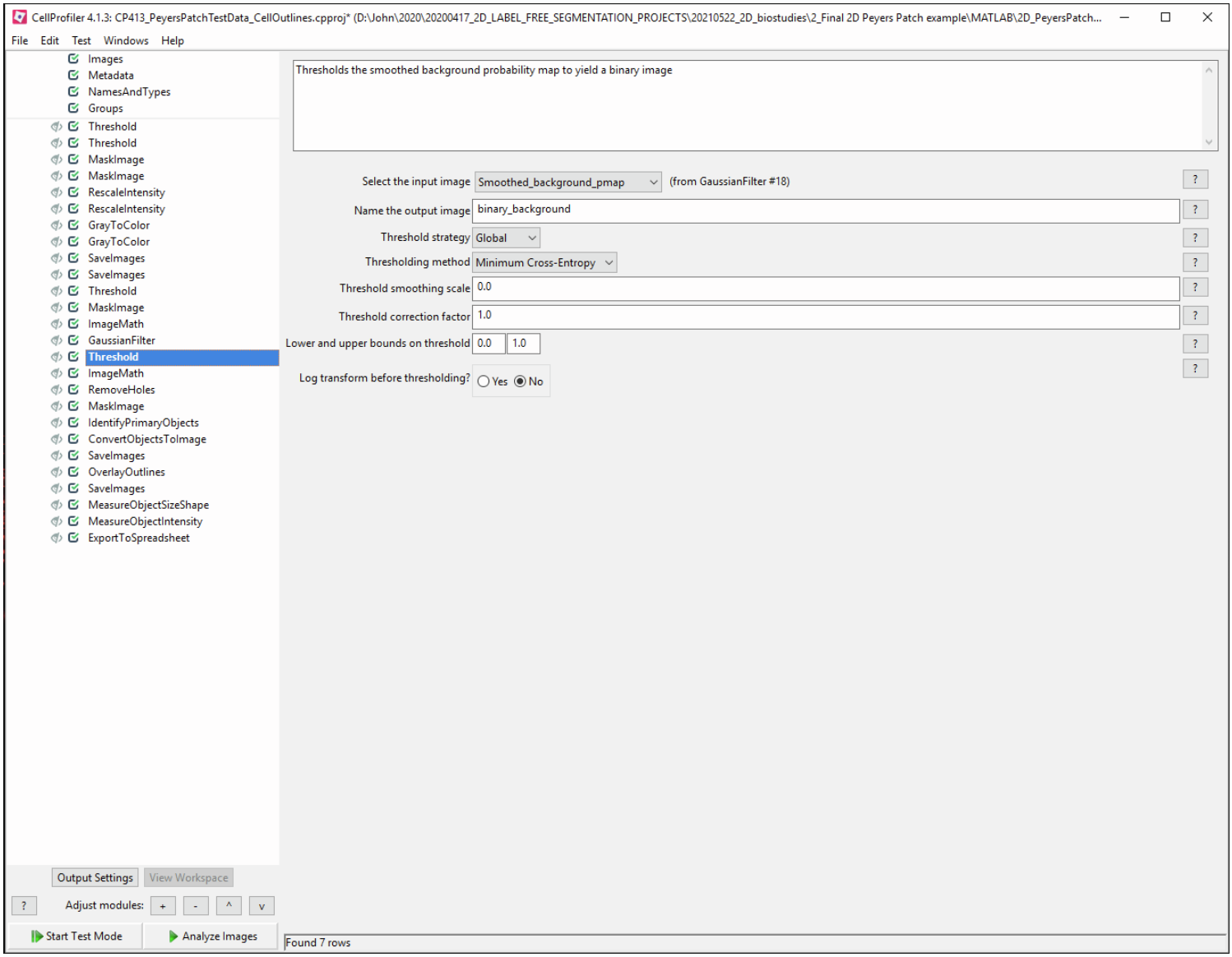
MaskImage
ImageMath
GaussianFilter
Threshold
ImageMath
RemoveHoles
MaskImage
IdentifyPrimaryObjects
ConvertObjectsToImage
SaveImages
OverlayOutlines
SaveImages
MeasureObjectSizeShape
MeasureObjectIntensity
ExportToSpreadsheet
Smooths the 'background/other' probability map
Select the input image
c7
(from NamesAndTypes)
Name the output image
Smoothed_background_pmap
Sigma
5
Output Settings
View Workspace
Adjust modules:
Start Test Mode
Analyze Images
Found 7 rows


CellProfiler 4.1.3: CP413_PeyersPatchTestData_CellOutlines.cpproj* (D:\John\2020\20200417_2D_LABEL_FREE_SEGMENTATION_PROJECTS\20210522_2D_biostudies\2_Final 2D Peyers Patch example\MATLAB\2D_PeyersPatch...
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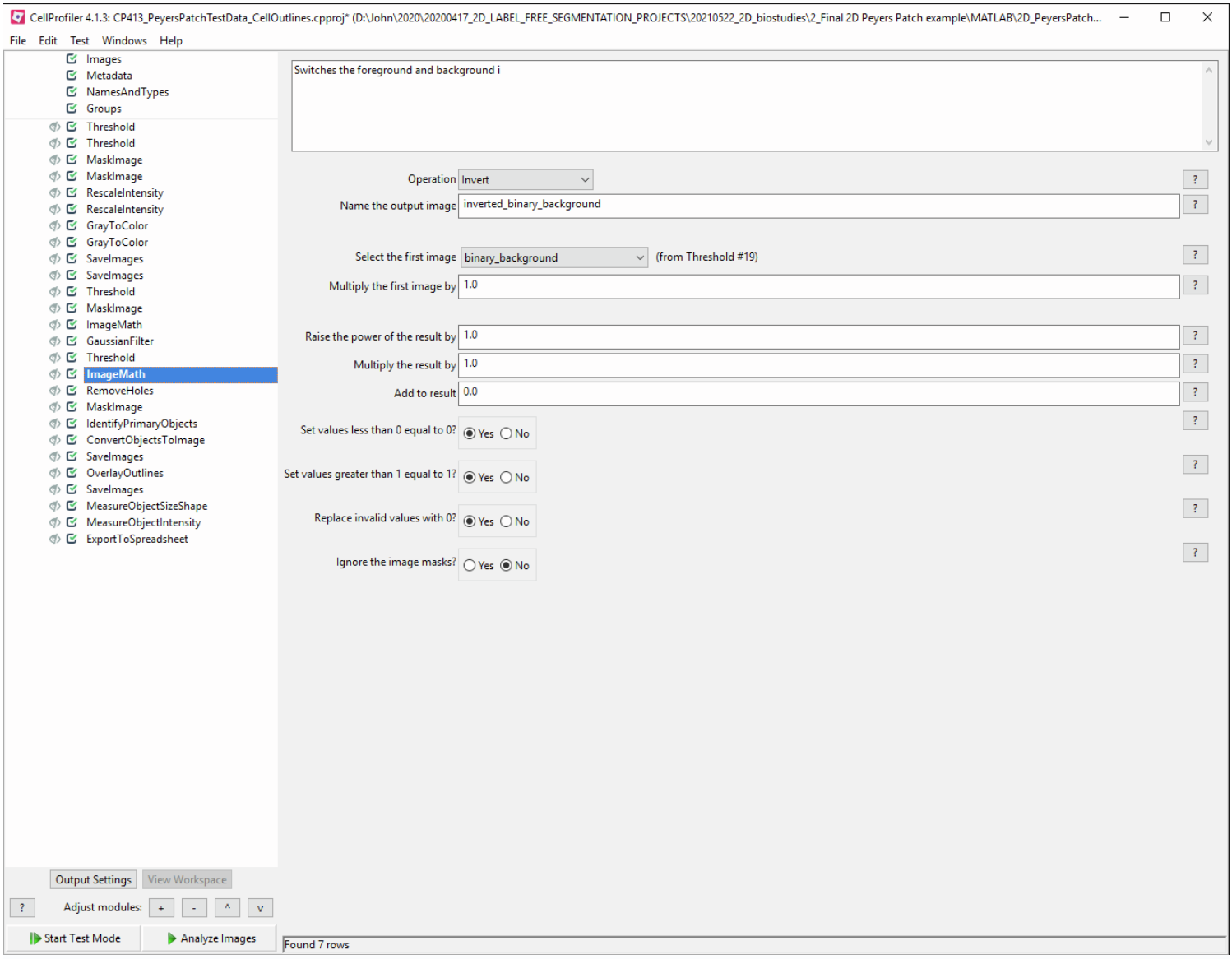
ImageMath
GaussianFilter
Threshold
ImageMath
RemoveHoles
MaskImage
IdentifyPrimaryObjects
ConvertObjectsToImage
SaveImages
OverlayOutlines
SaveImages
MeasureObjectSizeShape
MeasureObjectIntensity
ExportToSpreadsheet
Thresholds the smoothed background probability map to yield a binary image
Select the input image
Smoothed_background_pmap
(from GaussianFilter #18)
Name the output image
binary_background
Threshold strategy
Global
Thresholding method
Minimum Cross-Entropy
Threshold smoothing scale
0.0
Threshold correction factor
1.0
Lower and upper bounds on threshold
0.0
1.0
Log transform before thresholding?
Yes
No
Output Settings
View Workspace
Adjust modules:
Start Test Mode
Analyze Images
Found 7 rows


CellProfiler 4.1.3: CP413_PeyersPatchTestData_CellOutlines.cpproj* (D:\John\2020\20200417_2D_LABEL_FREE_SEGMENTATION_PROJECTS\20210522_2D_biostudies\2_Final 2D Peyers Patch example\MATLAB\2D_PeyersPatch...
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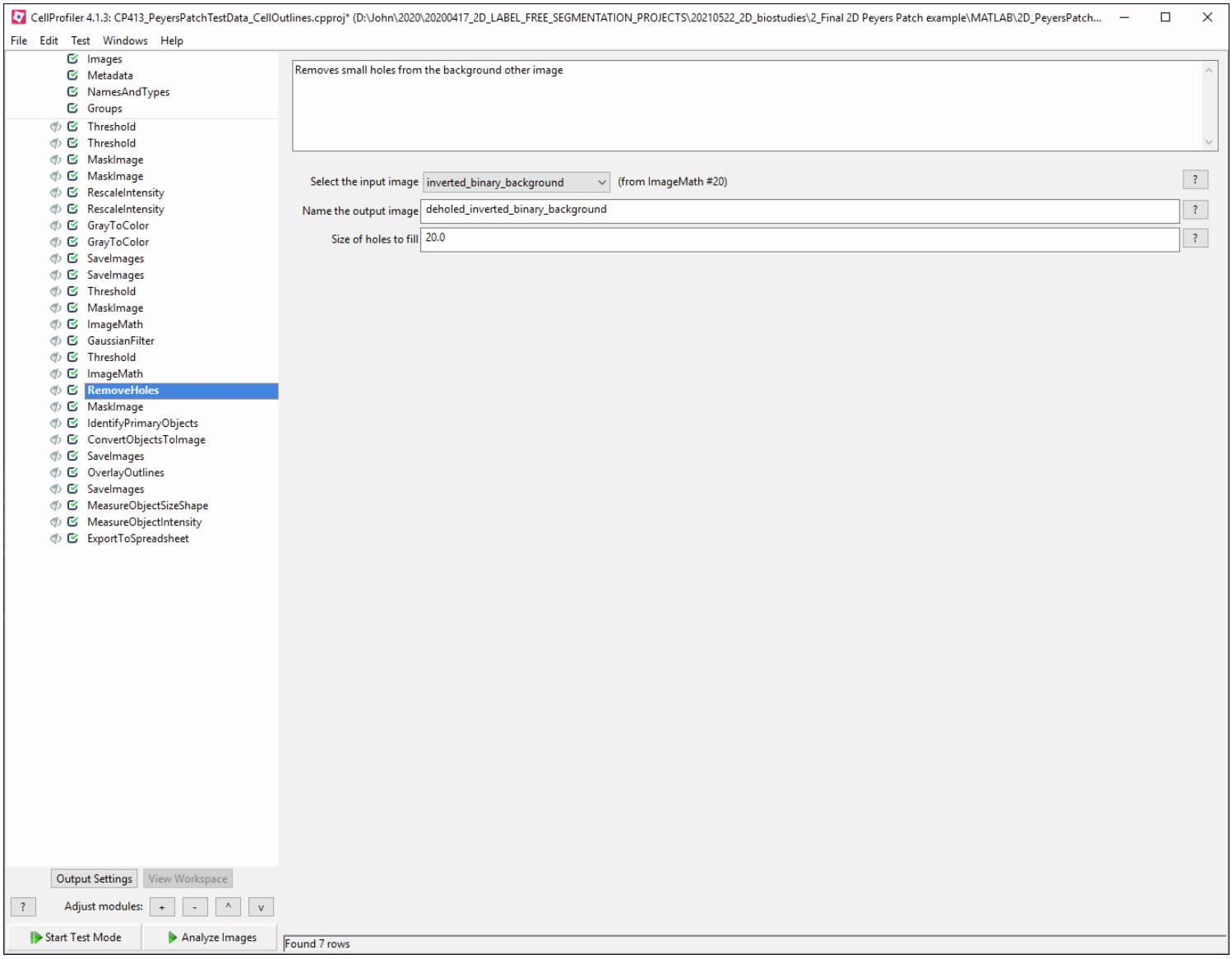
Threshold
ImageMath
RemoveHoles
MaskImage
IdentifyPrimaryObjects
ConvertObjectsToImage
SaveImages
OverlayOutlines
SaveImages
MeasureObjectSizeShape
MeasureObjectIntensity
ExportToSpreadsheet
Switches the foreground and background i
Operation Invert
Name the output image inverted_binary_background
Select the first image binary_background (from Threshold #19)
Multiply the first image by 1.0
Raise the power of the result by 1.0
Multiply the result by 1.0
Add to result 0.0
Set values less than 0 equal to 0? Yes No
Set values greater than 1 equal to 1? Yes No
Replace invalid values with 0? Yes No
Ignore the image masks? Yes No
Output Settings
View Workspace
Adjust modules:
Start Test Mode
Analyze Images
Found 7 rows


CellProfiler 4.1.3: CP413_PeyersPatchTestData_CellOutlines.cpproj* (D:\John\2020\20200417_2D_LABEL_FREE_SEGMENTATION_PROJECTS\20210522_2D_biostudies\2_Final 2D Peyers Patch example\MATLAB\2D_PeyersPatch...
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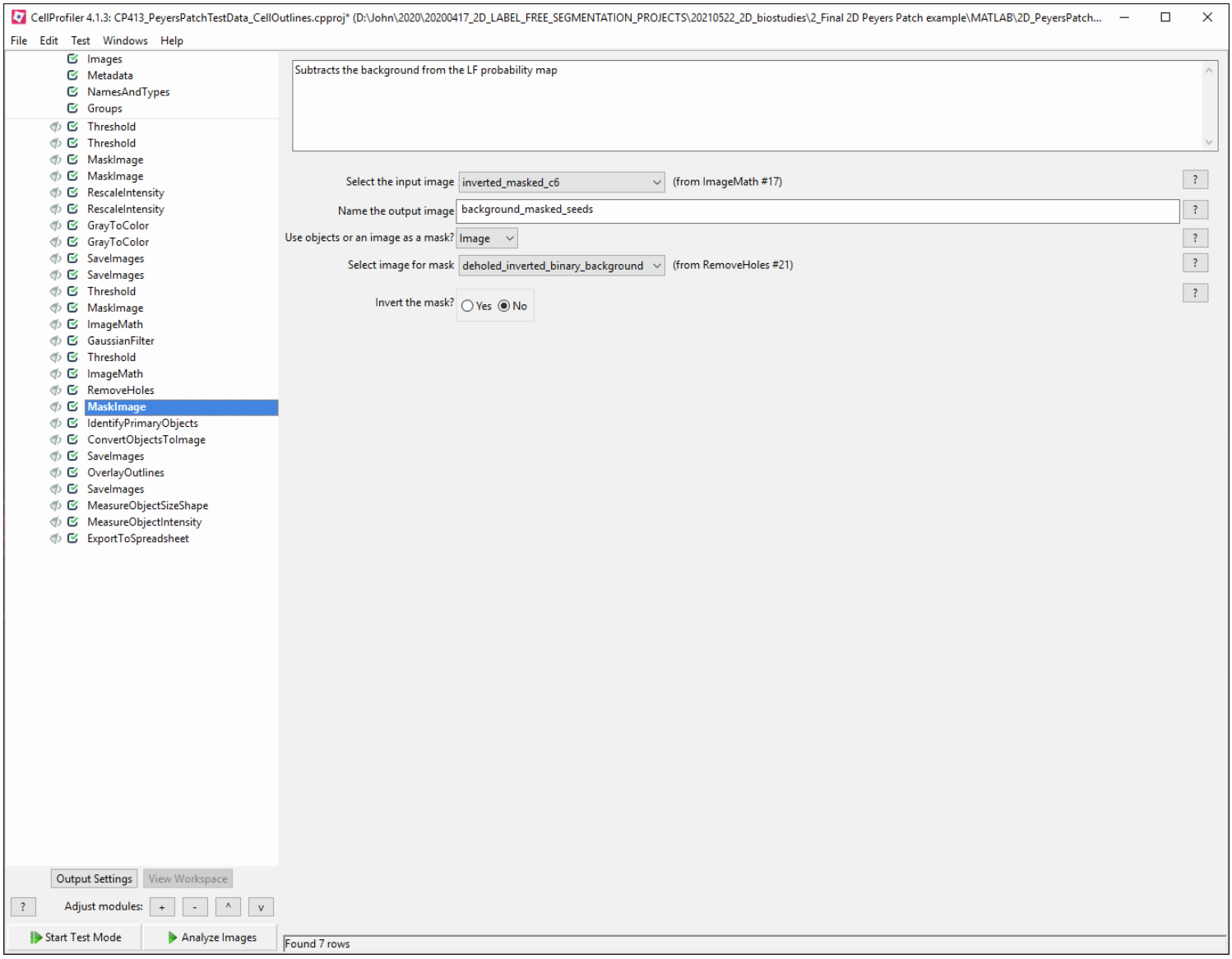
ImageMath
RemoveHoles
MaskImage
IdentifyPrimaryObjects
ConvertObjectsToImage
SaveImages
OverlayOutlines
SaveImages
MeasureObjectSizeShape
MeasureObjectIntensity
ExportToSpreadsheet
Removes small holes from the background other image
Select the input image
inverted_binary_background
(from ImageMath #20)
Name the output image
deholed_inverted_binary_background
Size of holes to fill
20.0
Output Settings
View Workspace
Adjust modules:
Start Test Mode
Analyze Images
Found 7 rows


CellProfiler 4.1.3: CP413_PeyersPatchTestData_CellOutlines.cpproj* (D:\John\2020\20200417_2D_LABEL_FREE_SEGMENTATION_PROJECTS\20210522_2D_biostudies\2_Final 2D Peyers Patch example\MATLAB\2D_PeyersPatch...
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IdentifyPrimaryObjects
ConvertObjectsToImage
SaveImages
OverlayOutlines
SaveImages
MeasureObjectSizeShape
MeasureObjectIntensity
ExportToSpreadsheet
Subtracts the background from the LF probability map
Select the input image
inverted_masked_c6
(from ImageMath #17)
Name the output image
background_masked_seeds
Use objects or an image as a mask?
Image
Select image for mask
deholed_inverted_binary_background
(from RemoveHoles #21)
Invert the mask?
Yes
No
Output Settings
View Workspace
Adjust modules:
Start Test Mode
Analyze Images
Found 7 rows

CellProfiler 4.1.3: CP413_PeyersPatchTestData_CellOutlines.cpproj* (D:\John\2020\20200417_2D_LABEL_FREE_SEGMENTATION_PROJECTS\20210522_2D_biostudies\2_Final 2D Peyers Patch example\MATLAB\2D_PeyersPatch...

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- GaussianFilter
- Threshold
- ImageMath
- RemoveHoles
- MaskImage
- **IdentifyPrimaryObjects**
- ConvertObjectsToImage
- SaveImages
- OverlayOutlines
- SaveImages
- MeasureObjectSizeShape
- MeasureObjectIntensity
- ExportToSpreadsheet

Output Settings | View Workspace

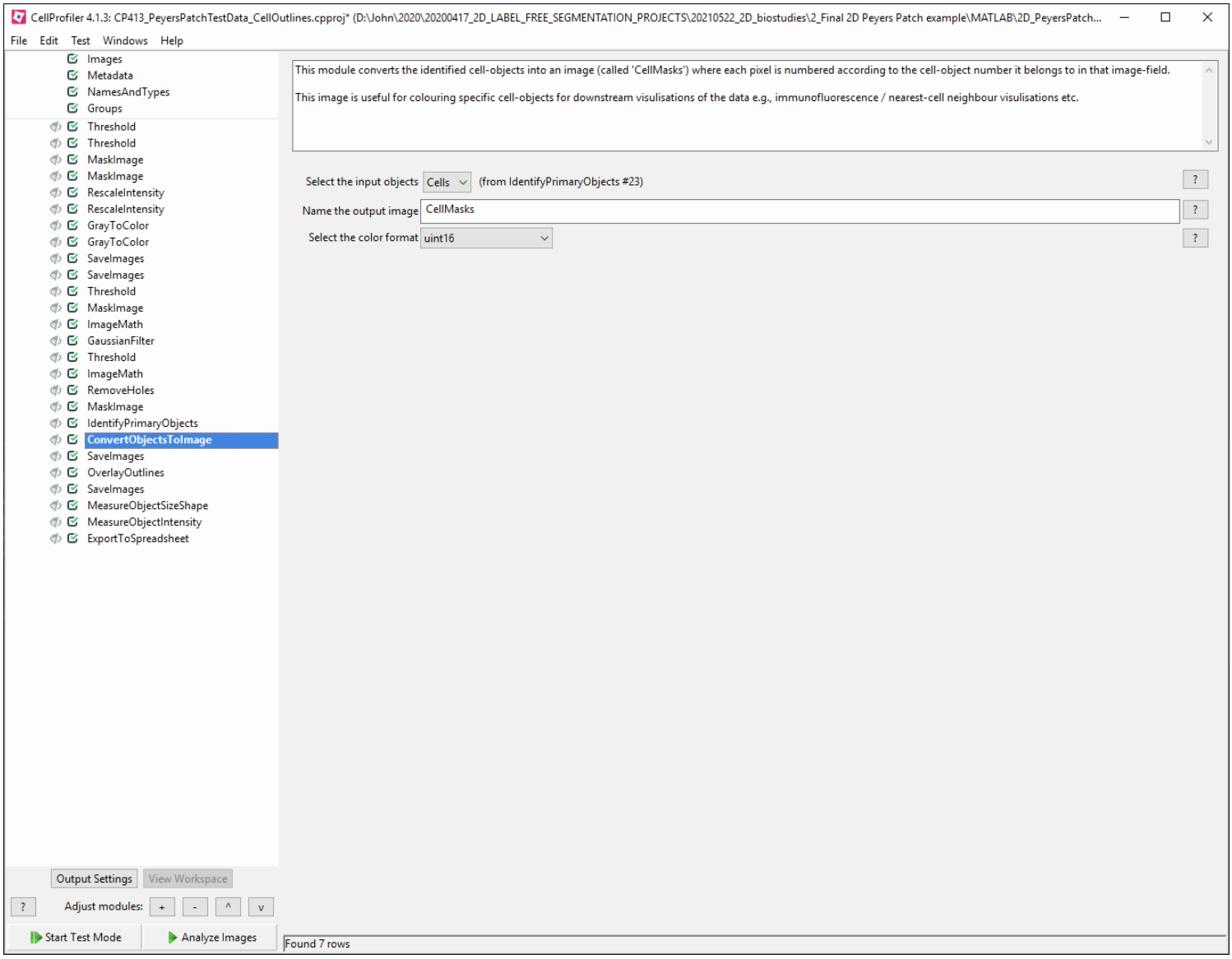
? Adjust modules: + - ^ v

Start Test Mode | Analyze Images

Label-free cell instance segmentation using the outputted probability maps from the Unet model

Use advanced settings?	(•) Yes ( ) No
Select the input image	background_masked_seeds (from MaskImage #22)
Name the primary objects to be identified	Cells
Typical diameter of objects, in pixel units (Min,Max)	26 59
Discard objects outside the diameter range?	( ) Yes (•) No
Discard objects touching the border of the image?	( ) Yes (•) No
Threshold strategy	Global
Thresholding method	Manual
Manual threshold	0.05
Threshold smoothing scale	22
Method to distinguish clumped objects	Intensity
Method to draw dividing lines between clumped objects	Intensity
Automatically calculate size of smoothing filter for declumping?	(•) Yes ( ) No
Automatically calculate minimum allowed distance between local maxima?	( ) Yes (•) No
Suppress local maxima that are closer than this minimum allowed distance	13
Speed up by using lower-resolution image to find local maxima?	(•) Yes ( ) No
Display accepted local maxima?	( ) Yes (•) No
Fill holes in identified objects?	After declumping only
Handling of objects if excessive number of objects identified	Continue

Found 7 rows


CellProfiler 4.1.3: CP413_PeyersPatchTestData_CellOutlines.cpproj* (D:\John\2020\20200417_2D_LABEL_FREE_SEGMENTATION_PROJECTS\20210522_2D_biostudies\2_Final 2D Peyers Patch example\MATLAB\2D_PeyersPatch...
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ImageMath
RemoveHoles
MaskImage
IdentifyPrimaryObjects
ConvertObjectsToImage
SaveImages
OverlayOutlines
SaveImages
MeasureObjectSizeShape
MeasureObjectIntensity
ExportToSpreadsheet
This module converts the identified cell-objects into an image (called 'CellMasks') where each pixel is numbered according to the cell-object number it belongs to in that image-field.
This image is useful for colouring specific cell-objects for downstream visulisations of the data e.g., immunofluorescence / nearest-cell neighbour visulisations etc.
Select the input objects
Cells
(from IdentifyPrimaryObjects #23)
Name the output image
CellMasks
Select the color format
uint16
Output Settings
View Workspace
Adjust modules:
Start Test Mode
Analyze Images
Found 7 rows

CellProfiler 4.1.3: CP413_PeyersPatchTestData_CellOutlines.cpproj* (D:\John\2020\20200417_2D_LABEL_FREE_SEGMENTATION_PROJECTS\20210522_2D_biostudies\2_Final 2D Peyers Patch example\MATLAB\2D_PeyersPatch...

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- GrayToColor
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- SaveImages
- Threshold
- MaskImage
- ImageMath
- GaussianFilter
- Threshold
- ImageMath
- RemoveHoles
- MaskImage
- IdentifyPrimaryObjects
- ConvertObjectsToImage
- **SaveImages**
- OverlayOutlines
- SaveImages
- MeasureObjectSizeShape
- MeasureObjectIntensity
- ExportToSpreadsheet

This module saves the image of the CellMasks as a NumPy array

Select the type of image to save: Image

Select the image to save: CellMasks (from ConvertObjectsToImage #24)

Select method for constructing file names: Sequential numbers

Enter file prefix: CellMasks

Number of digits: 3

Saved file format: npy

Output file location: Default Output Folder sub-folder (d:\\cellprofiler_out)

Sub-folder: CellMasks

Overwrite existing files without warning? (•) Yes ( ) No

When to save: Every cycle

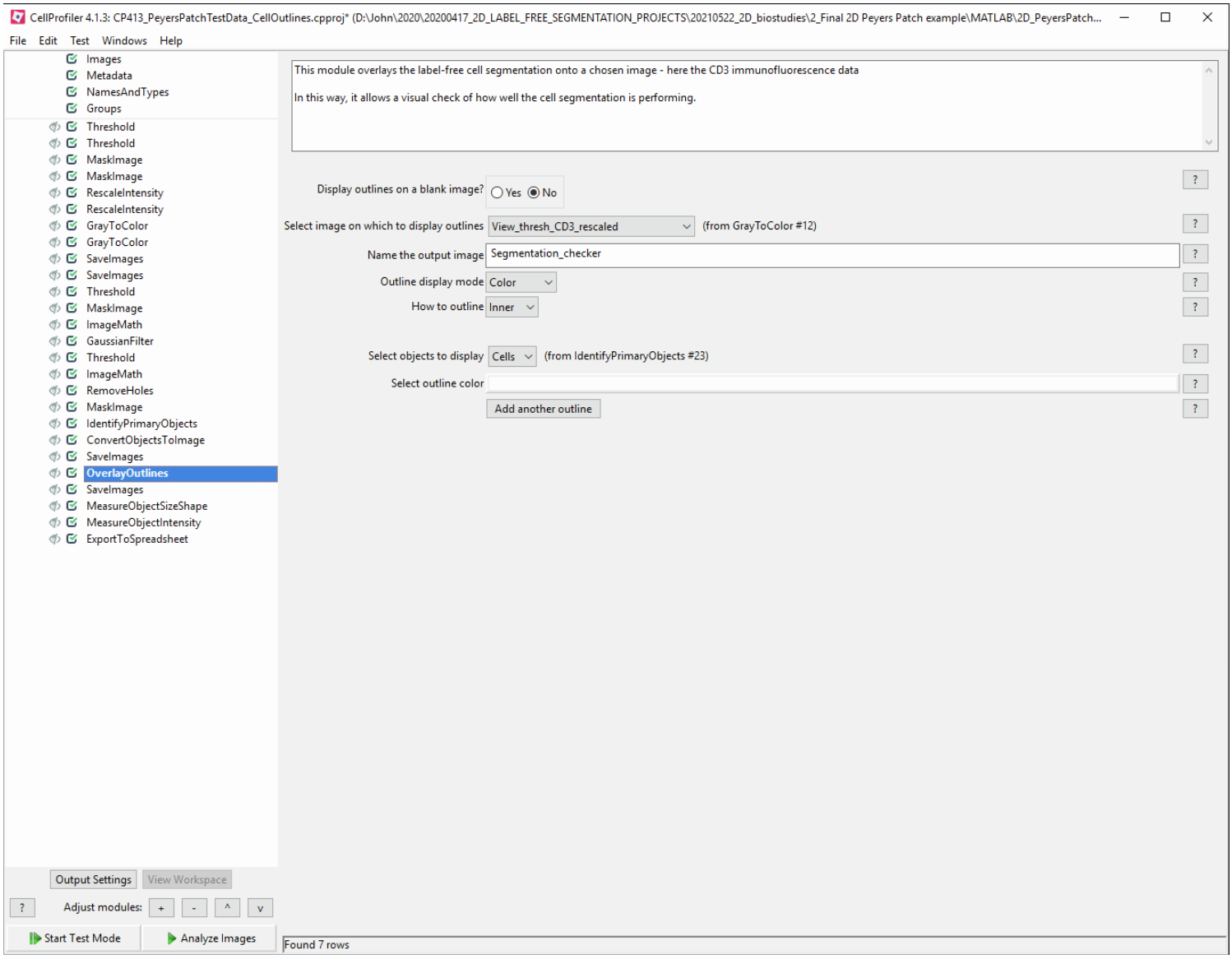
Record the file and path information to the saved image? ( ) Yes (•) No

Output Settings | View Workspace

? Adjust modules: + - ^ v

Start Test Mode | Analyze Images

Found 7 rows


CellProfiler 4.1.3: CP413_PeyersPatchTestData_CellOutlines.cpproj* (D:\John\2020\20200417_2D_LABEL_FREE_SEGMENTATION_PROJECTS\20210522_2D_biostudies\2_Final 2D Peyers Patch example\MATLAB\2D_PeyersPatch...
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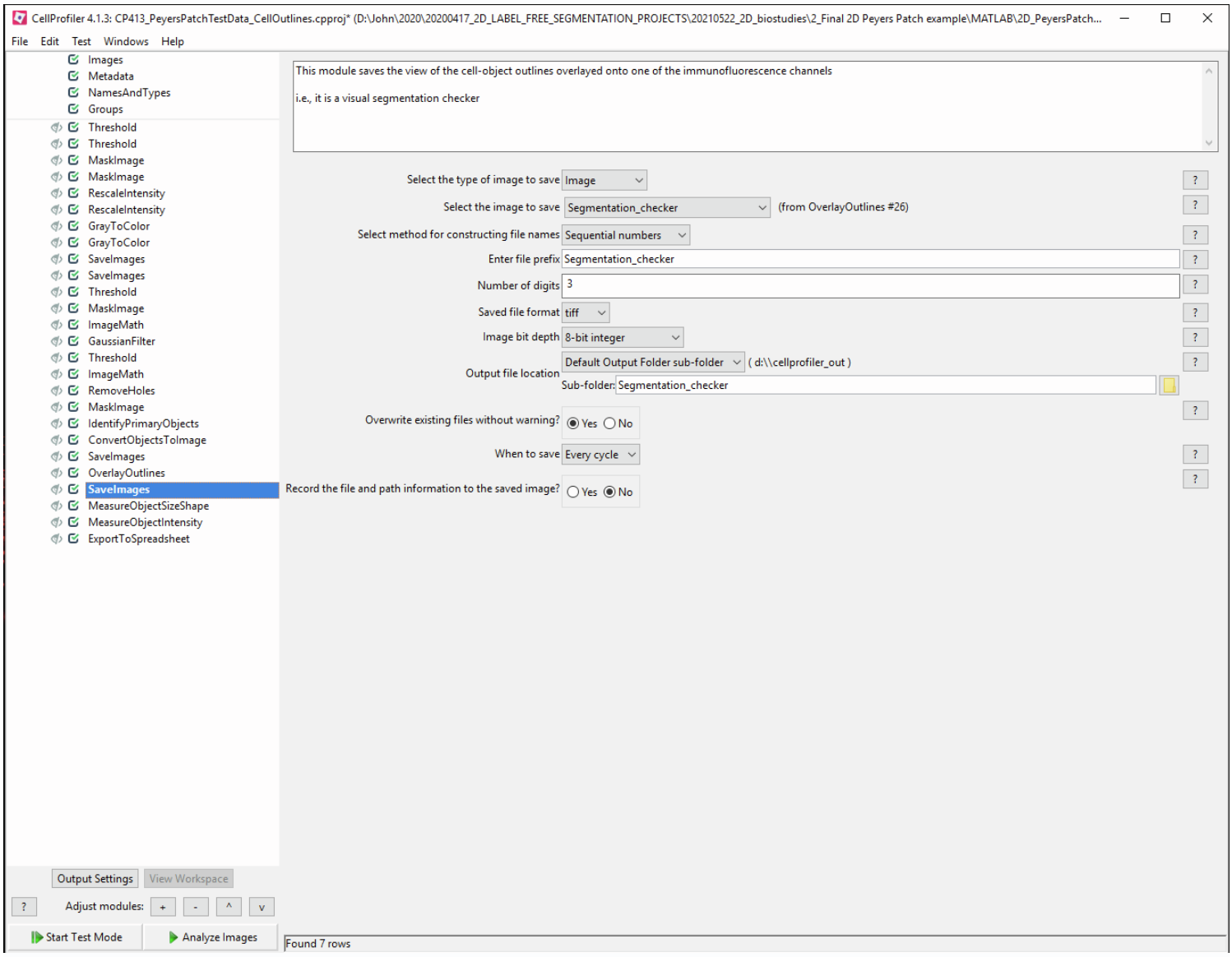
ExportToSpreadsheet
This module overlays the label-free cell segmentation onto a chosen image - here the CD3 immunofluorescence data
In this way, it allows a visual check of how well the cell segmentation is performing.
Display outlines on a blank image?
Yes
No
Select image on which to display outlines
View_thresh_CD3_rescaled
(from GrayToColor #12)
Name the output image
Segmentation_checker
Outline display mode
Color
How to outline
Inner
Select objects to display
Cells
(from IdentifyPrimaryObjects #23)
Select outline color
Add another outline
Output Settings
View Workspace
Adjust modules:
Start Test Mode
Analyze Images
Found 7 rows


CellProfiler 4.1.3: CP413_PeyersPatchTestData_CellOutlines.cpproj* (D:\John\2020\20200417_2D_LABEL_FREE_SEGMENTATION_PROJECTS\20210522_2D_biostudies\2_Final 2D Peyers Patch example\MATLAB\2D_PeyersPatch...
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IdentifyPrimaryObjects
ConvertObjectsToImage
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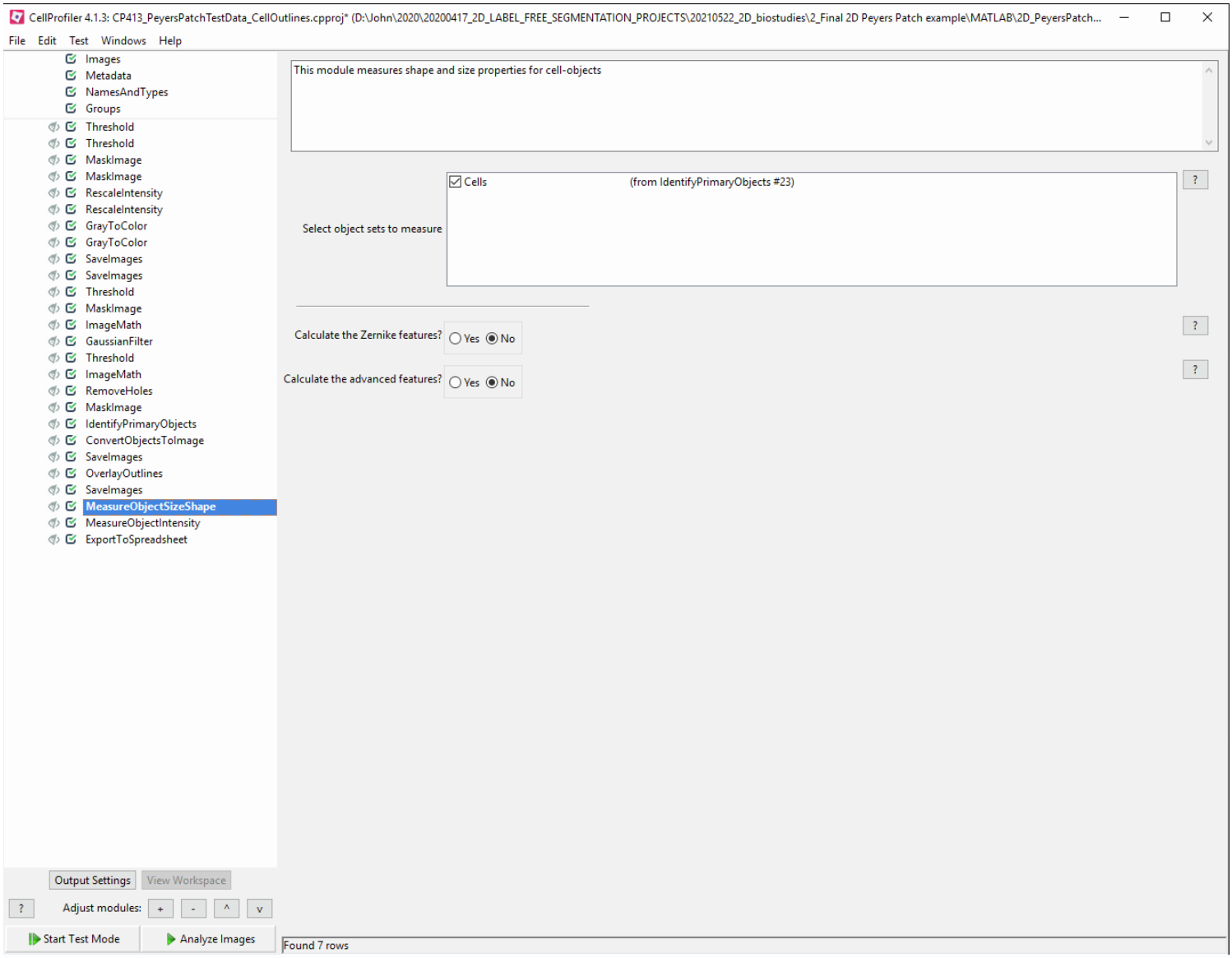
OverlayOutlines
SaveImages
MeasureObjectSizeShape
MeasureObjectIntensity
ExportToSpreadsheet
This module saves the view of the cell-object outlines overlayed onto one of the immunofluorescence channels
i.e., it is a visual segmentation checker
Select the type of image to save
Image
Select the image to save
Segmentation_checker
(from OverlayOutlines #26)
Select method for constructing file names
Sequential numbers
Enter file prefix
Segmentation_checker
Number of digits
3
Saved file format
tiff
Image bit depth
8-bit integer
Output file location
Default Output Folder sub-folder
(d:\\cellprofiler_out)
Sub-folder:
Segmentation_checker
Overwrite existing files without warning?
Yes
No
When to save
Every cycle
Record the file and path information to the saved image?
Yes
No
Output Settings
View Workspace
Adjust modules:
Start Test Mode
Analyze Images
Found 7 rows


CellProfiler 4.1.3: CP413_PeyersPatchTestData_CellOutlines.cpproj* (D:\John\2020\20200417_2D_LABEL_FREE_SEGMENTATION_PROJECTS\20210522_2D_biostudies\2_Final 2D Peyers Patch example\MATLAB\2D_PeyersPatch...
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ImageMath
RemoveHoles
MaskImage
IdentifyPrimaryObjects
ConvertObjectsToImage
SaveImages
OverlayOutlines
SaveImages
MeasureObjectSizeShape
MeasureObjectIntensity
ExportToSpreadsheet
This module measures shape and size properties for cell-objects
Select object sets to measure
Cells
(from IdentifyPrimaryObjects #23)
Calculate the Zernike features?
Yes
No
Calculate the advanced features?
Yes
No
Output Settings
View Workspace
Adjust modules:
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CellProfiler 4.1.3: CP413_PeyersPatchTestData_CellOutlines.cpproj* (D:\John\2020\20200417_2D_LABEL_FREE_SEGMENTATION_PROJECTS\20210522_2D_biostudies\2_Final 2D Peyers Patch example\MATLAB\2D_PeyersPatch...

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- RemoveHoles
- MaskImage
- IdentifyPrimaryObjects
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- MeasureObjectSizeShape
- **MeasureObjectIntensity**
- ExportToSpreadsheet

Output Settings | View Workspace

? Adjust modules: + - ^ v

Start Test Mode | Analyze Images

This module measures intensity properties in cell-objects for each of the images specified

e.g., amount of CD11c or CD3 expression in each cell object

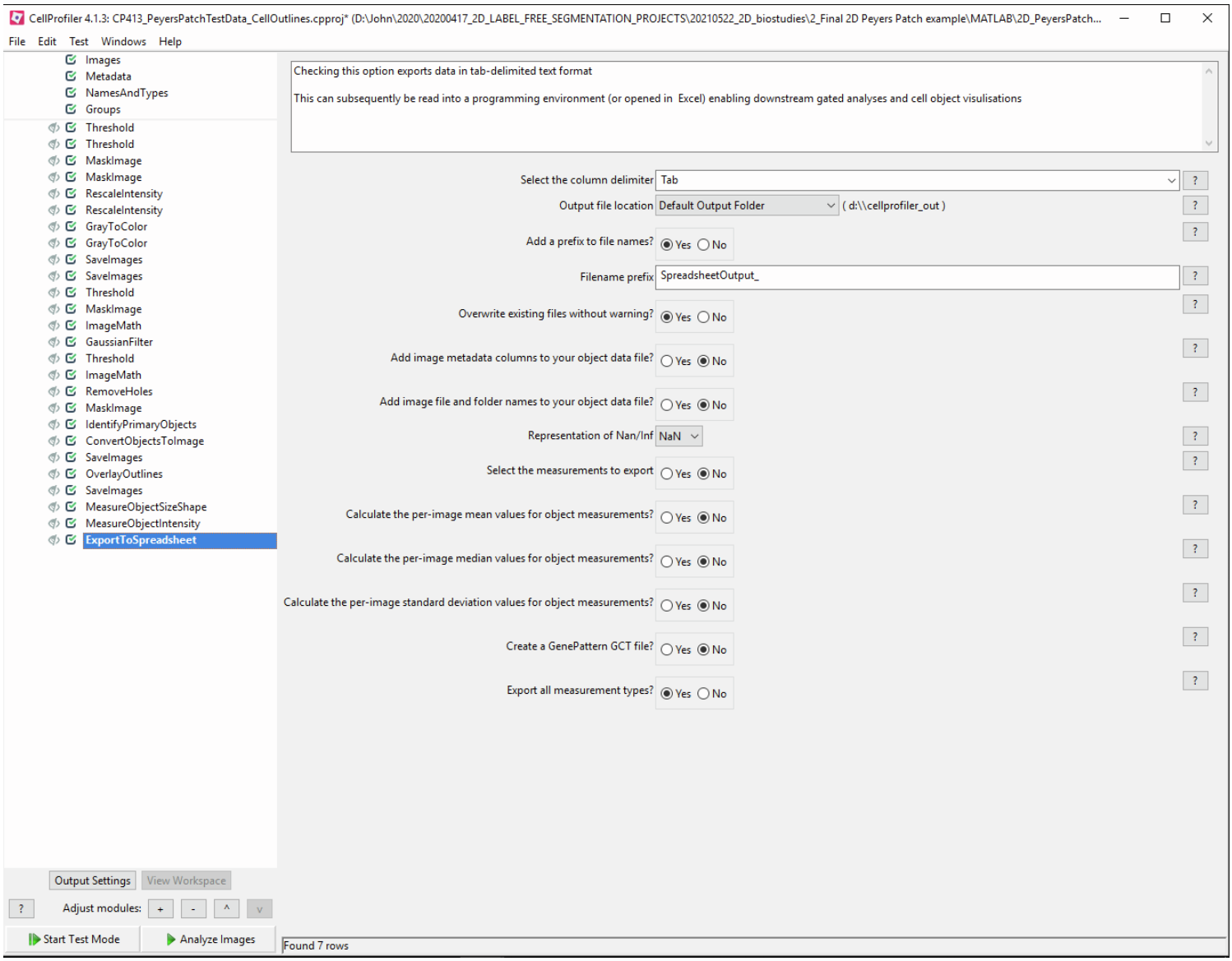
Select images to measure

- [ ] CellMasks (from ConvertObjectsToImage #24)
- [ ] Masked_c6 (from MaskImage #16)
- [ ] Segmentation_checker (from OverlayOutlines #26)
- [ ] Smoothed_background_pmap (from GaussianFilter #18)
- [ ] View_thresh_CD11c_rescaled (from GrayToColor #11)
- [ ] View_thresh_CD3_rescaled (from GrayToColor #12)
- [ ] background_masked_seeds (from MaskImage #22)

Select objects to measure

- [x] Cells (from IdentifyPrimaryObjects #23)

Found 7 rows


CellProfiler 4.1.3: CP413_PeyersPatchTestData_CellOutlines.cpproj* (D:\John\2020\20200417_2D_LABEL_FREE_SEGMENTATION_PROJECTS\20210522_2D_biostudies\2_Final 2D Peyers Patch example\MATLAB\2D_PeyersPatch...
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RemoveHoles
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IdentifyPrimaryObjects
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SaveImages
MeasureObjectSizeShape
MeasureObjectIntensity
ExportToSpreadsheet
Checking this option exports data in tab-delimited text format
This can subsequently be read into a programming environment (or opened in Excel) enabling downstream gated analyses and cell object visulisations
Select the column delimiter
Tab
Output file location
Default Output Folder
(d:\\cellprofiler_out)
Add a prefix to file names?
Yes No
Filename prefix
SpreadsheetOutput_
Overwrite existing files without warning?
Yes No
Add image metadata columns to your object data file?
Yes No
Add image file and folder names to your object data file?
Yes No
Representation of Nan/Inf
NaN
Select the measurements to export
Yes No
Calculate the per-image mean values for object measurements?
Yes No
Calculate the per-image median values for object measurements?
Yes No
Calculate the per-image standard deviation values for object measurements?
Yes No
Create a GenePattern GCT file?
Yes No
Export all measurement types?
Yes No
Output Settings
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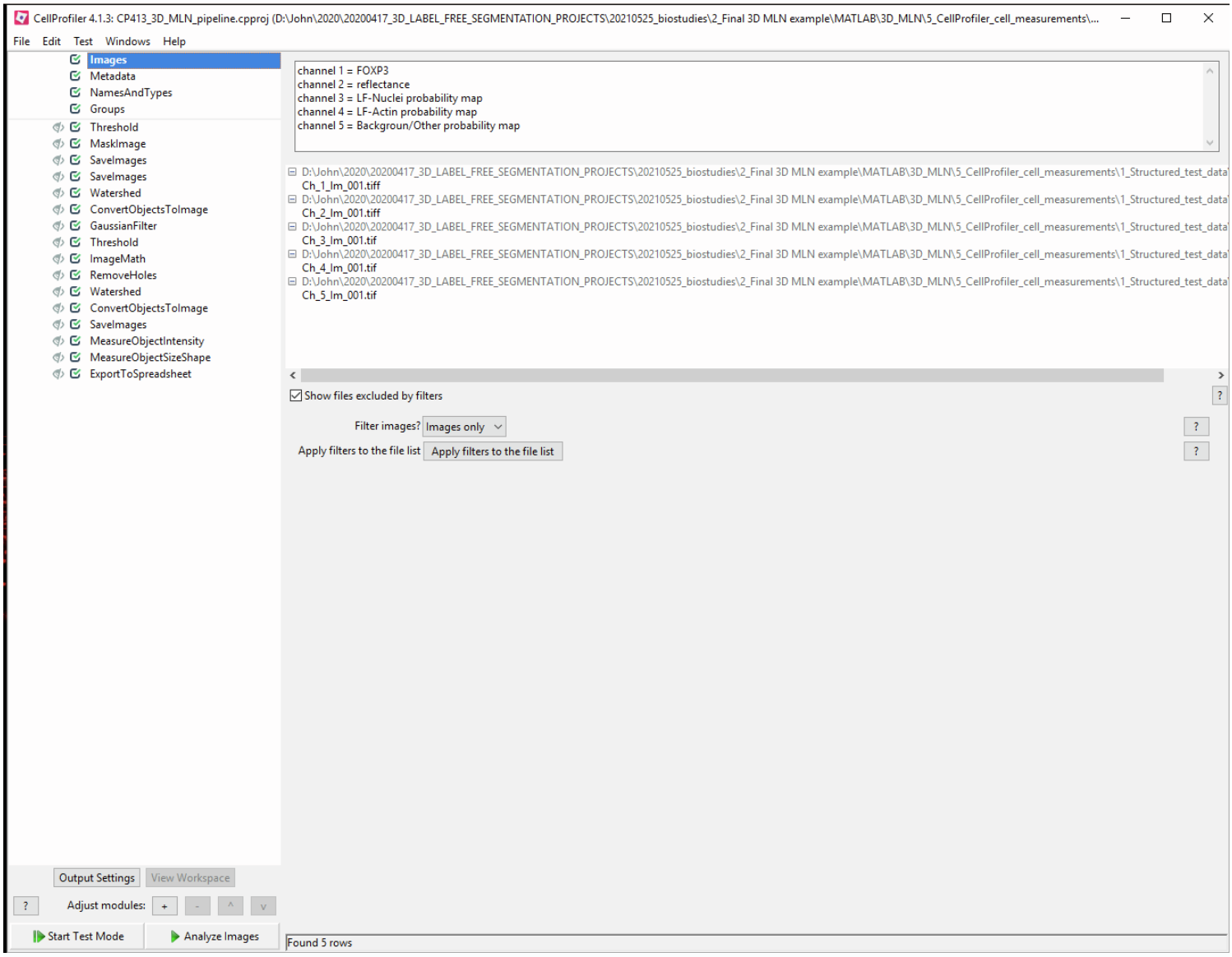
Adjust modules:
Start Test Mode
Analyze Images
Found 7 rows

3-D CellProfiler pipeline. This section presents screenshots of a CellProfiler image analysis pipeline used to achieve label-free cell segmentation in 3-D from the Unet network outputs and to measure the intensity and size/shape features of identified cell-objects. To use the image analysis pipeline with new image data, the 'IdentifyPrimaryObjects' module simply needs adjusting so that the 'typical diameter of objects' size-range matches the pixel scaling of the new images. For newcomers to CellProfiler, we recommend downloading the image-data and pipeline from BioStudies database https://www.ebi.ac.uk/biostudies/ under accession number S-BSST742. This enables the pipeline to be run with the data described in the manuscript and allows the user to see how each module works.



CellProfiler 4.1.3: CP413_3D_MLN_pipeline.cpproj (D:\John\2020\20200417_3D_LABEL_FREE_SEGMENTATION_PROJECTS\20210525_biostudies\2_Final 3D MLN example\MATLAB\3D_MLN\5_CellProfiler_cell_measurements\...

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- SaveImages
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- RemoveHoles
- Watershed
- ConvertObjectsToImage
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- MeasureObjectIntensity
- MeasureObjectSizeShape
- ExportToSpreadsheet

The Metadata module optionally allows you to extract information describing your images (i.e, metadata) which will be stored along with your measurements. This information can be contained in the file name and/or location, or in an external file.

Extract metadata? (•) Yes ( ) No

Metadata extraction method: Extract from file/folder names

Metadata source: File name

Regular expression to extract from file name: ^(?P<Ch>.*)_(?P<channel>[0-9])_(?P<Im>.*)_(?P<tile>[0-9]{1,3})

Extract metadata from: All images

Add another extraction method

Metadata data type: Text

Update	Path / URL	Series	Frame	Ch	FileLocation	channel	Im	tile
1	D:\John\2020\	0	0	Ch	file:///D:/Jo...1	1	Im	001
2	D:\John\2020\	0	0	Ch	file:///D:/Jo...2	2	Im	001
3	D:\John\2020\	0	0	Ch	file:///D:/Jo..._	3	Im	001
4	D:\John\2020\	0	0	Ch	file:///D:/Jo..._	4	Im	001
5	D:\John\2020\	0	0	Ch	file:///D:/Jo..._	5	Im	001

Output Settings | View Workspace

? Adjust modules: + - ^ v

Start Test Mode | Analyze Images

Found 5 rows

CellProfiler 4.1.3: CP413_3D_MLN_pipeline.cpproj (D:\John\2020\20200417_3D_LABEL_FREE_SEGMENTATION_PROJECTS\20210525_biostudies\2_Final 3D MLN example\MATLAB\3D_MLN\5_CellProfiler_cell_measurements\...

File Edit Test Windows Help

- Images
- Metadata
- NamesAndTypes
- Groups
- Threshold
- MaskImage
- SaveImages
- SaveImages
- Watershed
- ConvertObjectsToImage
- GaussianFilter
- Threshold
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- RemoveHoles
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The NamesAndTypes module allows you to assign a meaningful name to each image by which other modules will refer to it.

Assign a name to: Images matching rules

Process as 3D? (•) Yes ( ) No

Relative pixel spacing in X: 0.207

Relative pixel spacing in Y: 0.207

Relative pixel spacing in Z: 0.150

Select the rule criteria: Match All of the following rules
Metadata | Does | Have channel matching | 1

Name to assign these images: c1

Select the image type: Grayscale image

Duplicate this image

Select the rule criteria: Match All of the following rules
Metadata | Does | Have channel matching | 2

Name to assign these images: c2

Select the image type: Grayscale image

Duplicate this image

Remove this image

Select the rule criteria: Match All of the following rules
Metadata | Does | Have channel matching | 3

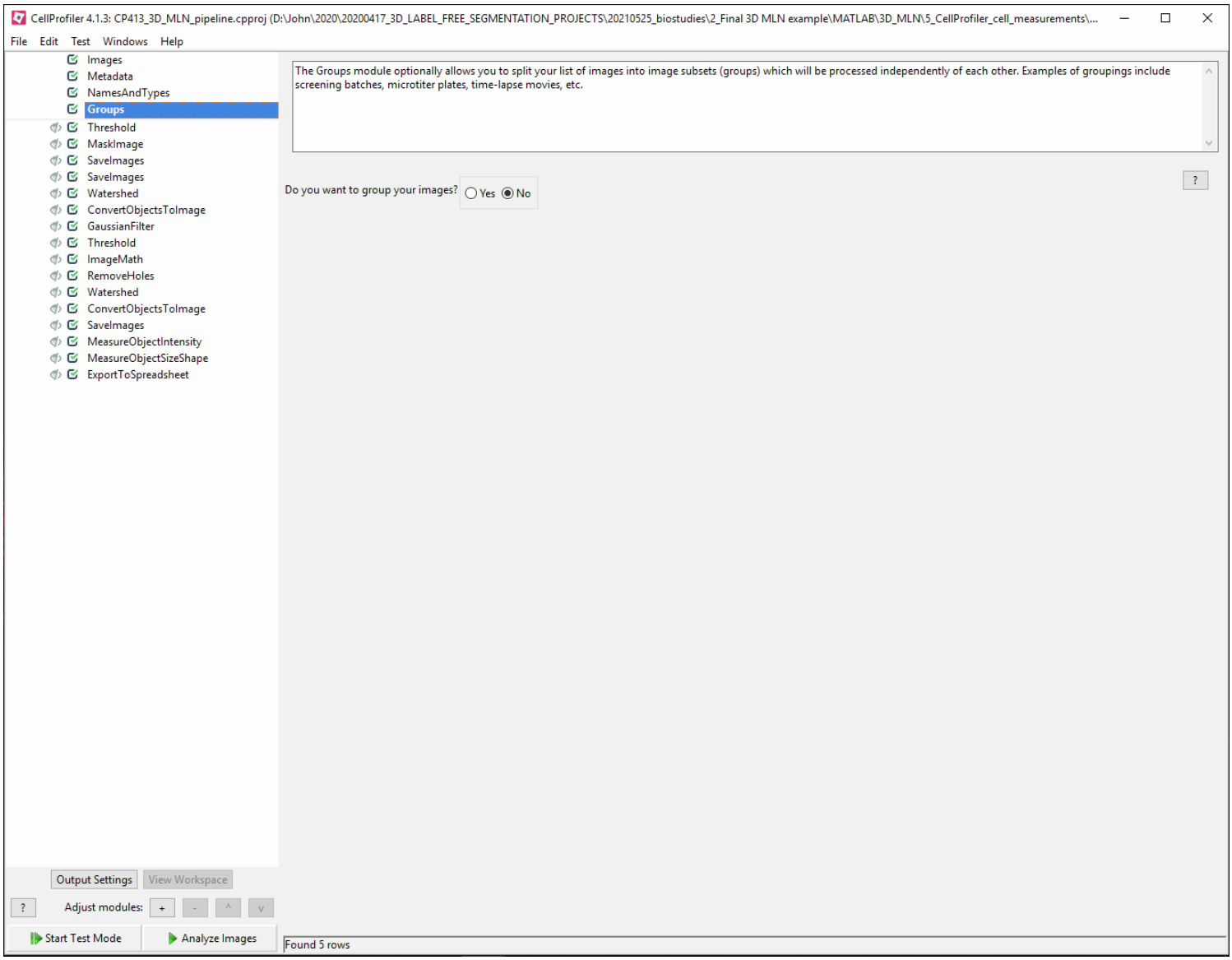
Update	c1	c2	c3	c4	c5
1	Ch_1_Im_001.tiff	Ch_2_Im_001.tiff	Ch_3_Im_001.tif	Ch_4_Im_001.tif	Ch_5_Im_001.tif

Output Settings | View Workspace

Adjust modules: + - ^ v

Start Test Mode | Analyze Images

Found 5 rows


CellProfiler 4.1.3: CP413_3D_MLN_pipeline.cpproj (D:\John\2020\20200417_3D_LABEL_FREE_SEGMENTATION_PROJECTS\20210525_biostudies\2_Final 3D MLN example\MATLAB\3D_MLN\5_CellProfiler_cell_measurements\...
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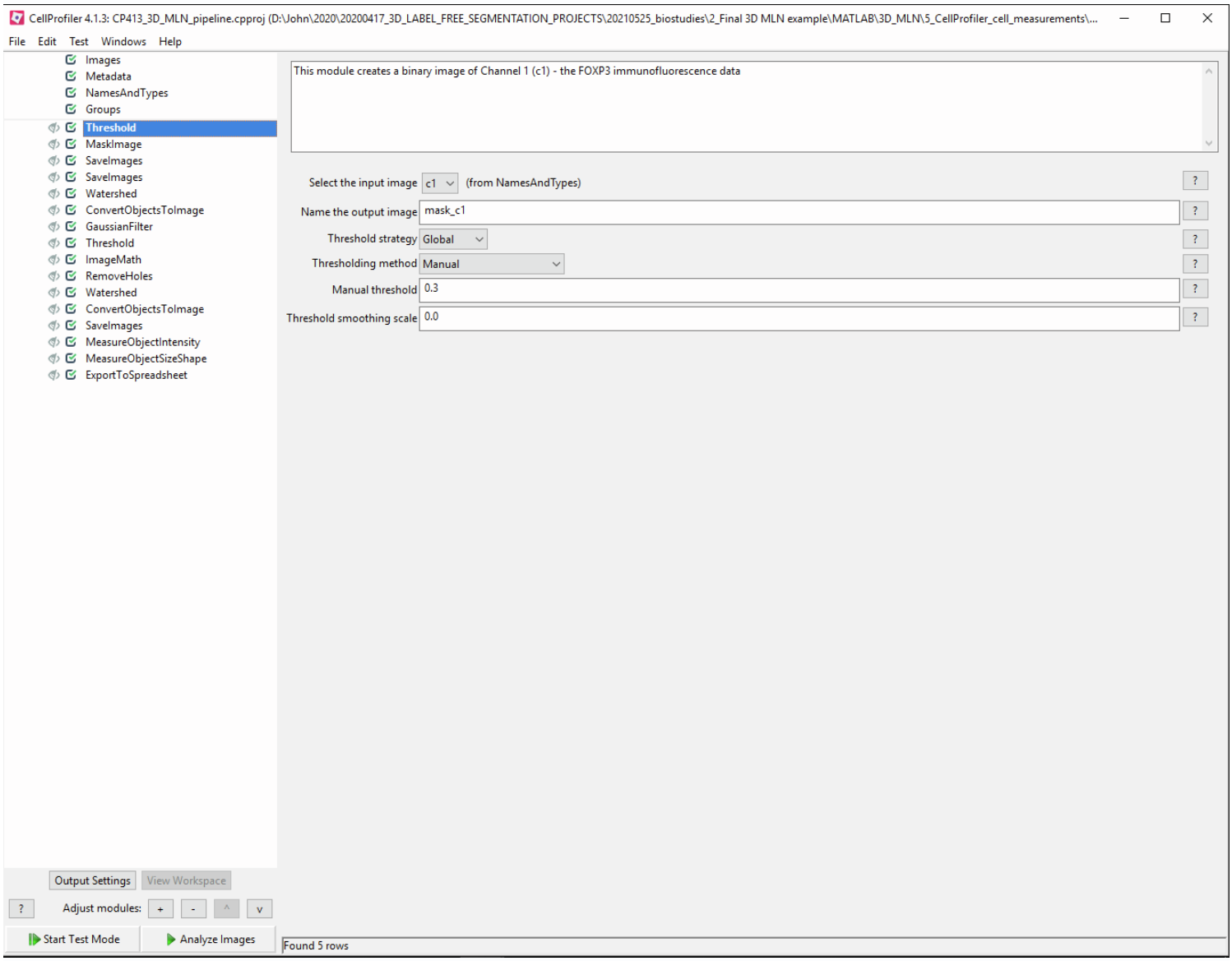
The Groups module optionally allows you to split your list of images into image subsets (groups) which will be processed independently of each other. Examples of groupings include screening batches, microtiter plates, time-lapse movies, etc.
Do you want to group your images?
Yes
No
?
Output Settings
View Workspace
?
Adjust modules:
+
-
^
v
Start Test Mode
Analyze Images
Found 5 rows


CellProfiler 4.1.3: CP413_3D_MLN_pipeline.cpproj (D:\John\2020\20200417_3D_LABEL_FREE_SEGMENTATION_PROJECTS\20210525_biostudies\2_Final 3D MLN example\MATLAB\3D_MLN\5_CellProfiler_cell_measurements\...
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MeasureObjectIntensity
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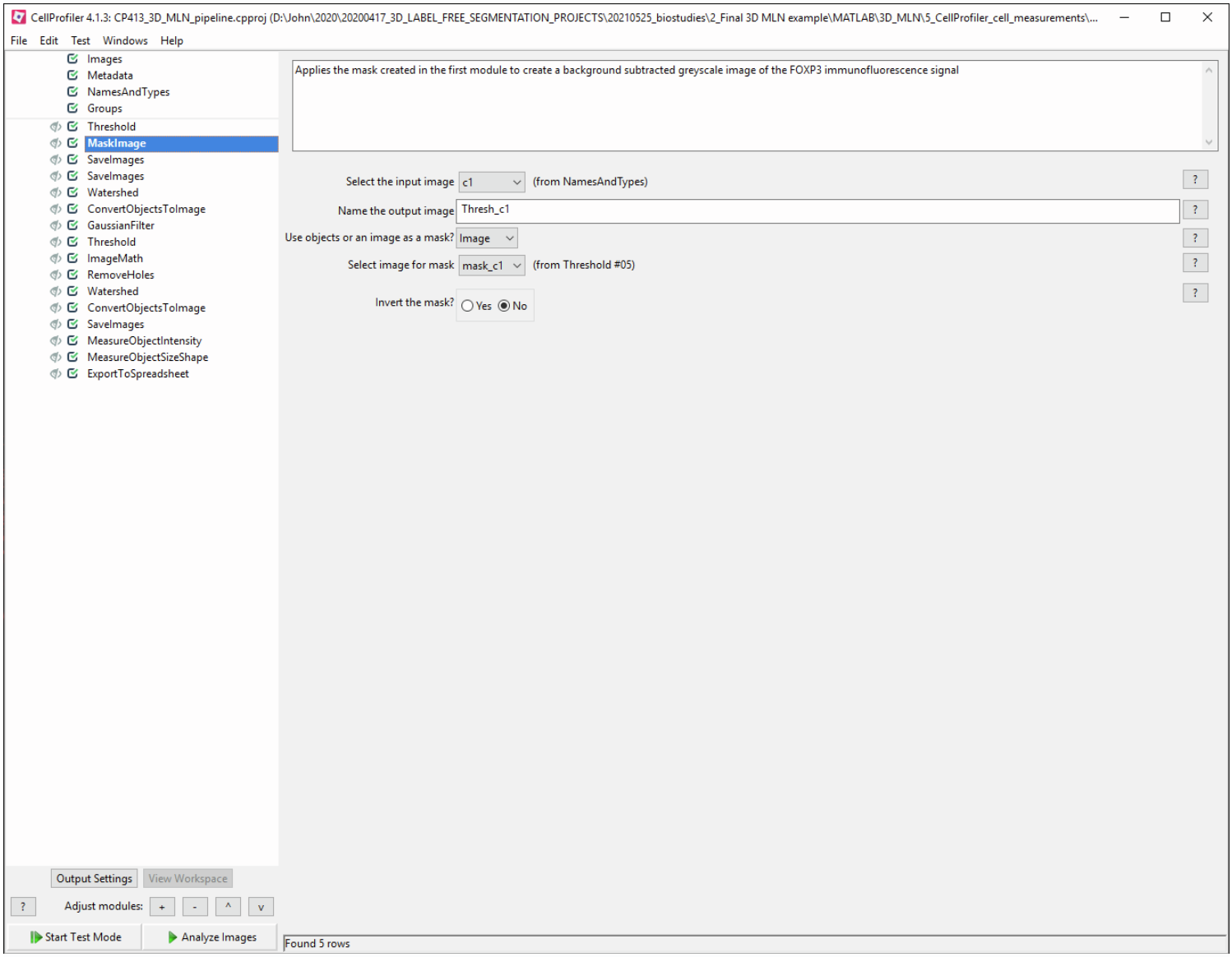
ExportToSpreadsheet
This module creates a binary image of Channel 1 (c1) - the FOXP3 immunofluorescence data
Select the input image
c1
(from NamesAndTypes)
Name the output image
mask_c1
Threshold strategy
Global
Thresholding method
Manual
Manual threshold
0.3
Threshold smoothing scale
0.0
Output Settings
View Workspace
Adjust modules:
Start Test Mode
Analyze Images
Found 5 rows


CellProfiler 4.1.3: CP413_3D_MLN_pipeline.cpproj (D:\John\2020\20200417_3D_LABEL_FREE_SEGMENTATION_PROJECTS\20210525_biostudies\2_Final 3D MLN example\MATLAB\3D_MLN\5_CellProfiler_cell_measurements\...
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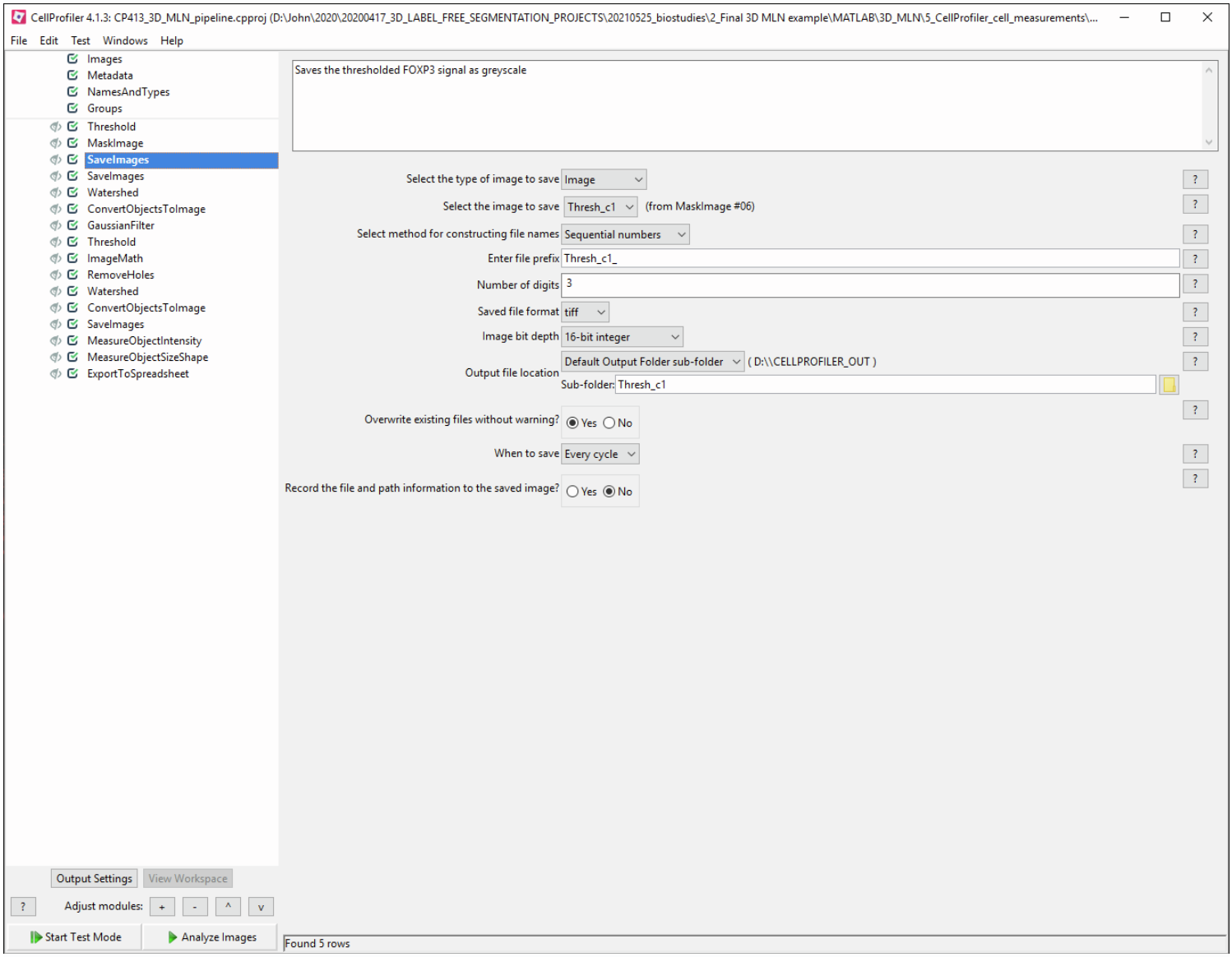
ExportToSpreadsheet
Applies the mask created in the first module to create a background subtracted greyscale image of the FOXP3 immunofluorescence signal
Select the input image c1 (from NamesAndTypes)
Name the output image Thresh_c1
Use objects or an image as a mask? Image
Select image for mask mask_c1 (from Threshold #05)
Invert the mask? Yes No
Output Settings
View Workspace
Adjust modules:
Start Test Mode
Analyze Images
Found 5 rows


CellProfiler 4.1.3: CP413_3D_MLN_pipeline.cpproj (D:\John\2020\20200417_3D_LABEL_FREE_SEGMENTATION_PROJECTS\20210525_biostudies\2_Final 3D MLN example\MATLAB\3D_MLN\5_CellProfiler_cell_measurements\...
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ConvertObjectsToImage
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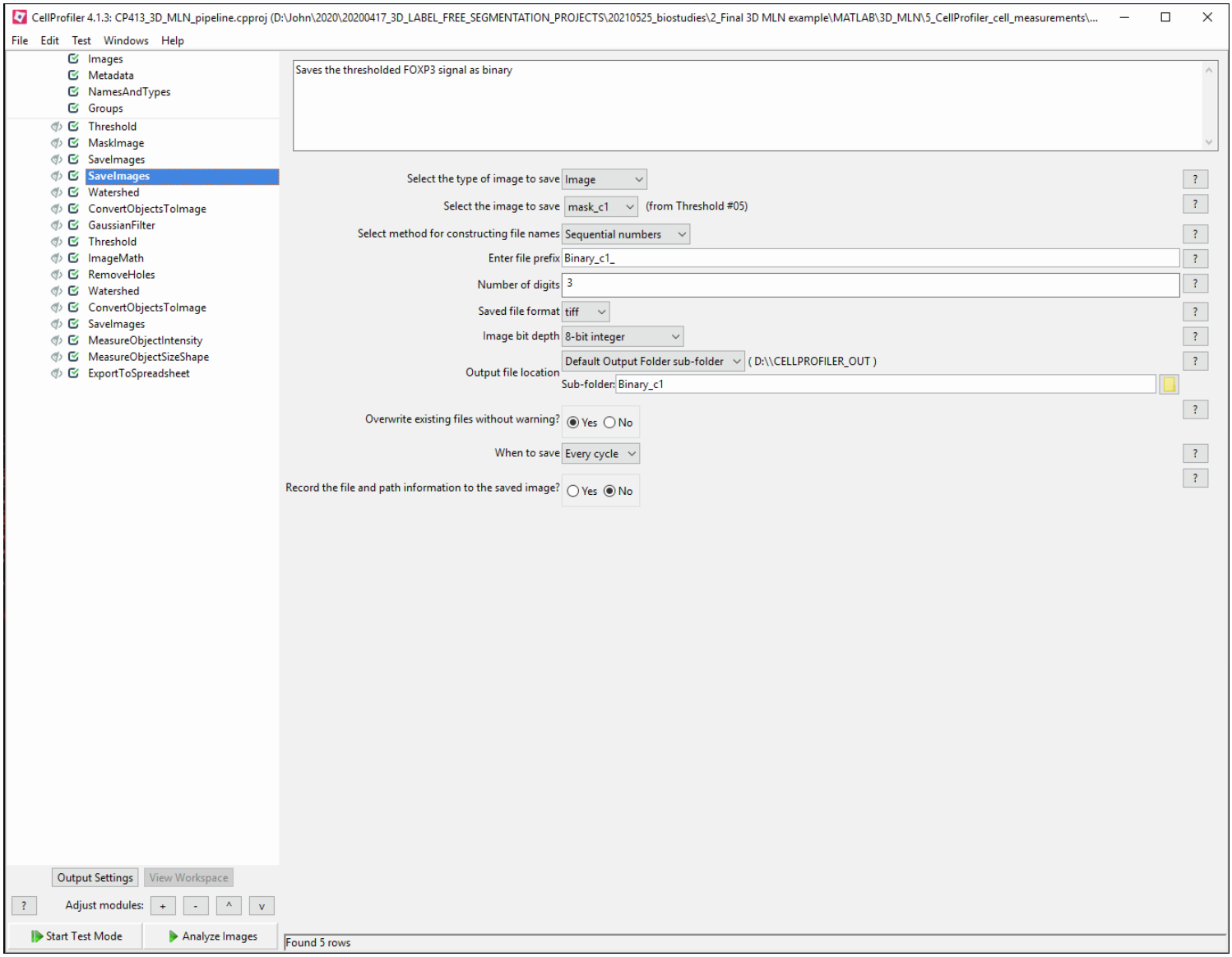
MeasureObjectIntensity
MeasureObjectSizeShape
ExportToSpreadsheet
Saves the thresholded FOXP3 signal as greyscale
Select the type of image to save
Image
Select the image to save
Thresh_c1
(from MaskImage #06)
Select method for constructing file names
Sequential numbers
Enter file prefix
Thresh_c1_
Number of digits
3
Saved file format
tiff
Image bit depth
16-bit integer
Output file location
Default Output Folder sub-folder
(D:\\CELLPROFILER_OUT)
Sub-folder:
Thresh_c1
Overwrite existing files without warning?
Yes
No
When to save
Every cycle
Record the file and path information to the saved image?
Yes
No
Output Settings
View Workspace
Adjust modules:
Start Test Mode
Analyze Images
Found 5 rows


CellProfiler 4.1.3: CP413_3D_MLN_pipeline.cpproj (D:\John\2020\20200417_3D_LABEL_FREE_SEGMENTATION_PROJECTS\20210525_biostudies\2_Final 3D MLN example\MATLAB\3D_MLN\5_CellProfiler_cell_measurements\...
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Watershed
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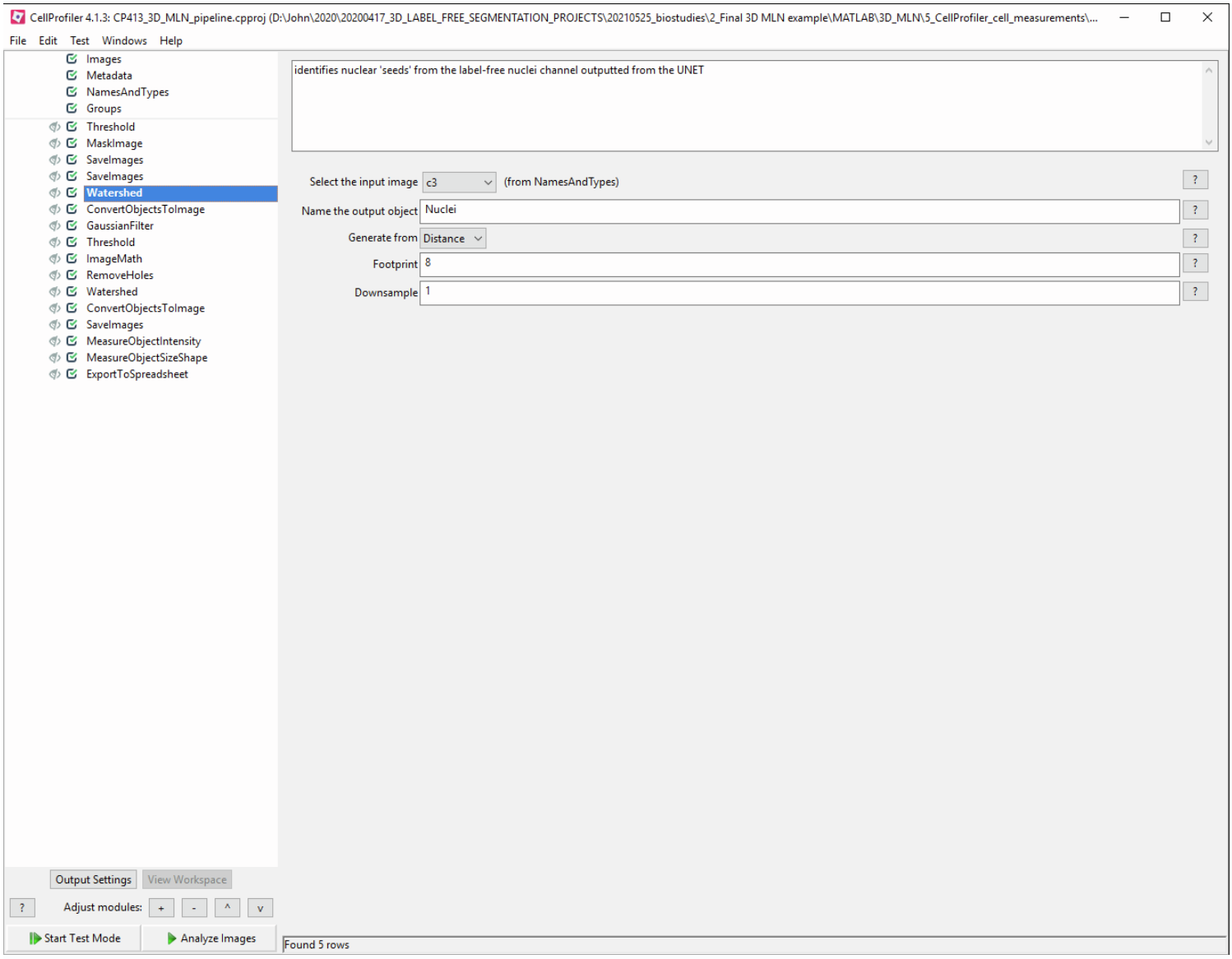
SaveImages
MeasureObjectIntensity
MeasureObjectSizeShape
ExportToSpreadsheet
Saves the thresholded FOXP3 signal as binary
Select the type of image to save
Image
Select the image to save
mask_c1
(from Threshold #05)
Select method for constructing file names
Sequential numbers
Enter file prefix
Binary_c1_
Number of digits
3
Saved file format
tiff
Image bit depth
8-bit integer
Output file location
Default Output Folder sub-folder
(D:\\CELLPROFILER_OUT)
Sub-folder:
Binary_c1
Overwrite existing files without warning?
Yes
No
When to save
Every cycle
Record the file and path information to the saved image?
Yes
No
Output Settings
View Workspace
Adjust modules:
Start Test Mode
Analyze Images
Found 5 rows


CellProfiler 4.1.3: CP413_3D_MLN_pipeline.cpproj (D:\John\2020\20200417_3D_LABEL_FREE_SEGMENTATION_PROJECTS\20210525_biostudies\2_Final 3D MLN example\MATLAB\3D_MLN\5_CellProfiler_cell_measurements\...
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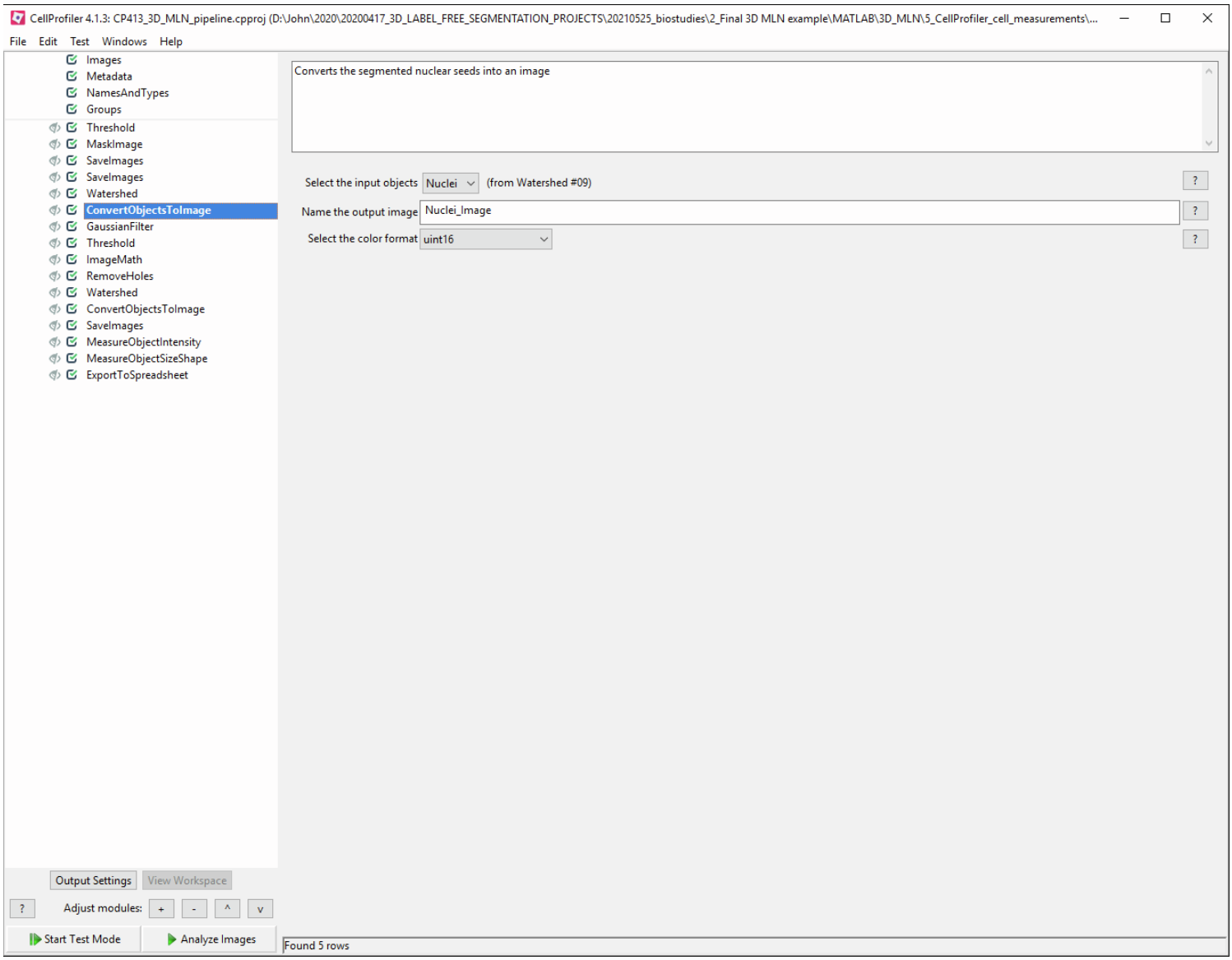
MeasureObjectSizeShape
ExportToSpreadsheet
identifies nuclear 'seeds' from the label-free nuclei channel outputted from the UNET
Select the input image
c3
(from NamesAndTypes)
Name the output object
Nuclei
Generate from
Distance
Footprint
8
Downsample
1
Output Settings
View Workspace
Adjust modules:
Start Test Mode
Analyze Images
Found 5 rows


CellProfiler 4.1.3: CP413_3D_MLN_pipeline.cpproj (D:\John\2020\20200417_3D_LABEL_FREE_SEGMENTATION_PROJECTS\20210525_biostudies\2_Final 3D MLN example\MATLAB\3D_MLN\5_CellProfiler_cell_measurements\...
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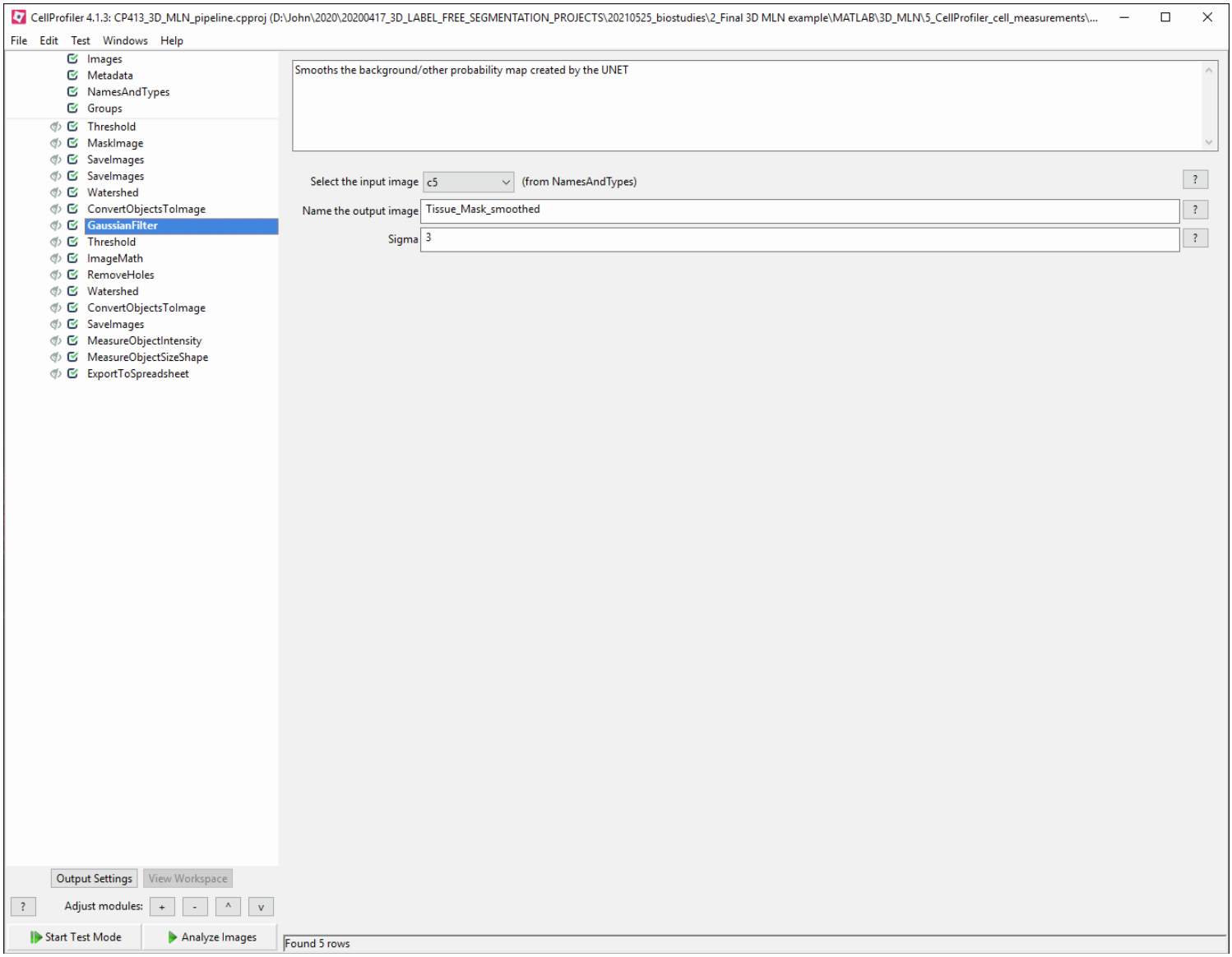
MeasureObjectIntensity
MeasureObjectSizeShape
ExportToSpreadsheet
Converts the segmented nuclear seeds into an image
Select the input objects
Nuclei
(from Watershed #09)
Name the output image
Nuclei_Image
Select the color format
uint16
Output Settings
View Workspace
Adjust modules:
Start Test Mode
Analyze Images
Found 5 rows


CellProfiler 4.1.3: CP413_3D_MLN_pipeline.cpproj (D:\John\2020\20200417_3D_LABEL_FREE_SEGMENTATION_PROJECTS\20210525_biostudies\2_Final 3D MLN example\MATLAB\3D_MLN\5_CellProfiler_cell_measurements\...
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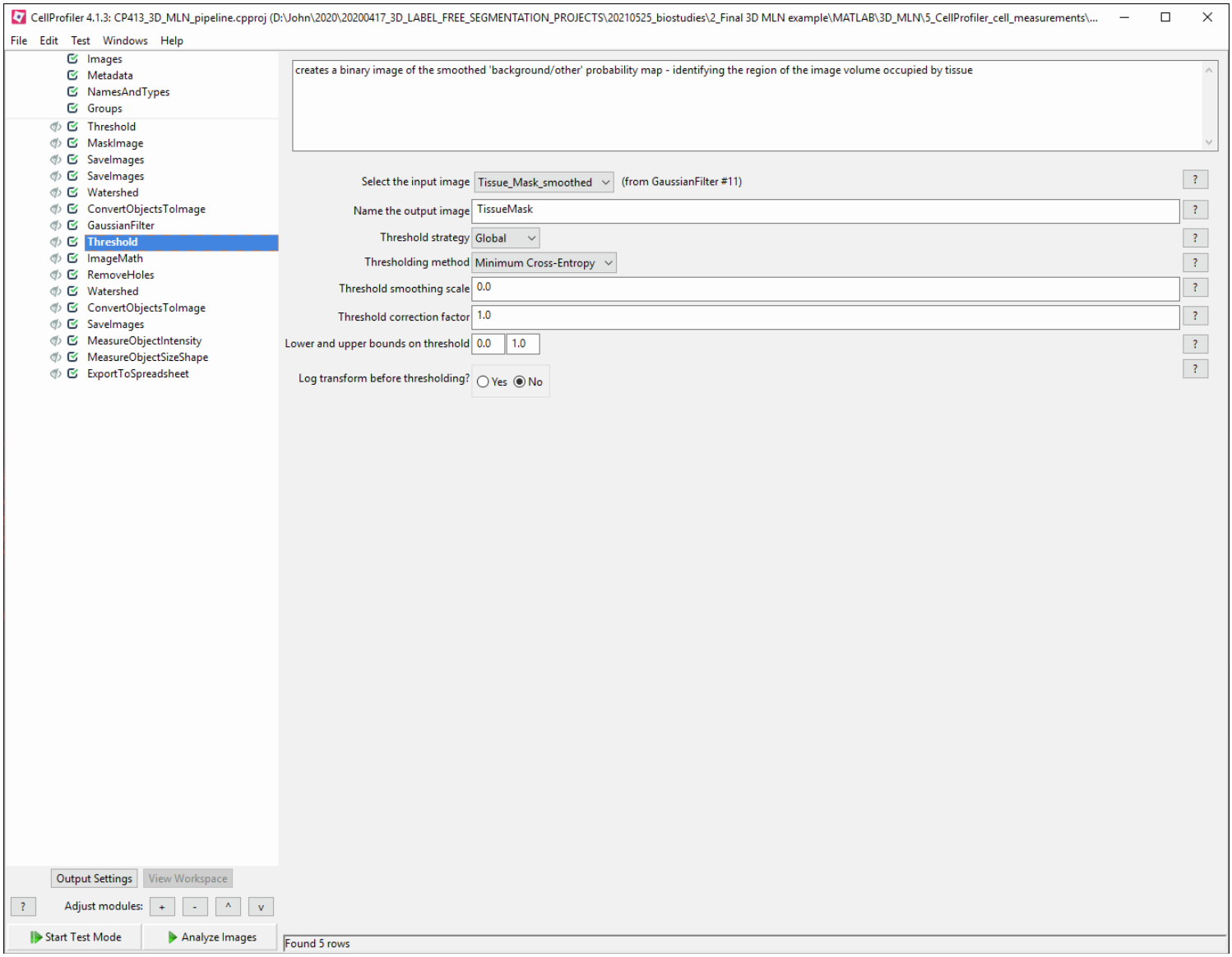
ExportToSpreadsheet
Smooths the background/other probability map created by the UNET
Select the input image
c5
(from NamesAndTypes)
Name the output image
Tissue_Mask_smoothed
Sigma
3
Output Settings
View Workspace
Adjust modules:
Start Test Mode
Analyze Images
Found 5 rows


CellProfiler 4.1.3: CP413_3D_MLN_pipeline.cpproj (D:\John\2020\20200417_3D_LABEL_FREE_SEGMENTATION_PROJECTS\20210525_biostudies\2_Final 3D MLN example\MATLAB\3D_MLN\5_CellProfiler_cell_measurements\...
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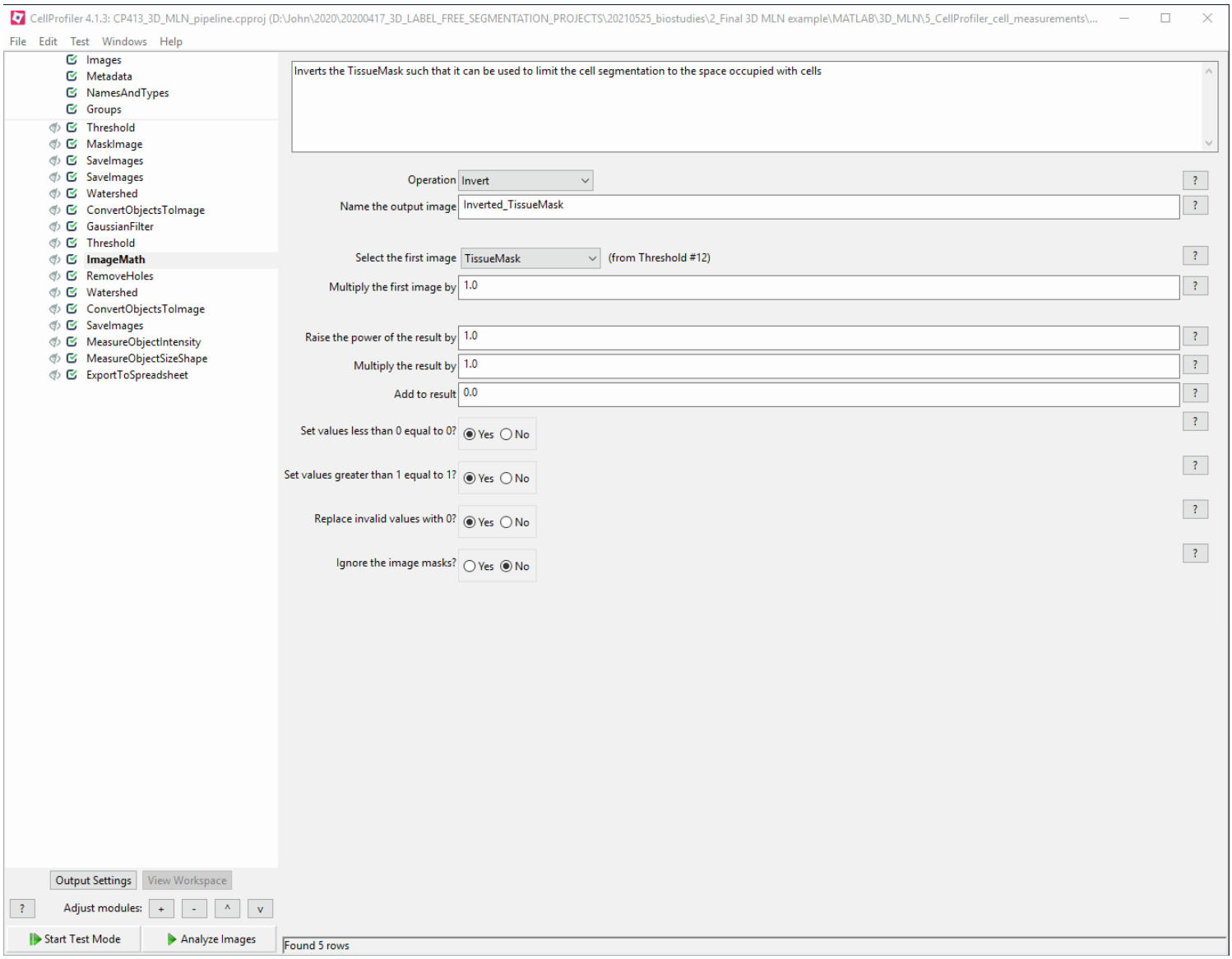
ExportToSpreadsheet
creates a binary image of the smoothed 'background/other' probability map - identifying the region of the image volume occupied by tissue
Select the input image
Tissue_Mask_smoothed
(from GaussianFilter #11)
Name the output image
TissueMask
Threshold strategy
Global
Thresholding method
Minimum Cross-Entropy
Threshold smoothing scale
0.0
Threshold correction factor
1.0
Lower and upper bounds on threshold
0.0
1.0
Log transform before thresholding?
Yes
No
Output Settings
View Workspace
Adjust modules:
Start Test Mode
Analyze Images
Found 5 rows


CellProfiler 4.1.3: CP413_3D_MLN_pipeline.cpproj (D:\John\2020\20200417_3D_LABEL_FREE_SEGMENTATION_PROJECTS\20210525_biostudies\2_Final 3D MLN example\MATLAB\3D_MLN\5_CellProfiler_cell_measurements\...
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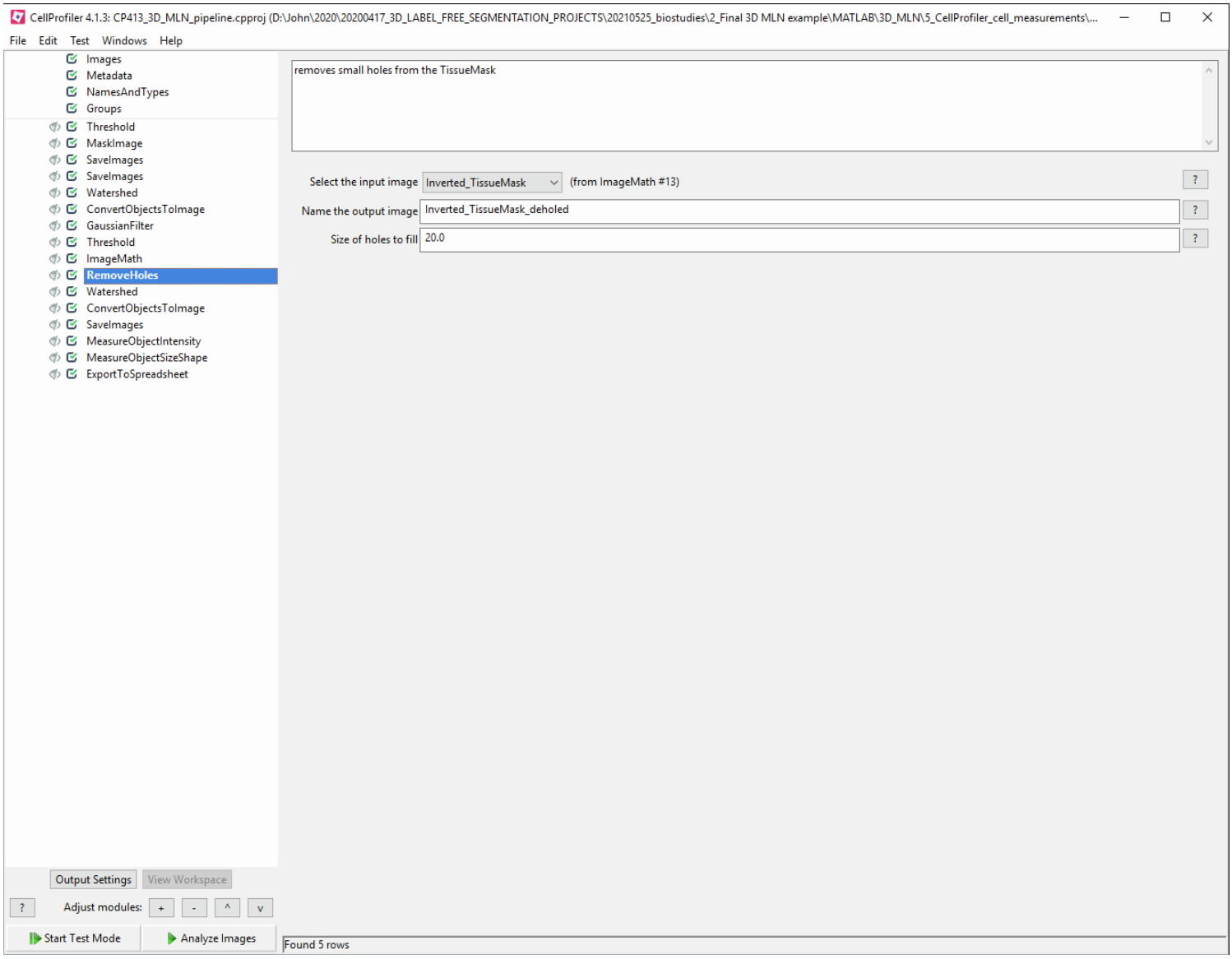
Inverts the TissueMask such that it can be used to limit the cell segmentation to the space occupied with cells
Operation Invert
Name the output image Inverted_TissueMask
Select the first image TissueMask (from Threshold #12)
Multiply the first image by 1.0
Raise the power of the result by 1.0
Multiply the result by 1.0
Add to result 0.0
Set values less than 0 equal to 0? Yes No
Set values greater than 1 equal to 1? Yes No
Replace invalid values with 0? Yes No
Ignore the image masks? Yes No
Output Settings
View Workspace
Adjust modules:
Start Test Mode
Analyze Images
Found 5 rows


CellProfiler 4.1.3: CP413_3D_MLN_pipeline.cpproj (D:\John\2020\20200417_3D_LABEL_FREE_SEGMENTATION_PROJECTS\20210525_biostudies\2_Final 3D MLN example\MATLAB\3D_MLN\5_CellProfiler_cell_measurements\...
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removes small holes from the TissueMask
Select the input image
Inverted_TissueMask
(from ImageMath #13)
Name the output image
Inverted_TissueMask_deholed
Size of holes to fill
20.0
Output Settings
View Workspace
Adjust modules:
Start Test Mode
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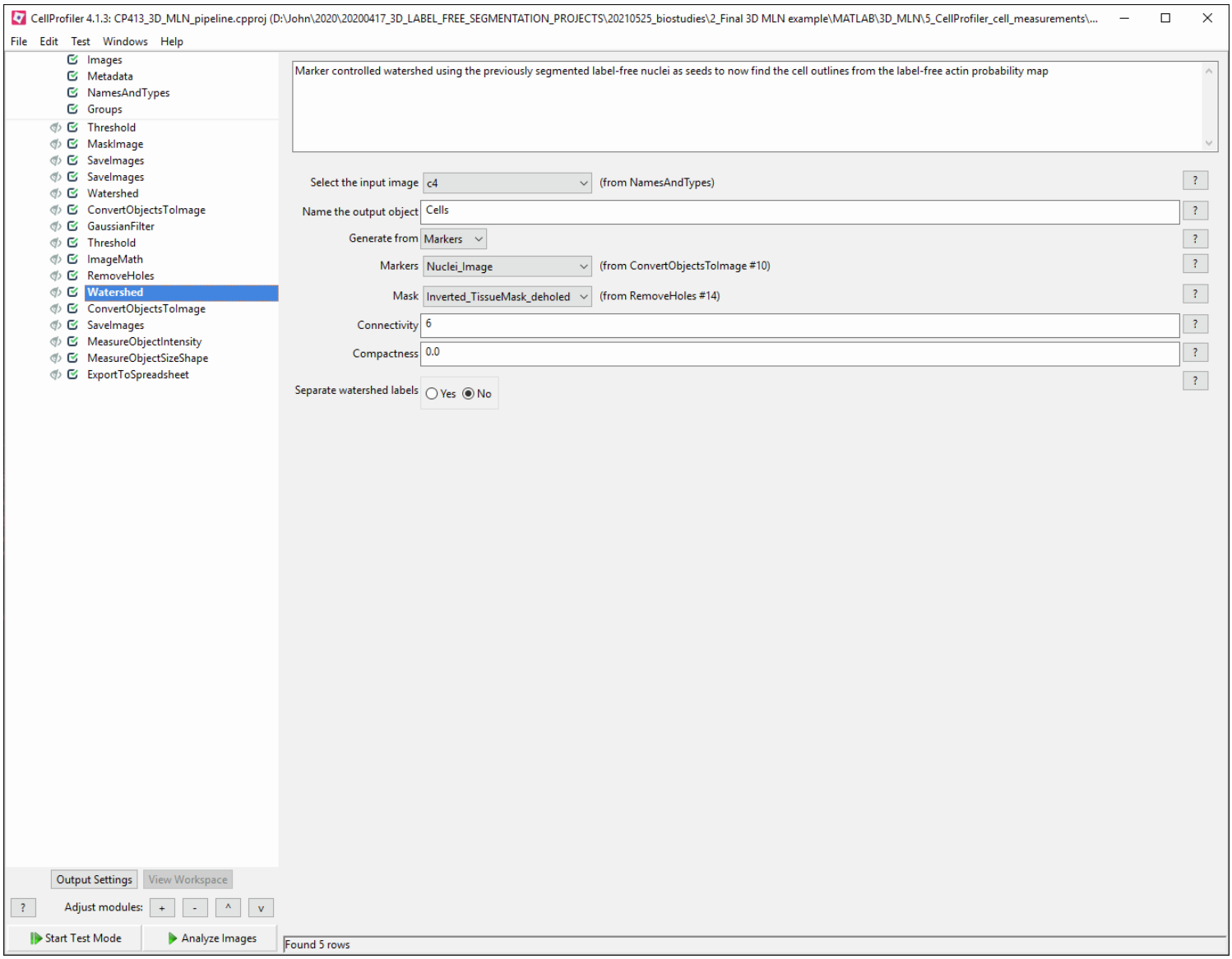
Found 5 rows


CellProfiler 4.1.3: CP413_3D_MLN_pipeline.cpproj (D:\John\2020\20200417_3D_LABEL_FREE_SEGMENTATION_PROJECTS\20210525_biostudies\2_Final 3D MLN example\MATLAB\3D_MLN\5_CellProfiler_cell_measurements\...
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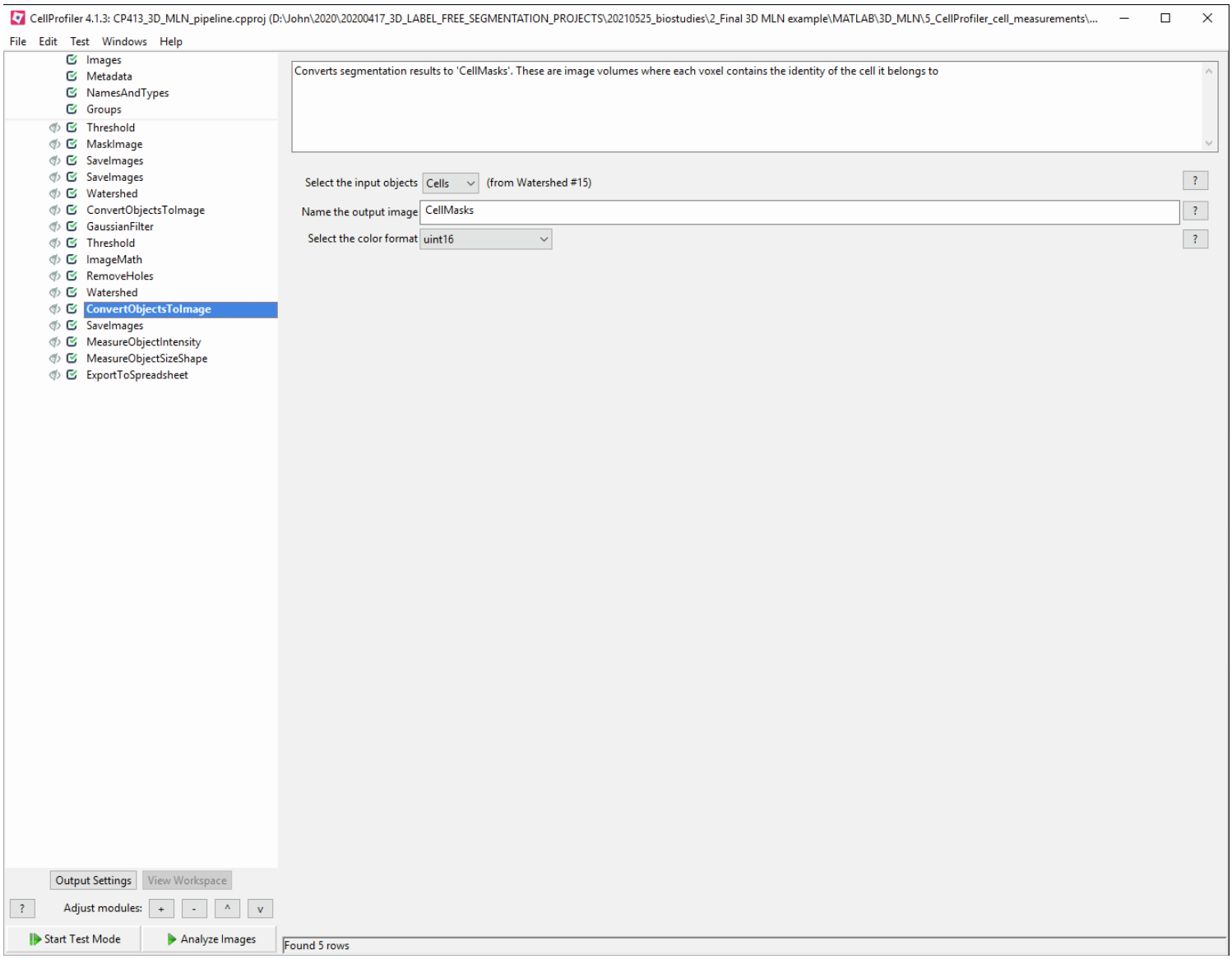
ExportToSpreadsheet
Marker controlled watershed using the previously segmented label-free nuclei as seeds to now find the cell outlines from the label-free actin probability map
Select the input image
c4
(from NamesAndTypes)
Name the output object
Cells
Generate from
Markers
Markers
Nuclei_Image
(from ConvertObjectsToImage #10)
Mask
Inverted_TissueMask_deholed
(from RemoveHoles #14)
Connectivity
6
Compactness
0.0
Separate watershed labels
Yes
No
Output Settings
View Workspace
Adjust modules:
Start Test Mode
Analyze Images
Found 5 rows


CellProfiler 4.1.3: CP413_3D_MLN_pipeline.cpproj (D:\John\2020\20200417_3D_LABEL_FREE_SEGMENTATION_PROJECTS\20210525_biostudies\2_Final 3D MLN example\MATLAB\3D_MLN\5_CellProfiler_cell_measurements\...
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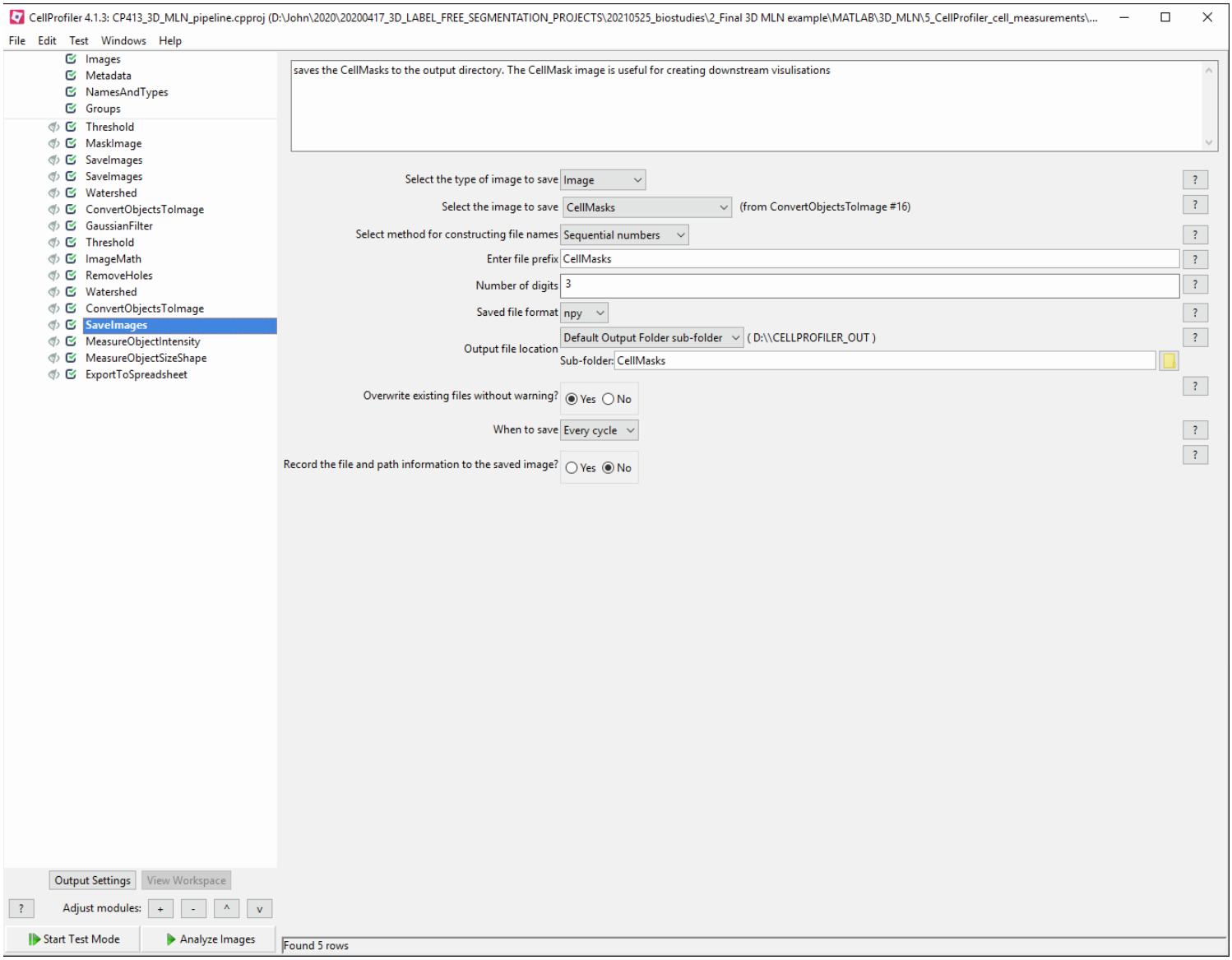
ExportToSpreadsheet
Converts segmentation results to 'CellMasks'. These are image volumes where each voxel contains the identity of the cell it belongs to
Select the input objects
Cells
(from Watershed #15)
Name the output image
CellMasks
Select the color format
uint16
Output Settings
View Workspace
Adjust modules:
Start Test Mode
Analyze Images
Found 5 rows


CellProfiler 4.1.3: CP413_3D_MLN_pipeline.cpproj (D:\John\2020\20200417_3D_LABEL_FREE_SEGMENTATION_PROJECTS\20210525_biostudies\2_Final 3D MLN example\MATLAB\3D_MLN\5_CellProfiler_cell_measurements\...
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ExportToSpreadsheet
saves the CellMasks to the output directory. The CellMask image is useful for creating downstream visulisations
Select the type of image to save
Image
Select the image to save
CellMasks
(from ConvertObjectsToImage #16)
Select method for constructing file names
Sequential numbers
Enter file prefix
CellMasks
Number of digits
3
Saved file format
npy
Output file location
Default Output Folder sub-folder
(D:\\CELLPROFILER_OUT)
Sub-folder:
CellMasks
Overwrite existing files without warning?
Yes
No
When to save
Every cycle
Record the file and path information to the saved image?
Yes
No
Output Settings
View Workspace
Adjust modules:
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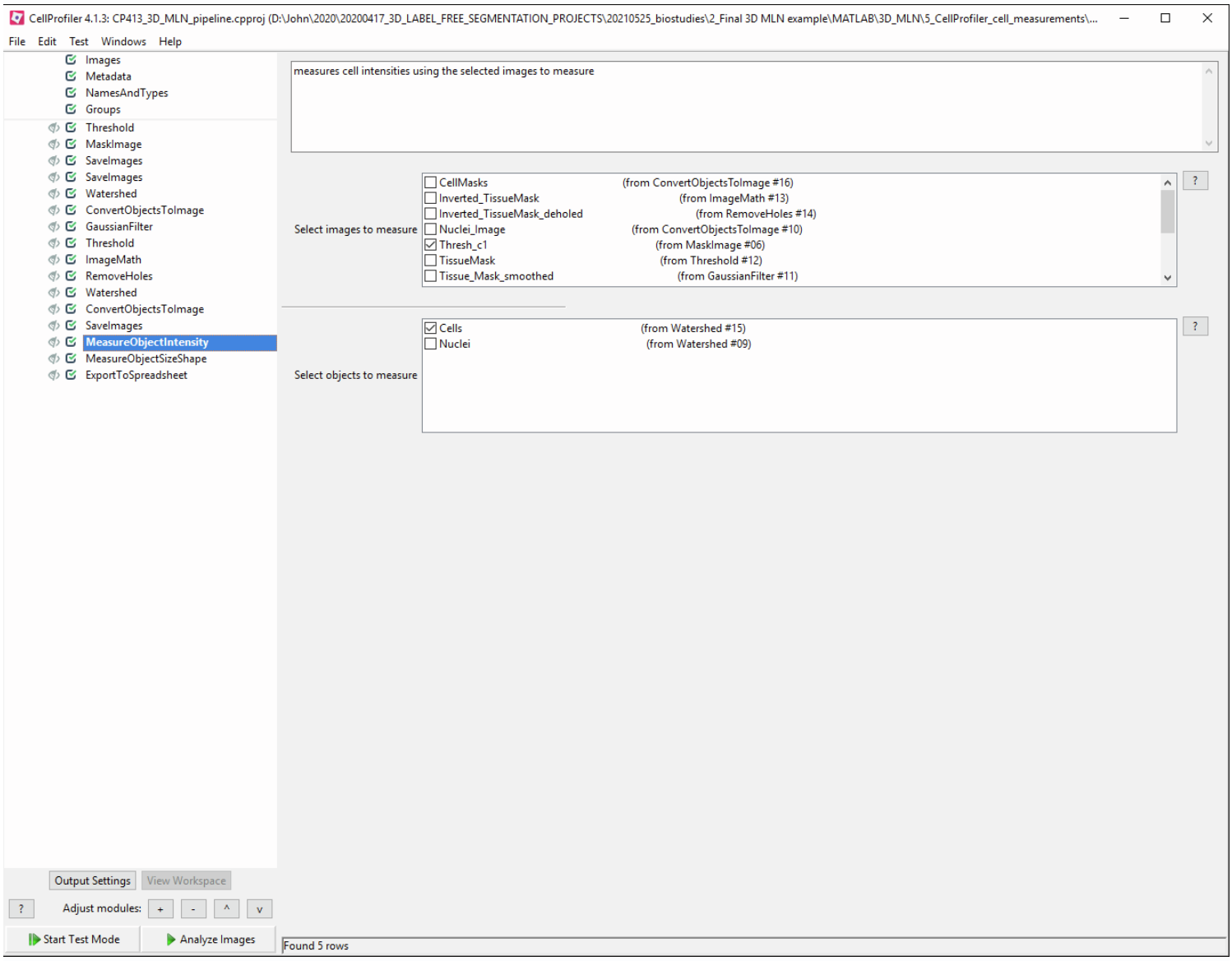
Found 5 rows


CellProfiler 4.1.3: CP413_3D_MLN_pipeline.cpproj (D:\John\2020\20200417_3D_LABEL_FREE_SEGMENTATION_PROJECTS\20210525_biostudies\2_Final 3D MLN example\MATLAB\3D_MLN\5_CellProfiler_cell_measurements\...
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measures cell intensities using the selected images to measure
Select images to measure
CellMasks (from ConvertObjectsToImage #16)
Inverted_TissueMask (from ImageMath #13)
Inverted_TissueMask_deholed (from RemoveHoles #14)
Nuclei_Image (from ConvertObjectsToImage #10)
Thresh_c1 (from MaskImage #06)
TissueMask (from Threshold #12)
Tissue_Mask_smoothed (from GaussianFilter #11)
Select objects to measure
Cells (from Watershed #15)
Nuclei (from Watershed #09)
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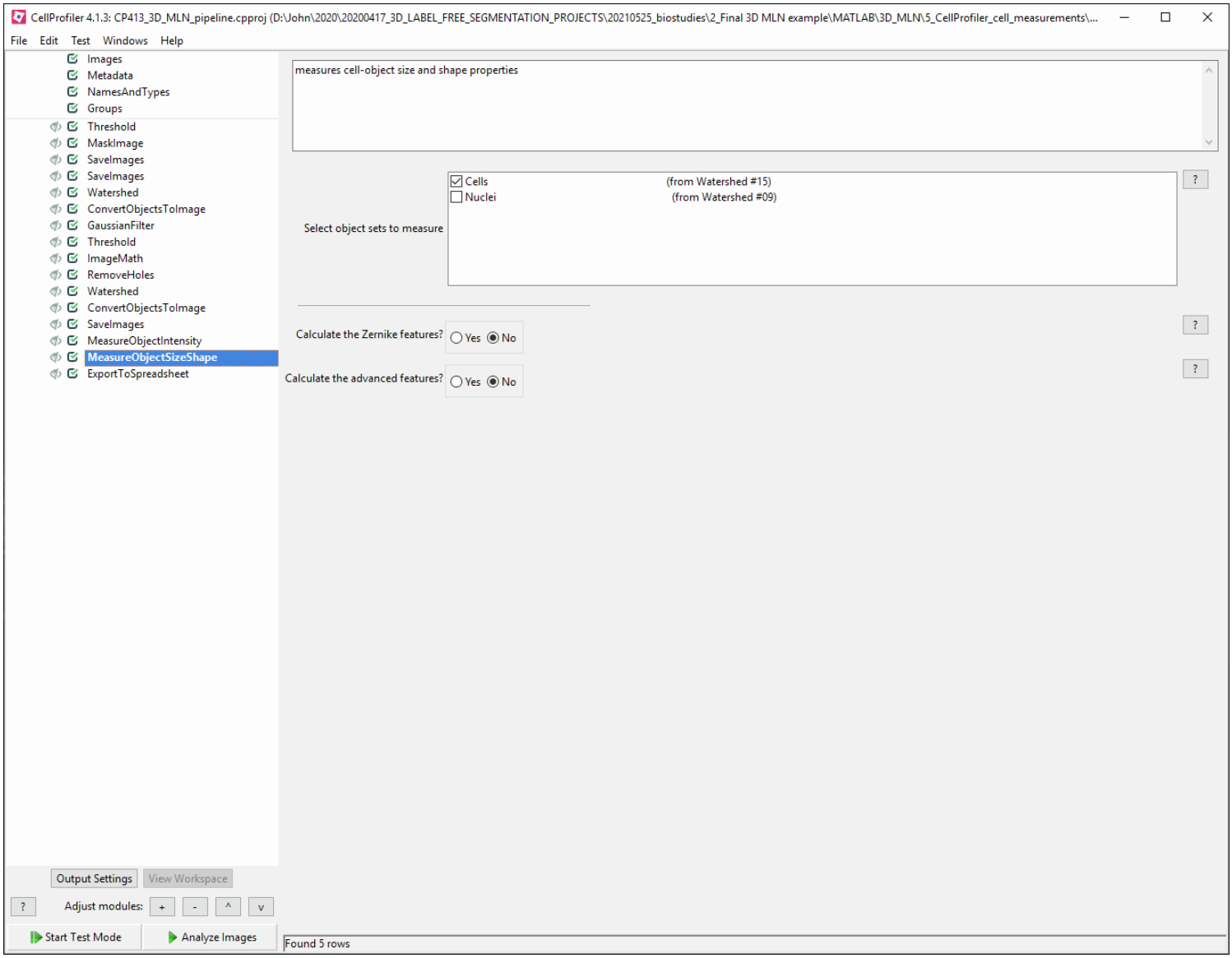
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ExportToSpreadsheet
measures cell-object size and shape properties
Select object sets to measure
Cells (from Watershed #15)
Nuclei (from Watershed #09)
Calculate the Zernike features? Yes No
Calculate the advanced features? Yes No
Output Settings
View Workspace
Adjust modules:
Start Test Mode
Analyze Images
Found 5 rows

CellProfiler 4.1.3: CP413_3D_MLN_pipeline.cpproj (D:\John\2020\20200417_3D_LABEL_FREE_SEGMENTATION_PROJECTS\20210525_biostudies\2_Final 3D MLN example\MATLAB\3D_MLN\5_CellProfiler_cell_measurements\...

File Edit Test Windows Help

- Images
- Metadata
- NamesAndTypes
- Groups
- Threshold
- MaskImage
- SaveImages
- SaveImages
- Watershed
- ConvertObjectsToImage
- GaussianFilter
- Threshold
- ImageMath
- RemoveHoles
- Watershed
- ConvertObjectsToImage
- SaveImages
- MeasureObjectIntensity
- MeasureObjectSizeShape
- **ExportToSpreadsheet**

outputs cell measurements in tab delimited text format

Select the column delimiter: Tab

Output file location: Default Output Folder (D:\\CELLPROFILER_OUT)

Add a prefix to file names? (•) Yes ( ) No

Filename prefix: MyExpt_

Overwrite existing files without warning? (•) Yes ( ) No

Add image metadata columns to your object data file? ( ) Yes (•) No

Add image file and folder names to your object data file? ( ) Yes (•) No

Representation of Nan/Inf: NaN

Select the measurements to export ( ) Yes (•) No

Calculate the per-image mean values for object measurements? ( ) Yes (•) No

Calculate the per-image median values for object measurements? ( ) Yes (•) No

Calculate the per-image standard deviation values for object measurements? ( ) Yes (•) No

Create a GenePattern GCT file? ( ) Yes (•) No

Export all measurement types? (•) Yes ( ) No

Output Settings | View Workspace

? Adjust modules: + - ^ v

Start Test Mode | Analyze Images

Found 5 rows