Deep learning approach for quantification of organelles and misfolded polypeptides delivery within degradative compartments

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Review Timeline:

Submission Date: 2020-04-27
Accepted: 2020-05-06

Editor-in-Chief: Matthew Welch

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

RE: Manuscript #E20-04-0269
TITLE: "Deep learning approach for quantification of organelles and misfolded polypeptides delivery within degradative compartments"

Dear Maurizio,

Thank you for considering Molecular Biology of the Cell for your submission after sending the manuscript to Review Commons for peer-review by three experts in the field. I believe that you have addressed the concerns of the reviewers in a satisfactory manner, and I am therefore very happy to let you know that your manuscript is now accepted for publication in Molecular Biology of the Cell.

Congratulations for a very nice paper!

Best regards,

Jean Gruenberg

Monitoring Editor
Molecular Biology of the Cell

Dear Prof. Molinari:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

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Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
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Reviewer #1

(Evidence, reproducibility and clarity (Required)):

Here Morone et al. develop a deep-learning (DL) based image analysis tool - LysoQuant - to segment lysosomal structures from cells (although practically this would be suited to many types of subcellular structures that localize as largely circular puncta). The authors compare the performance of this tool to classical machine learning based approaches, as well as human operators.

The authors conclude that LysoQuant outperforms both classical machine learning based methods and human operators, especially in terms of time take to segment.

I think this manuscript does a good job at describing the development of a tool that has a great deal of potential to be used by the wider community. As someone that performs single cell phenotypic analysis on a routine basis, I want to give it a try.

> **We would like to thank the Reviewer for the positive comments. All data needed to evaluate the conclusions in the paper are present in the paper and/or in the Supplementary Information. The described plugin, the training network and model are available on the github platform (https://github.com/irb-imagingfacility/lysoquant) and through the ImageJ update site. Training datasets of the experiments will be available on request. Additional data related to this paper may be requested from the authors.**

Some comments:

(1) I believe that LysoQuant is effective at segmentation in the cases described here. But notably, this work uses a very small dataset which appears to be all acquired by the same group on the same microscope using a single cell line.

This does put into question the robustness of LysoQuant to detect structures across images of different cell types with different staining, resolution, and quality.

I have little doubt LysoQuant is working well for this dataset, but it would be much better if the authors could demonstrate its utility across datasets.

I do not want to overburden authors with revisions, but deployment of LysoQuant on additional datasets would be an addition that would significantly increase the impact of this work.

> **We thank the referee for these suggestions. Due to activity shut down as a consequence of the COVID-19 pandemics, we were unable to involve other groups in using LysoQuant for quantification of their experiments. However, we challenged and confirmed the robustness of LysoQuant by performing quantitative assessments in another cell line and by using different organelle markers and images of different resolution and quality.**
> **As for an additional cell line used to test LysoQuant performance, we selected Human Embryonic Kidney 293 (HEK293) cells because they are widely used by the cell biology community. Moreover, imaging studies with these cells are particularly challenging due to their small size and reduced adherence to glass coverslips (compared to the mouse embryonic fibroblasts (MEF) used in the previous and in this submission). The new Fig. 6 proves the excellent performance of LysoQuant both in accuracy and rapidity of the analyses also when performed in HEK293 cells (page 12 of the new manuscript).**

We also performed, as suggested, experiments, where lysosomes were stained differently. Rather than exploiting the endogenous LAMP1 antigen, we overexpressed an ectopic marker of the catabolic organelles (i.e, GFP-RAB7, in Fig. 6). Moreover, in addition to the cargo markers ATZ, SEC62 (both endogenously and ectopically expressed), and ectopically expressed SEC62-LIR (Figs. 1-5 and Supplementary Figures), in this resubmission we add two additional ER markers (namely FAM134B and FAM134B-LIR, Fig. 6), which were detected by their HA-epitope tag. Finally, we refer to Supplementary Figure 3 for analyses of LysoQuant performance on images with different resolution and quality (Page 10, last paragraph).

(2) I applaud the authors for making LysoQuant available in ImageJ.

The described plugin, the training network and model are available on the github platform (https://github.com/irb-imagingfacility/lysoquant) and through the ImageJ update site. Training datasets of the experiments will be available on request. Additional data related to this paper may be requested from the authors.

(3) As far as I can determine LysoQuant is still relatively slow, and probably not yet suited for high-throughput studies.

We thank the reviewer for this comment that raises an important point. Collinet et al (Nature, 2010) performed a phenotypic profile of the human genome with respect to transferrin and epidermal growth factor endocytosis. They reported that their image analysis approximately required 1.8 CPU hours/image. LysoQuant completes analysis of each image in 0.53 ± 0.04 min to 1.45 ± 0.04 min (depending on the number of pixels of the starting image, page 10 and Supplementary Figure 3C). This corresponds to 0.008 to 0.024 CPU hours/image, which is consistent with the rate required for high-throughput studies (Carpenter *et al.*, Genome Biol 7, R100 (2006)). This information has been added in Results (page 10) and in the final paragraph of the Discussion.

(4) I do not doubt the superiority of LysoQuant compared to the machine learning based methods or human operators as described here. But, I can't help but wonder if in the case of the classical machine learning based methods the authors truly tried to optimize their performance on the segmentation task. In addition, the authors make comments such as: "Not unexpectedly therefore, the performance of machine learning approaches remained well-below human accuracy" in reference to other studies. However, based on our own expertise ML-based tools can have excellent performance on complex image analysis tasks.
I just question whether this study truly represents a rigorous comparison of the best available tools.

Based on referees comments and suggestions, we performed an extensive set up phase for the machine and deep learning-based methods available in the literature including the state-of-the-art algorithms developed for the quantification of endocytic organelles. This part has a dedicated sub-chapter (Detection and classification of EL: available approaches) in the Results section (pages 5-6), Material and Methods section (pages 15-16), and data are shown in Figure 1, in Supplementary Figure 1 and Table 1.

All in all, the unsatisfactory results obtained with the approaches presented above led us to develop a more reliable and accurate methods for rapid, automatic segmentation and quantification of EL features.

(5) For a general audience I find the introduction very jargon heavy (i.e. with descriptions of different degradation pathways)

We have re-elaborated the introduction. However, since our aim has been to develop a quantitative tool to be then employed on few practical examples, we decided to leave in the text a "simplified" explanation of the pathways under investigation. References are given in the text to literature that explains these pathways in more detail.

Reviewer #1 (Significance (Required)):

Improved image analysis tools are required as our ability to image cellular and sub-cellular structures continues to advance. Specifically, simply detecting cellular structures is still a technical challenge. Deep-learning based methods represent an exciting means by which increase the performance of image segmentation. This study describes the development of a potentially useful tool to segment small punctuate structures such as lysosomes. This could have widespread utility.

Although a flurry of deep-learning based analysis tools have been recently developed, there is still few that use deep-learning for cell biology, thus I think this work could have some impact.

The audience of this work would be both cell biologists and computational biologist interest in cell image analysis.

We have extensive expertise in the use of machine-learning based methods to analyse cellular phenotypes, and are now developing deep-learning based tools for digital pathology.

Reviewer #2

(Evidence, reproducibility and clarity (Required)):

The manuscript by Morone et al. reports the application of the Deep Learning approach to the quantification of endo-lysosomal compartments. The authors test two types of implementation of deep neural network U-Net and found that the larger one matches the quality of manual segmentation. The application of U-Net architecture for segmentation of biological images is not novel per se. However, the approach of sub-sampling images to fixed pixel size with linear interpolation, which allows decreasing network insensitivity to image acquisition, and the demonstration of its robustness, are interesting results.

We would like to thank the Reviewer for the positive comments.

Reviewer #2 (Significance (Required)):

However, the authors did not compare the proposed approach with state-of-the-art algorithms, which have been previously developed for the quantification of endocytic organelles (see, for example, Helmuth et al, J.Struct.Biol, 2009; de Chaumont et al, Nature Methods, 2012; Collinet et al, 2010; Aguet et al, Dev.Cell, 2013). The authors referred to general-purpose segmentation algorithms, which are obviously sub-optimal in the particular case. Although Deep Learning is state-of-the-art in image analysis, the authors ought to do a comparison with previous methods.

Based on referees comments and suggestions, we performed an extensive set up phase for the machine and deep learning-based methods available in the literature including

the mentioned state-of-the-art algorithms developed for the quantification of endocytic organelles, notably those cited in Helmut et al., de Chaumont et al., Auguet et al. This part has a dedicated sub-chapter (Detection and classification of EL: available approaches) in the Results section (pages 5-6), Material and Methods section (pages 15-16), and data are shown in Figure 1, in Supplementary Figure 1 and Table 1.
The analysis described in Helmuth et al, J.Struct.Biol, 2009 is implemented in an ImageJ plugin called Squassh. The usage procedure for this plugin is also described in Ritk et al, Nat. Protocols 2014. When applied to our images, this analysis protocol fails to segment individual ELs with their lumen (which is an essential requirement for the discrimination of cargo loaded and empty EL). A representative image was added in Fig. 1G (where we now highlight true positives, false negatives and false positives) and Supplementary Fig. 1G of the revised manuscript.

Icy software has been used for the segmentation of processes of endocytosis (de Chaumont et al, 2012) with the help of the Spot Detection plug-in. When applied to our images (LAMP1 channel), it fails to define the inner and outer regions of the EL lumen and to separate individual EL. A representative image is shown in Fig. 1H and Supplementary Fig. 1H. In this revised version we describe in detail the results of this plugin.

MotionTracking, the software reported in Collinet et al, 2010, is a software especially designed for the assessment of a phenotypic profile of the human genome with respect to transferrin and epidermal growth factor endocytosis. After acquisition with a confocal spinning disk microscope, the first step of the analysis is image segmentation of the endosomes. However, when applied to our representative image, it failed to recognize any object inside the LAMP1 channel.

The analysis reported in Auguet et al, 2013 was developed for images of endosomes with a size smaller or comparable with microscope resolution. The analysis is optimized for time lapse videos acquired in total internal reflection (TIRF) microscopy and relies on the optical acquisition parameters to estimate the clathrin-coated structures localization from a 2D Gaussian fitting of signal spots on a varying background. For this reason, this analysis is not suited to address the tasks covered by LysoQuant. The segmentation output of this analysis has now been included to the manuscript in Fig. 1I and Supplementary Fig. 1I.

The present approach for single cell analysis is claimed to be 30-fold faster than the manual one, but it is not faster than the previously published algorithms for analysis of fluorescence images of endosomes/lysosomes.

The speed is not the only key advantage of LysoQuant. As the reviewer writes, other methods report similar analysis speeds (e.g., Ritk et al., 2014). However, when we compare performances of other methods, none of them achieves those of LysoQuant in segmenting and classifying cargo loaded and empty EL (see explanations above, Results section (pages 5-6), Material and Methods section (pages 15-16), and data are shown in Figure 1, in Supplementary Figure 1 and Table 1).

The advantage of U-Net is the classification of segmented endosomes on "loaded" and "empty" in relation to specific cargo. However, this result can be obtained by existing object-based colocalization algorithms (for example, Helmuth et al., BMC Bioinformatics 2010, Woodcroft et al, Cytometry A, 2009. These have to be compared with the proposed one.

We thank the reviewer for this comment. We compared the performance of LysoQuant in segmenting and classifying cargo loaded and empty EL with the algorithms suggested in his/her comment. These object-based colocalization approaches are designed to assess the single or double positivity of spots of evenly distributed signal. As such, they proved inadequate to distinguish the different scenarios found in the biological problem addressed in our study, where the subcellular-structure-under investigation (protein or organelle) can be found inside the EL lumen, unevenly distributed, or in puncta on the outer membrane of the EL.
More precisely, the approach described in Helmut et al, 2010 performs in-depth statistical analyses of sub-resolved co-localization images which, for the reason described before, results in a poor individuation of the single EL position and shape. Results of this strategy are provided in the revised version of manuscript, Fig. 1I and Supplementary Fig. 1I.
Woodcroft et al segmentation procedure relies on the automatic or manual set of a fluorescence threshold and a watershed algorithm. This approach is equivalent to the one presented in Fig. 1B (Automatic Threshold & Watershed) and fails to distinguish EL with varying intensity levels. Therefore, also this approach underperforms compared to LysoQuant. Representative images are provided in Fig. 1J and Supplementary Fig. 1J of the revised manuscript.

Minor point:

In the main text, the calculation time is claimed to be per cell, but in the legend to Sup.Fig.2 it is claimed to be per image.

Times are calculated per image. We apologize for this typo and we corrected it in the revised version of the manuscript.

Reviewer #3

(Evidence, reproducibility and clarity (Required)):

Summary:

Morone et al present a deep learning-based system for detection and segmentation of endolysosomal compartments with and without cargo. A U-net is used for segmentation, results are compared with manual annotations. The method appears to be working very well.

We would like to thank the Reviewer for the positive comments.

Major comments:

-Are the key conclusions convincing?
The key conclusion is that the segmentation problem can be solved with a U-net. This has been shown convincingly, but it is not an unexpected finding.

We applied a state-of-the-art deep learning architecture which has been applied to many medical and light microscopy images (Bulten, Bandi et al., 2019, Nguyen,

Uhlmann et al., 2019, Oktay & Gurses, 2019, van der Heyden, Wohlfahrt et al., 2019, Van Valen, Kudo et al., 2016, Zhuang, Li et al., 2019). To our knowledge this is the first time that the U-Net architecture has been applied to the segmentation and classification of endolysosomes (EL). In the submitted version, we showed that many common segmentation approaches perform less than our modified U-Net architecture in the task. In this revision, we also added other segmentation algorithms specifically designed for the segmentation of EL, showing that they perform also less than U-Net. Interestingly, the application of vanilla U-Net (as reported in Falk et al.), although it improves the segmentation output, it is prone to many false negatives and false positives, as well as some misclassification errors, while the modifications to the U-Net architecture that we proposed in the manuscript ultimately provided a segmentation performance as good as the one achieved by manual quantification with three operators. These results are shown in the output images and graphs of the revised Supplementary Fig. 2, as compared to Fig. 3. Furthermore, the application of other deep learning-based methods, such as the Stardist plugin which was suggested by the reviewer, did not perform better than our proposed approach (see in-depth response below). Thus, neither the available common and task-specific approaches to segmentation were able to perform as good as the approach proposed in the manuscript. For this reason, we think that LysoQuant could be helpful to the cell biology community.

-Should the authors qualify some of their claims as preliminary or speculative, or remove them altogether?
No

-Would additional experiments be essential to support the claims of the paper? Request additional experiments only where necessary for the paper as it is, and do not ask authors to open new lines of experimentation.
A comparison to other deep learning-based methods is missing. What about the official U-net Fiji plugin from Falk et al? The StarDist Fiji plugin?

Based on referees comments and suggestions, we performed an extensive set up phase for the machine and deep learning-based methods available in the literature including the state-of-the-art algorithms developed for the quantification of endocytic organelles. This part has a dedicated sub-chapter (Detection and classification of EL: available approaches) in the Results section (pages 5-6), Material and Methods section (pages 15-16), and data are shown in Figure 1, in Supplementary Figure 1 and Table 1.

Our approach is based on the official U-net Fiji plugin from which we inherited the segmentation framework. When compared with the vanilla U-Net architecture (as reported in Falk et al.), we found that although it improves the segmentation output over state-of-the-art available approaches, it is prone to many false negatives and false positives, as well as some misclassification errors, while the modifications to the U-Net architecture that we proposed in the manuscript ultimately provided a segmentation performance as good as the one achieved by manual quantification with three operators. These results are shown in the output images and graphs of the revised Supplementary Fig. 2, as compared to Fig. 3. Furthermore, the application of other deep learning-based methods, such as the Stardist plugin which was suggested by the reviewer, did not perform better than our proposed approach (see in-depth response above). in Supplementary Fig. 2, to which we now added the segmentation output.

StarDist is a technique originally developed to segment nuclei through the use of star-convex polygons. A lightweight U-Net convolutional neural network predicts for every pixel a polygon for the cell instance at that position. Final instances are then selected via Non-Maximum Suppression (NMS). We trained this network with the provided Jupyter notebooks and our training dataset. Model was trained for 400 epochs. We then calculated the NMS factors for this training and exported the model to the Stardist Fiji plugin, with which we performed the segmentation. Results show that Stardist recognizes most of the EL but exaggerates their actual size, resulting in a poor shape definition which would prevent the recognition of differences in shape factors and the overlapping of EL boundaries when in close proximity. This data is now added in Fig. 1L and Supplementary Fig. 1L.

-Are the data and the methods presented in such a way that they can be reproduced?
The code is not provided, the trained network weights are not provided, the method would have to be fully reimplemented for reproduction. It is also not clear to me from the data availability statement if all training and validation data will be released. If not, the paper is essentially irreproducible.

The described plugin, the training network and model are available on the github platform (https://github.com/irb-imagingfacility/lysoquant) and through the ImageJ update site. Training datasets of the experiments will be available on request. Additional data related to this paper may be requested from the authors.

-I do not quite understand what the authors refer to as "novel DL approach" on page 4. As far as I can see, a vanilla U-net is used with an additional depth level, with no special loss or training procedure.

The introduction (pages 3-4) has been re-written to simplify it (as per request of referee 1). We found that linearly upscaling the training images to convert them to a subresolution pixel size increased the robustness of the results (see page 10 and Supplementary Fig. 3A-B), allowing the segmentation of images with very different resolutions. The additional layers in the network architecture, as shown in Fig. 2B, while reducing the number of base features from 64 to 16 improved the segmentation quality. In fact, when vanilla U-Net was trained from scratch with the same training dataset, it yielded a worse performance, as shown in Supplementary Figure 2. In this revised manuscript, we added to Supplementary Figure 2 the segmentation output of this vanilla U-Net, highlighting both for the empty- and loaded-EL classes the false positives and false negatives.

-A Fiji plugin is mentioned in the text, but has not been made available, not even to thee reviewers. It is also not clear what the plugin can do: inference? training? fine-tuning? How is the GPU accessed? How are the models deployed?

The described plugin, the training network and model are available on the github platform (https://github.com/irb-imagingfacility/lysoquant) and through the ImageJ update site. Training datasets of the experiments will be available on request. Additional data related to this paper may be requested from the authors.

The plugin performs all the steps of the analysis except for the DL segmentation. It takes as an input the ROIs of the selected cells and the channels to analyze. It then performs the conversion to RGB, clears the signal outside the selected ROIs (Fig. 2Bc)

and calls the U-Net plugin, which normalizes the image (Fig. 2Bd) and performs the segmentation with the specified weight file. The segmented image (Fig. 2Be) is then recalled by the plugin and for each class (Fig. 2Bf) the objects above the minimum size (Fig. 2Bg) are quantified (Fig. 2Bh-i) through the use of the Analyze Particles class. Summary measurements are then presented to the user. This information has now been added to the Materials and Methods.

Minor comments:

- In Figure 1, where failure modes of "shallow" algorithms are shown, the errors need to be highlighted. The figure should also show the results of the proposed approach on thee data from Fig. 1A

We thank the reviewer for the comment. In this revised version, in Fig. 1 we compared the results with manual segmentation and highlighted false positives and false negatives, while providing the segmentation output in Supplementary Fig. 1. We also added to Fig. 1 the F1 score and IoU values for each approach.

Reviewer #3 (Significance (Required)):

- Describe the nature and significance of the advance (e.g. conceptual, technical, clinical) for the field.
As far as I can see, the work does not introduce any novel algorithms or methods. The contribution is thus on the technical side, but the described plugin has not been released, and its technical side has not been described in much detail.

The modifications to the U-Net architecture that we proposed in the manuscript improved the segmentation performance over the state-of-the-art available approaches, notably the classic and machine-learning approaches and those involving deep learning, such as Stardist and vanilla U-net. The proposed architecture and method ultimately provided a segmentation performance as good as the one achieved by manual quantification with three operators. We made publicly available on github the plugin, its code, and will make available the trained network file and the datasets related to the experiments presented according to the publisher's guidelines.

- Place the work in the context of the existing literature (provide references, where appropriate).

The work proposes to apply a U-net to a segmentation problem in 2D light microscopy images. Not being from the EL field, I do not know if this particular application has been introduced before, but similar structures have been segmented in the literature (see, for example, the Falk et al. Nature Methods paper describing the U-net plugin).

To our knowledge, this is the first time that deep learning is employed in the segmentation of individual intracellular structures (endolysosomes) and their cargo. We provided evidence that LysoQuant yields better results than previous methods. The use of U-Net architecture (Falk et al. 2019) resulted in F1 score significantly lower than the two best available approaches Squassh and Colocalisation Pipeline, page 8 and Supplementary Fig. 2) and was therefore not suitable. Adding additional levels to this architecture and reducing the number of base features greatly improved the results, yielding significantly better values on all metrics as shown in Fig. 2.

- State what audience might be interested in and influenced by the reported findings.

Biologists in need for user-friendly segmentation methods for EL and its cargo.

- Define your field of expertise with a few keywords to help the authors contextualize your point of view. Indicate if there are any parts of the paper that you do not have sufficient expertise to evaluate.

I am from the computer vision domain.

RE: Manuscript #E20-04-0269
TITLE: "Deep learning approach for quantification of organelles and misfolded polypeptides delivery within degradative compartments"

Dear Maurizio,

Thank you for considering Molecular Biology of the Cell for your submission after sending the manuscript to Review Commons for peer-review by three experts in the field. I believe that you have addressed the concerns of the reviewers in a satisfactory manner, and I am therefore very happy to let you know that your manuscript is now accepted for publication in Molecular Biology of the Cell.

Congratulations for a very nice paper!

Best regards,

Jean Gruenberg

Monitoring Editor
Molecular Biology of the Cell

Dear Prof. Molinari:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

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