

Supplementary information

Multiphoton intravital microscopy of rodents

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Multiphoton intravital microscopy of rodents

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Supplementary Table 1: Image analysis software tools. Non-exhaustive list of software tools that are available for image analysis detailing major functions and example applications.

Analysis Step	Software	Workflow/Major functions	Applications
Motion correction	IVM Artifact Reduction Tool (IMART)	Achieve alignment and similarity by rigid registration of images to correct for overall movement followed by non-rigid registration to correct for small-scale aberrations/movements	Stabilization of IVM data from kidney and liver ¹
	Intravital_Microscopy_Toolbox (ImageJ Macro)	Generation of a dissimilarity score between images of a series and reference frame with subsequent exclusion of frames with too high scores, also includes x-y alignment Additional features for image processing include noise reduction or intensity projections	Stabilization of IVM data from brain, spinal cord, sciatic and enteric nerves ²
	Sequential Imaging Analysis (SIMA, Python package)	Frame-by-frame alignment in relation to reference template (average of already aligned frames) followed by algorithm/model-based correction (Hidden Markov Model and Viterbi-based algorithm) Additional applications for automated segmentation and signal extraction	Motion correction of brain IVM data, ROI registration across longitudinal IVM in the same animal ^{3,4}
	Galene	Extraction of individual photon arrival times followed by frame reconstruction based on intensity and rigid realignment to a reference frame to correct for overall movement and displacement estimation and fitting to correct for small scale aberrations	FLIM-FRET IVM of intestinal crypts, pancreas and cancer cell extravasation in the liver as well as FLIM IVM of the skin in human volunteers ⁵
	StackReg and HyperstackReg (ImageJ/Fiji plugins)	Alignment of grey-scale or single-channel image stacks/series where each image serves as a template for alignment of subsequent image. Further development has led to HyperStackReg, which allows alignment of multichannel image stacks/series. 4D imaging data sets formatted as Hyperstacks within Fiji can be registered over time using a sub-pixel registration algorithm that minimizes the mean square difference of intensities between each subsequent time point. Registration may be based upon the intensities of one or more-color channels.	Alignment/registration, removal of residual PET/MRI imaging data as well as residual XY drift within intravital images ⁶⁻⁸
Segmentation	Two Photon Processor (MATLAB package), Search for Neural Cells Accelerated (SeNeCA)	Real-time cell segmentation frame-by-frame with cell re-identification in subsequent frames. Additional image processing functions for real-time segmentation and annotation	Cell segmentation in brain IVM data ⁹
Segmentation and quantification	Volumetric Tissue Exploration and Analysis (VTEA)	Nuclear segmentation, cell identification and cell quantification	3D <i>in situ</i> cytometry applications shown for kidney samples ¹⁰
	DeepSynth	nuclear segmentation based on deep learning approaches	Cell segmentation in volumetric data of fluorescently stained kidney, liver and intestinal samples ¹¹

	Sphere Estimation Network (SphEsNet)	automated segmentation of nuclei and quantification of size	Nuclei segmentation and quantification in kidney and intestine ¹²
	Jelly filling framework	segmentation of 3D data into lumen and boundaries upon multitarget labelling in 3D imaging data	3D imaging data from kidney imaging, liver imaging and human breast mammographies ¹³
	CellProfiler	Cell/nuclear segmentation followed by quantification of cellular or subcellular size, shape, intensity and texture	Calcium IVM in mouse brain ¹⁴
	Icy	free, open-source and open community bioimage informatics platform to process (visualize, annotate and quantify) imaging data	Presentation of software tool and features ¹⁵
	N/A	Nuclei segmentation and automatic processing	Classification of FUCCI cell cycle imaging data ¹⁶
	PIVlab	Data segmentation and plotting of velocity vectors	Quantification of tissue flow (speed and directionality) in embryos ¹⁷
Deconvolution, tracking/quantification, 3D rendering	BioImageXD	Open-source general-purpose tool incorporating a range of processing tools including deconvolution, tracking/quantification, 3D rendering and measurements	Assessment of integrin clustering, movement and internalization ¹⁸
Segmentation, tracking and lineage tracing	Lineage Editing and Validation (LEVER)	free open-source software for segmentation, tracking and lineage tracing of cells (lineage trees) as well as quantification of cell shape, size, location and movement	Longitudinal imaging of neural progenitor cells ¹⁹
	N/A	Open-source platform for segmentation and tracking of nuclei for reconstruction of cell lineages	Show applications in embryonic organisms, e.g., Drosophila, zebrafish and mouse ²⁰
Cell tracking and lineage tracing	tTt and qTfy	tTt tool for cell tracking and qTfy tool for segmentation/quantification of cellular and molecular features (e.g., fluorescence, ratio of fluorescence from different channels, tracking of cellular/subcellular localization)	Tracking and tracing of embryonic stem cells ²¹
	TrackMate (Fiji plugin)	Cell/particle tracking and lineage tracing	Tracing of cell lineages and tracking of molecular clusters ^{22,23}
3D rendering of imaging data	Voxx	Voxel-based 3D imaging program for 3D rendering of IVM data that does not require high-end computing power, allows almost real-time rendering	Rendering of kidney and heart imaging data ²⁴
Quantification/prediction of branching morphogenesis	Models of branching and annihilating random walks (BARWs)	Modelling of branching morphogenesis based on local rules	Quantification/prediction of branching morphogenesis in murine mammary and kidney as well as human adult prostate ²⁵
Denoising	BM3D algorithm	De-noising tool for IVM data, based on local and non-local redundancy in images	IVM of nanoparticles and gut mucosa ²⁶

		(morphological features) followed by image grouping and de-noising via algorithm	
ECM analysis	Gray-Level Co-occurrence Matrix (GLCM) analysis (MATLAB-based)	Quantification of matrix fiber organization using texture analysis tool	SHG imaging data of fibrillar collagen I from healthy and diseased skin samples ²⁷
	N/A	Computational fluid dynamics (CFD) approach to analyze and quantify ECM features	Analysis of de-cellularized heart ECM samples and quantification of dynamic changes in ECM upon heart failure ²⁸
	TWOMBLI (Fiji Macro)	Analysis of ECM networks with potential to also be used for analysis of other networks	Quantification of ECM features in cell-derived matrices (CDMs) mouse tissues and patient/tissue biopsies ²⁹
	Fiber Finding Algorithm (FFA)	Identification and tracking of individual ECM fibers	Collagen gels ³⁰
Analysis of vasculature	N/A	Algorithm to calculate / derive red blood cell (RBC) velocity and determine volumetric flow rates, radial velocity profiles, wall shear rate and stress	Assessment of dynamic behavior and interactions of/between blood cells and vessel walls ³¹
	Spatial Temporal Analysis of Fieldwise Flow (STAFF, Fiji-based)	automated analysis of microvascular flow	Analysis of microvascular flow IVM in liver ^{32,33}
	N/A	Vessel diameter calculation as full width at half-maximum (FWHM) of vasculature	Quantification of vascular features in the live brain (cranial window) ³⁴
	Relative Velocity, Direction, and Morphology (RVDM)	Use of FWHM method to quantify vessel diameter with calibration of beads of known size, generation of 3D velocity maps, velocity assessment based on changes in RBC shape (distortion from round shape [elongation]) linked to scan speed in relation to blood flow velocity and direction	Fluorescent data and also looking at vessel leakage, using example of IVM of (micro)vasculature in ear dermis ³⁵
Removal of noise/endogenous fluorescence	Digital Enhancement of Fibers with Noise Elimination (DEFINE, open-source macro for Fiji)	Relies on broad emission range of endogenous fluorescence, acquisition of images using fluorescent channels and lower wavelength autofluorescence (where fluorophores do not emit anymore) followed by subtraction of endogenous fluorescence using DEFINE	Imaging of axonal projections in the brain ³⁶
Spectral unmixing/removal of endogenous fluorescence	Learning Unsupervised Means of Spectra (LUMoS)	Spectral unmixing tool based on unsupervised learning of the spectral signatures of fluorophores	Spectral unmixing as well as reduction/removal of endogenous fluorescence ³⁷
Phasor-based segmentation of FLIM data	Kmeans FLIM phasors	Unbiased phasor approach using unsupervised machine learning (K-means clustering), shown to outperform manual selection	FLIM imaging data of the kidney ³⁸
FLIM data analysis	FLIMfit	Phasor based data segmentation to remove endogenous fluorescence, global fitting of FLIM data to monoexponential or multiexponential decays, generation of	Fitting/quantification/analysis of FRET biosensor imaging, Homo-FRET/anisotropy

		intensity merged FLIM images and FLIM images without intensity information	imaging and metabolic FLIM data ^{5, 39, 40}
Spectral unmixing	HyperSpectral Phasor (HySP)	Conversion and mapping of each photon across fluorescence spectrum on phasor plot	Unmixing of 5D hyperspectral imaging data with up to 7 signals ⁴¹
Removal of endogenous fluorescence and FLIM data analysis	Phasor application	Transformation of histograms of time delays for each pixel into phasor/vector followed by 2D mapping of phasors per pixel	Segmentation/analysis of imaging data from FPs, FRET biosensors as well as segmentation/removal of endogenous fluorescence ^{42,43}

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