Scalable fluorescence microscopic assays for mitochondrial physiology

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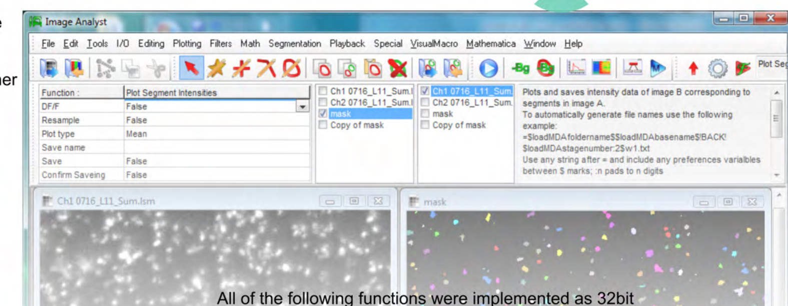
Fluorescence microscopic tools are widely used for assessing mitochondrial biology, however quantitative assay of parameters such as organelle dynamics and the mitochondrial membrane potential are not trivial. Microscope motorization and high-content imaging systems established the need for scalable assays that are robust and are able to evaluate biological parameters in an automated, unsupervised manner...

Image Analyst MKII - a biologist friendly image processing environment edged for mitochondrial studies

Image Analyst MKII is a designated image analysis tool for fluorescence time lapse microscopy. This tool calculates and extract graphs of biological parameters from image sequences, like ion concentrations, organelle shape changes and motion. Image Analyst MKII implements in a user friendly manner recently published algorithms for:

- mitochondrial swelling (Gerencser *et al.*, 2008 Biophys J)
- organelle motility (Gerencser & Nicholls, 2008 Biophys J)
- mitochondrion to cell volume ratio (Gerencser *et al.*, 2012 J Physiol)
- mitochondrial and plasma membrane potential (Gerencser *et al.*, 2012 J Physiol)
- Ca^{2+} rationing, Ca^{2+} waves (Gerencser & Adam-Vizi., 2005 Biophys J)
- FRET (Gerencser et al., 2009 J.Neurochem)
- Segmentation-based time-lapse intensity measurements (Choi SW et al., 2009 J. Neurochem; 2011 J. Neurosci.)
- Automation for handling multi dimensional data sets and microplates:



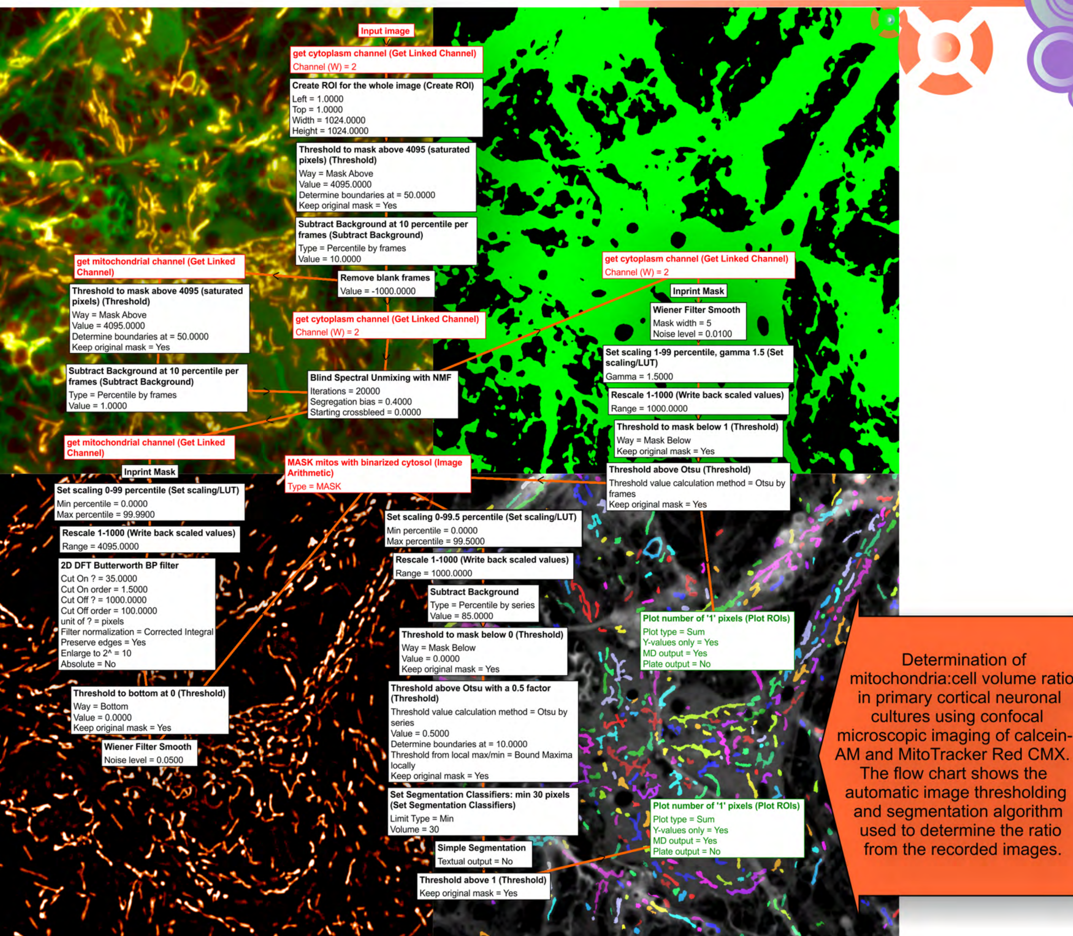
All of the following functions were implemented as 32bit floating point calculation and support high-speed image analysis on multi-core PC-s.

Flow chart-based automation makes design of custom workflow fast and easy

Software download & protocols: www.imageanalyst.net

Analysis type	Image Analyst MKII	ImageJ w/ plugins	Cell Profiler	Nikon Elements (v3.2)
Time lapse / physiology oriented	**Yes**	**Not (poorly implemented)**	**No time analysis**	**Yes**
Known, documented algorithms	Published	yes (open source)	Yes (open source)	Macro language is badly documented
Wide range of input formats	yes, through Bio-formats	yes, through Bio-formats	yes, through Bio-formats	Some
Image registration, and spectral unmixing	Unsupervised subpixel alignment for channels and time lapses	yes	Through ImageJ	Manual markers for channels, Automatic for time?
Floating point calculations	**All**	**32f in core functions and in certain plugins**	**In ImageJ plugins**	**No**
Dedicated background subtraction algorithms	**Yes, interpolated percentile, ROI, mean below percentile**	**No dedicated, some in plugins (no floating point)**	**Yes**	**Image, value, region**
Spatial and temporal filtering	Both, SG, Differentiation, versatile spectral DFT	Versatile with plugins, some temporal, Bandpass only DFT	Spatial, with Image J plugins	Spatial only, DFT is **Bandpass only and poorly implemented**
Segmentation based analysis	Yes Grayscale Watershed / Local Thresholding	yes	Yes, very versatile	Yes Binary watershed only, No local threshold
Spectral Unmixing	Blind, measured coefficients For any channels	yes	Through ImageJ	For spectral detection
Automation	Flowchart macro (parallel), Mathematica	Fiji macro, Matlab, R interoperatibility	Pipeline (linear)	C-like macro language
Automatic Thresholding	**Otsu, Percentile, Local flood fill**	**Otsu, Maximum Entropy…**	**Yes, very versatile**	**Some**
Multi core-support	**Yes**	**No**	**No**	**Yes**

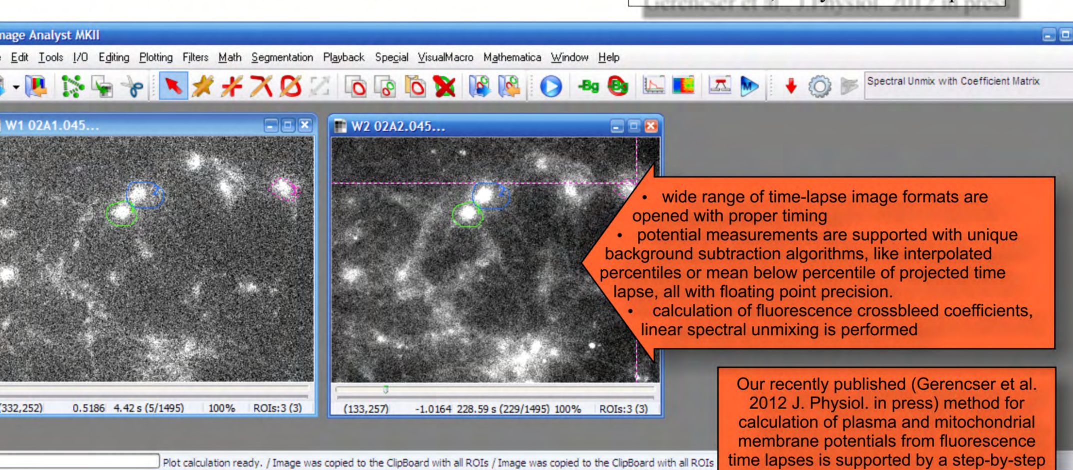
Mitochondria:cell volume ratio and in situ mitochondrial membrane potential determination



Determination of mitochondria:cell volume ratio in primary cortical neuronal cultures using confocal microscopic imaging of calcein-AM and MitoTracker Red CMX. The flow chart shows the automatic image thresholding and segmentation algorithm used to determine the ratio from the recorded images.

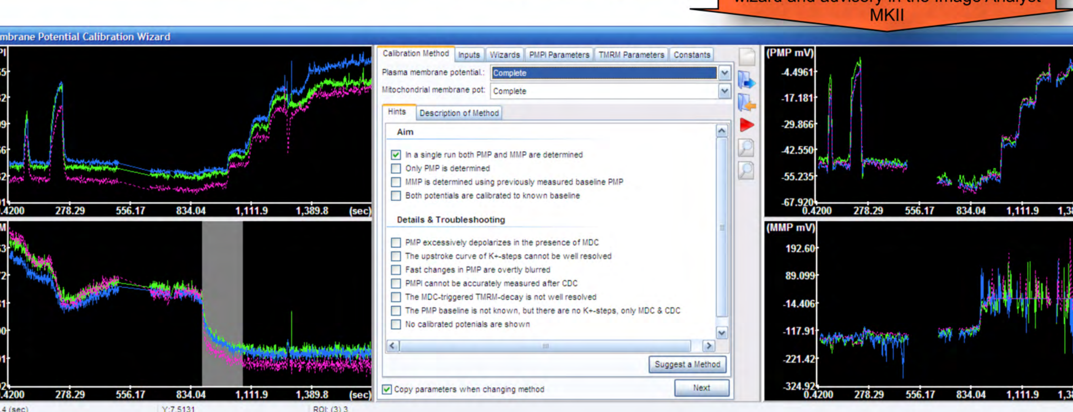
- This is the first micro-scale method to enable measurement of differences in mitochondrial membrane potential between cells with different properties, e.g. size, mitochondrial density and plasma membrane potential, including cases when plasma membrane potential fluctuates.
- To this end we have derived a model of fluorescent potentiometric probe dynamics, and on these principles we introduce an absolute quantitative method for assaying mitochondrial membrane potential in millivolts in individual cultured cells.
- Mitochondrial membrane potential in cultured rat cortical neurons is −139 mV at rest. In response to electrical stimulation of the cells, it is regulated between −108 mV and −158 mV by concerted increases in energy demand and metabolic activation.
- Microscopy requirements:
  - wide-field, confocal or z-focusing microplate reader (volume and activity coefficient ratios require confocal microscopy)
- single cell or cell population imaging at low resolution, low signal to noise ratio
- probes:
  - TMRM: tetramethylrhodamine methyl ester: lipophilic cation: mitochondrial probe
  - PMPI: plasma membrane potential indicator (#R8042 FLIPR Membrane Potential Assay Explorer Kit from Molecular Devices)

Gerencser et al., J.Physiol. 2012 in press



- wide range of time-lapse image formats are opened with proper timing
- potential measurements are supported with unique background subtraction algorithms, like interpolated percentiles or mean below percentile of projected time lapse, all with floating point precision
- calculation of fluorescence crossbleed coefficients, linear spectral unmixing is performed

Our recently published (Gerencser et al. 2012 J. Physiol. in press) method for calculation of plasma and mitochondrial membrane potentials from fluorescence time lapses is supported by a step-by-step wizard and advisory in the Image Analyst MKII



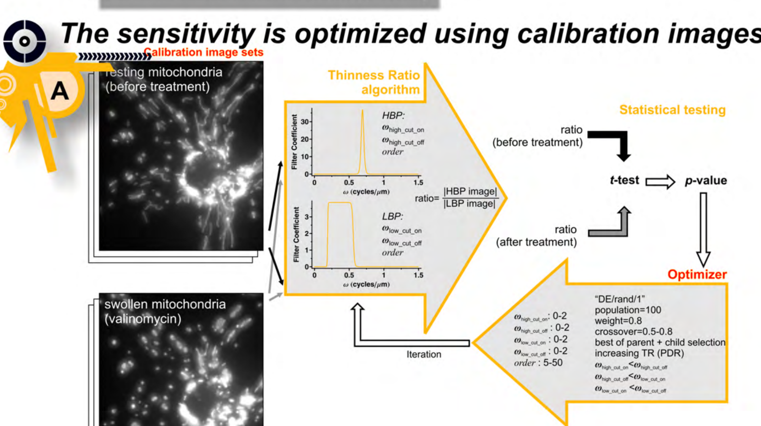
Mitochondrial Shape Changes at Superresolution Level

- The thinness ratio technique measures diameter changes of *in situ* mitochondria.
- The thinness ratio technique is based on band pass spatial filtering in *Fourier* domain. Band pass filtering measures the intensity of puncti and filaments with a given range of diameters. The relative contribution of fluorescence intensities of thinner and thicker structures is measured by a set of two band pass spatial filters and ratioing of filtered intensities.

Gerencser et al. 2008 Biophys J. 95:2583-98.

- Microscopy requirements:
  - wide-field, confocal or two-photon
  - single image planes or z-projections
  - mitochondria labeled with targeted GFP variants
  - Nyquist resolution or less, no oversampling (~0.1 µm/pixel)
- Advantages:
  - The technique is sensitive to diameter increases at 10 nm scale
  - Irrespective of overlaps and motion of mitochondria
  - Selective for mitochondrial swelling over fission

The sensitivity is optimized using calibration images

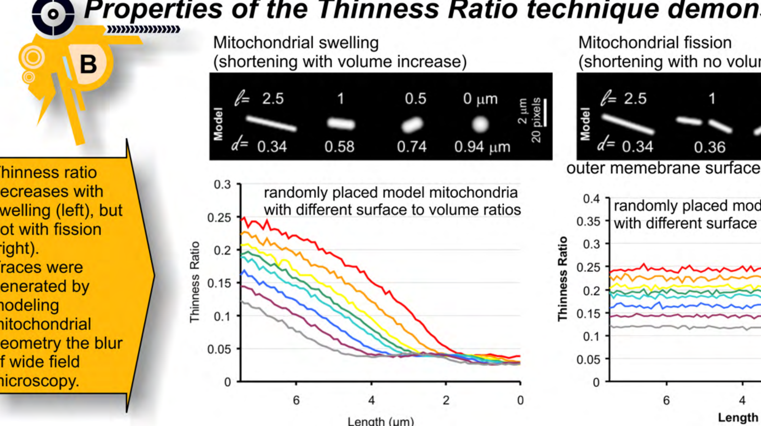


The high frequency band pass (HBP) and a low frequency band pass (LBP) spatial filters are generated by an optimization algorithm maximizing the sensitivity of the Thinness Ratio calculation towards the biological change depicted by the calibration image. A user friendly implementation of this algorithm is available in Image Analyst MKII (see on the right).

Built-in Differential Evolution-based optimizer to generate high frequency band pass and low frequency band pass filter functions based on recorded calibration images.

The Thinness Ratio calculation is performed by a flow chart-based, visually simple, yet powerful macro operation.

Properties of the Thinness Ratio technique demonstrated by model images



Thinness ratio decreases with swelling (left), but not with fission (right). Traces were generated by modeling mitochondrial geometry the blur of wide field microscopy.

Organelle Transport by Instantaneous Velocity Determination

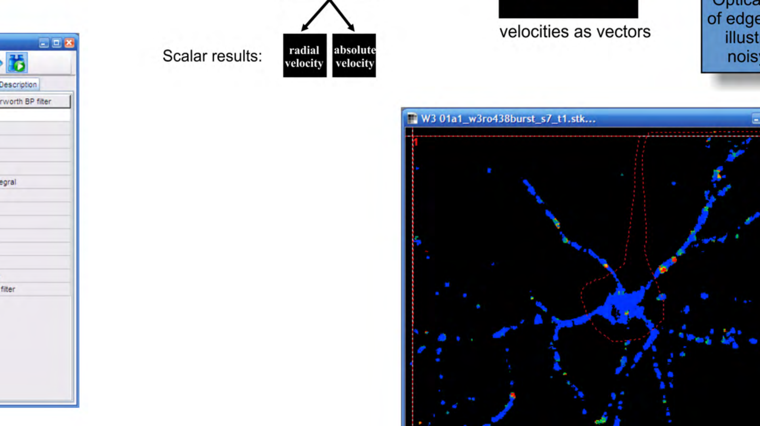
- Velocity vectors, anterograde/retrograde velocities, mean absolute velocities, transport/wiggling motion are determined from pairs of images using Optical Flow calculation.
- The Optical Flow reflects the real physical motion of the imaged objects between frames of an image sequence.
- Microscopy requirements:
  - wide-field, confocal or two-photon
  - mitochondria labeled with targeted GFP variants or MitoTrackers
  - pairs of frames (short time lapses) of single image planes
  - submaximal resolution (~0.3 µm/pixel)
- Advantages:
  - velocities determined from a pair of low light level images, photodynamic oxidative artifacts evoked by fluorescence excitation.
  - The instantaneous velocity determination can be exploited in multiplexed and microplate acquisitions
  - motion measurement is irrespective of shapes and overlaps between mitochondria

Gerencser & Nicholls 2008 Biophys J. 95:3079-99

Multiplexing velocity measurements in multiwell plates

Optical Flow is recorded as repeated time lapses (Zeiss, Metamorph), repeated channel acquisition (Elements, see above) or z-stacks with zero increments. Image Analyst supports multi dimensional image sets and the above variations in data structure.

Image processing steps and properties of Optical Flow



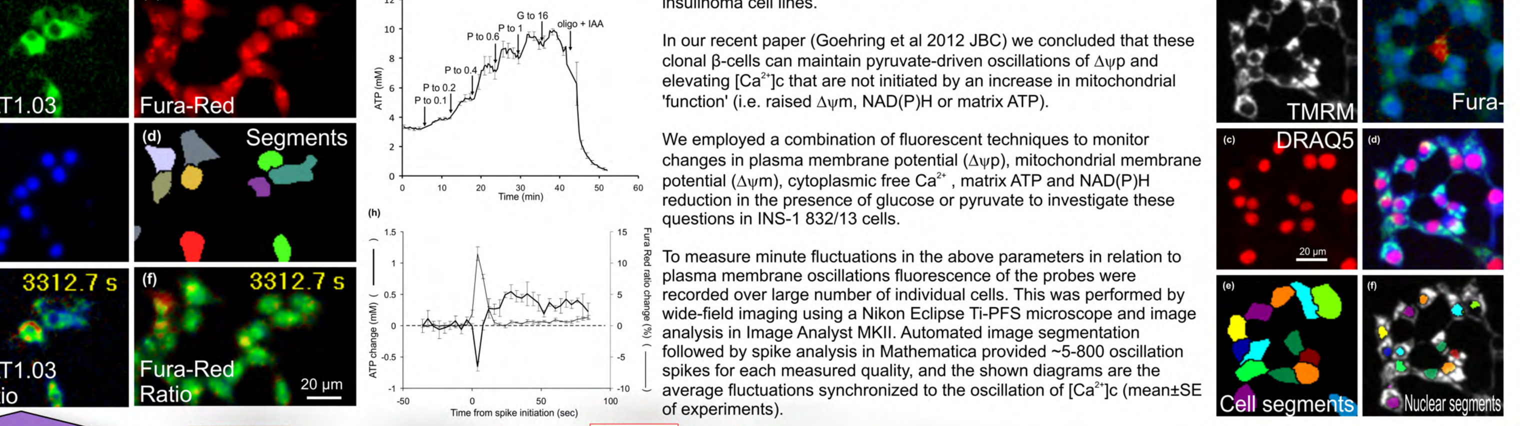
Optical Flow senses subpixel dislocations of edges of object. The blue and red traces illustrate profiles of computer modeled noisy objects moved by 1/8 of a pixel.

Parameters of Optical Flow calculation follow accurately Gerencser & Nicholls 2008 Biophys J. In a general case the user calculates detector noise variance values by the "Sensor Noise Characteristics" and enters the pixel size to obtain velocities in µm/sec in Optical Flow images.

Optical Flow is calculated during loading of the image sequence and shown as a pseudocolor image, where pixel values encode velocities in µm/sec. Areas where no significant edges were found are masked (black). This image is available for ROI mean calculation or for measuring velocities of objects by segmenting intensity images.

Velocities over ROIs or segmented objects are plotted against time, exported as text files or directly transported to Excel. Automation for multiwell plates.

Fluctuations of mitochondrial membrane potential and $[ATP]_{mito}$ in insulinoma cells



Oscillations in plasma membrane potential ($\Delta\psi_p$) play a central role in glucose-induced insulin secretion from pancreatic β-cells and related insulinoma cell lines.

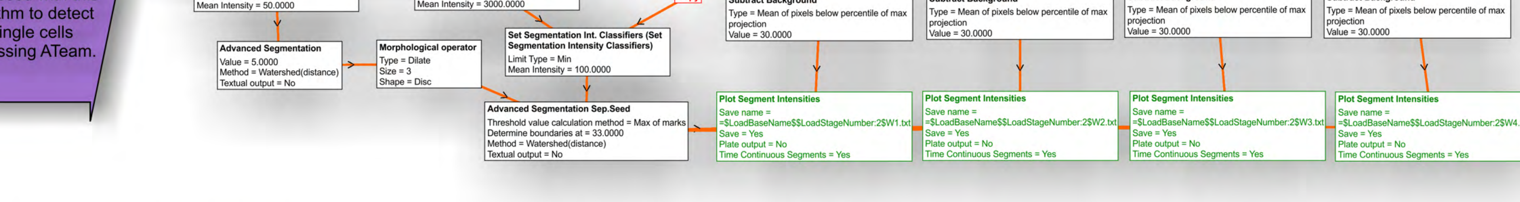
In our recent paper (Goehring et al 2012 JBC) we concluded that these clonal β-cells can maintain pyruvate-driven oscillations of $\Delta\psi_p$ and elevating $[Ca^{2+}]_c$ that are not initiated by an increase in mitochondrial 'function' (i.e. raised $\Delta\psi_m$, NAD(P)H or matrix ATP).

We employed a combination of fluorescent techniques to monitor changes in plasma membrane potential ($\Delta\psi_p$), mitochondrial membrane potential ($\Delta\psi_m$), cytoplasmic free Ca^{2+}, matrix ATP and NAD(P)H reduction in the presence of glucose or pyruvate to investigate these questions in INS-1 832/13 cells.

To measure minute fluctuations in the above parameters in relation to plasma membrane oscillations fluorescence of the probes were recorded over large number of individual cells. This was performed by wide-field imaging using a Nikon Eclipse Ti-PFS microscope and image analysis in Image Analyst MKII. Automated image segmentation followed by spike analysis in Mathematica provided ~5-800 oscillation spikes for each measured quality, and the shown diagrams are the average fluctuations synchronized to the oscillation of $[Ca^{2+}]_c$ (mean±SE of experiments).

Pyruvate titration of mito-ATeam[1] in INS-1 832/13 cells. Matrix ATP concentration in INS-1 832/13 cells as a function of substrate concentration and during $[Ca^{2+}]_c$ spikes in the presence of 0.4 mM pyruvate.

Changes in $\Delta\Psi_m$ associated with $[Ca^{2+}]_c$ spike in the presence of pyruvate. TMRM was used in quench-mode, and the fluctuations in TMRM fluorescence were sensitively detected over nuclear areas of cells.



ATeam-Fura-Red samples were post-stained by Hoechst dye, and processed with this algorithm to detect only single cells expressing ATeam.

Image segmentation was performed automated in Image Analyst MKII with a similar workflow to the one on the left.

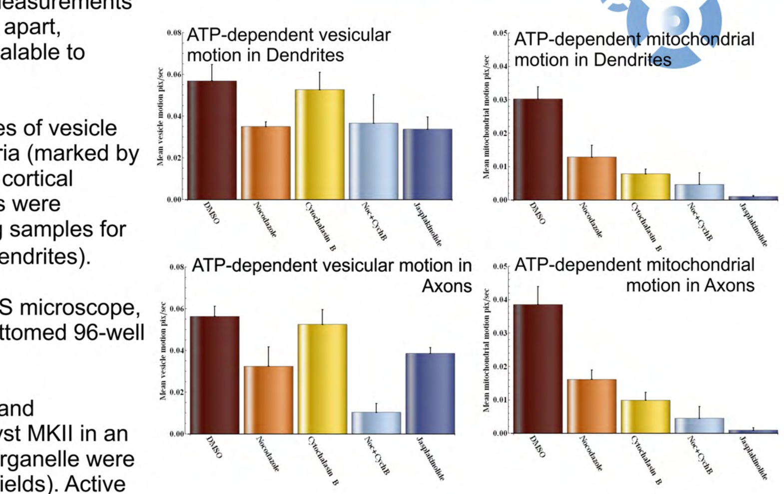
Mitochondrial and Vesicle Transport in Dendrites and Axons using Instantaneous Velocity Determination in 96-well plates

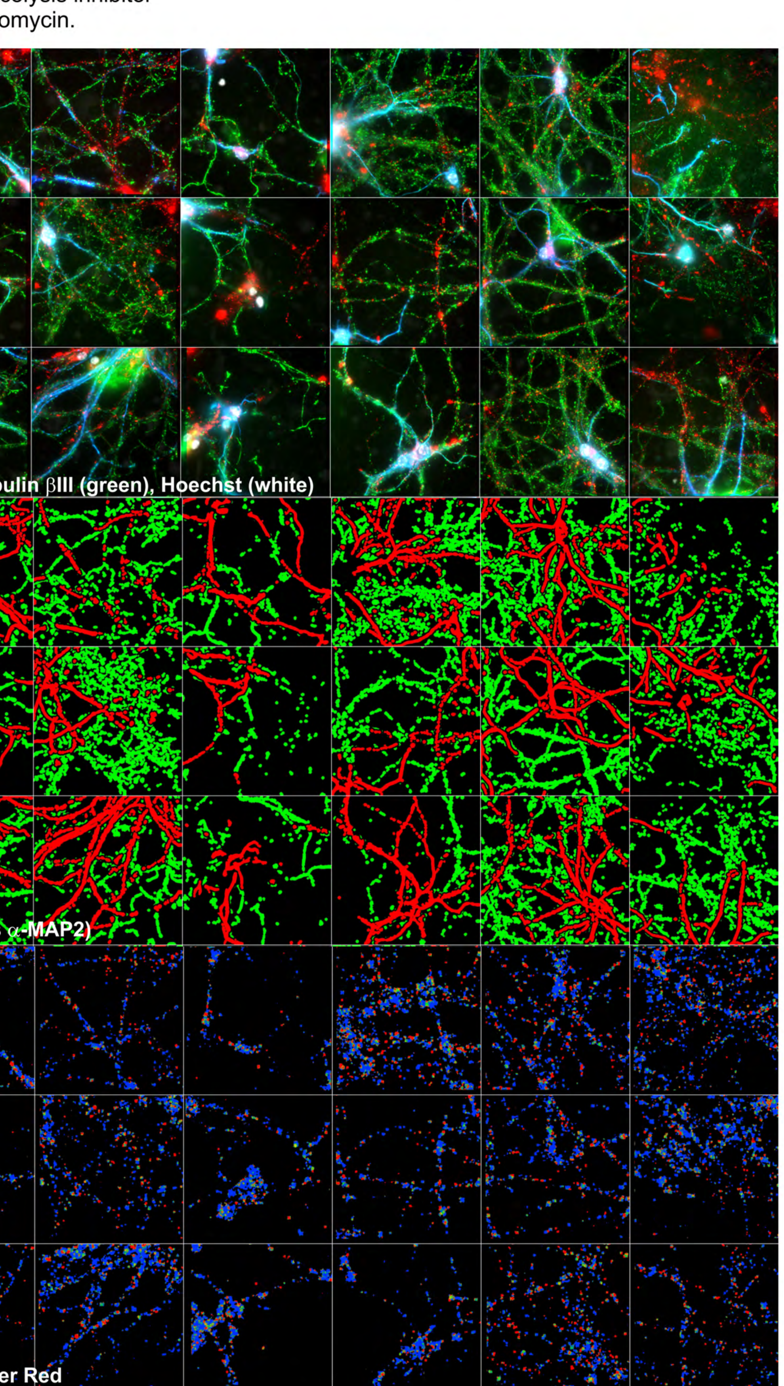
Velocity measurement using Optical Flow offers instantaneous velocity measurements from pairs of frames recorded 0.5-1 s apart, therefore this assay is conveniently scalable to microplates.

In this example we measured mean velocities of vesicle (marked by LysoTracker Red) or mitochondria (marked by MitoTracker Deep Red) trafficking in mouse cortical cultures. Velocities in axons and in dendrites were selectively detected by post-immunostaining samples for MAP2 (dendrites) and Tubulin βIII (axons+dendrites).

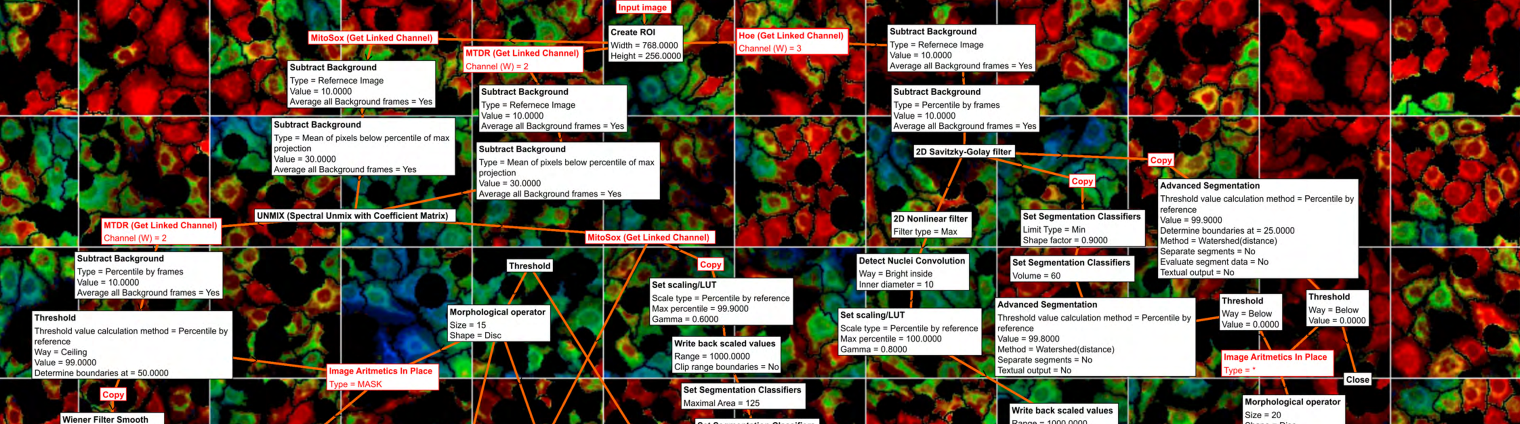
For image acquisition a Nikon Eclipse Ti-PFS microscope, 60x Plan Apo VC oil lens and coverglass bottomed 96-well plate (reading out 30 wells) was used.

Optical Flow calculation, image registration and thresholding were performed in Image Analyst MKII in an automated workflow. Data shown for each organelle were recorded in a single run (mean±SE of view fields). Active motion was determined by application of glycolysis inhibitor iodoacetate plus ATP-synthase inhibitor oligomycin.





Mitochondrial superoxide level assay using MitoSOX:MitoTracker Deep Red ratioing, and debris avoidance



Using MitoSOX:MitoTracker Deep Red ratioing and image segmentation for measuring mitochondrial superoxide levels intends to:

- cancel effects of geometric differences between different cell types, when compared
- cancel the effects of different mitochondrial densities
- cancel the effect of plasma and mitochondrial membrane potential
- avoid measuring dead-cell debris, that produces MnTMPyP-insensitive (not superoxide mediated) increase in MitoSOX fluorescence.

The algorithm supports unsupervised processing of microplates as an end point assay.

Microplates are preincubated in normal cell culture medium MitoSOX:MitoTracker Deep Red, and then imaged in Hoechst dye and MnTMPyP containing clear buffer.