Novel fluorescence microscopic assays for mitochondrial swelling, transport and membrane potentials in adherent cultured cells

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...

Akos A. Gerencser, David G. Nicholls, Martin D. Brand

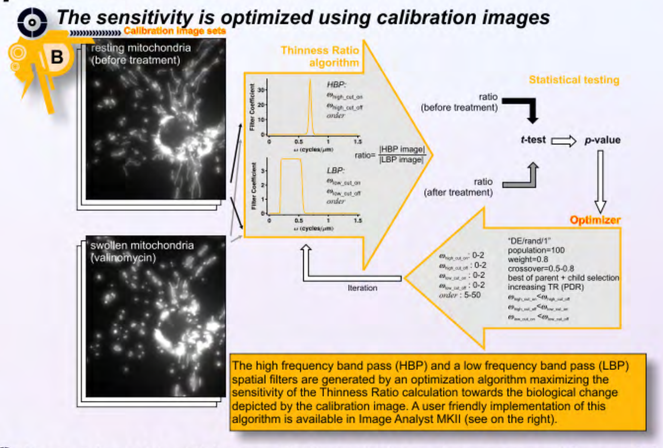
Buck Institute for Age Research, Novato, California, 94945, USA;
e-mail: agerencser@buckinstitute.org

Mitochondrial Shape Changes at Superresolution Level

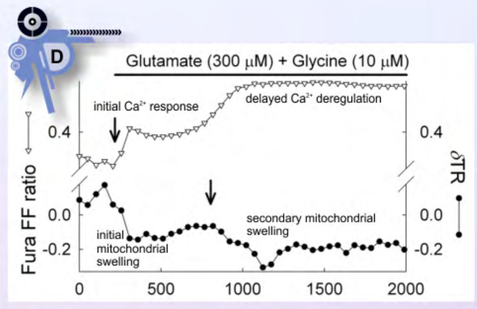
- The thinness ratio technique is a sensitive assay of diameter changes of *in situ* mitochondria.
- The thinness ratio technique is based on band pass spatial filtering in *Fourier* domain. Band pass filtering measures the intensity of puncti and filaments with a given range of diameters. The relative 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.
- 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

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

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).



Cultured mouse cortical neurons expressing mitochondrially targeted DsRed2 and loaded with the Ca^{2+} dye Fura-FF were excitotoxicly stimulated with the combination of glutamate + glycin to open NMDA receptors. The first and second drops of the TR always coincided with the initial response to glutamate and followed the delayed Ca^{2+} deregulation, respectively.

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

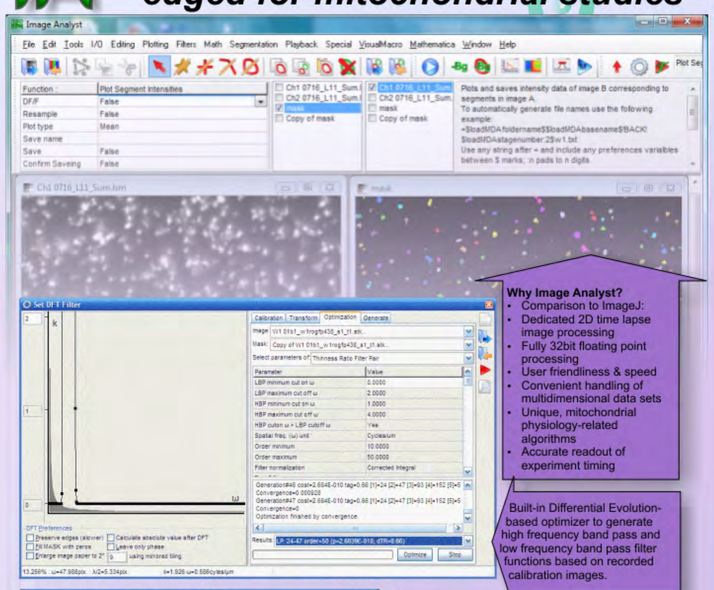
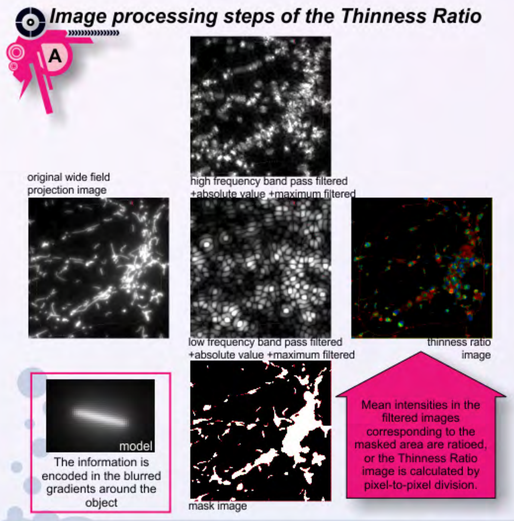
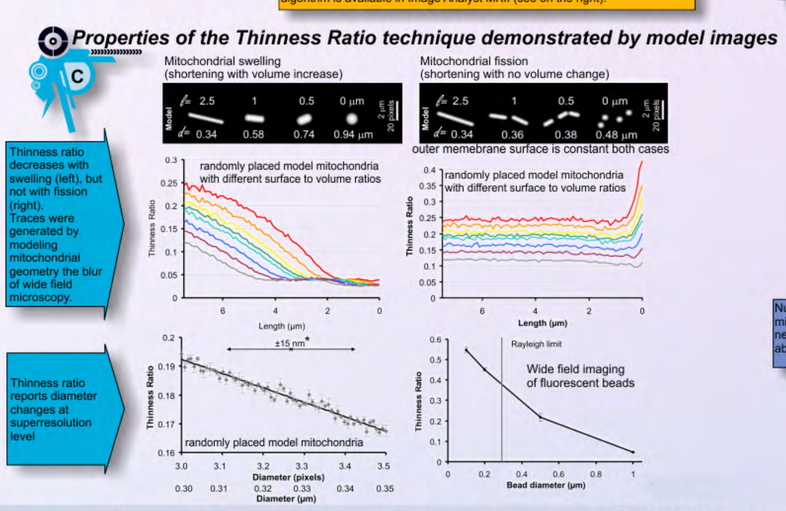
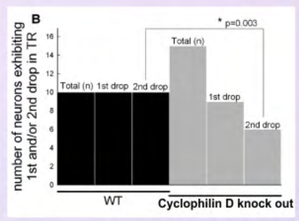


Image processing steps of the Thinness Ratio



Properties of the Thinness Ratio technique demonstrated by model images





Numbers of 1st and 2nd drops of the Thinness Ratio marks initial and delayed mitochondrial swelling. Mitochondrial swelling was detected in all observed neurons from wild type mice. Mitochondria did not swell after glutamate addition in about half of the neurons looked from Cyclophilin D knock out mice.

Doczi et al. 2011. J. Biol. Chem. 2011 286:6345-53

Mitochondrial 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

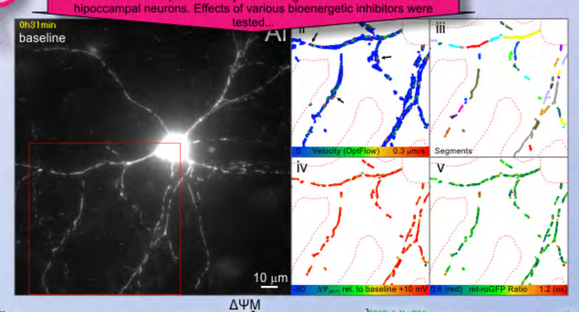
Gerencser & Nicholls 2008 Biophys J. 95:3079-99

Single Mitochondrion Analysis

Relative mitochondrial membrane potential, redoxGFP redox potential, mitochondrial velocities and size were analyzed on single mitochondrion level in rat cultured hippocampal neurons.

Cell Population Analysis

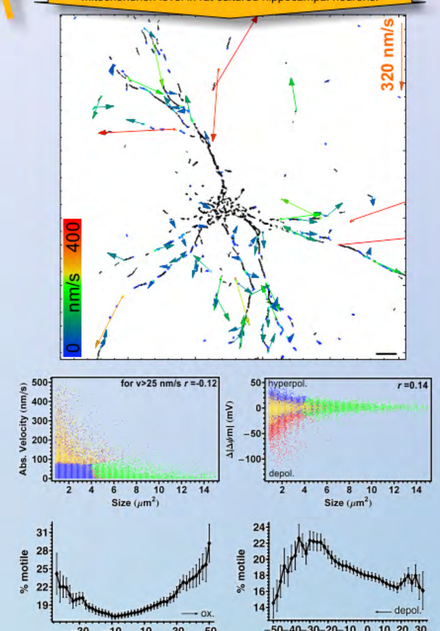
Mitochondrial membrane potential, redoxGFP redox potential mitochondrial velocities and shape was analyzed on single cell level in rat cultured hippocampal neurons. Effects of various bioenergetic inhibitors were tested...



Is mitochondrial motion governed by [ATP] or by ΔΨM?
- Velocities decreased by the expected ATP depletion and were insensitive to ΔΨM.

Does bioenergetics govern the direction and motility of individual mitochondria?
- Anterograde and retrograde transport were always balanced in all conditions

Can mitochondrial swelling explain decreased motility?
- Bioenergetical inhibition triggered shortening and thickening of mitochondria. However when pyruvate was present (-glucose + oligomycin) mitochondria shrinked and slowed, therefore swelling is not a primary cause.



Is there a correlation between the size and the velocity of mitochondria?
- The negative sign of the correlation coefficient indicates that larger mitochondria are slower.

Does the matrix thiol redox potential or the membrane potential predict the direction and motility of individual mitochondria?
- More oxidized and less polarized mitochondria have a tendency to move faster
- Motility was increased by depolarization until ~ -40 mV but mitochondria more depolarized than this threshold had lower motility.
- No correlation could be detected between the direction of movement and either ΔE_{mOGFP} or ΔΨM

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

Image processing steps and properties of Optical Flow

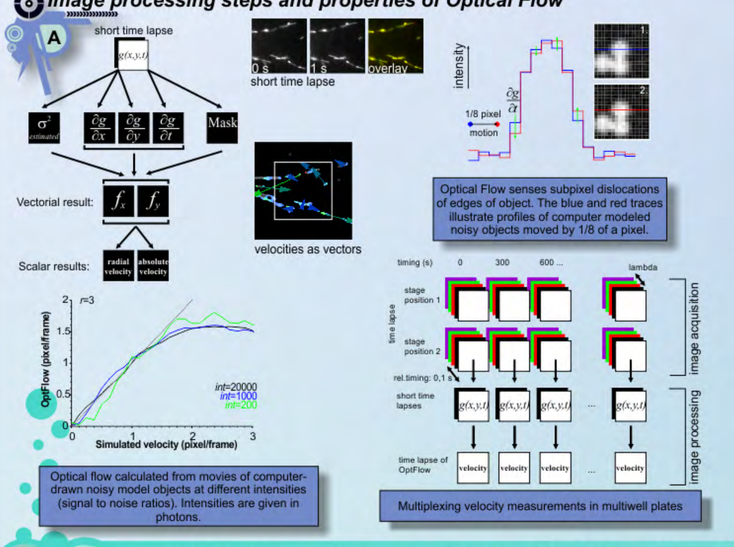
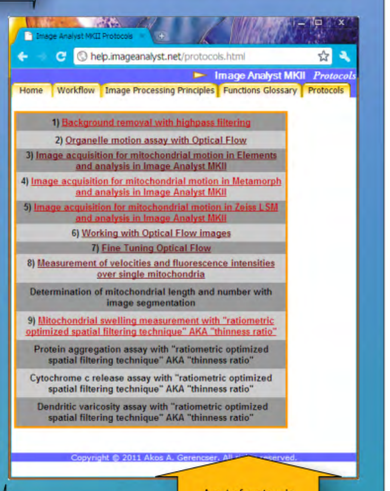
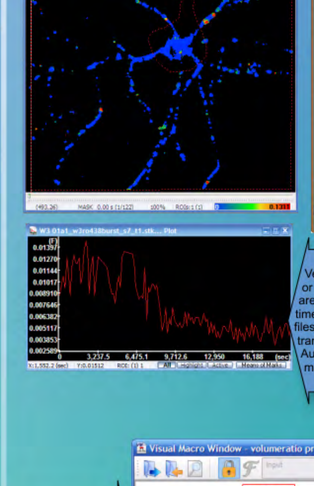


Image Analyst MKII Protocols



A set of protocols detailing image acquisition for Thinness Ratio and Optical Flow calculation for different microscope systems and image analysis using Image Analyst is available on the net.

www.imageanalyst.net



This poster is available for download at http://www.imageanalyst.net/tutorials.html

Mitochondrial Membrane Potential in Absolute Millivolts

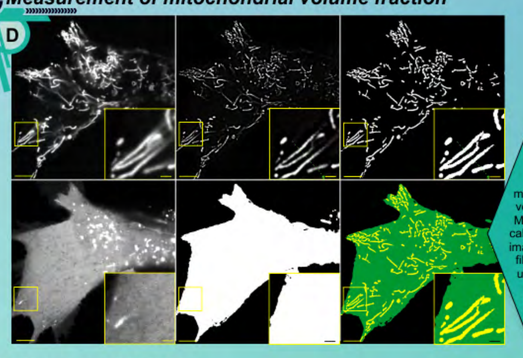
- Mitochondrial membrane potential is measured in millivolts in monolayer cell cultures. Cell types with different geometry can be compared.
- Mitochondrial membrane potential can be determined in the presence of fluctuating plasma membrane potential.
- Potentials are determied by the solution of a biophysical model, that counts with the effects of plasma and mitochondrial membrane potential on TMRM fluorescence with its slow redistribution through the plasma membrane, its low- and high-affinity binding to cellular compartments, volume ratios, auto- and background fluorescence.
- Microscopy requirements:
  - wide-field or confocal, 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)

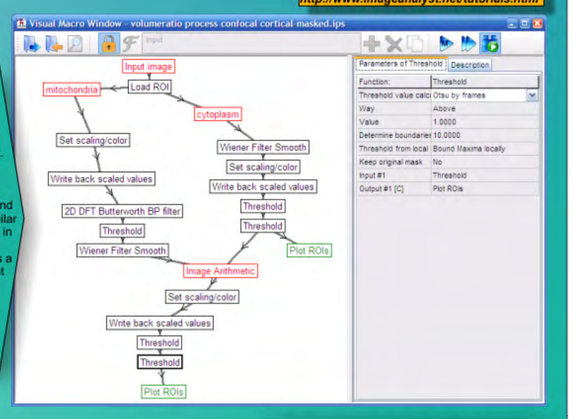
Novel features*
- plasma membrane potential dependent kinetics of probe uptake and release (Eyring rate theory, barrier model)
- activity coefficients (applied for chemical activity and nonspecific, low-affinity binding of the probes to cellular components)
- background fluorescence (f_x) that includes: nonspecific, high-affinity binding of the probes to cellular components, autofluorescence and local fluorescence background
- The apparent (effective) charge of TMRM and PMPI is measured, and used for all calculations
- The effect of tetraphenylboron (TPB) on equilibrium properties of TMRM and PMPI was asessed, PMPI follows Nernstian distribution

* compared to Nicholls 2006 J.Biol.Chem. 281, 14864-14874

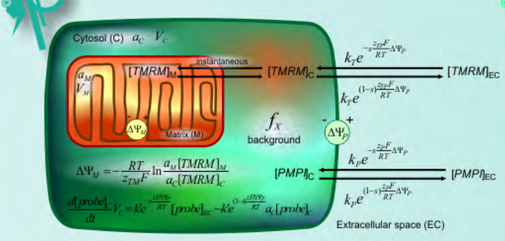
unpublished / preliminary use in: Birket et. al. Journal of Cell Science, 2011 124:348-58, Chinopoulos et. al. FASEB J 2010

Measurement of mitochondrial volume fraction



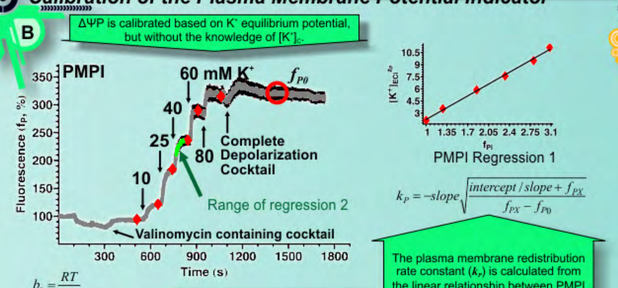


Biophysical model of TMRM accumulation in cells



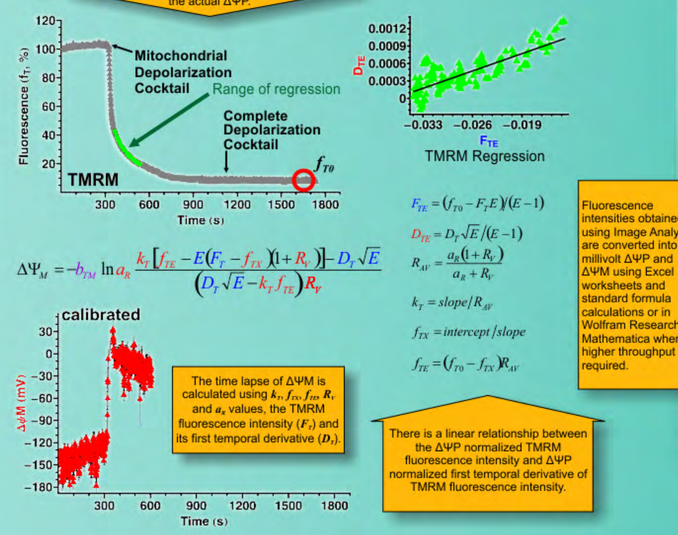
Calibration of the Plasma Membrane Potential Indicator

ΔΨP is calibrated based on K^+ equilibrium potential, but without the knowledge of $[K^+]_C$.

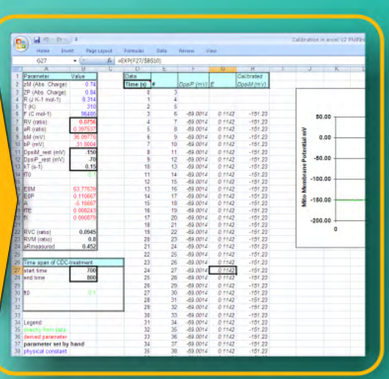


Calibration of TMRM

ΔΨM is calibrated based on the efflux kinetics of TMRM following an acute and complete mitochondrial depolarization. This decay of TMRM fluorescence is determined by the previously existing resting ΔψM, the volume and activity coefficient ratios and the actual ΔΨP.



Fluorescence intensities obtained using Image Analyst are converted into millivolt ΔΨP and ΔΨM using Excel worksheets and standard formula calculations or in Wolfram Research Mathematica when higher throughput is required.



The calibration is performed in Excel or in Mathematica.

Publications using Image Analyst MKII and its predecessors:

- **Gerencser, A.A.** and Adam-Vizi, V. (2001). Selective, High-Resolution Fluorescence Imaging of Mitochondrial Ca2+ Concentration. Cell Calcium. 30:5:311-321.
- **Gerencser, A.A.**, Doczi, J., Töröcsik, B., Bossy-Wetzel, E., and Adam-Vizi, V. (2008). Mitochondrial Swelling Measurement in Situ by Optimized Spatial Filtering: Astrocyte-Neuron Differences. Biophys J. Sep;95(5):2583-98.
- **Gerencser A. A.** and Nicholls D. G. (2008) Measurement of Instantaneous Velocity Vectors of Organelle Transport: Mitochondrial Transport and Bioenergetics in Hippocampal Neurons. Biophys J. 2008 Sep 15;95(6):3079-99.
- Choi S.W., **Gerencser A.A.**, Nicholls D.G. (2009) Bioenergetic analysis of isolated cerebrocortical nerve terminals on a microgram scale: spare respiratory capacity and stochastic mitochondrial failure. J Neurochem. 2009 May;109(4):1179-91
- **Gerencser A. A.**, Mark KA, Hubbard AE, Divakaruni AS, Mehrabian Z, Nicholls DG, Polster BM (2009) Real-time Visualization of Cytoplasmic Calpain Activation and Calcium Deregulation in Acute Glutamate Excitotoxicity. J Neurochem. 2009 Aug;110(3):990-1004
- Konrad C, Kiss G, Töröcsik B, Lábár JL, **Gerencser AA**, Mándi M, Adam-Vizi V, Chinopoulos C. (2011) A distinct sequence in the adenine nucleotide translocase from Artemia franciscana embryos is associated with insensitivity to bongkrekate and atypical effects of adenine nucleotides on Ca(2+) uptake and sequestration. FEBS J. 2011 Mar;278(5):822-36
- Doczi J, Turiak L, Vajda S, Mandi M, Torocsik B, **Gerencser AA**, Kiss G, Konrad C, Adam-Vizi V, Chinopoulos C. (2011) Complex contribution of cyclophilin D to Ca2+-induced permeability transition in brain mitochondria, with relation to the bioenergetic state. J. Biol. Chem. 2011 Feb 25;286(8):6345-53
- Choi SW, **Gerencser AA**, Lee DW, Rajagopalan S, Nicholls DG, Andersen JK & Brand MD. Intrinsic bioenergetic properties and stress-sensitivity of dopaminergic synaptosomes. J. Neurosci. 2011 Mar 23;31(12):4524-34
- Birket MJ, Orr AL, **Gerencser AA**, Madden DT, Vitelli C, Swistowski A, Brand MD, and Zeng X. A reduction in ATP demand and mitochondrial activity with neural differentiation of human embryonic stem cells. Journal of Cell Science, 2011 24:348-58.
- Alavez S, Vantipalli MC, Zucker DJ, Klang IM, Lithgow GJ. Amyloid-binding compounds maintain protein homeostasis during ageing and extend lifespan. Nature. 2011 472:226-9

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