Quantitative multi-parameter microscopy of mitochondrial function in living cells

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Quantitative life cell technology currently running at our department

**FLUORESCENT CHEMICAL REPORTERS**
- Rhodamine 123 (dynamics)
- TMRM et al. ($\Delta \psi$, dynamics, PTP)
- Mitotrackers (dynamics)
- Rhod-2 et al. (mito Ca$^{2+}$)
- Fura-2 et al. (cyto Ca$^{2+}$)
- BCECF et al. (cyto pH)
- Hydroethidine / MitoSOX RED (superoxide)
- CM-DCCFDA (ROS)
- ER tracker (dynamics)
- C11 BODIPY (lipid peroxidation)
- NAD(P)H autofluorescence
- FADH$_2$ autofluorescence

**FLUORESCENT PROTEINS**
- GFP et al. (dynamics)
- GFP-protein et al. (protein dynamics)
- roGFP1 (thiol redox status)
- pHluorin (mito pH)
- D1ER (ER Ca$^{2+}$)
- Pericam (Ca$^{2+}$)
- Photo-activatable Dendra2 (mito/protein dynamics)

**BIOLUMINESCENCE**
- Targeted aequorin (Ca$^{2+}$)
- Targeted luciferase (ATP)

**SOFTWARE**
- Image analysis:
  - Image Pro Plus
  - Metamorph
  - Imaris
  - Volocity
  - ImageJ
- Numerical analysis:
  - Origin Pro
  - Excel
- Modeling:
  - MATLAB/Simulink
  - Figure preparation
  - CorelDraw

**IMAGING APPROACHES & DATA HANDLING**
- Multispectral confocal laser scanning microscopy
- Videorate confocal laser scanning microscopy
- CCD camera videomicroscopy
- Bioluminescence recording (PMT)
- Fluorescence correlation spectroscopy
- FRAP/FLIP microscopy
- Curve fitting
- Mathematical cell/data modeling
Fluorescence: Jablonski diagram

Fluorescence

Absorption

Higher energy and vibration state

Lowest singlet excited state

Ground state

Bleaching

Absorption

Irreversible oxidation (bleaching)
Fluorescence: Stokes shift

$E = h \cdot f = \frac{h}{\lambda}$

Stokes shift

Excitation

Emission

Wavelength
The green fluorescent protein family

Shaner et al., J. Cell Sci., 2007
The green fluorescent protein family

Shaner et al., J. Cell Sci., 2007
Protein tagging with GFP

Getting the fluorescent proteins in the cell: Using the Gateway® system (Invitrogen)
Fluorescence imaging principle

Dichroic mirror  Barrier filter  Light source

Exciter filter  Transmi

Objective

Fluorescent sensor measurement

Adapted from: www.olympus.com and www.omega.com
XYZ-resolution of various imaging approaches

Koopman et al., AJP, 2008
“Not images but numbers”: The key paradigm for quantitative image analysis

SO FOCUS ON:
- Optimal cell quality
- Optimal reporter selection
- Optimal staining approach
- Optimal image acquisition

Optimal image quality

Best quality numerical data
What is a good life cell image?

The ‘easy’ answer: “That depends on the question you want to address”

**GENERAL RECOMMENDATIONS**
- Keep your cells happy at all time
- Avoid photobleaching / damage: Correct fluorophore, lamp/laser intensity
- Use the correct objective: Magnification, numerical aperture, coverslip correction!
- Acquire a low noise image with a good signal-to-noise ratio: signal averaging
- Use an optically aligned microscopy system

**MORE SPECIFIC RECOMMENDATIONS:**

*I want to measure the intensity of a signal:*
- Keep your settings identical between experiments
- Cell thickness changes?
- Ratio imaging possible?

*I want to measure co-localization*
- No optical pixel shift (optical quality of the imaging system)
- No bleed through between wavelengths (fluorophore choice, spectral unmixing)
- How to calculate? (currently the best: The Manders Algorithm)
Which fluorescent cation to choose?

\[ \text{PMF} = \Delta \psi - \frac{2.303 \cdot RT \cdot \Delta pH}{F} \]

\[ \Delta \psi (\text{mV}) = \frac{2.303 \cdot RT}{zF} \log \left( \frac{C_m}{C_{\text{cyt}}} \right) \]

\[ C_m = C_{\text{ext}} \exp \left[ - (\Delta V + \Delta \psi) zF/RT \right] \]

\[ \Delta \psi = -60 \cdot 10^{\log \left( 7.6 \cdot F_{\text{mit}}/F_{\text{nuc}} \right)} \]

Koopman et al., Methods, 2008
Acquiring the optimal image: Black-level and intensity gain

(a) Photomultiplier Signal vs Distance

(b) Amplitude vs Negative Offset Voltage Applied

(c) Gain Applied
Stability of the TMRM signal

A

TMRM Leakage

B

TMRM Leakage

C

TMRM Illumination

D

TMRM Illumination

Distelmaier et al., Cytometry A, 2008
Automated quantification of mitochondrial parameters
Automated quantification of mitochondrial parameters: Algorithm

Automated quantification of mitochondrial parameters
How does a spatial filter work?

A) Image

Central pixel = recalculated

B) Image

Gray values

Kernel

<table>
<thead>
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<th>20</th>
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3x3 Top-Hat

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<tr>
<th>-1</th>
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<td>-1</td>
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C) Original image

7x7 Top-Hat (3x)

New central pixel value =
[-1*10-1*20-1*30
-1*40+8*50-1*60
-1*70-1*80-1*90]/
[-1-1-1+8-1-1-1]
Meaning of aspect ratio (AR) and formfactor (F)

Koopman et al., 2005a, AJP Cell Phys.
Effect of noise in the images

Koopman et al., 2006, Cytometry A
Branching mitogram

<table>
<thead>
<tr>
<th>Class</th>
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<th>Objects</th>
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<td>56</td>
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<td>3</td>
<td>49</td>
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<td>2</td>
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Koopman et al., 2005a, AJP Cell Phys.
Koopman et al., 2005b, AJP Cell Phys.
Koopman et al., 2006, Cytometry A
Spatial visualization of mitochondrial area and structure

Mitochondrial area

Degree of branching

Distelmaier et al. (submitted)
Automated determination of mitochondrial position

\[ D = \sqrt{(x_m - x_n)^2 + (y_m - y_n)^2} \]
Quantification of $\Delta \Psi$ during more depolarized conditions

Distelmaier et al., Cytometry, 2007
Human complex I deficiency: \( \Delta \psi \) is depolarized

Distelmaier et al. (Submitted)
BD pathway 855

R123-stained live cells: 2D Z-Stack

Optimize contrast (1)

Median filter 3x3, S1, P1 (2)

- Avg min/max intensity
- Z=2.00
- No autosharp (3)

Background subtraction (4)

3D post-processing

Optimize contrast (5)

3D stack calculation

- 3D-Stack (6)

Quantification
- Volume (V)
- Surface area (A)
  - A/V ratio
  - Shape
- Connectivity (4D)
- Dynamics (4D)
- Signaling (4D)
The next challenge: Quantifying mitochondrial shape and function in multiple dimensions

<table>
<thead>
<tr>
<th>‘Dimensionality’ of the data</th>
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<tbody>
<tr>
<td>- Spatial dimensions ((x,y,z))</td>
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<tr>
<td>- Time ((t))</td>
</tr>
<tr>
<td>- Multiple reporters ((n\geq1))</td>
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<tr>
<td>- Multiple parameters from same reporter ((n\geq1))</td>
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<table>
<thead>
<tr>
<th>Quantification</th>
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<tbody>
<tr>
<td>- Volume ((V)), Surface area ((A)), (A/V) ratio</td>
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<tr>
<td>- Shape</td>
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<tr>
<td>- Colocalization (4D)</td>
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<tr>
<td>- Connectivity (4D): Intra/inter mitochondrial, with ER</td>
</tr>
<tr>
<td>- Dynamics, 3D tracking (4D)</td>
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<td>- Signaling (4D)</td>
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<table>
<thead>
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<th>Example</th>
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<tbody>
<tr>
<td>- Mitochondria stained (GFP), ER stained (RFP): 2 Dimensions</td>
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<tr>
<td>- Make 3D stacks in time: 4 Dimensions</td>
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<tr>
<td>- Determine co-localization and shape 3 Dimensions</td>
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</table>
Quantifying mitochondrial shape and function in higher dimensions: The right software?

MCF7 Breast Carcinoma Cells. LAMP1 (Alexa 568- Red), P32 (Alexa 488- Green), Nuclei (DAPI- Blue). Courtesy of Ed Monosov- Burnham Institute, La Jolla, CA.

Figure 2. Nuclear volumes (blue) created through the use of the Surface Value slider. Blue boxes indicate selected volumes in Figure 3.
Quantifying mitochondrial shape and function in higher dimensions: The right software?

Figure 3. The Volume Measurements Data Table

Figure 4. The Volume Measurements Options Menu
Photoconvertible FPs

A

Neutral Phenol
Native Species (Dark)

PA-GFP
PS-CFP2

Photoactivation
405 nm

Anionic Phenolate

Decarboxylation

B

Native Species (Green)

Kaede
KikGR
Eos
Dendra2

Photoconversion
405 nm

C

trans Chromophore

cis-trans Isomeration

Dronpa
mTFP0.7
KFP1

Dark State

Fluorescent State

Shaner et al., J. Cell Sci., 2007
Dendra2 photoconversion mechanism

Neutral state

Anionic (green) state

Red state

Chudakov et al., Nature Protocols, 2007