Science Webinar Series

TECHNIQUES AND METHODS IN LIVE-CELL IMAGING: Practical Advice for Microscopy-based Research

18 July, 2012

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TECHNIQUES AND METHODS IN LIVE-CELL IMAGING: Practical Advice for Microscopy-based Research

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Brought to you by the Science/AAAS Custom Publishing Office

Participating Experts:

Vytas Bindokas, Ph.D.
University of Chicago
Chicago, IL

Simon C. Watkins, Ph.D.
University of Pittsburgh School of Medicine
Pittsburgh, PA

Tomasz Zal, Ph.D.
MD Anderson Cancer Center
Houston, TX

Sponsored by:

Leica Microsystems
Fundamentals of Live Cell Microscopy

Biology is dynamic

Vytas Bindokas, Ph.D.
The University of Chicago
Integrated Light Microscopy Core Facility
Beware of the “Observer Effect”—
The act of making a measurement affects the system being measured.

Levels of Complexity:
Whole organism
Tissue
Cellular
Subcellular

Here’s the problem…

(Keeping the “live”
In live cell microscopy!)

Cartoon credit: Shirley Bond
Approaches: Transmitted light & Fluorescence

Brightfield (transmitted) light is suitable for colored specimens, but **even clear materials** can be visualized by contrast generation techniques including: phase contrast, differential interference contrast (DIC), Hoffman modulation, etc.

Fluorescence imaging creates a bright signal against a dark background—something we can easily see/detect. (Fluorescence excitation and emission colors are distinct, thus isolatable)

**Huge diversity of assays**

Multiple targets can be visualized using vital stains, native materials, fluorescent proteins, etc.

**Common uses:** cell tracking, gross structure, fine details

**Tip:** use an automated shutter to limit light exposure only to actual data collection
General Rule 1: Use the least light, the brightest probes, the most sensitive detectors.

Avoid: Over-magnification
Long Illumination Dwell
Some of the best sensitivity detectors: back-thinned EM-CCD (up to 93% efficiency)
Drawbacks: high $$$, large and few pixels
Positives: wide dynamic range, very fast, high signal-to-noise content

Pixel wars! Mega pixels are really better, right?
Speed wars! Do I need video rate (30 fps) capability? ?? A 5-second exposure?
Bit wars! Do I need a full 16 bits of brightness data? 8, 10, 12, 14 bit?

KEY FACTOR: Signal-to-noise

Example: mitochondria distinguishable at just 3 intensity units above background

Hamamatsu Orca-Flash 2.8 sCMOS

Resolution wars! Standard vs. confocal vs. superresolution microscopy? (weigh how much detail is needed, how much light is available / tolerated)
Problems:

**Short wavelengths (UV light, blue light) and High Energy (high-power lamps and lasers) damage cells**

Fixes: Limit intensity (ND 2), limit exposure / dwell time, limit number of images, *choose redder probes*

---

**Some fluorophores copiously generate reactive oxygen species**

- e.g., B vitamins (esp. riboflavin) in full cell media, some dyes

Fixes: change probes, use partial media or saline, try radical quenchers/scavengers

---

**Focus drift**

Fixes: equilibrate all temperatures before starting, maintain dish volume, automate self-focus, do 3-D

---

**Mammalian cells require observation at 37°C and bicarbonate buffers require 5% CO₂**

Fixes: use alternate buffers, *use incubators*
Temperature and Incubation control

Two Fast 3-D Capture Options: Galvo, Piezo

Heated dish

Relatively short term (hours)

Objective heater

[full jacket, (but) Mini CO₂ chamber]
Do: Multiple fields of view.

Concurrent runs save time and money.
Moving targets are really difficult!

Problem: objects moves out of view

Swim/crawl motion:

- **Trap** in high viscosity: agarose, methyl cellulose, thermogels [e.g., CyGEL is liquid when cold, gels on warming]
- **Restrict** to shallow volume [trap in low volume; microfluidic channels]
- Active **Track**ing: programmed XY stage + software. E.g., Matrix (Leica), self-built (Ben Arous et al., J Neurosci Methods 187:229-234 2010)
- Increase optical section **Thick**ness (keep more in focus per time point), Use lower **magnification** (large view area)

Relative motion, multiple targets:

Problem: objects move out of register in signal/color channels […]like “head and tail lights”]

- Multiple simultaneous detectors (image splitters, multiple cameras, etc)
Problems: High magnification required; confocal bleaching; over-expression alters behavior.

Low-light LIVE confocal imaging of *E. coli* expressing GFP-FtsZ ring construct.

~FIX: Fast scanning, frame accumulation, line averaging, lessen confocality.
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Live cell imaging, breaking speed barriers

Simon C. Watkins Ph.D
Why do we need to go fast

• Motion analysis of:
  – Endocytosis (3 color TIRF, 9/fps)
  – Calcium and physiologic measurement (10-50 fps)
  – Motion
    • Organismal (C-elegans)
    • Cellular (sperm)
    • Blood flow
    • Cilia beat
Speed limiters: the microscope

• Slowest component- port changer- solved
• Slowest component- objective changer- not needed
• Slower component - cube changer- solved
• Light shuttering- solved
• Light color- solved
• Cameras- solved
Changing camera ports

• Why?
  – Combining modalities (TIRF/Confocal for eg)
  – Ports need to be on the same side of the microscope such that images can be registered in real time
  – Galvo port switchers are available with up to 3 positions
  – See for example Intelligent Imaging Innovations www.intelligent-imaging.com
Changing colors

If fluorescence and brightfield are mixed, a transmitted light shutter is needed also.

It is absolutely essential to have a filterwheel controlling excitation light, this must have neutral density filters and shutters. Using this device the cells are only exposed to light during photography.

Generally two wheels in tandem, one wheel has primary barriers filters the other neutral density, therefore it is possible to optimize light wavelength and intensity.

Also limit O2 based toxicity by putting oxygen scavengers in the culture medium (oxyrase, ascorbic acid).
Mercury vs Argon arc lamps 1:

- Mercury arc lamps are brighter
- Xenon has a flatter spectrum and is therefore more quantitative for ratiometry and other applications
<table>
<thead>
<tr>
<th>Nominal wavelength</th>
<th>Open Power</th>
<th>Filtered power</th>
</tr>
</thead>
<tbody>
<tr>
<td>390nm</td>
<td>220</td>
<td>185</td>
</tr>
<tr>
<td>440nm</td>
<td>322</td>
<td>190</td>
</tr>
<tr>
<td>475nm</td>
<td>191</td>
<td>147</td>
</tr>
<tr>
<td>514nm</td>
<td>191</td>
<td>141</td>
</tr>
<tr>
<td>550nm</td>
<td>366</td>
<td>220</td>
</tr>
<tr>
<td>575nm</td>
<td>800</td>
<td>230</td>
</tr>
<tr>
<td>632nm</td>
<td>265</td>
<td>208</td>
</tr>
</tbody>
</table>
LED sources 2

• Many colors
  – no UV/ Fura

• Fast on/off (0.25 ms)
  – no need for a shutter.

• Continuous attenuation
  – No need for neutral density filters

• Can be combined with matched multiband filter cubes
  – No need to change filter cubes for up to 4 colors
Cameras

- Need to design camera with advantages of frame transfer though with higher speed, less noise, and high quantum efficiency.

A major limitation is that for each active row of pixels a masked transfer row exists too. This halves the number of lines on the chip, and hence halves the light gathering power of the chip.

Micro-lens technology has solved this problem. Light that would normally fall on the mask is redirected to the active areas of the chip by a plastic lens.
Electron Multiplier CCD

• Strong points
  – No-photocathode.
    • Wide range of sensitivity.
    • Good uniformity.
    • Good resolution.
  – No damage from excess light
  – High frame rate.
  – Binning, Sub-array.

• Weak points
  - Multiplication noise reduces effective Q.E. by 50%
  - Gain and gain fluctuation are temperature dependent
  - Residual image can interfere with single photon imaging
Camera limitations

• Cooled CCD cameras and EM CCDs are still relatively slow
• Frame rates still max at around 20 mHz (20 million pixels/second)
• Increase frame rates by binning
  – adjacent arrays of pixels are added together
  – 2x2, 4x4 for eg, increases frame rate but decreases resolution
  – Sub array (only collect a portion of the chip)
• Ultimately speed is limited because of heat leading to noise when reading fast
CCD Sensor

CMOS Sensor

Complementary metal–oxide–semiconductor (CMOS)
Active pixel sensor (aps)
Newest CMOS technology

Buried Channel Photodiodes

Column amps with CDS

Digital data out

On chip digitizers
CMOS continued

• Full frame (2048x2048) readout 100 fps
• Pixels read out in complete columns 2048 pixels (5 microseconds/column)
• Sub arrays 2048X200 pixels 1000 fps
• Noise is much better
  – Pixel level 1.03 electrons/pixel/second
  – Still have variance at the pixel level and at the column level
  – Technology is improving daily
• Made by
  – Andor
  – PCO edge
  – Hamamatsu
Combining LED w/CMOS

• With triggering
  – where the camera controls other devices
• Can change colors very quickly
  – Sub millisecond vs 50ms for a filter wheel
• Can turn light off very quickly
  – 0.25 millisecond
• Can now be used for transmitted light imaging
  – (made by Prior)
Can be combined with diode sources to generate truly high speed multicolor imaging

2 colors 83 fps

Diodes have 0.25ms rise time

<table>
<thead>
<tr>
<th>Pixel count</th>
<th># 3 color images/sec</th>
</tr>
</thead>
<tbody>
<tr>
<td>2048x2048</td>
<td>23</td>
</tr>
<tr>
<td>1024x2048</td>
<td>33</td>
</tr>
<tr>
<td>512x2048</td>
<td>51</td>
</tr>
<tr>
<td>256x2048</td>
<td>111</td>
</tr>
</tbody>
</table>
Confocal microscopy and speed

- Resonant scanners (Nikon, Leica) offer some speed advantages but still use a single point detector.
- Array detectors offer much more sensitivity than PMTS and offer an advantage when combined with multipinhole confocal.
- Several commercial solutions currently available with different implementation of the multipinhole approach.
Multipinhole confocals are commonly much more sensitive and lead to less phototoxicity

- This is primarily because of the sensitivity of the detector.
- To optimize sensitivity (and speed) need adjustable pinholes and triggered detection

Prairie Sweptfield confocal head

7 adjustable pinholes/slits
Speed is limited by the detector

• Because of sensitivity and amplification, EMCCDs still more or less essential
• Triggering very important
• Piezo Z control essential to build high speed Z stacks
  – MCL and PI
• Can now do 2 color 30 Z slice stacks/second.
Summary

• Solving speed issues is the new frontier in live cell imaging
• Most problems are now solved and widefield can go truly fast with no moving parts
• New microscope stands will have many implemented components with speed at their core.
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Leica Microsystems
Live cell imaging

• Optimizing the optics for aqueous samples
• Imaging single cell dynamics in vivo
• Imaging molecular interactions
• How to relate cell dynamics to molecular-level information in the same cells?
  ✓ Dynamics-Immunosignal Correlative (DISC) Microscopy
Aqueous specimens require special optics

Fixed and mounted
- Slide
- Mounting n=1.525
- Cover Glass n=1.525
- Oil n=1.525
- 63x NA 1.4 oil

LIVE
- Medium n=1.333
- Cell n=1.333-1.420
- Water n=1.333
- Silicone n=1.404
- 63x NA 1.2 W 0.17 correction collar

Oil immersion: Thickness of the cover glass is relatively unimportant

Water immersion objectives minimize the spherical aberration (SA)
- Thickness of the glass bottom is critical
- Use the collar to correct for C.G. and temp.
- Water evaporation is problematic for long term time lapse - avoided by new silicone immersion objectives
Aqueous specimens require special optics

- Autofocus hardware is available from several manufacturers
- Typically based on reflection from the glass/water interface
- Allows long term time lapse – no drift for hours, days
Live cell imaging in vivo

Water dipping configuration minimizes the SA and affords access

- Used for Electrophysiology, Microinjection and **In Vivo**
- Requires yet different objectives
- But: no autofocus by reflection
- Tissue surface may move too much

→ The upright configuration is versatile, especially with a suction holder
→ Good to have two objectives: one for dipping and one for the cover glass

Medium $n=1.333$

The scattered photons can contribute to image formation, thereby increasing sensitivity
Multiplex 2-photon Excitation

<table>
<thead>
<tr>
<th>PMT</th>
<th>Fluorophore</th>
<th>Excitation</th>
<th>Emission</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDD1</td>
<td>SHG (collagen)</td>
<td>870 nm</td>
<td>435 nm</td>
</tr>
<tr>
<td>NDD1</td>
<td>mCerulean (tumor)</td>
<td>870 nm</td>
<td>~ 470 nm</td>
</tr>
<tr>
<td>NDD2</td>
<td>YFP (dendritic cells)</td>
<td>990 nm</td>
<td>~ 530 nm</td>
</tr>
<tr>
<td>NDD2</td>
<td>DsRed (T cells)</td>
<td>990 nm</td>
<td>~ 570 nm</td>
</tr>
</tbody>
</table>
No Multiphoton for In Vivo? Confocal may do just fine

<table>
<thead>
<tr>
<th></th>
<th>Multiphoton</th>
<th>Confocal (point-scan or spinning disc)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Depth in tissue</td>
<td>$\sim 300\ \mu m$ (typical)</td>
<td>$\sim 100\ \mu m$ (typical, often sufficient)</td>
</tr>
<tr>
<td>Photobleaching and Phototoxicity</td>
<td>Confined to the focal plane but occurs nevertheless</td>
<td>Extends beyond the focal plane but may be quite low</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Negligible at low laser powers and high scan speeds: <strong>Resonant scanning is wonderful!</strong></td>
</tr>
<tr>
<td>Spectral selectivity</td>
<td>Only 2-4 channels (if a single laser)</td>
<td>&gt;4 channels (based on emission <em>and</em> excitation)</td>
</tr>
<tr>
<td>Resolution</td>
<td>Slightly worse than confocal</td>
<td>Can be traded for sensitivity and depth</td>
</tr>
<tr>
<td>Intrinsic contrast</td>
<td>• Autofluorescence</td>
<td>• Autofluorescence</td>
</tr>
<tr>
<td></td>
<td>• <strong>Second/Third Harmonic</strong></td>
<td>• <strong>Reflection</strong></td>
</tr>
<tr>
<td>Cost</td>
<td>Very high</td>
<td>High</td>
</tr>
</tbody>
</table>

→Either modality can be used in vivo with advantages; good idea to have both
Imaging molecular interactions in living cells

Sensitized Emission (SE)

Donor Lifetime (FLIM)

Polarization

FRET

Cross-Correlation

FCCM

Bimolecular Fluorescence Complementation

FRET

Spatial and temporal resolution

thermodynamic quantitation

equal to Förster radius (~10nm)

Sensitivity, quantitation

Spatial resolution

FCCM

BiFC

r < focal diameter (~200nm)

r < linker length (~50nm)

BiFC

MD Anderson Cancer Center

THE UNIVERSITY OF TEXAS
FRET by Sensitized Emission

- Fast and gentle – preferred for live cells
- How to calculate the $E$?

Three images are necessary:

<table>
<thead>
<tr>
<th>Image 1</th>
<th>Donor Ex</th>
<th>Donor Em</th>
<th>D +bkgd +some Bleedthrough</th>
</tr>
</thead>
<tbody>
<tr>
<td>Image 2</td>
<td>Donor Ex</td>
<td>Acceptor Em</td>
<td>SE +bkgd +more Bleedthrough</td>
</tr>
<tr>
<td>Image 3</td>
<td>Acceptor Ex</td>
<td>Acceptor Em</td>
<td>A (+ guess what)</td>
</tr>
</tbody>
</table>

$E = \frac{SE}{SE + G*D}$

Zal et al., Biophys. J., 2004

- Images 1 and 2 are simultaneous using dual cameras or image splitter, then Image 3 after fast change of excitation → no motion artifacts
- Image 3 is necessary to eliminate Bleedthrough (and to evaluate acceptor presence)
- “G” is the Gordon coefficient – system dependent
Visualization and Quantitative Analysis of Cell Dynamics

- **Translational motility**
  - faster or slower?
  - random or directional?
  - steady or with arrests?

- **Shape dynamics** – a type of non-translational motility

- **Substance Concentration and Transport**
  - calcium fluxes, pH, translocation of signaling proteins

- **3D visualization** (projections, rotations, fly-through, sectioning)

- **Free software**: Fiji = ImageJ (Cumbersome but powerful: a large number of plugins is available, including for 3D visualization and tracking)

- >$$ $$, $$$: Imaris, Volocity, Huygens, Slidebook, Metamorph, and other commercial packages are powerful and easy to use
Dynamics-Immunosignal Correlation (DISC) Microscopy

A) Live cell/in vivo microscopy

B) 1. Flash fixation w. PFA
2. Immunofluorescence microscopy

C) Landmark alignment in silico, soft-body correction

Dynamics-ImmunoSignal Correlation

Chodaczek et al., Nature Immunol., 2012
DISC Microscopy

LAST LIVE FRAME

20x obj, dry, NA=0.7

AFTER FIXATION

40x obj, oil, NA=1.2
DISC Microscopy

LAST LIVE FRAME

AFTER FIXATION

20x obj, dry, NA=0.7

40x obj, oil, NA=1.2
DISC Microscopy
DISC Microscopy

CD8 T cells
CD4 T cells
*tumor*

30 min tracking

Motility arrest (%)

- CD8
- CD4

p = 0.0028
Summary

• Special optics for aqueous samples is expensive but critical

• Imaging single cells in vivo
  • The upright configuration is versatile – especially with a suction-based tissue holder
  • Adjustable objectives corrected both for water dipping and cover glass are advantageous
  • Multiphoton is great, but confocal may be sufficient

• Imaging molecular interactions: FRET by sensitized emission is gentle on live cells, fast and quantitative

• Dealing with the data: the weakest link in the chain

• Dynamics-Immunosignal Correlative (DISC) Microscopy allows relating the cell dynamics to a wide range of molecular events in the same cells (Chodaczeck et al Nat. Immunol. 2012)
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Q&A

To submit your questions, type them into the text box and click Submit.

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