Fluorescence Imaging of Live Cells

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1. History of Modern Fluorescence Imaging
2. Basic Principles of Various Modes of Fluorescence Imaging
3. Future Development
Useful Web Sites

• http://probes.invitrogen.com/handbook (basic fluorescence and probes)
• http://www.microscopyu.com/articles/fluorescence (microscopy)
• http://www.olympusmicro.com/primer/techniques/fluorescence/fluorhome.html (microscopy)
• http://www.chroma.com/resources/handbook.php (filters)
• http://www.clontech.com/support/brochures.asp (fluorescent proteins)
Books


Searching for the Simplest Model to Account for Diverse Forms of Cell Shape and Migration

Neuron (16.5 hours)
Gary Banker

Fibroblast (1 hour)
Vic Small

Keratocyte (12 min)
Kurt Anderson
Fluorescence Microscopy

• Started at the turn of the 20\textsuperscript{th} century as one of the contrast generating methods

• Early applications used primarily histological stains, conceptually similar to bright field microscopy

• Noted for its introduced labels against a dark background, and the associated specificity and sensitivity

• Molecular targeting started with the introduction of fluorescently tagged antibodies by Coons in 1950, turning it from a morphological into a molecular tool
What is Fluorescence

Emission of photons as a molecule returns from singlet excitation state to ground state.
Anatomy of Organic Fluorescent Probes

- Reactive/binding moiety (biotin)
- Alexa-546 fluorophore
- Spacer
Immunofluorescence

primary antibody: rabbit antibody directed against antigen A

secondary antibodies: marker-coupled antibodies directed against rabbit antibodies

marker
Factors that Affect Fluorescence Microscopy

- **Photobleaching** – fading of fluorescence, as a result of chemical reactions of the excited state. Typically involves triplet state and oxygen and irreversible.

- **Photodamage** – creation of toxic molecules by excited fluorophores. Typically involves photobleaching and free radical generation.

- **Quenching** – non-radiative processes that dissipate the energy of the excited state. Typically involves non-covalent molecular interactions and reversible.

- **Autofluorescence** – fluorescence emitted by molecules other than the probes. Could be from co-enzymes, media, glass, immersion oil.

- **Background fluorescence** – diffuse photons that may originate from autofluorescence or from out of focus probes.

- **Noise** – statistical uncertainties associated with the detection of fluorescence. Typically appear as fine grains.
Bottlenecks of Early Fluorescence Optics

Optics – Dark field Illumination
   High background, Limiting objective NA, oil immersion

Probes – Few
   Bleaching, Limited reaction targets, Limited spectral ranges

Detectors – Photographic film
   Low sensitivity, Long exposure
Legacy (Diascopic) Design for Fluorescence Microscopy

Figure 1

(bright field) (dark field)
Optical Design in Epi-Fluorescence Microscopy

Dichromatic Mirror Function in Reflected Light Fluorescence Illumination

Figure 2

Excitation Illumination
Reflected Light Illumination
Condenser and Objective
Specimen
Fluorescence Emission
Bottlenecks of Early Fluorescence Optics

Optics – Dark field Illumination
High background, Limiting objective NA, oil immersion
Epi-Illuminator (physics)

Probes – FITC
Bleaching, Limited reaction targets, Limited spectral ranges
New bleaching-resistant dyes, sulfhydryl reagent, Indicator dyes, etc (chemistry)

Detectors – Photographic film
Low sensitivity, Long exposure, Tedious
Intensified video cameras (engineering)
Fluorescent Probes of Calcium Ion

![Chemical structure of a fluorescent probe]

<table>
<thead>
<tr>
<th>Indicator</th>
<th>$K_d(\text{Ca}^{2+})$</th>
<th>$R^2$</th>
<th>$R^7$</th>
<th>$R^5$</th>
<th>$R^6$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluo-3</td>
<td>0.39 μM</td>
<td>Cl</td>
<td>Cl</td>
<td>CH₃</td>
<td>H</td>
</tr>
</tbody>
</table>

![Fluorescence emission spectrum]

Ex = 488 nm
Fluorescent Analog Cytochemistry

- Protein Purification
- Fluorescent Labeling
- In vitro characterization
- Microinjection
- Image recording

Assembly of cortical actin following fertilization of sea urchin eggs, 1979

Microinjection of IAF-actin and recording with tri-X film
Applications of Fluorescent Analog Cytochemistry

Early Movies of Actin-Myosin Dynamics

Alpha-actinin in invadopodia of transformed NRK cells, 1987

Myosin II in lamella 3T3 fibroblasts, 1989
Beyond Simple Imaging
Marking Structures with Laser Beam for Visualizing Transport

Wang, 1985
Injection of iatr-actin into IMR33 cells, detection with a Venus intensified video camera, recording with a film camera positioned in front of a TV monitor

Small, 1981
Brave New World of Fluorescence Microscopy
Limitations of Fluorescent Analog Cytochemistry

- Efforts – protein purification, labeling, characterization
- Skills – microinjection
- Limitations – dependence on exchange, inaccessibility to membrane structures, inside organelles, etc

Fluorescent Proteins

![Fluorescent Proteins](image)
Natural Fluorescent Proteins

GFP
Barrel of 11 antiparallel beta sheets
27kDa
30Å in diameter
40Å in length
Fluorophore located in the middle (red), protected by the barrel

Formation of the Fluorophore
Involving cyclization of Gly67 Tyr66 Ser65 by autocatalysis requiring oxygen
Imaging Beyond Simple Localization

• Spatial
Detecting conformation and molecular interactions – FRET

• Temporal
Detecting random molecular movements – FCS, FRAP

• Spatial-Temporal
Detecting structural transport and assembly – FSM, STICS

• New Modalities
Traction forces
Rate Constant of FRET

\[ K_T = \left(\frac{1}{\tau_D}\right) \cdot \left[\frac{R_0}{r}\right]^6 \]

\[ R_0 = 2.11 \times 10^{-2} \cdot \left[\kappa^2 \cdot J(\lambda) \cdot \eta^{-4} \cdot Q_D\right]^{1/6} \]

\[ J = \text{spectral overlap integral} \]

\[ \kappa^2 = (\cos \theta_T - 3 \cos \theta_D \cos \theta_A)^2 = (\sin \theta_D \sin \theta_A \cos \phi - 2 \cos \theta_D \cos \theta_A)^2 \]

\[ Q_D = \text{donor QE} \]

\[ \eta = \text{medium refractive index} \]
Effects of FRET on the Emission Spectrum

Donor Quench

Acceptor Sensitized fluorescence

[Graph showing fluorescence intensity vs. wavelength and relative intensities]
FRET Biosensor for the Small GTPase Activity
Measuring Protein Mobility

Fluorescence Recovery after Photobleaching (FRAP) and
Fluorescence Loss in Photobleaching (FLIP)

FRAP

FLIP

Methods 37:154 (2005)
Fluorescence Correlation Spectroscopy for Measuring Protein Mobility and Association

\[ G(\tau) = \frac{N(t)N(t+\tau)}{N(t)^2} - 1 \]  \hspace{1cm} \text{Eq.1}

\[ G(0) = \frac{\text{Var}(F(t))}{(\text{Avg}(F(t))^2) = \frac{1}{N} \]  \hspace{1cm} \text{Eq.2}
Fluorescent Speckle Microscopy

Dimer Pool

Dual-wavelength FSM of microtubules (green) and actin (red):
Microtubules are coupled to actin movements
32 x 48 um
38 min elapsed time
Applications of Photoactivated Fluorescence

Dynamics

Pulse-Chase

Lineage

5-carboxymethoxy-2-nitrobenzyl caging group

UV

fluorescein

Caged fluorescein (Molecular Probes)
Photoconversion of GFP – Fluorescence Induction

A “neutral” and “anionic” structures are depicted, showing the conversion of CO₂ at ~400 nm.

B-C: Normalized absorbance and fluorescence spectra for PA-GFP and WEGFP pre- and post-activation.

D: Comparison of normalized absorbance and fluorescence spectra between PA-GFP and WEGFP.
Photoconversion – Switching Color and Intensity

Figure 4: Photoconversion Spectral Profiles and Images of PS-CFP
- Non-Converted PS-CFP Absorption and Emission
- Photoconverted PS-CFP Absorption and Emission

Tracking PS-CFP Labeled Cells
(a) (b) (c) (d)

Reversible Photoswitching of Dronpa Fluorescent Protein

Figure 9:
(a) Photoswitching
- Excitation Wavelength: 488 nm, 405 nm
- Image Capture Points
- Fluorescence Intensity vs. Time (Seconds)

(b) Time (Seconds)
- Fluorescence Intensity
Overcoming The Resolution Limit

- **Optical**
  - Confocal Optics – Spinning disk, 1P, 2P
  - TIRF Optics

- **Computational**
  - Deconvolution

- **Combined Approaches**
  - Air disk positioning, PALM/STORM
  - Structured Illumination Microscopy
Illumination Intensity

\[ I(z) = I(o)e^{-z/d} \]

Where

\[ d = \frac{\lambda}{4 \pi} \left[ n(1)^2 \sin^2 \theta^2 - n(2)^2 \right]^{-1/2} \]
Molecular Imaging with TIRF

S/N = 35

S/N = 1.3

(Ron Vale Lab)
Correlation
Mathematical Approach to Match Patterns

Intensity Array: 2 4 6 8 6 4 2

Template 1: 1 2 3 4 3 2 1
Template 2: 1 1 2 2 3 3 4

Score against Template 1
2*1 + 4*2 + 6*3 + 8*4 + 6*3 + 4*2 + 2*1 = 88

Score against Template 2
2*1 + 4*1 + 6*2 + 8*2 + 6*3 + 4*3 + 2*4 = 72

Higher score, better match
Correlation-Based Feature Detection
Super-resolution of Fiber Structures

Raw image

Detect line segments with line kernels along 8 directions

\[ K^0 = \begin{bmatrix} 1 & 2 & 4 & 2 & 1 \\ 1 & 2 & 4 & 2 & 1 \\ 1 & 2 & 4 & 2 & 1 \\ 1 & 2 & 4 & 2 & 1 \\ 1 & 2 & 4 & 2 & 1 \end{bmatrix} \text{ etc} \]

Threshold images

Add images in all orientations
Flux of Actin Filaments Toward the Equator

![Image of actin filaments with a kymograph showing movement over time.](image.png)
Spatial Temporal Correlation Microscopy
Detecting Structure Flow
Spatiotemporal Image Correlation Analysis of Sub-Equatorial Fluxes of Actin Filaments

Mian Zhou
Microscopy of Traction Forces

Bead displacement

Vectors of bead displacement
Traction Force Microscopy

Vectorial plot

Color rendering
Key Areas for Development

• Probes & Sensors – “brighter” probes, new parameters

• Biological Context – intravital imaging, physiological mimetics

• Informatics – automation, high-throughput imaging, information extraction, modeling
Tangled Linear Polymers as Intracellular Pressure Sensors
Myosin II Dependent Cytoplasmic Forces

Max asp ratio = 7 (along cell axis)
Max speed = $8.5 \times 10^{-2}$ micron/sec

Blebbistatin (myosin II inhibitor)    T. Iwasaki
Cell Migration in Collagen Gels
Lagging Cell Body When Tracking Collagen Fibers
Cell Migration along Adhesive Strips
Stable Front and Contractile Rear
Dynamics of Myosin II during Early Cytokinesis
Temporal Differential Microscopy

Detecting Normalized Rate of Intensity Changes

$$D_i = \frac{(I_{t+dt} - I_t)}{(I_{t+dt} + I_t)}$$
Discrete, Transient Domains of Myosin Assembly

Metaphase

Early Cytokinesis

TIRF

TDM
Suppression of Myosin Disassembly along the Equator
Separating Information from Noise

Multiresolution decomposition into Signal and Noise

Noise matching characteristics?

- no
- yes

Done

Original  Signal  Noise
From “Seeing” to “Understanding”

• What Biologists Can See with Light Microscopy -
  • Spatial and temporal details of chemical and physical properties encoded by optical sensors

• What Biologists Need for Understanding -
  • The rules of interactions that lead to biological functions

• Images Represent Manifestations or Consequences of Functions, Not the Function Itself

• Images Represent Local Information Presented in Tandem, Functions Often Require the Integration Across Space and Time
Understanding by Modeling

Circuit parameters?

Equations of Functional Circuit

Measurable Characteristics

Mathematical Optimization

Live Cell Imaging

Measurable Characteristics

Manual Match

Simulated Cell

Molecular parameters

Equations of Molecular Interactions

Measurable Characteristics

Manual Match

Simulated Cell

no match

match
Modeling Microscope Images
Based on Biological Mechanisms and Instrumental Characteristics

Experimental images of microtubules in yeast spindle

Simulated images

Concluding Remarks
Learning from the Recent History of Light Microscopy

• Breakthroughs often come from merging physical, biological, chemical, and engineering approaches.
• Much untapped potential exists in other disciplines, waiting to be imported for fluorescence microscopy.
• Simple schemes have the greatest potential for the widest, fastest dissemination and impact.