Fluorescence Imaging of Live Cells

Yu-li Wang

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

Useful Web Sites

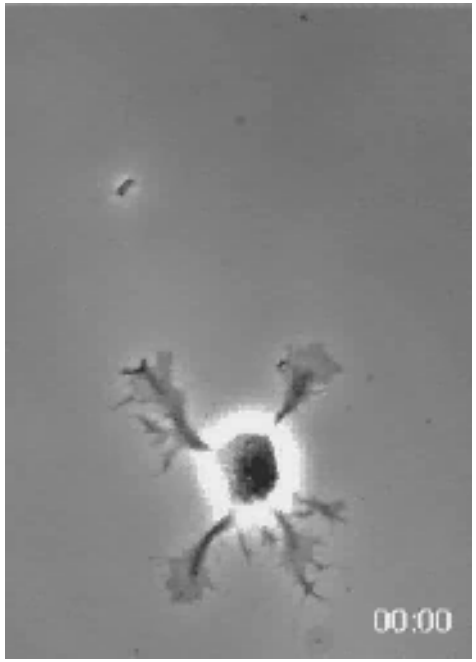
- http://probes.invitrogen.com/handbook (basic fluorescence and probes)
- http://www.microscopyu.com/articles/fluorescence (microscopy)
- http://www.olympusmicro.com/primer/techniques/fluorescence/fluorhome.html (microscopy)
- http://www.chroma.com/resources/handbook.php (filters)
- http://www.clontech.com/support/brochures.asp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

Books

- Imaging in Neuroscience and Development, A Laboratory Manual (R. Yuste, F. Lanni, A. Konnerth eds.) Cold Spring Harbor Lab Press, (2005).
- Live Cell Imaging, A Laboratory Manual (R.D. Goldman, D.L. Spector, eds) Cold Spring Harbor Lab Press, (2005).
- Methods in Enzymology Vol. 360-361, Biophotonics (Gerard Marriott and Ian Parker, eds) Academic Press, (2003).

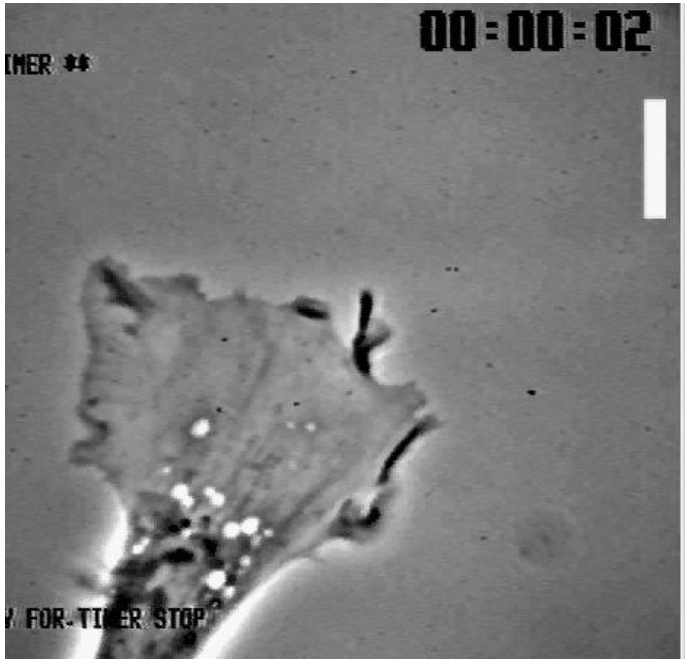
Searching for the Simplest Model

to Account for Diverse Forms of Cell Shape and Migration



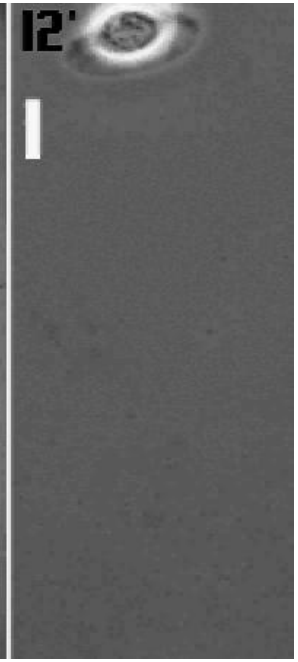
Neuron (16.5 hours)

Gary Banker



Fibroblast (1 hour)

Vic Small



Keratocyte (12 min)

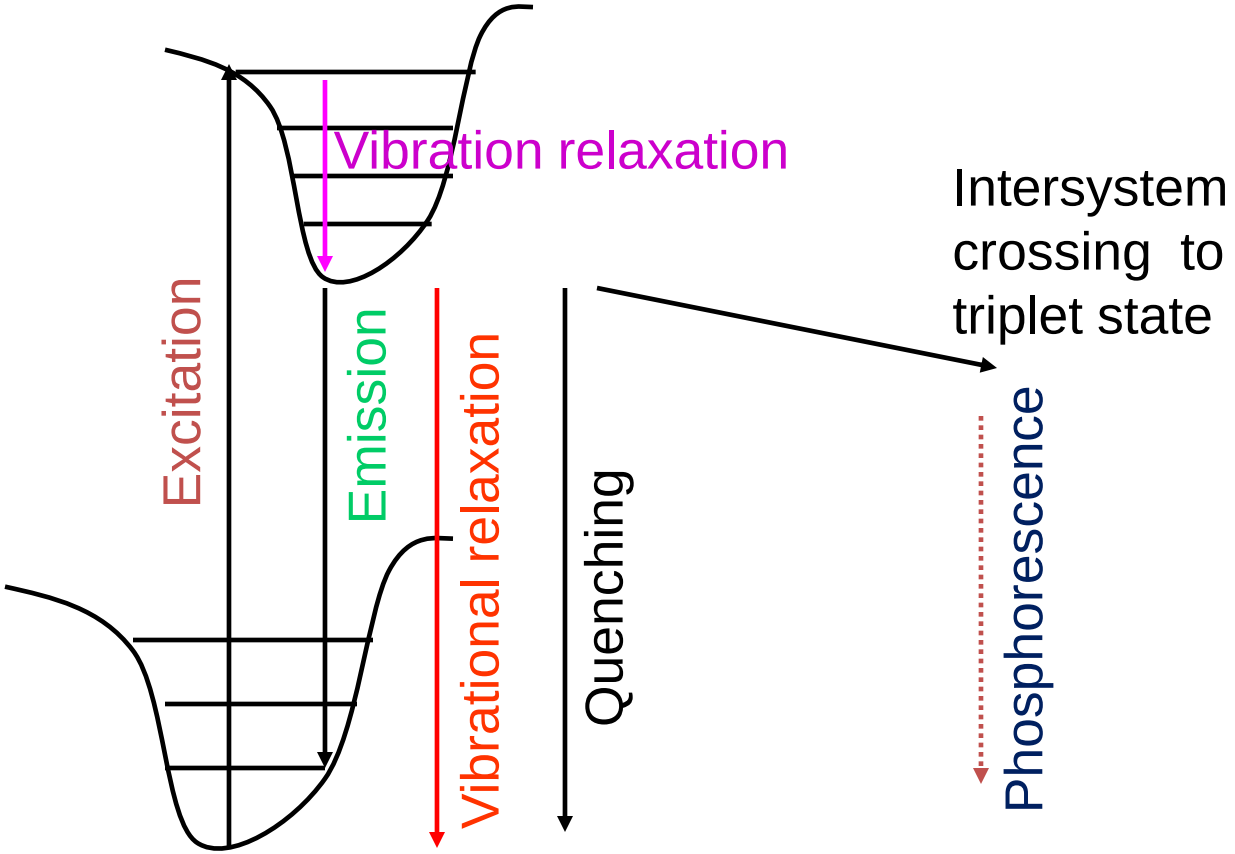
Kurt Anderson

Fluorescence Microscopy

- Started at the turn of the 20th 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

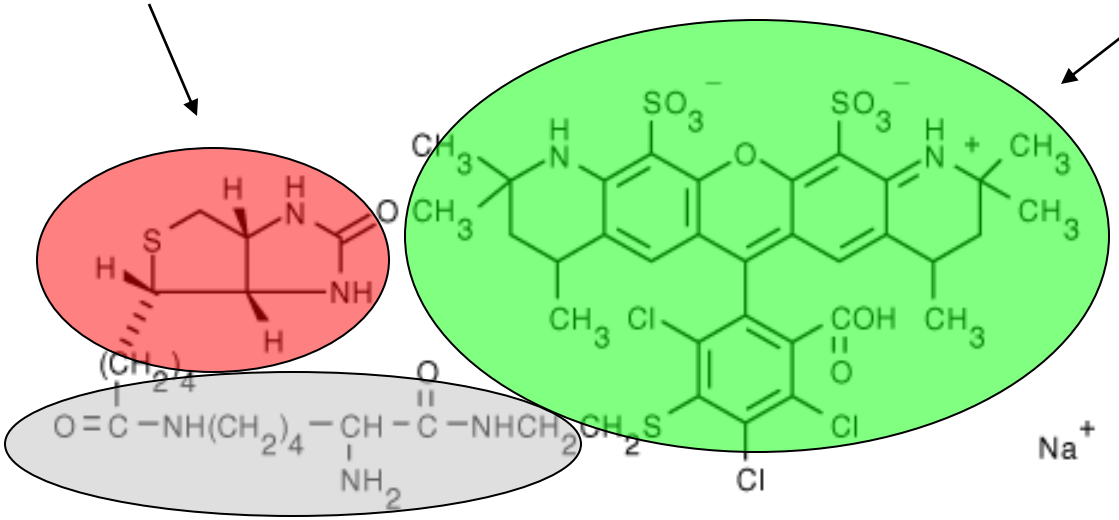
Emission of photons as a molecule returns from singlet excitation state to ground state



Anatomy of Organic Fluorescent Probes

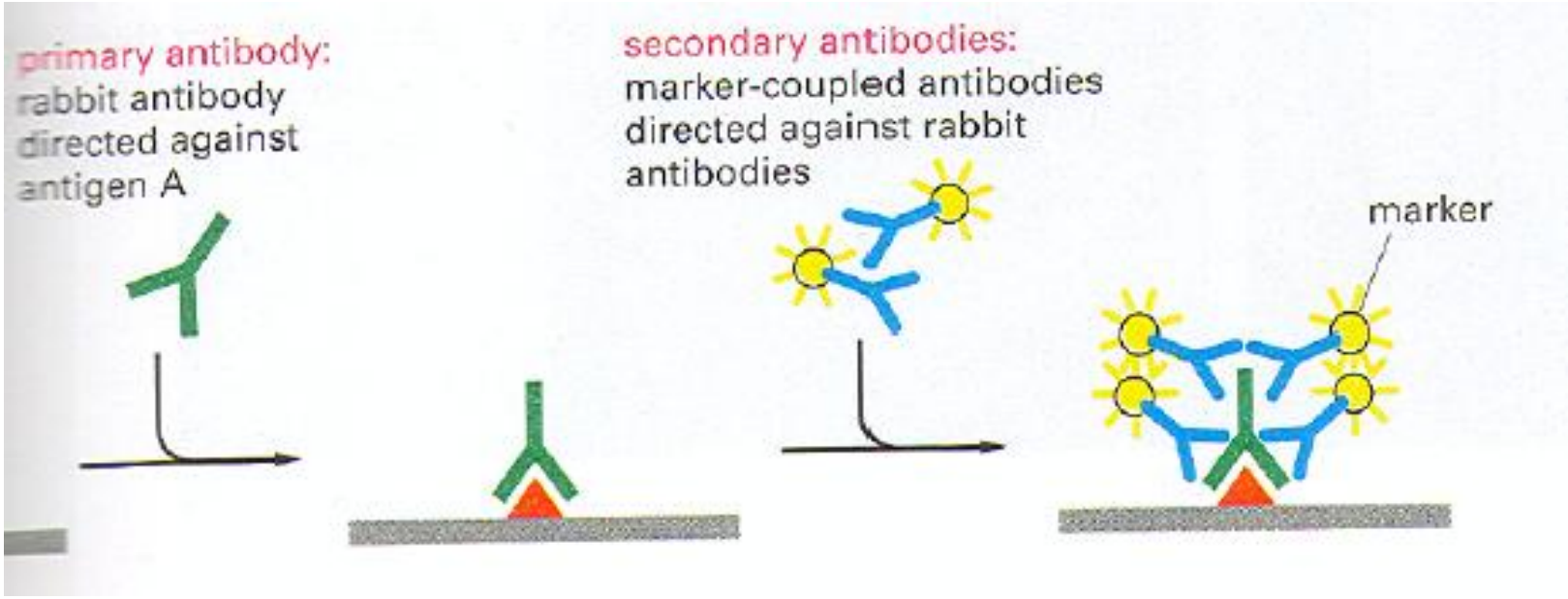
Reactive/binding moiety (biotin)

Alexa-546 fluorophore



Spacer

Immunofluorescence



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

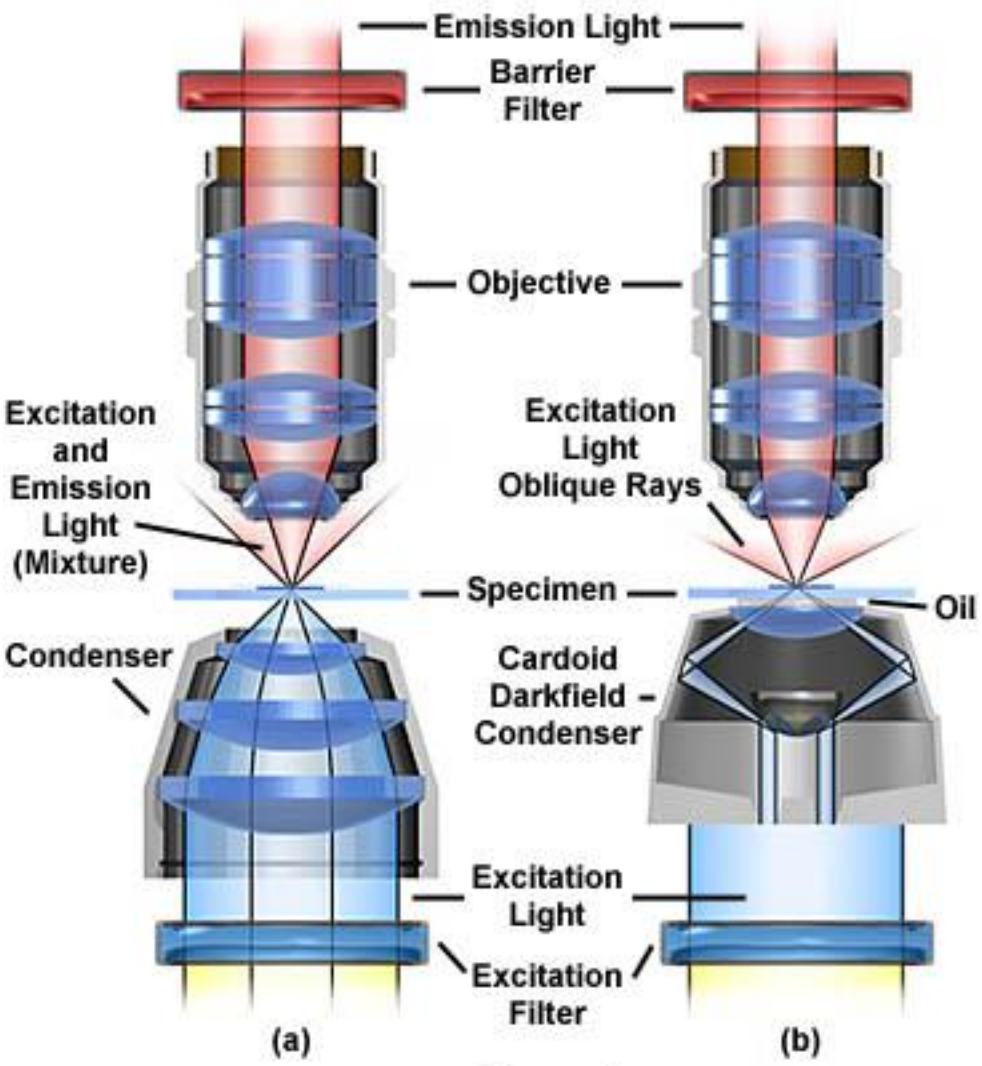


Figure 1

(bright field) (dark field)

Optical Design in Epi-Fluorescence Microscopy

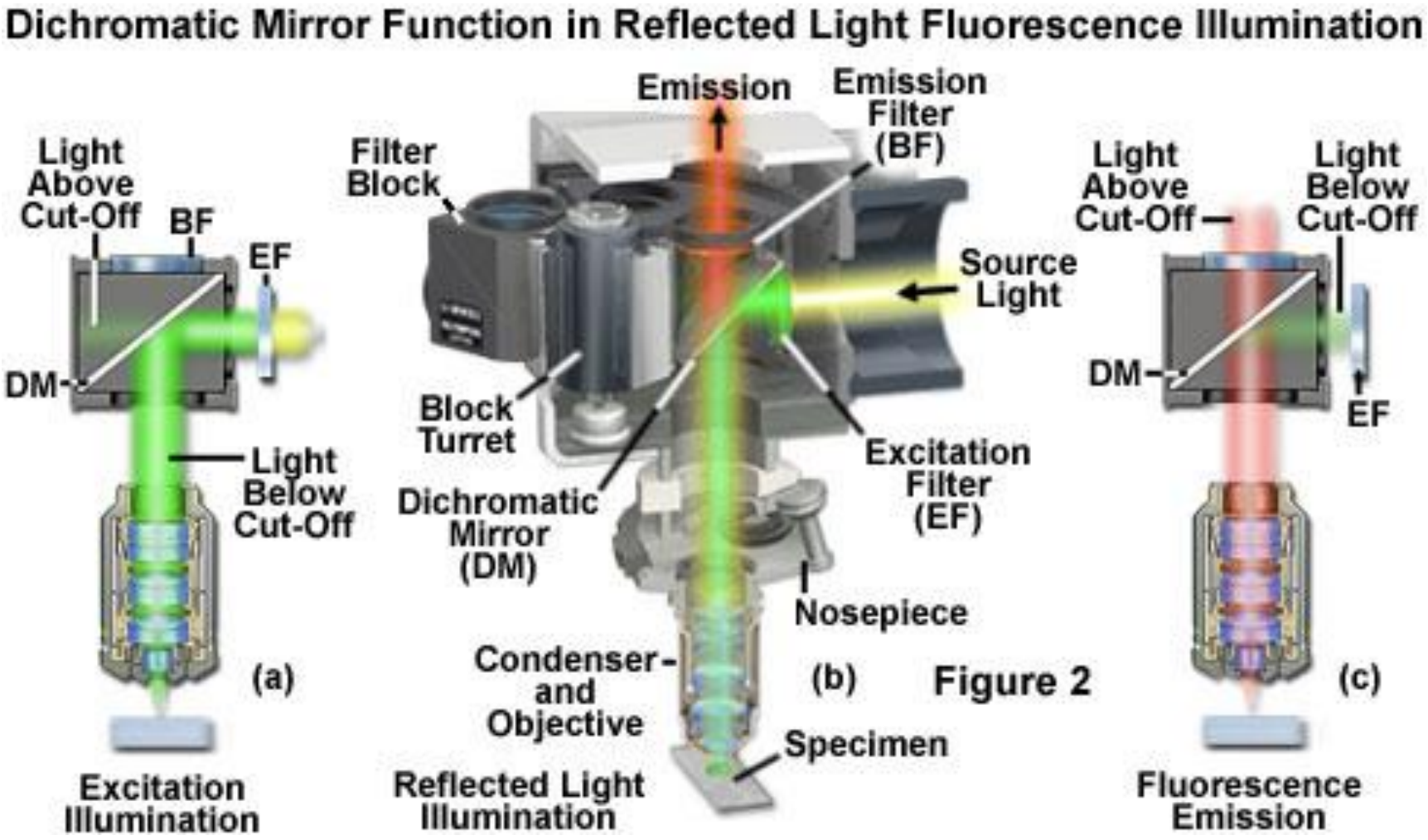


Figure 2

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

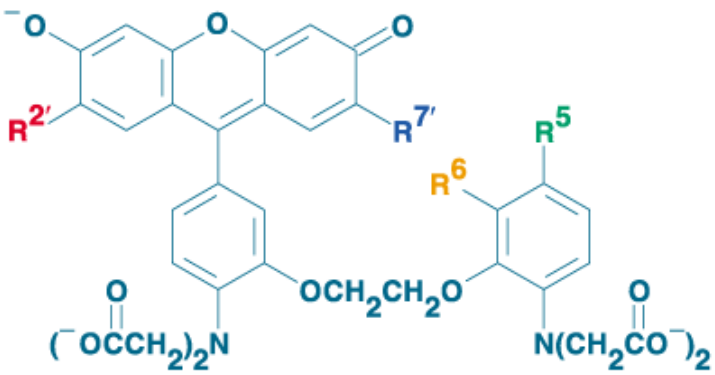
New bleaching-resistant dyes, sulfhydryl reagent, Indicator dyes, etc (chemistry)

Detectors – Photographic film

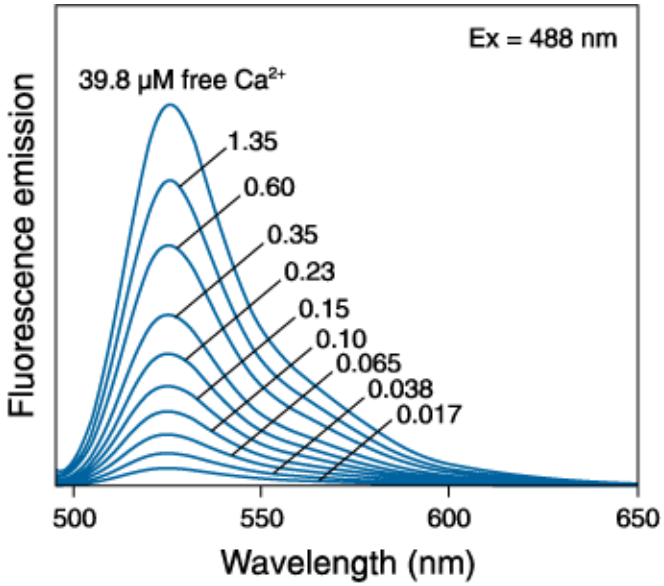
Low sensitivity, Long exposure, Tedious

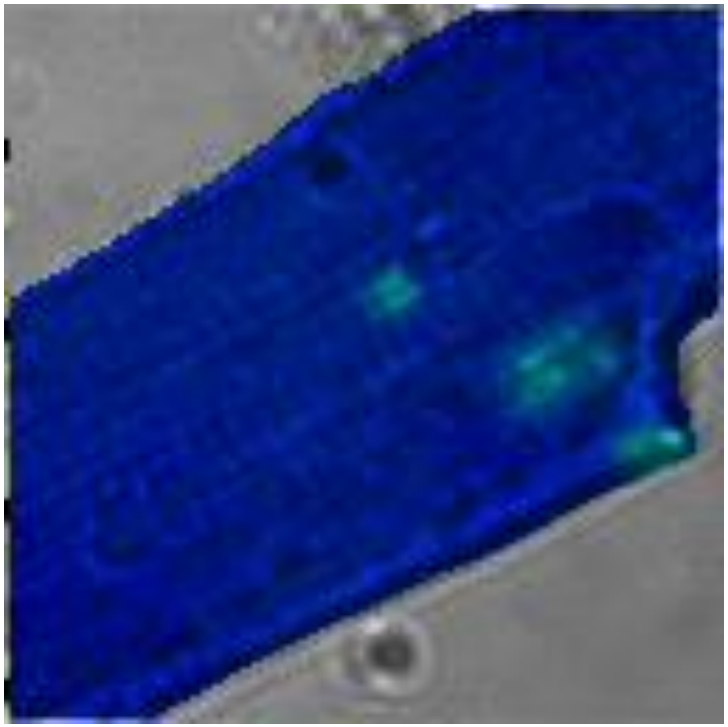
Intensified video cameras (engineering)

Fluorescent Probes of Calcium Ion



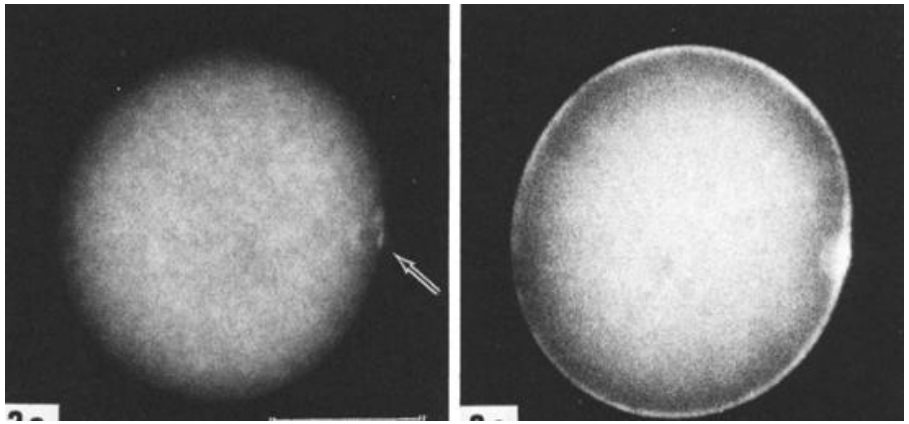
Indicator	$K_d(Ca^{2+})$	$R^{2'}$	$R^{7'}$	R^5	R^6
Fluo-3	0.39 μM	Cl	Cl	CH_3	H





Fluorescent Analog Cytochemistry

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

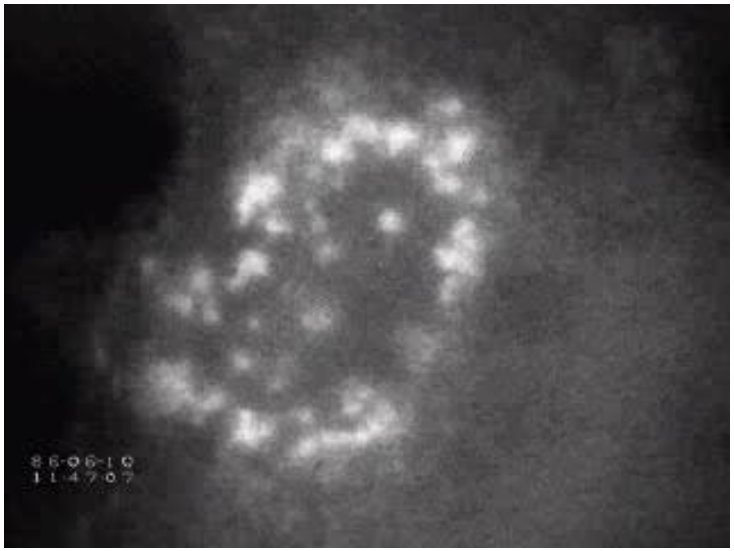


Assembly of cortical actin following fertilization of sea urchin eggs, 1979

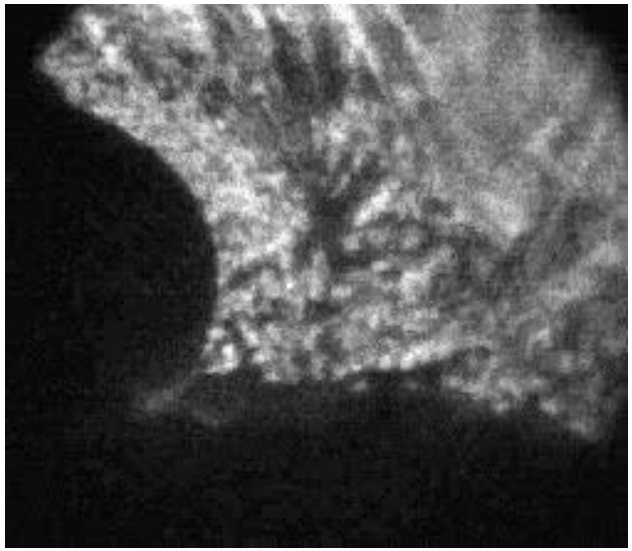
Microinjection of IAF-actin and recording with tri-X film

Applications of Fluorescent Analog Cytochemistry

Early Movies of Actin-Myosin Dynamics



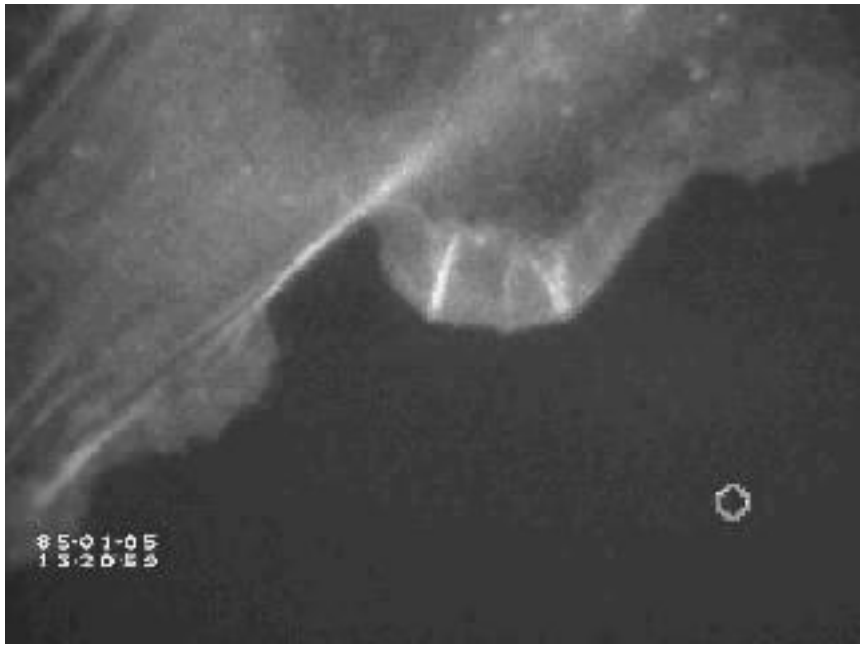
Alpha-actinin in invadopodia of transformed NRK cells, 1987



Myosin II in lamella 3T3 fibroblasts, 1989

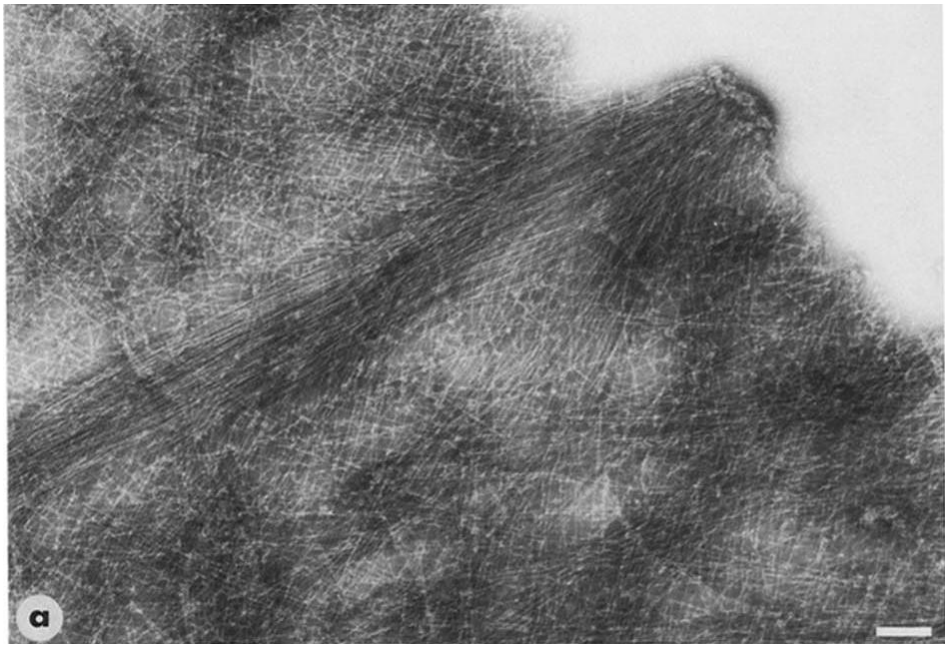
Beyond Simple Imaging

Marking Structures with Laser Beam for Visualizing Transport



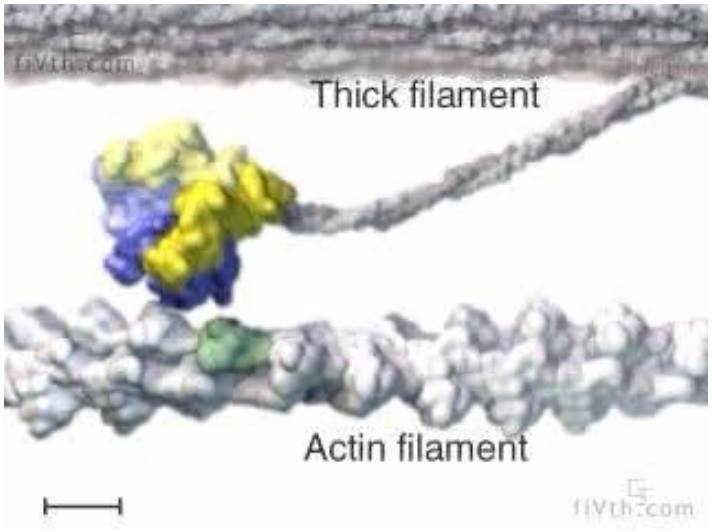
Wang, 1985

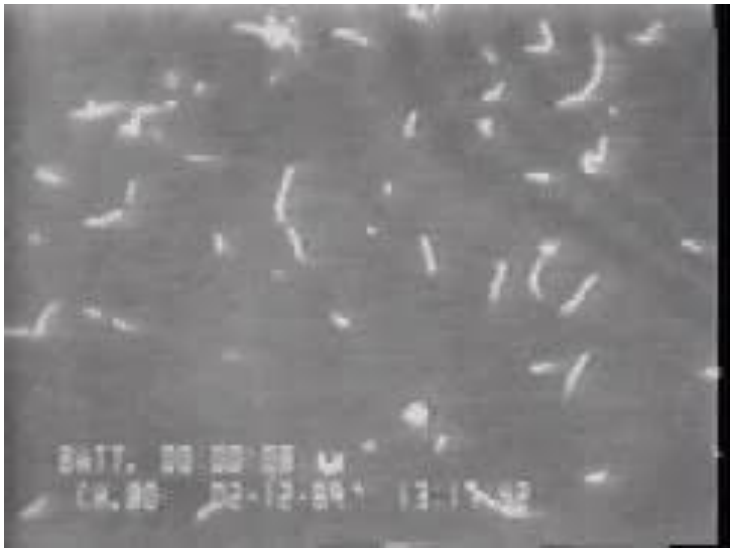
Injection of iatr-actin into IMR33 cells, detection with a Venus intensified video camera, recording with a film camera positioned in front of a TV monitor



Small, 1981

Brave New World of Fluorescence Microscopy

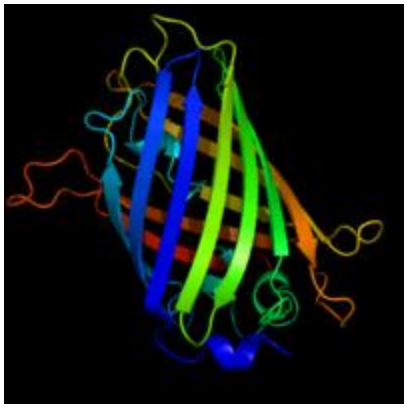




Limitations of Fluorescent Analog Cytochemistry

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

- Fluorescent Proteins



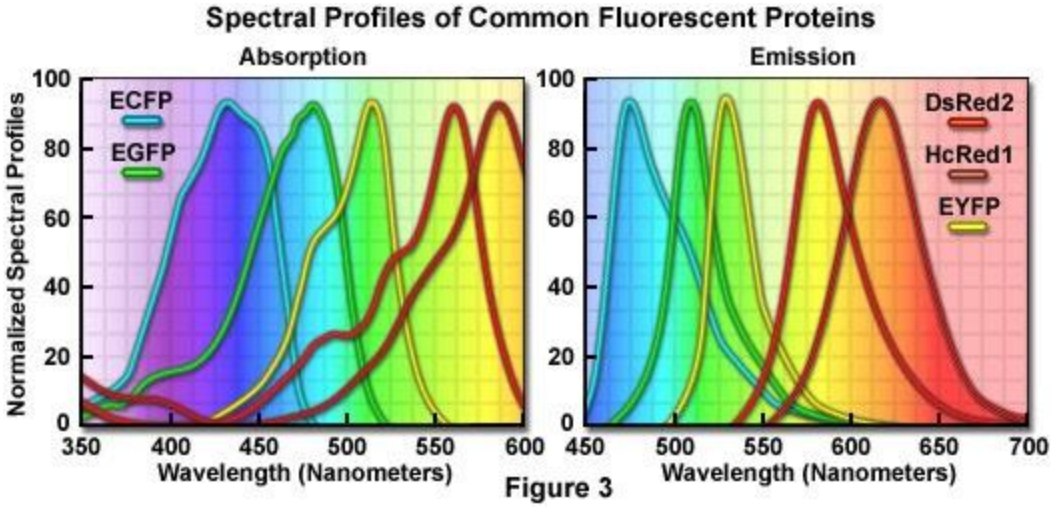
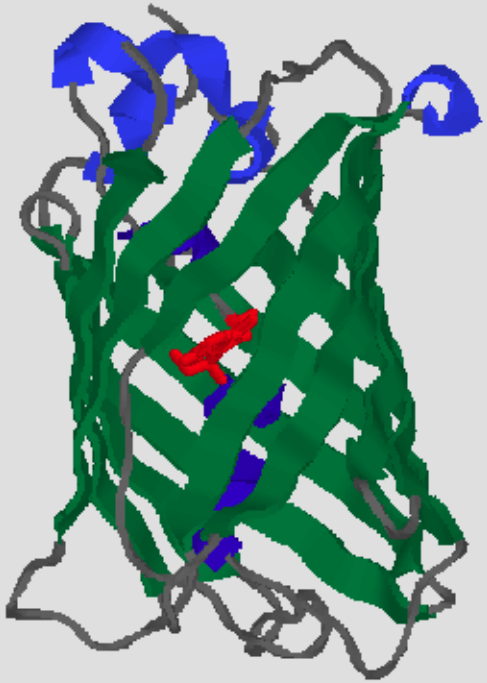


Figure 3

Natural Fluorescent Proteins



GFP

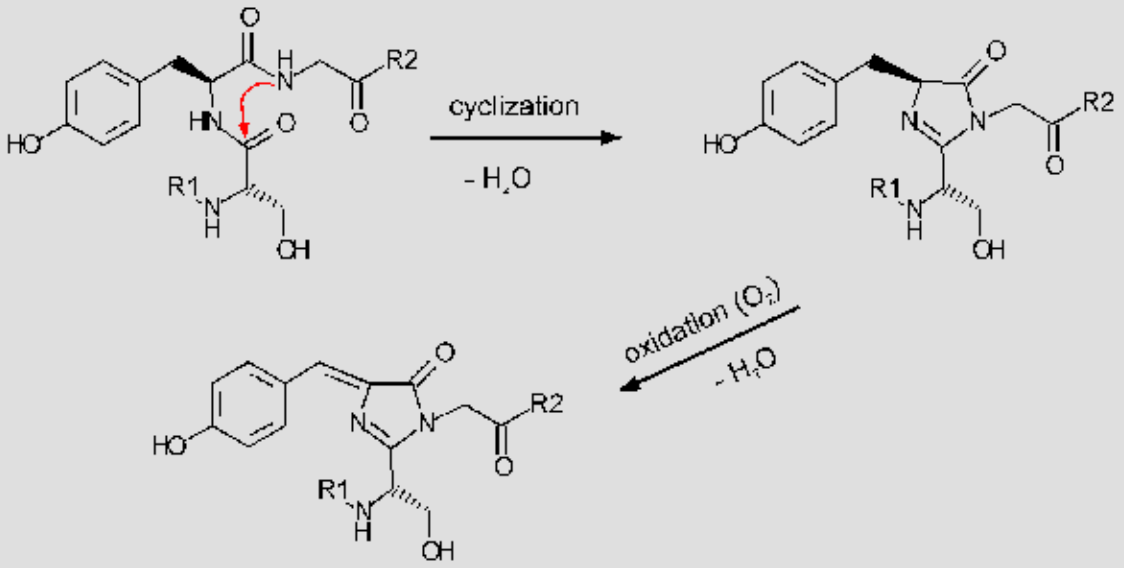
Barrel of 11 antiparallel beta sheets

27kDa

30A in diameter

40A 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

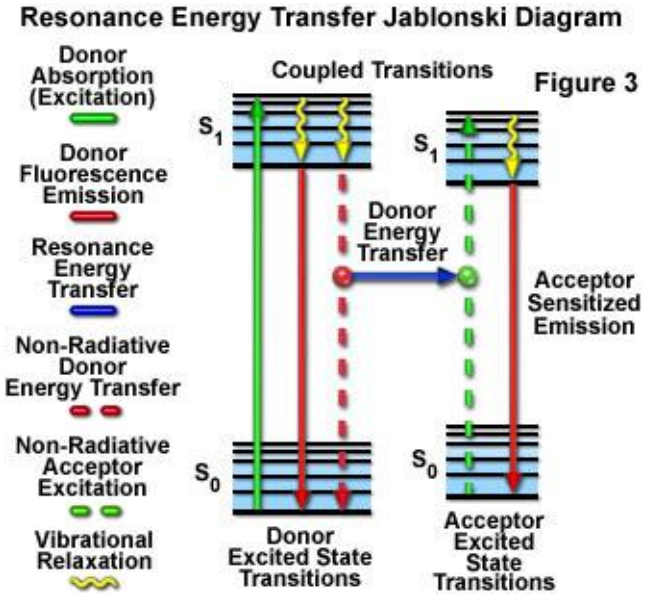
- Spatial-Temporal

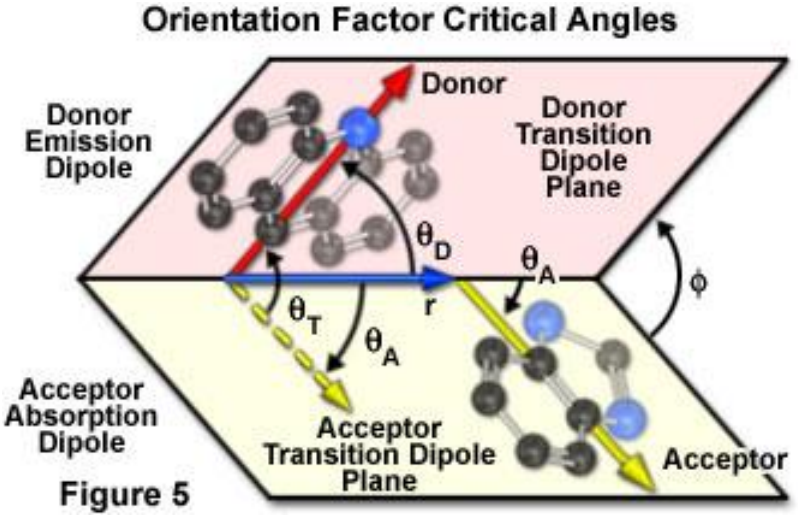
Detecting structural transport and assembly – FSM, STICS

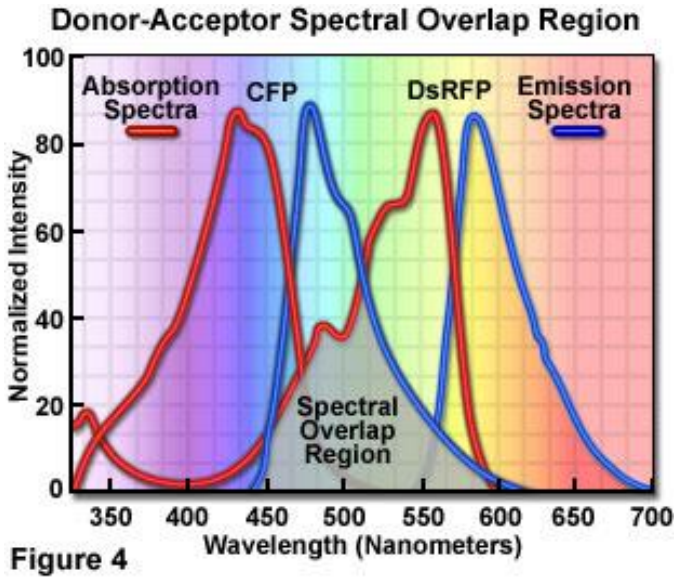
- New Modalities

Traction forces

Fluorescence Resonance Energy Transfer (FRET)







Rate Constant of FRET

$K_T = (1/\tau_D) \bullet [R_0/r]^6$

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

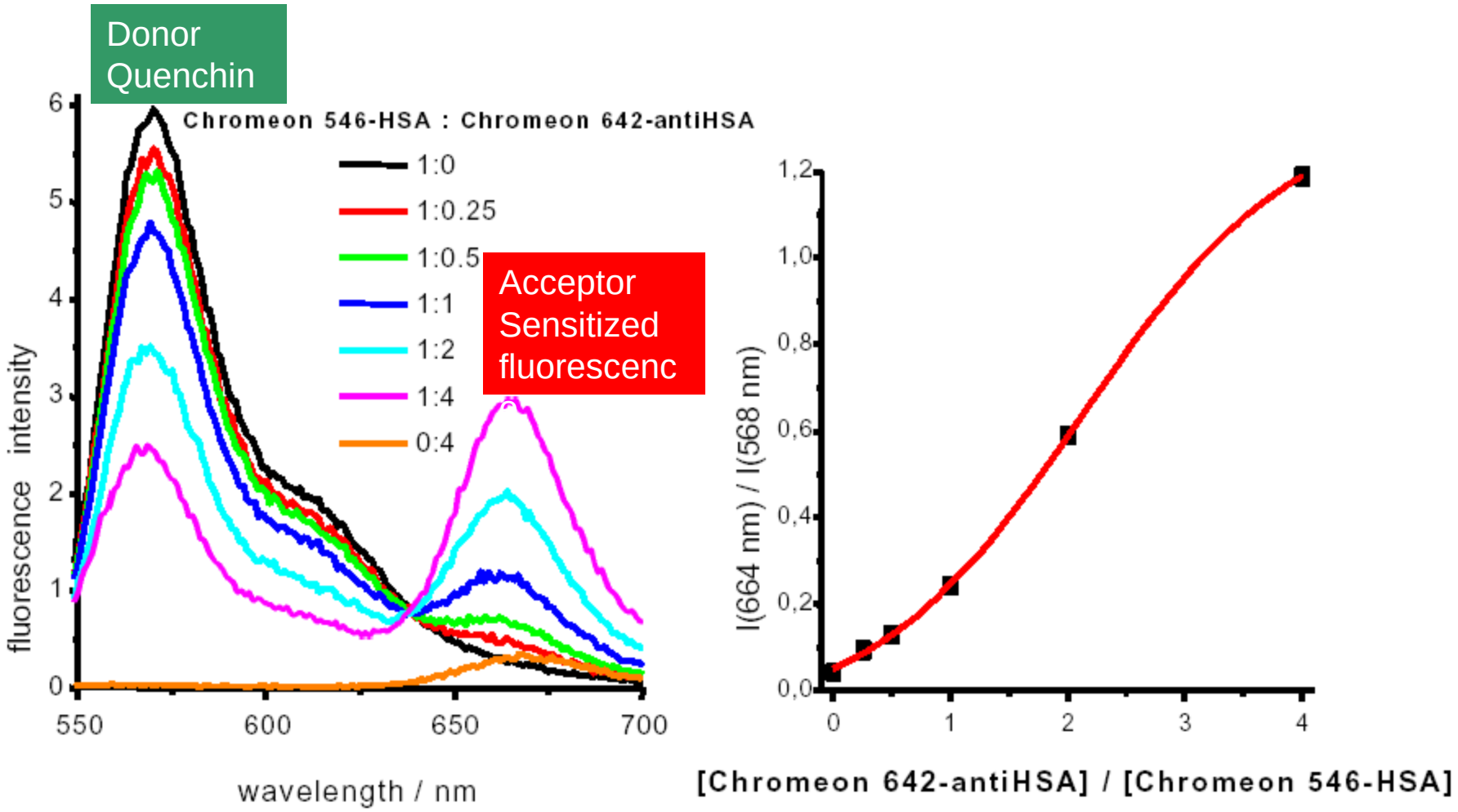
J = spectral overlap integral

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

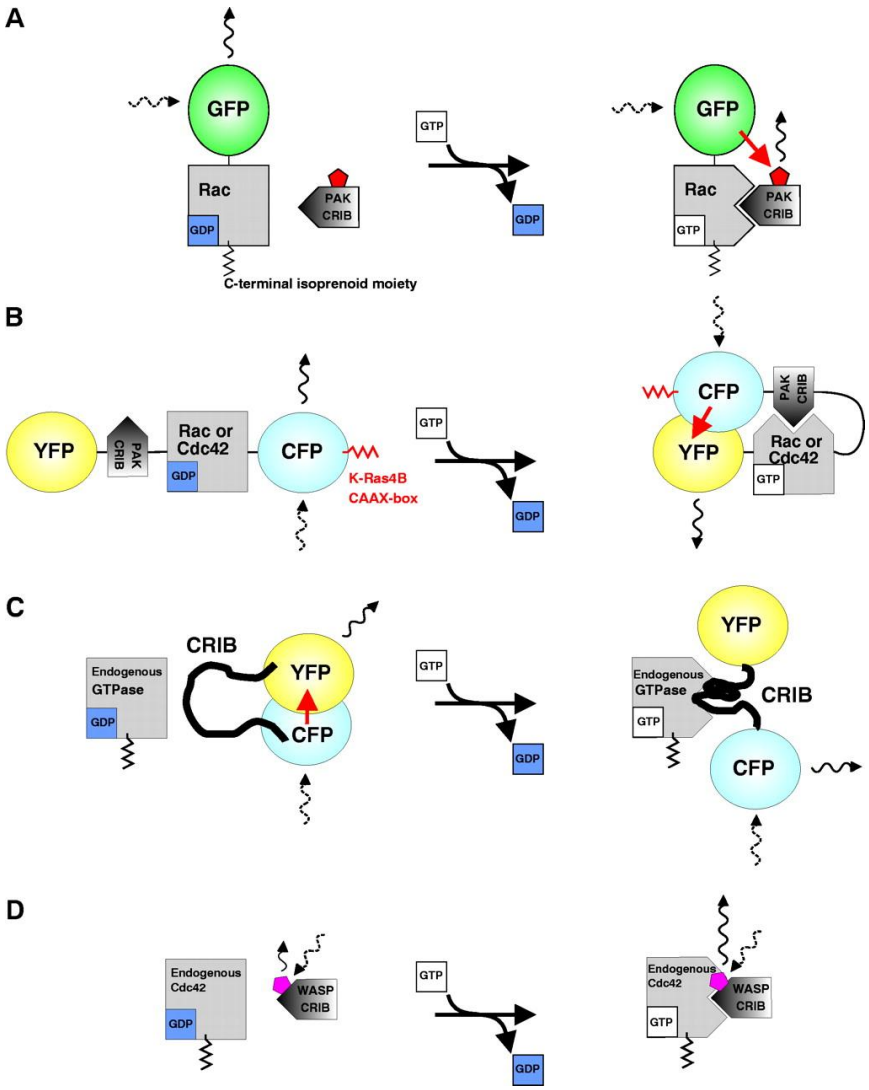
Q_D = donor QE

η = medium refractive index

Effects of FRET on the Emission Spectrum



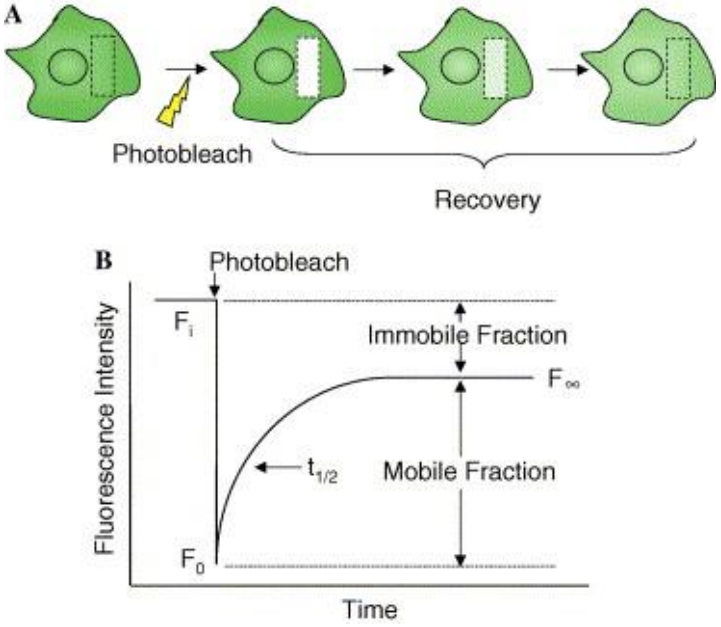
FRET Biosensor for the Small GTPase Activity



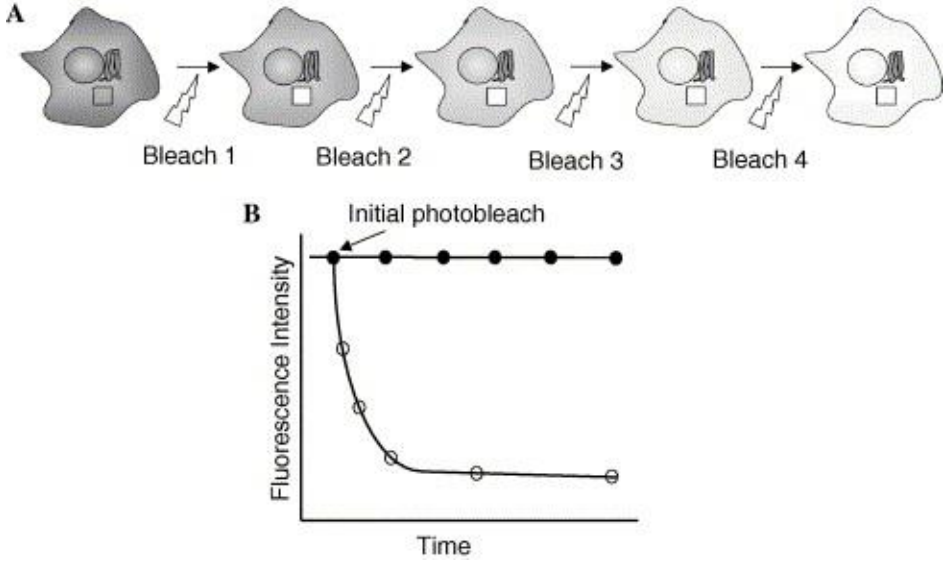
J. Cell Sci. 117:1313 (2004)

Measuring Protein Mobility

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



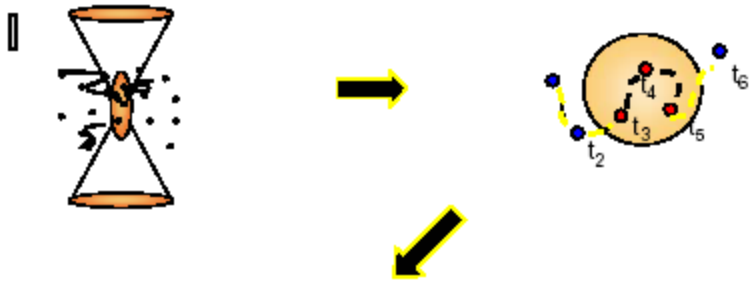
FRAP

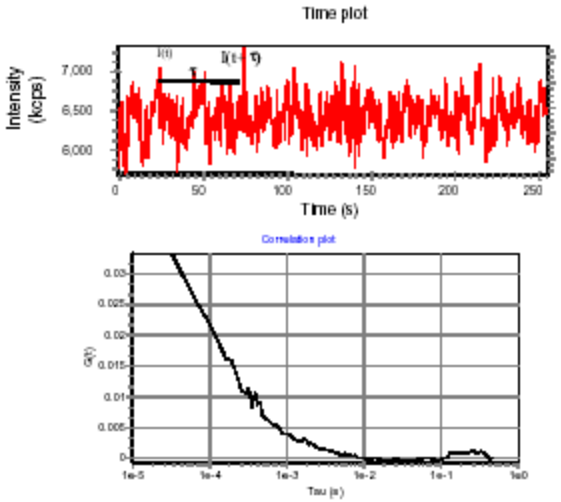


FLIP

Methods 37:154 (2005)

Fluorescence Correlation Spectroscopy for Measuring Protein Mobility and Association

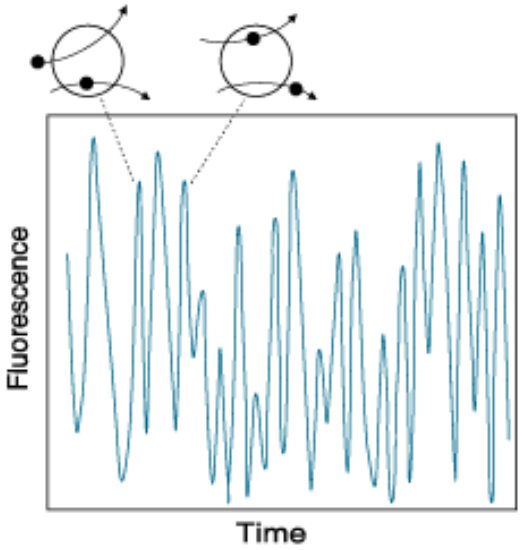


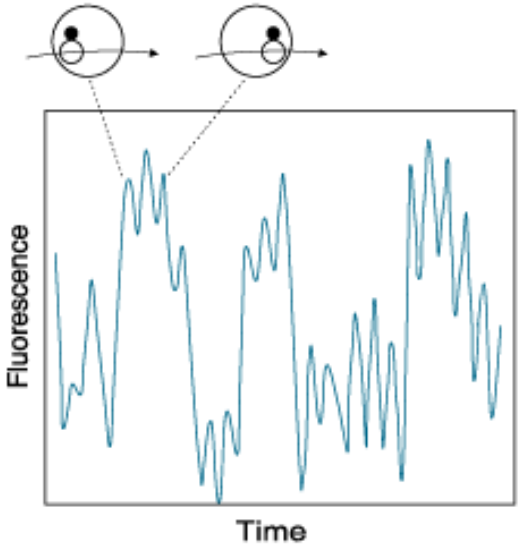


Autocorrelation Function

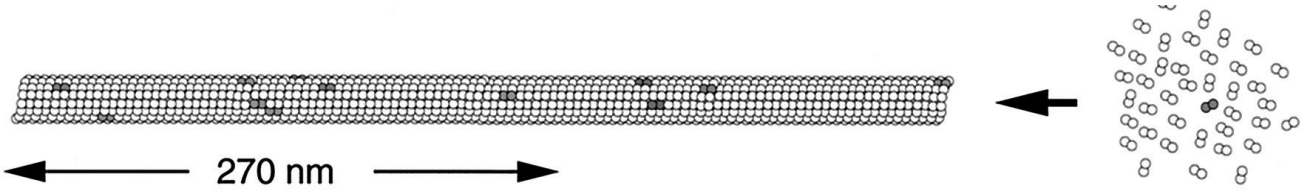
$$G_i(\tau) = \frac{N(t)N(t+\tau)}{N(t)^2} - 1 \qquad \text{Eq.1}$$

$$G(0) = \frac{Var(F(t))}{(Avg(F_i(t)))^2} = \frac{1}{N} \qquad \text{Eq.2}$$

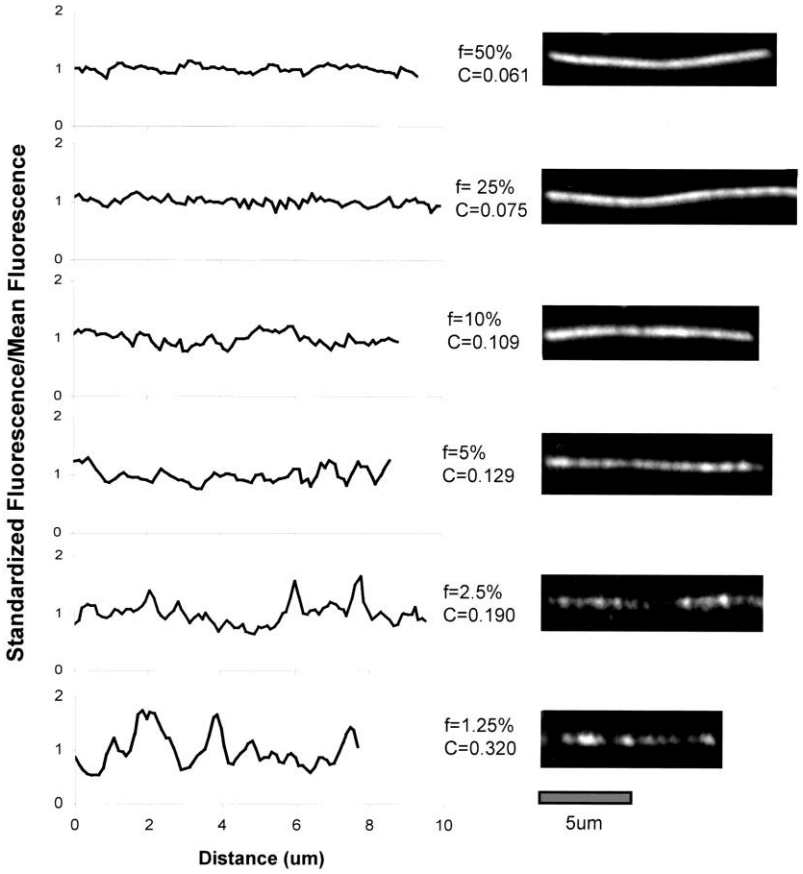




Fluorescent Speckle Microscopy



Dimer Pool



Dual-wavelength FSM of microtubules (green) and actin (red):

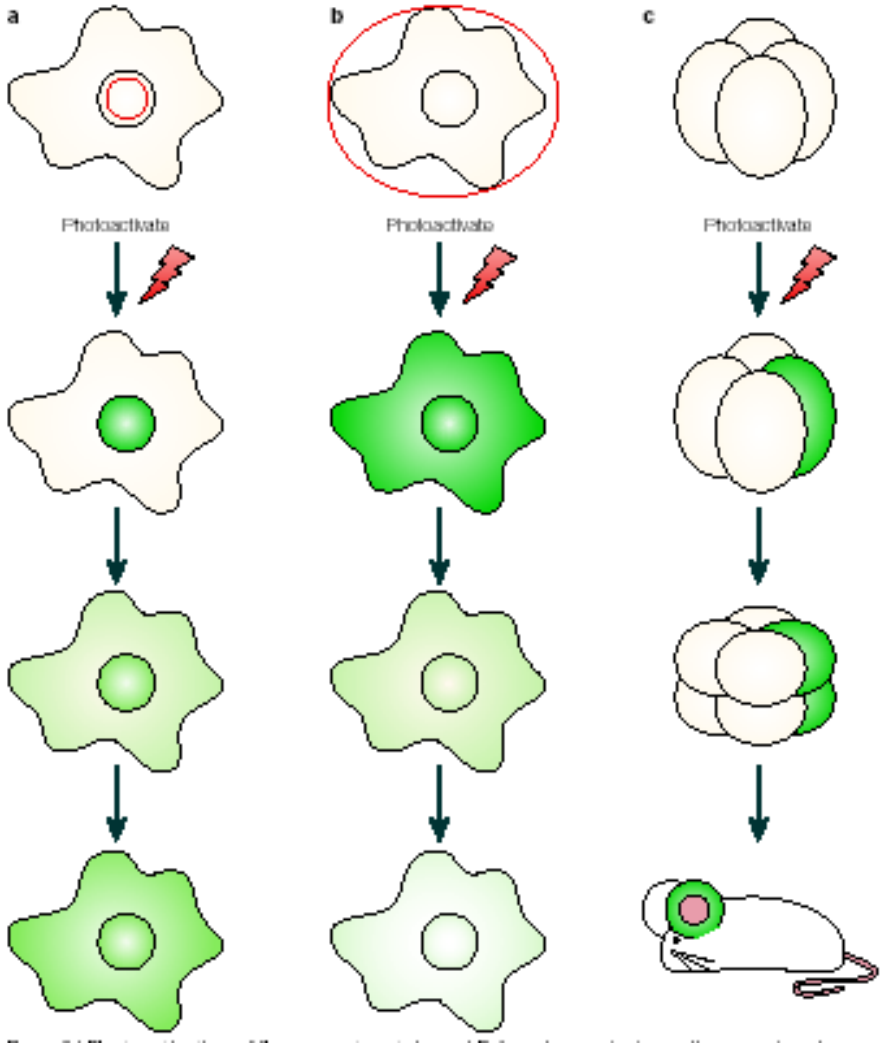
Microtubules are coupled to actin movements

32 x 48 um

38 min elapsed time

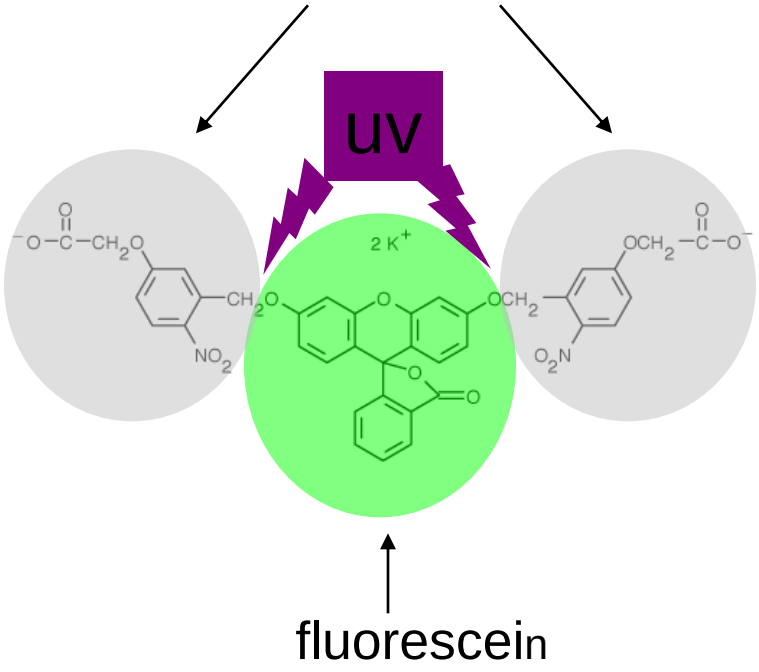
Biophys J. 75:2059-2069 (1998)

Applications of Photoactivated Fluorescence



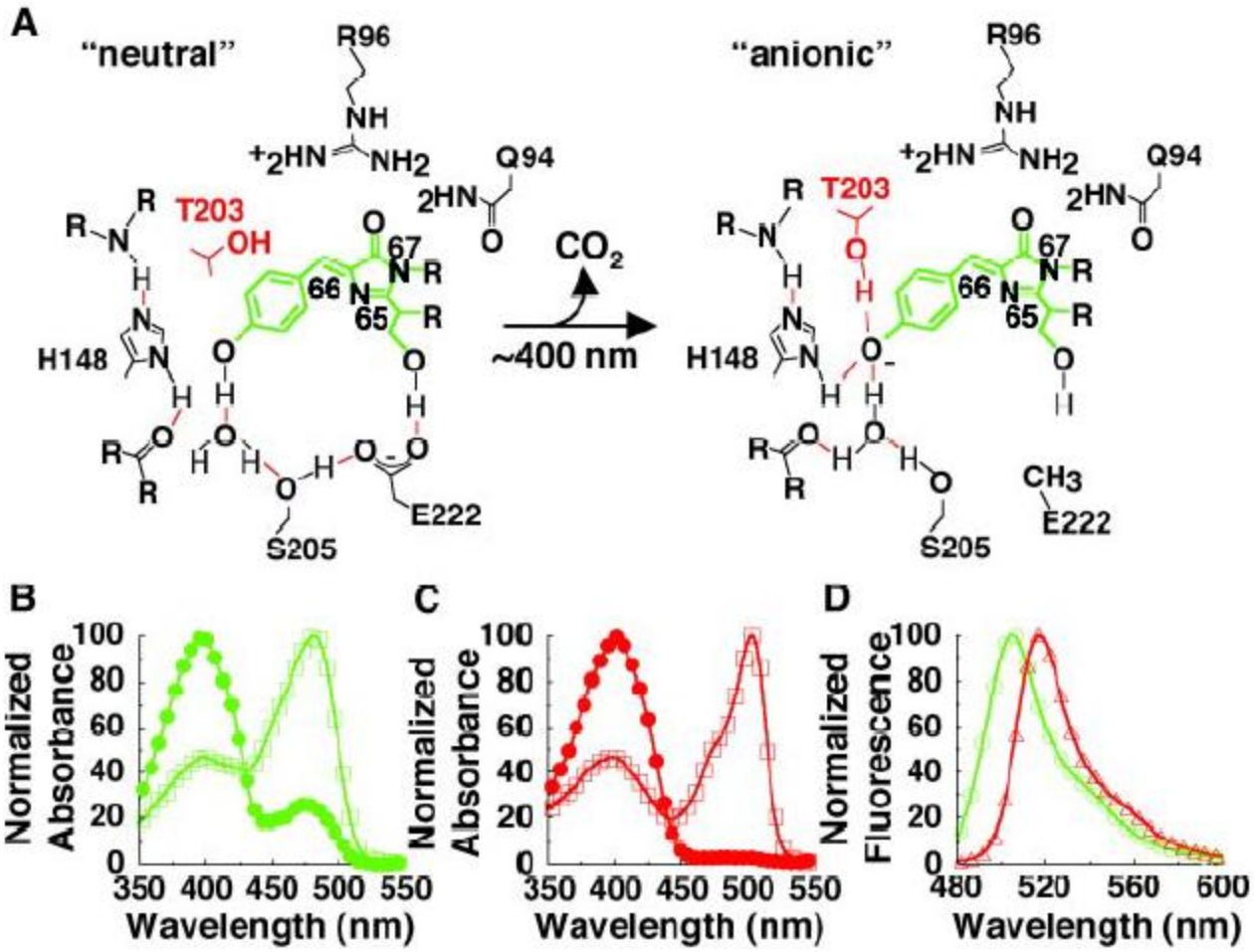
Dynamics Pulse-Chase Lineage

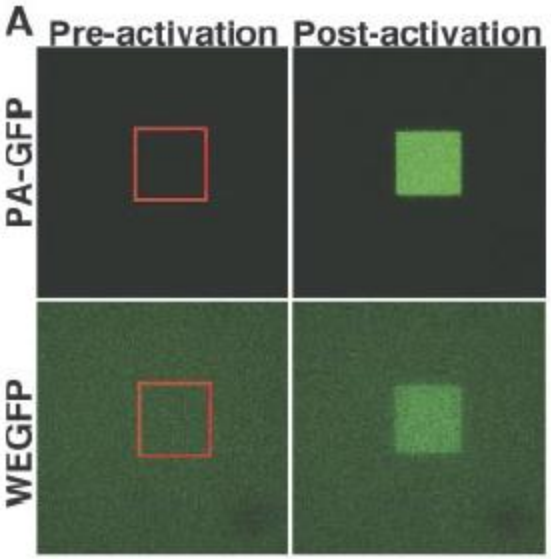
5-carboxymethoxy-2-nitrobenzyl caging group



Caged fluorescein (Molecular Probes

Photoconversion of GFP – Fluorescence Induction





Science 297:1873 (2002)

Photoconversion – Switching Color and Intensity

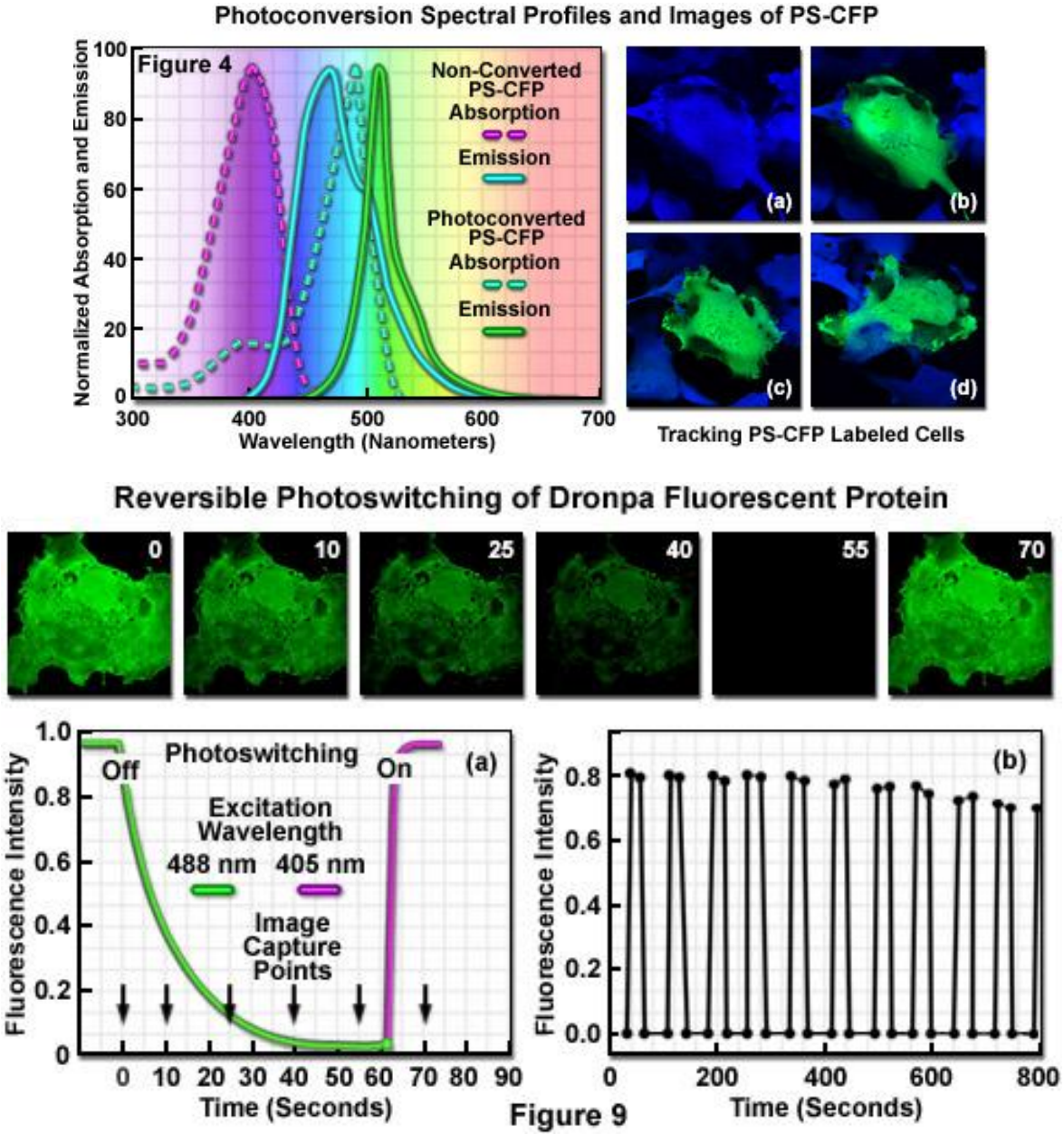
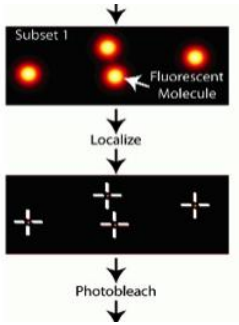


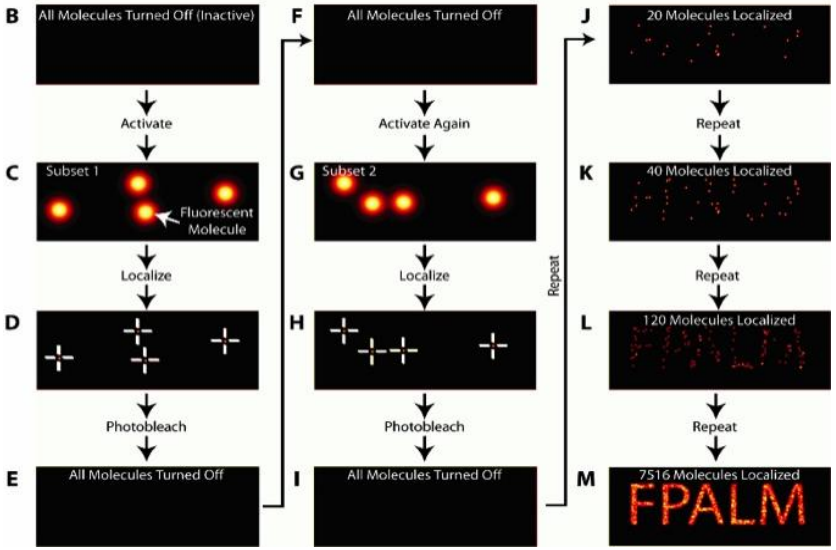
Figure 9

Overcoming The Resolution Limit

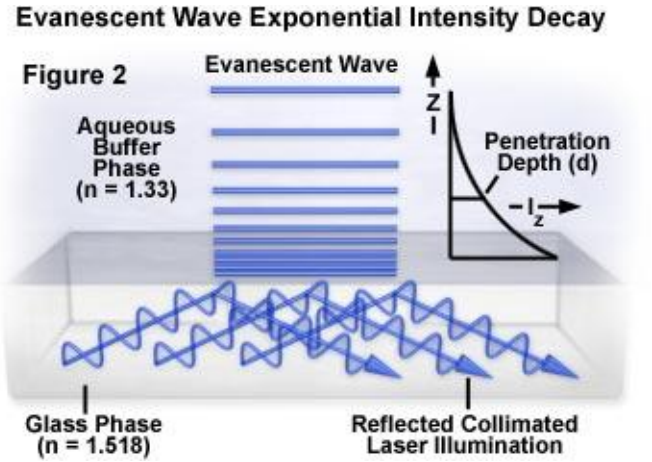
- Optical
  - Confocal Optics – Spinning disk, 1P, 2P
  - TIRF Optics
- Computational
  - Deconvolution



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



Total Internal Reflection Fluorescence Microscopy

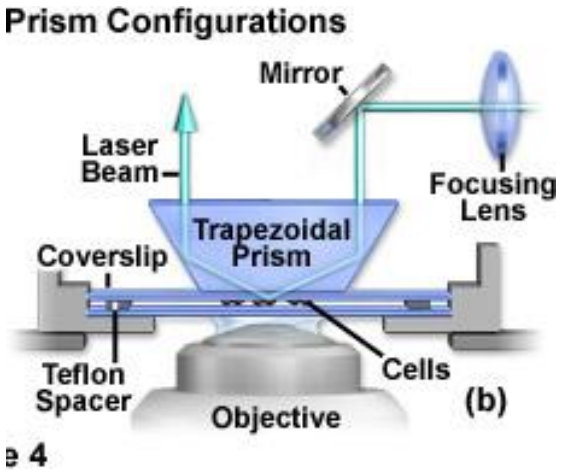


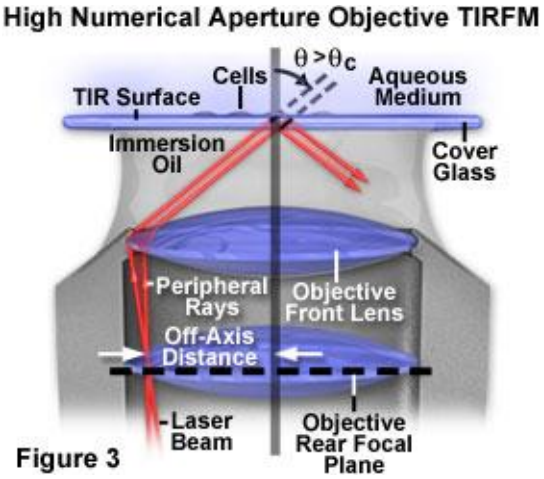
Illumination Intensity

$I(z) = I(o)e^{-z/d}$

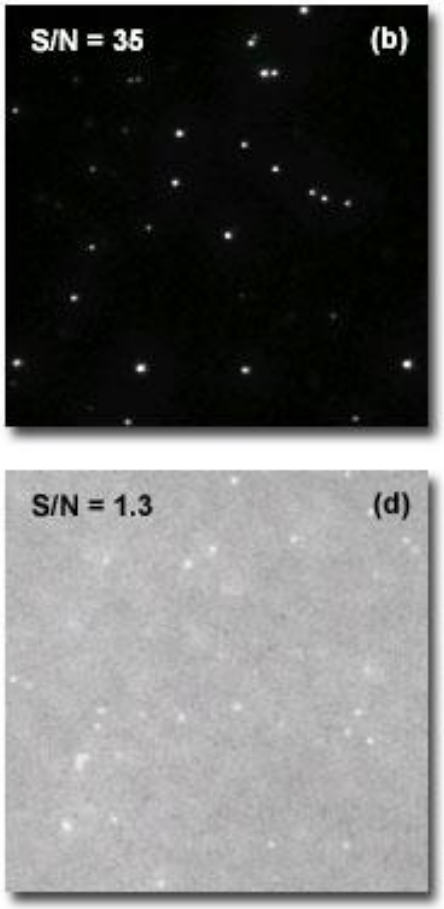
Where

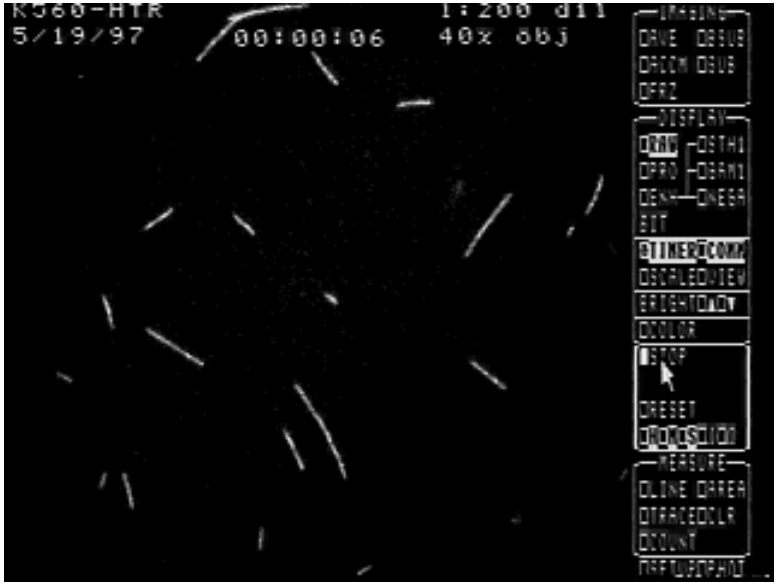
$d = \lambda / 4\pi [n(1)^2 \sin\theta^2 - n(2)^2]^{-1/2}$





Molecular Imaging with TIRF





(Ron Vale Lab)

Correlation

Mathematical Approach to Match Patterns

Intensity Array: 2 4 6 8 6 4 2

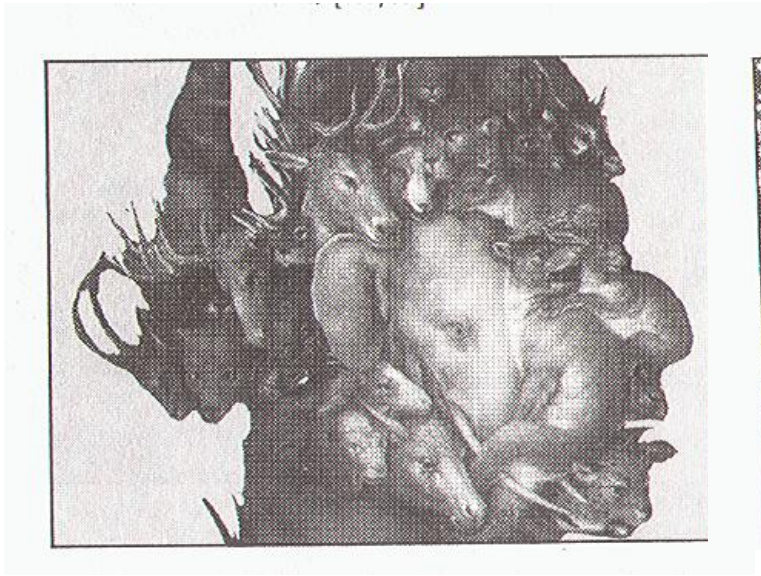
Template 1: 1 2 3 4 3 2 1
Template 2: 1 1 2 2 3 3 4

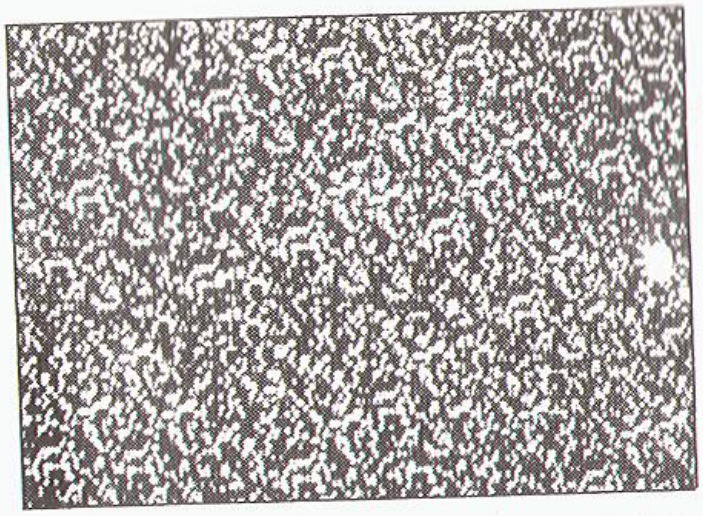
Score against Template 1
2*1 + 4*2 + 6*3 + 8*4 + 6*3 + 4*2 + 2*1 = 88

Higher score, better match

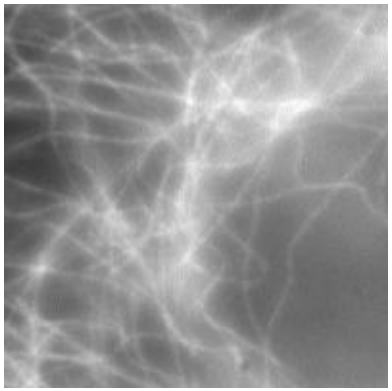
Score against Template 2
2*1 + 4*1 + 6*2 + 8*2 + 6*3 + 4*3 + 2*4 = 72

Correlation-Based Feature Detection

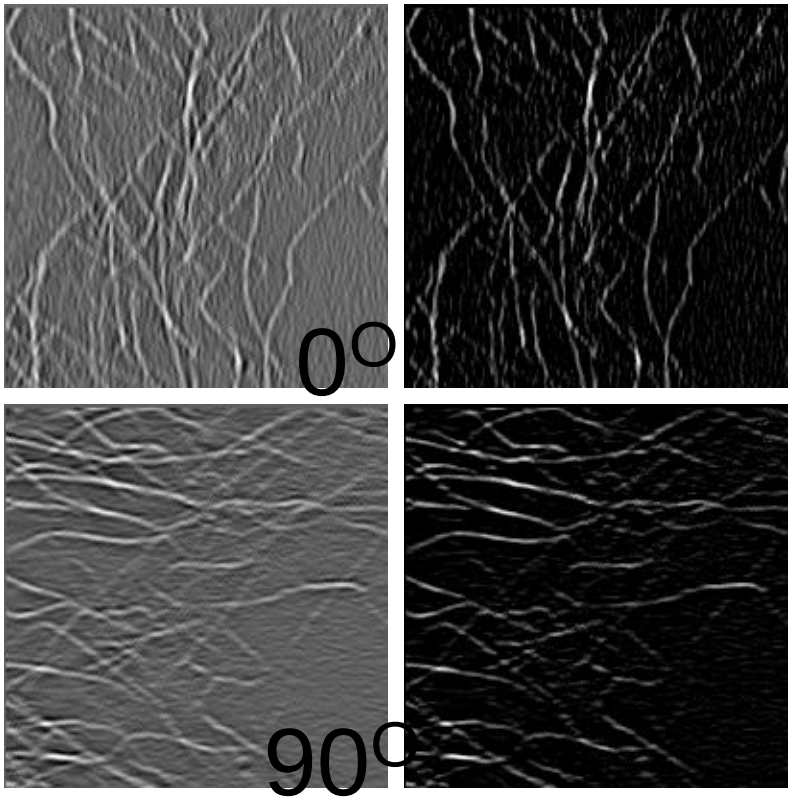




Super-resolution of Fiber Structures

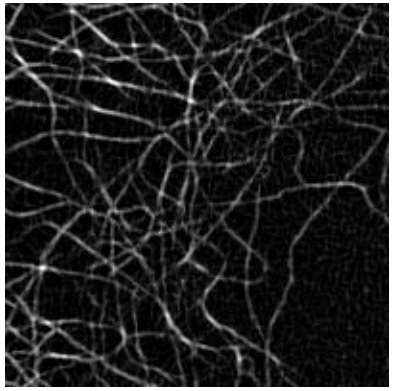


Raw image



Detect line segments with line kernels along 8 directions

Threshold images



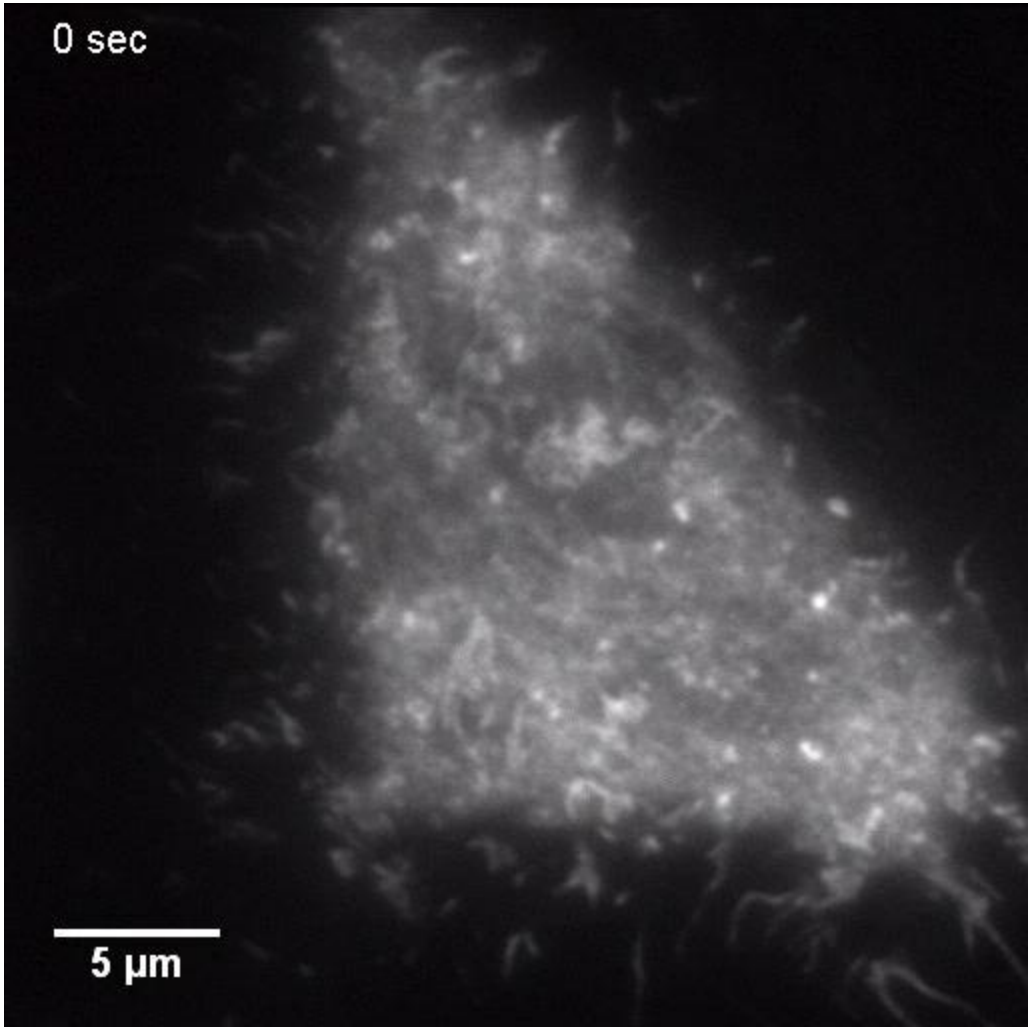
Add images in all orientations

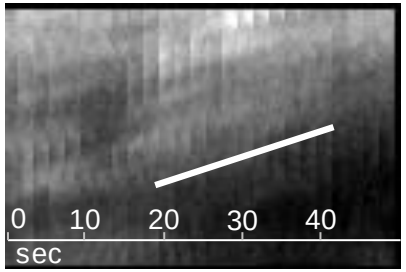
$K^0 =$

$$\begin{vmatrix} 1 & 2 & 4 & 2 & 1 \\ 1 & 2 & 4 & 2 & 1 \\ 1 & 2 & 4 & 2 & 1 \\ 1 & 2 & 4 & 2 & 1 \\ 1 & 2 & 4 & 2 & 1 \end{vmatrix}$$

etc

Flux of Actin Filaments Toward the Equator

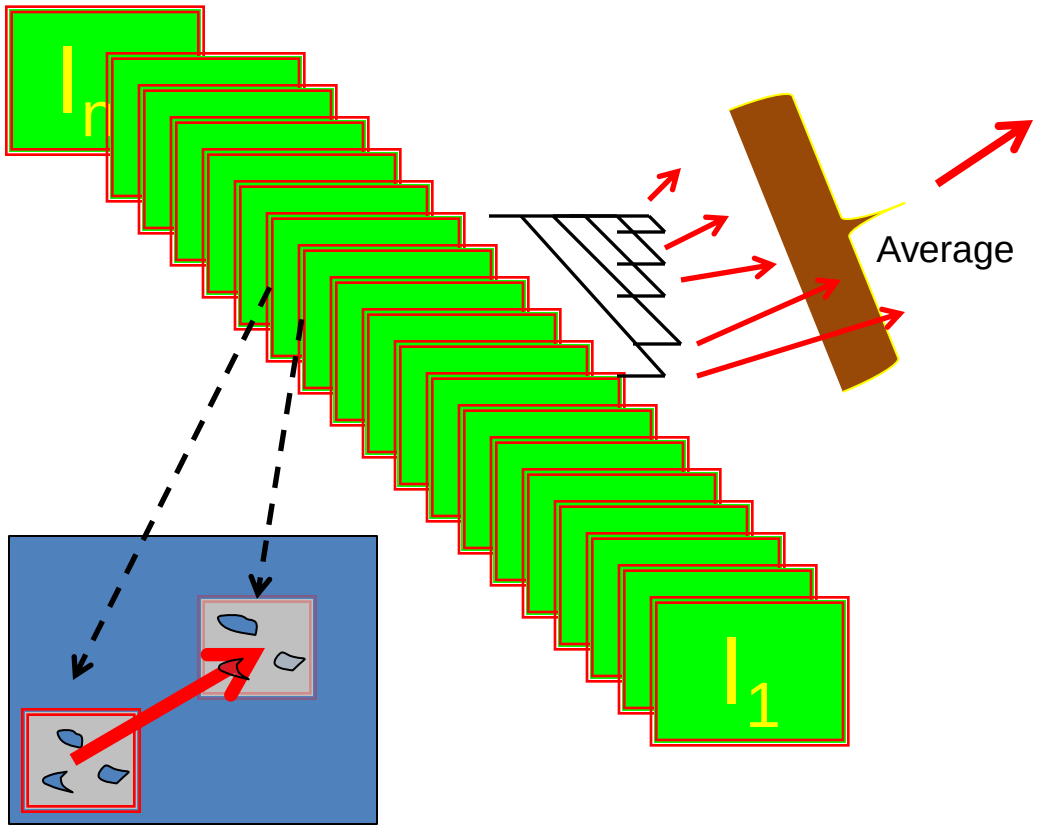




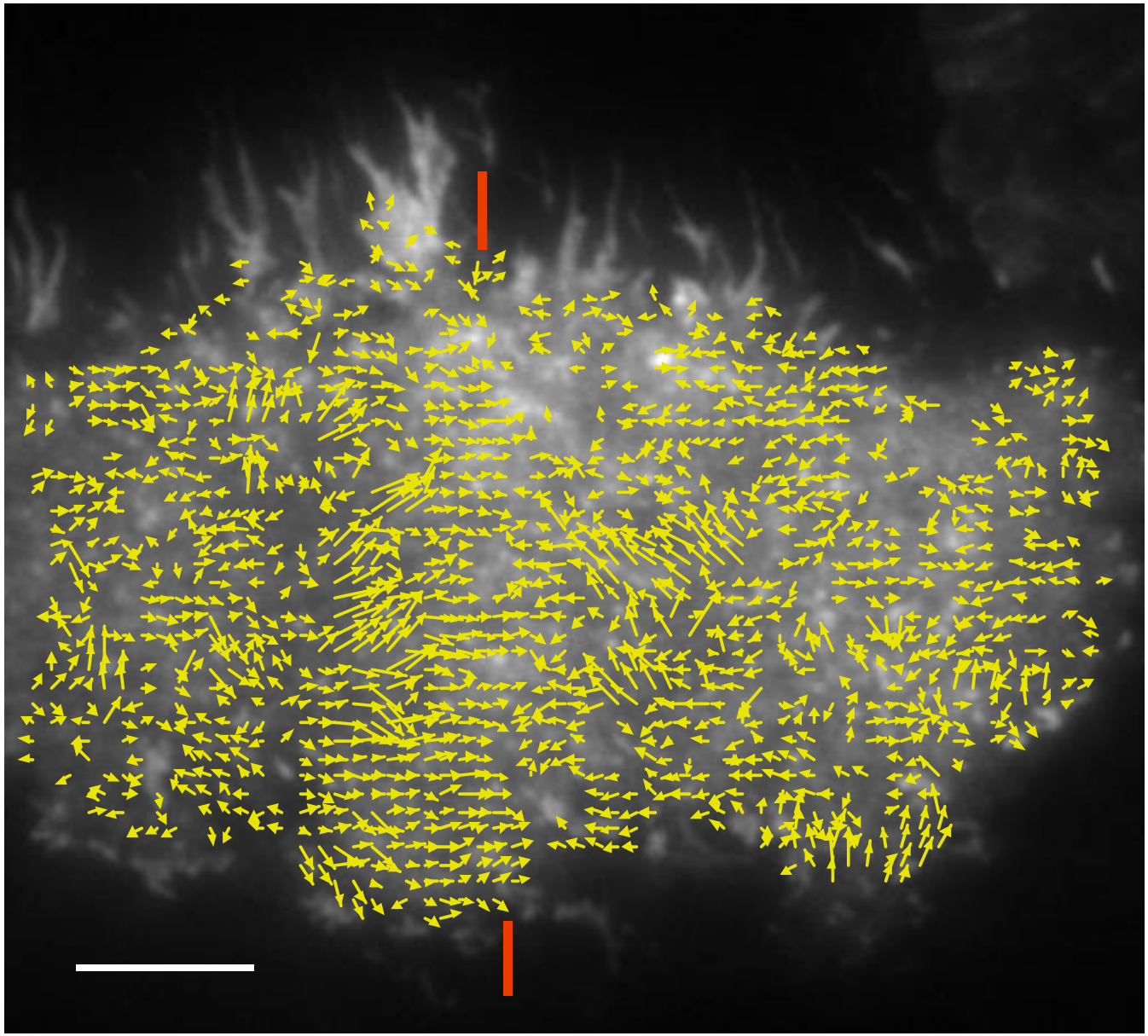
kymograph

Spatial Temporal Correlation Microscopy

Detecting Structure Flow

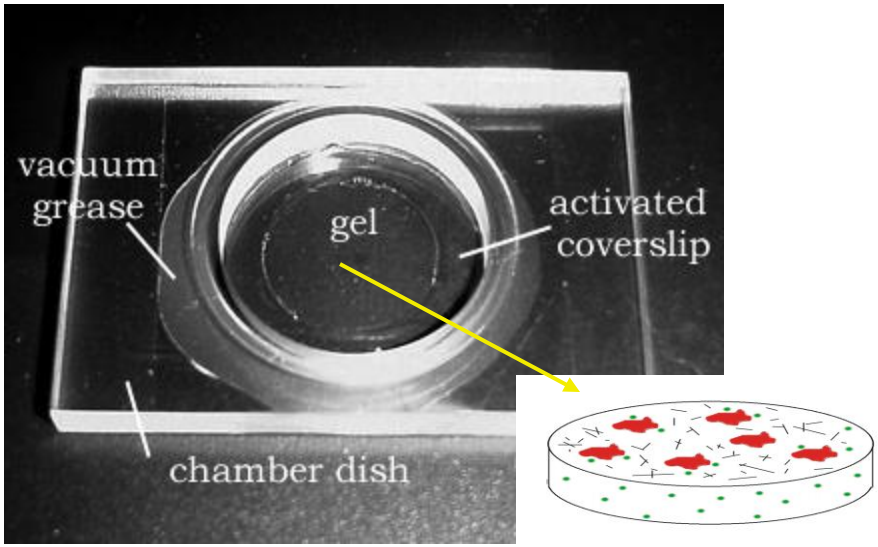


Spatiotemporal Image Correlation Analysis of Sub-Equatorial Fluxes of Actin Filaments

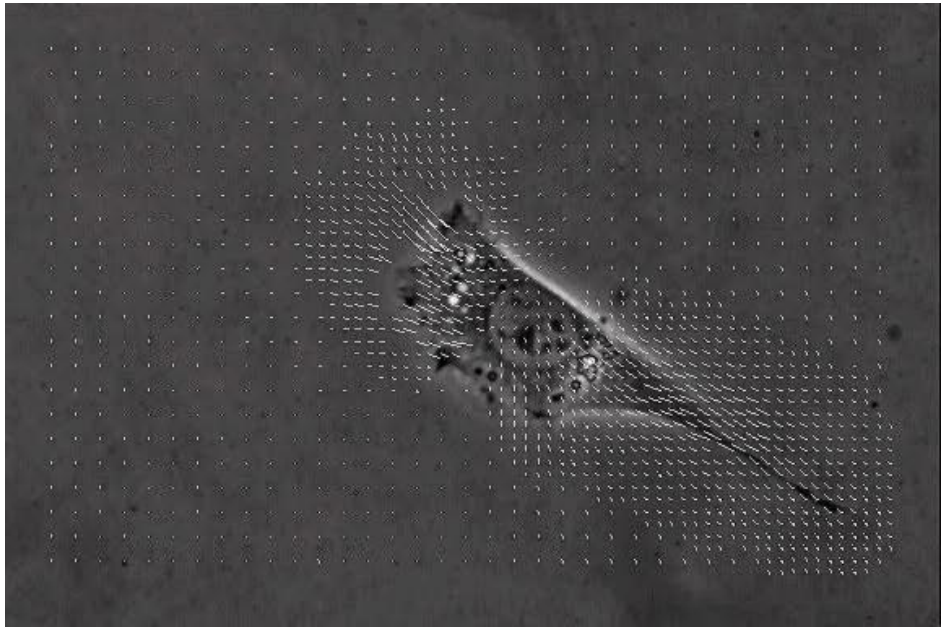


Mian Zhou

Microscopy of Traction Forces



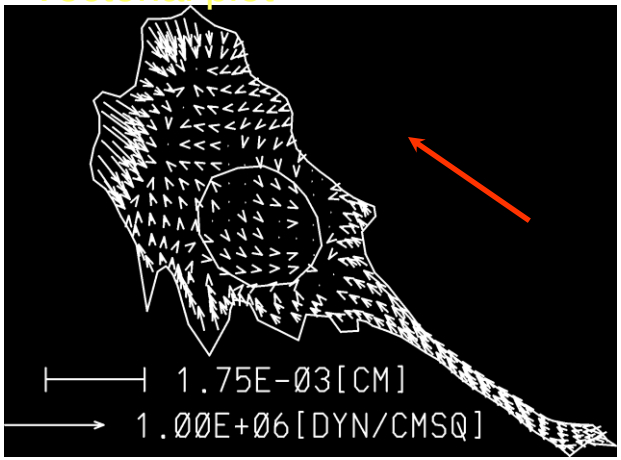
Bead displacement



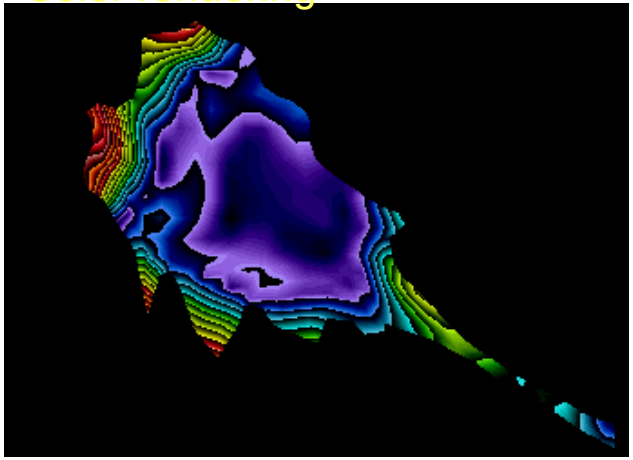
Vectors of bead displacement

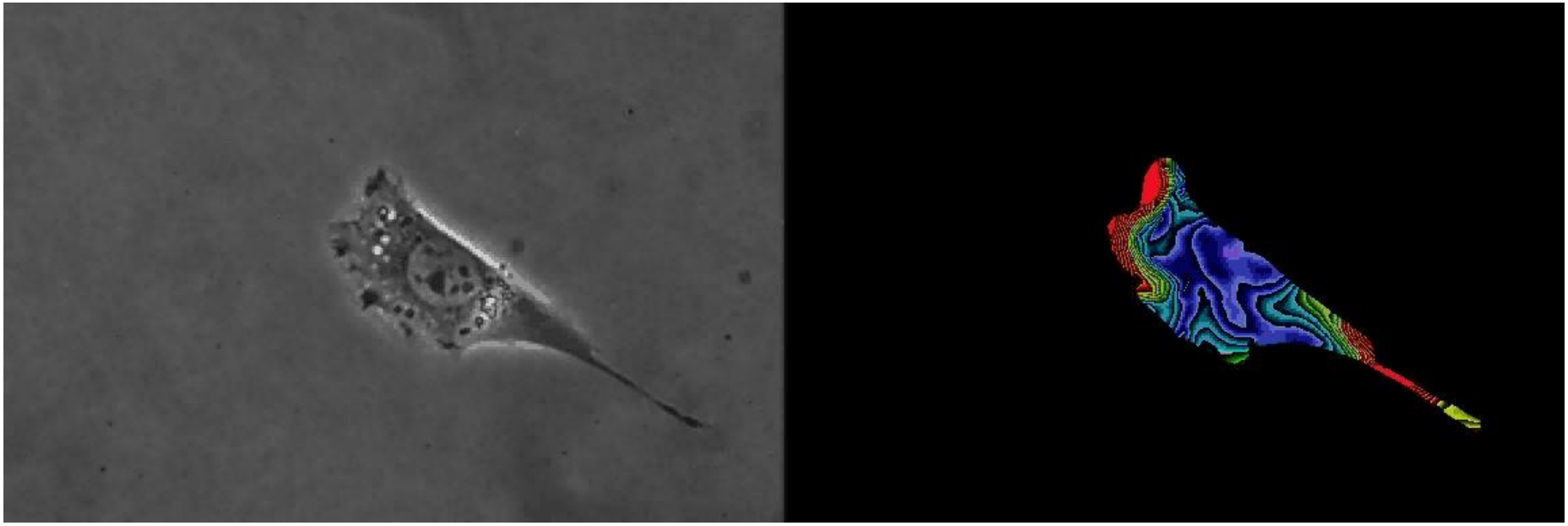
Traction Force Microscopy

Vectorial plot



Color rendering

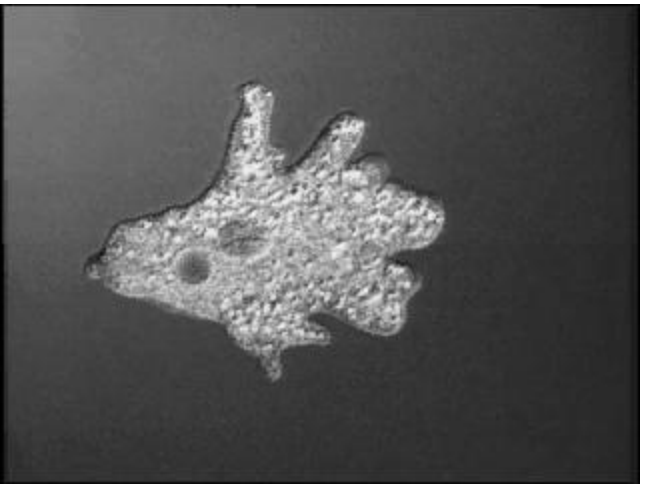


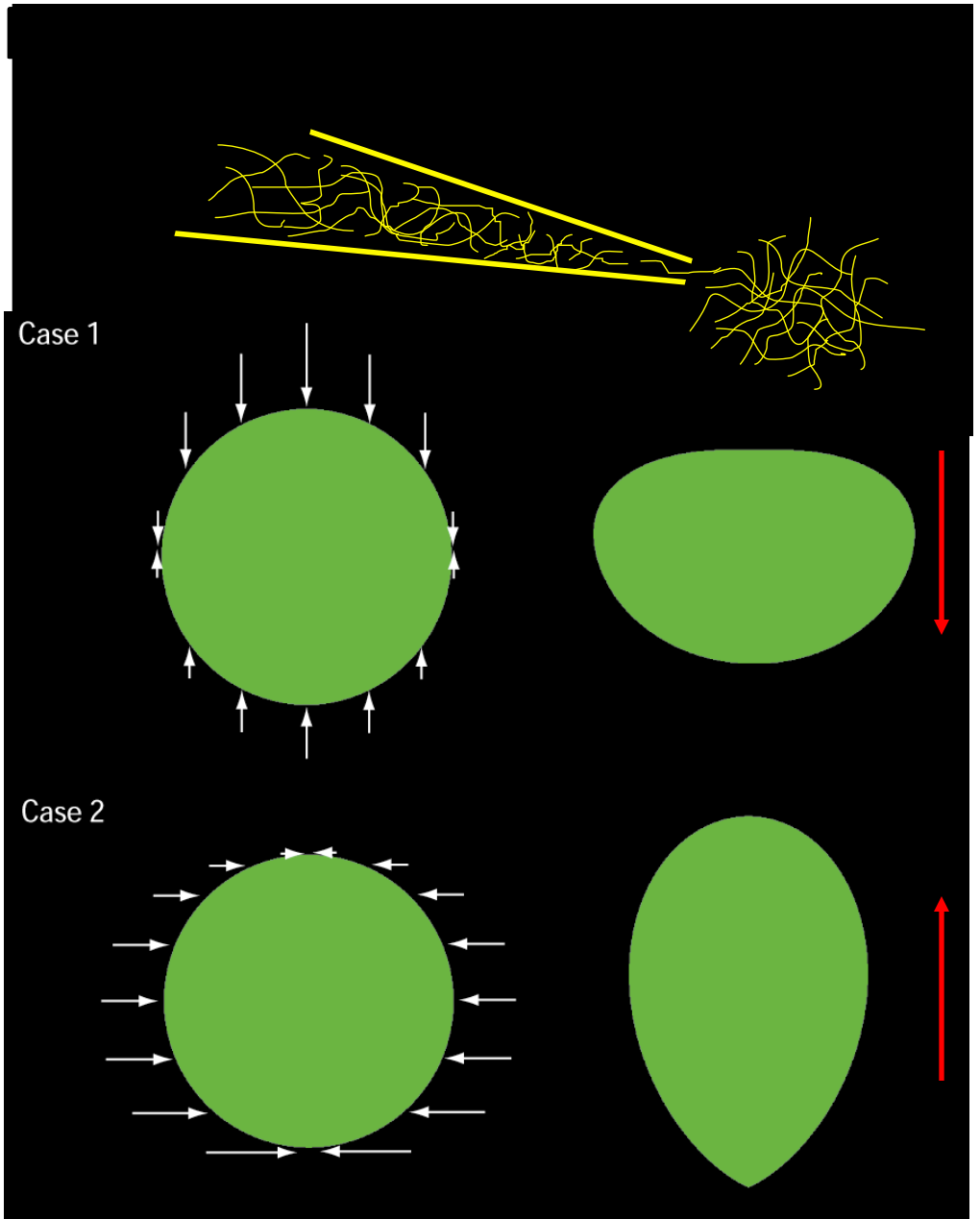


Key Areas for Development

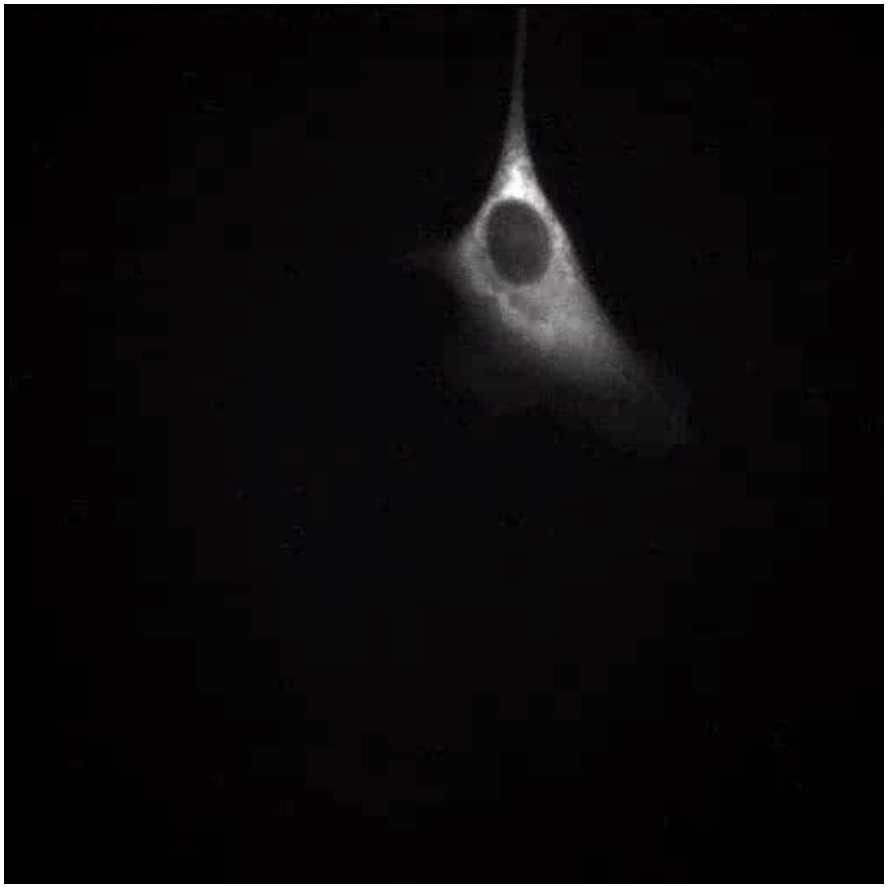
- Probes & Sensors – “brighter” probes, new parameters
- Biological Context – intravital imaging, physiological mimetics
- Informatics – automation, high-throughput imaging, information extraction, modeling

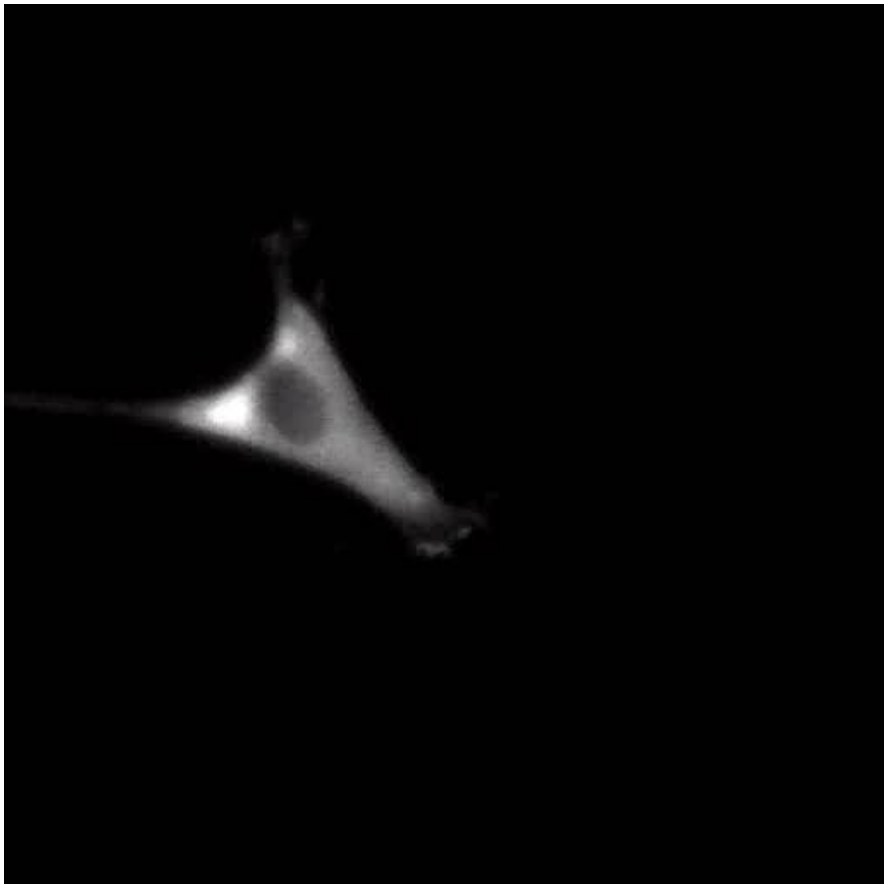
Tangled Linear Polymers as Intracellular





Myosin II Dependent Cytoplasmic Forces





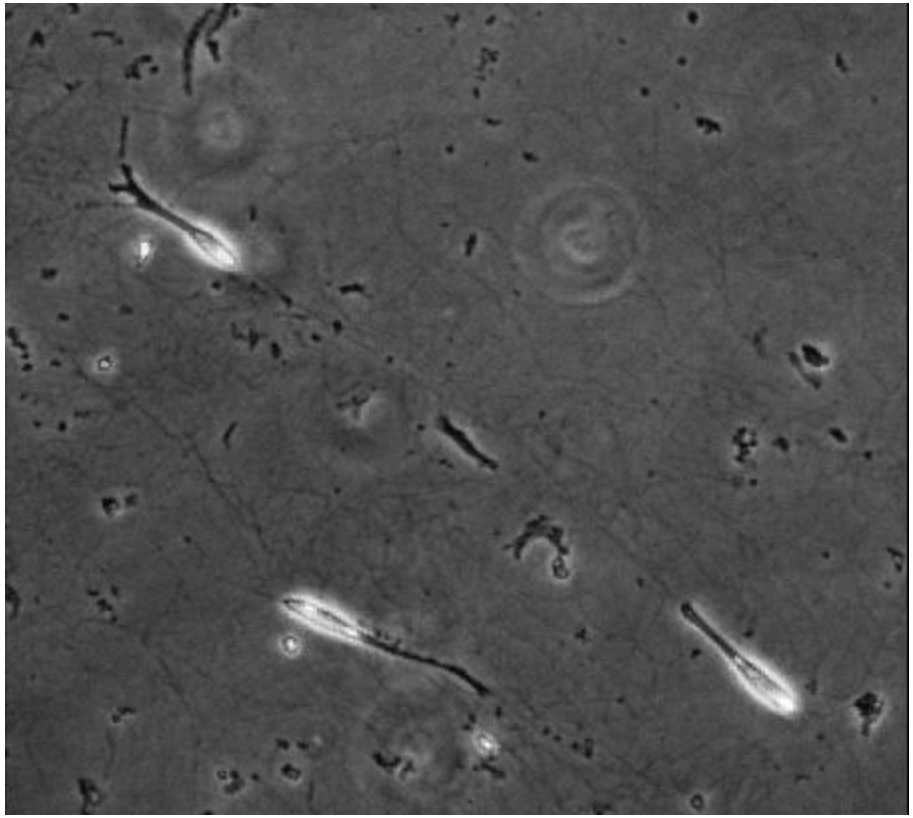
Max asp ratio = 7 (along cell axis)
Max speed = 8.5 x 10^{-2} micron/sec

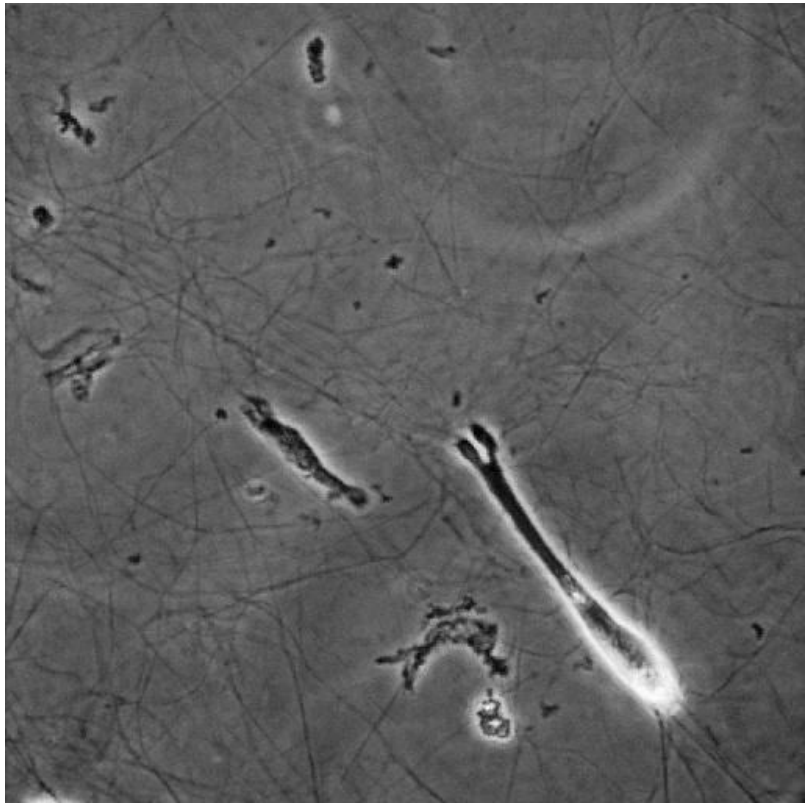
Blebbistatin
(myosin II inhibitor)

T. Iwasaki

Cell Migration in Collagen Gels

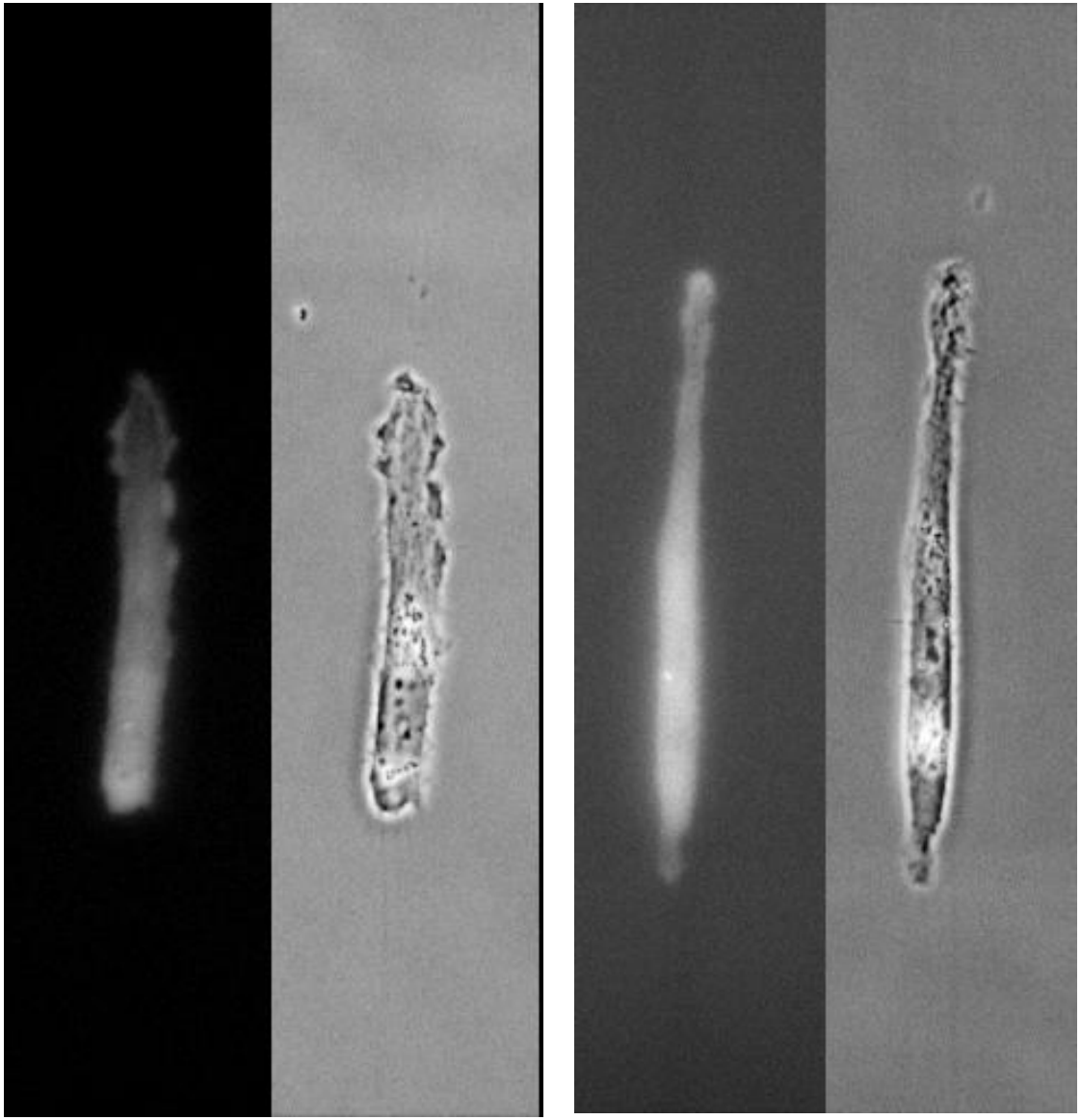
Lagging Cell Body When Tracking Collagen Fibers



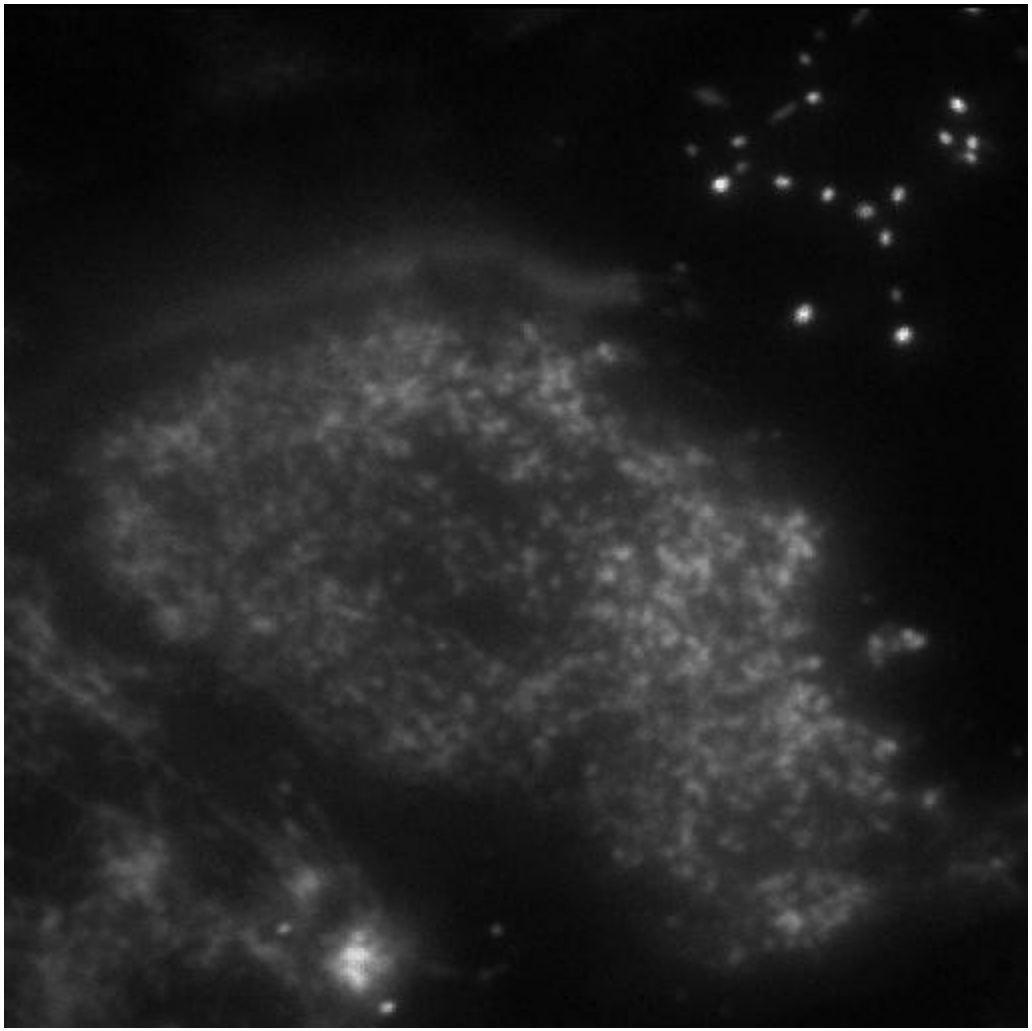


Cell Migration along Adhesive Strips

Stable Front and Contractile Rear

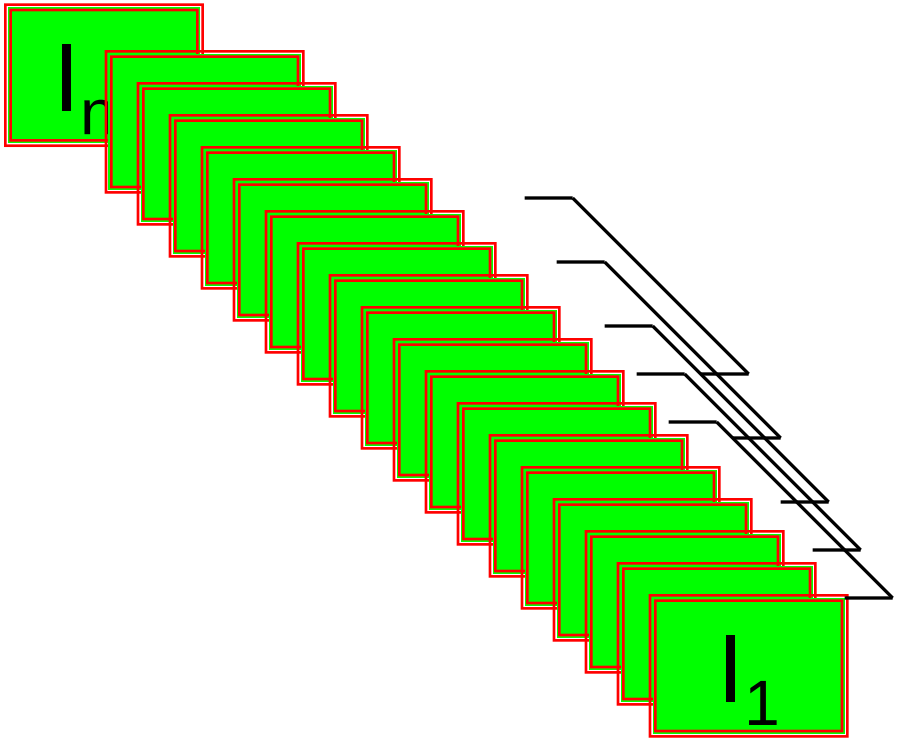


Dynamics of Myosin II during Early Cytokinesis



Temporal Differential Microscopy

Detecting Normalized Rate of Intensity Changes

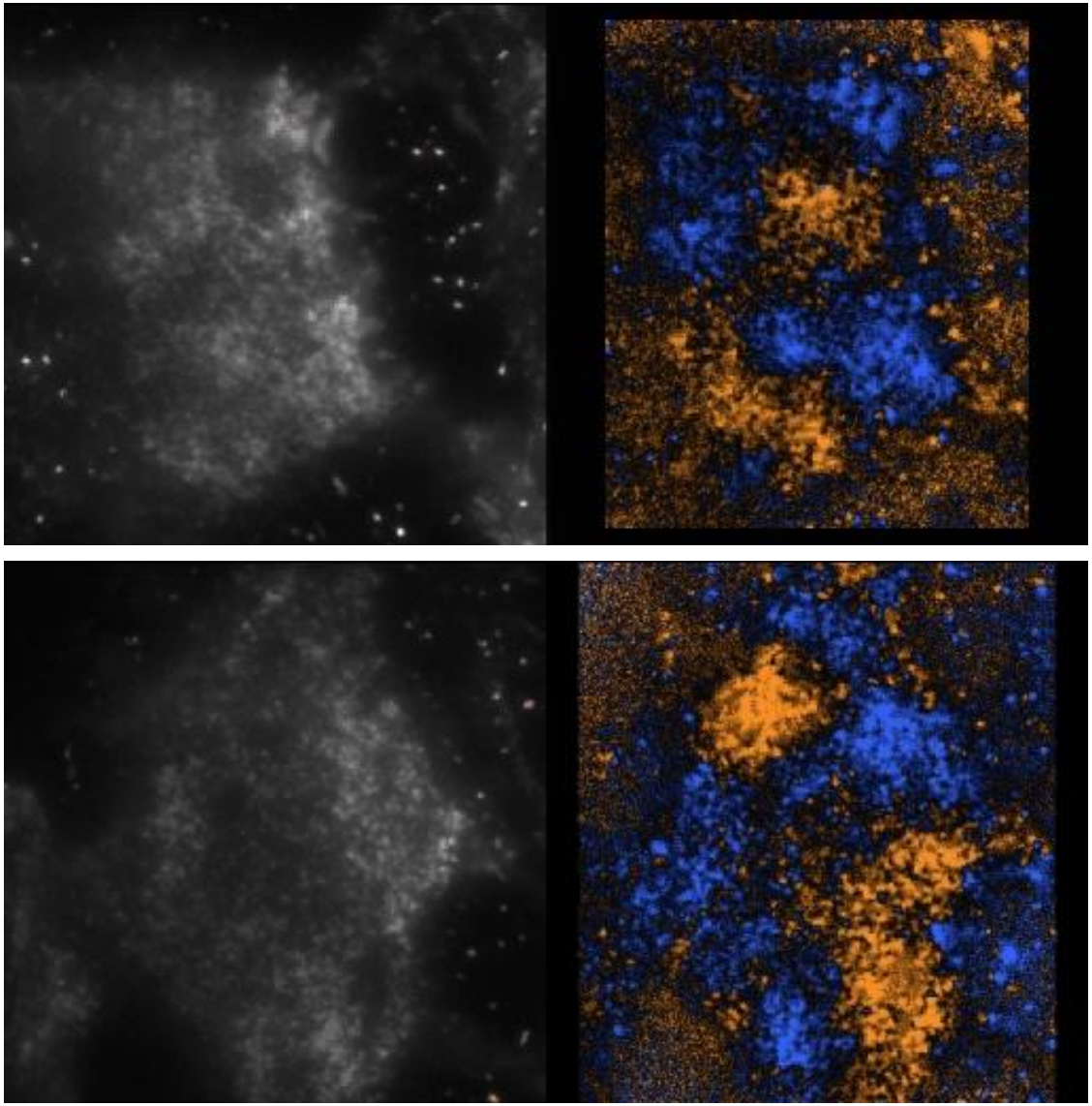


$$D_i = (I_{t+dt} - I_t)/(I_{t+dt} + I_t)$$

Discrete, Transient Domains of Myosin Assembly

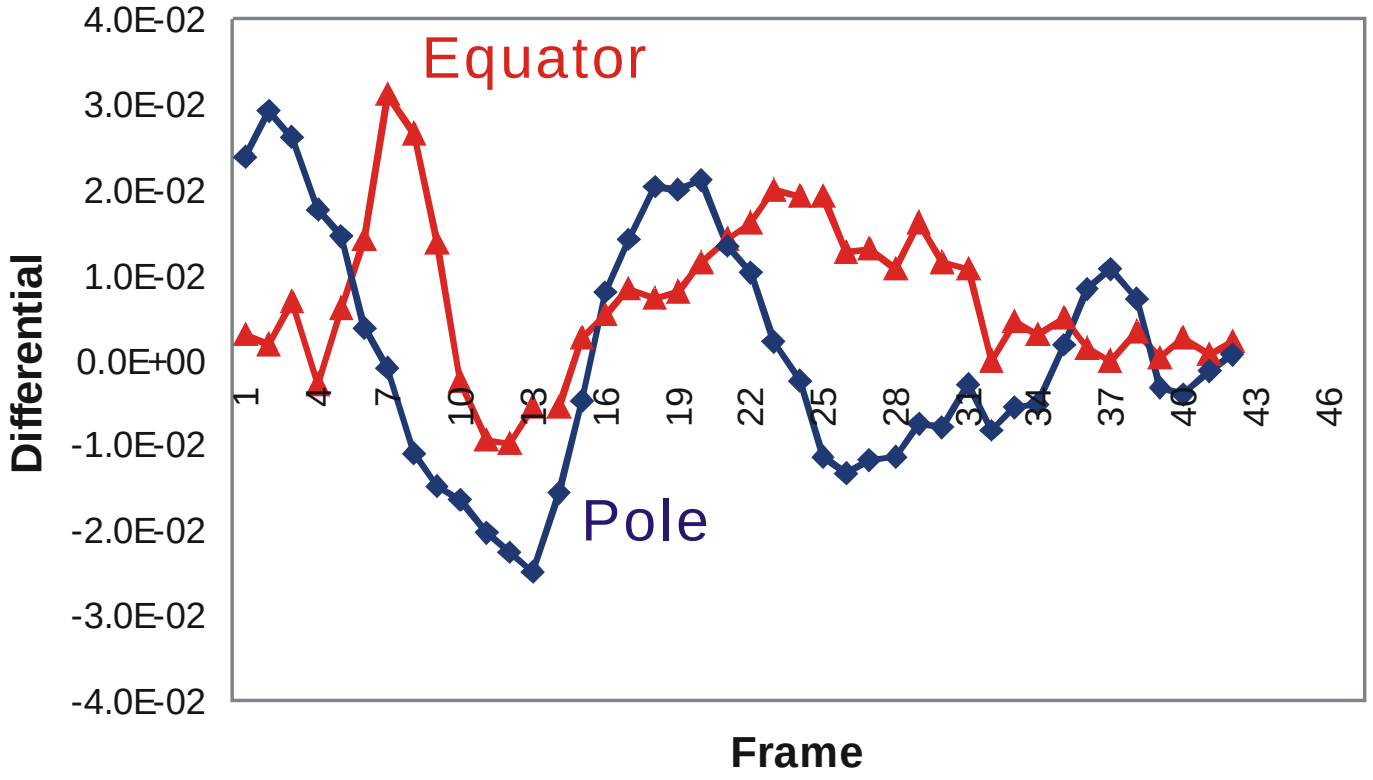
Metaphase

Early Cytokinesis



TIRF TDM

Suppression of Myosin Disassembly along the Equator



Separating Information from Noise

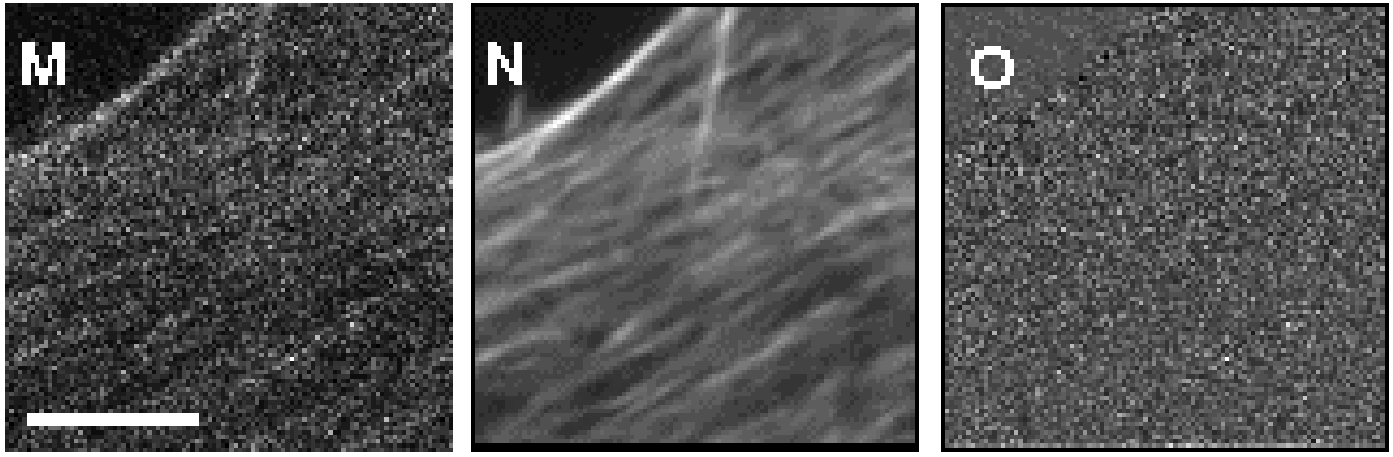
Multiresolution decomposition into Signal and Noise

↓

Noise matching characteristics?

no ↑

yes → Done

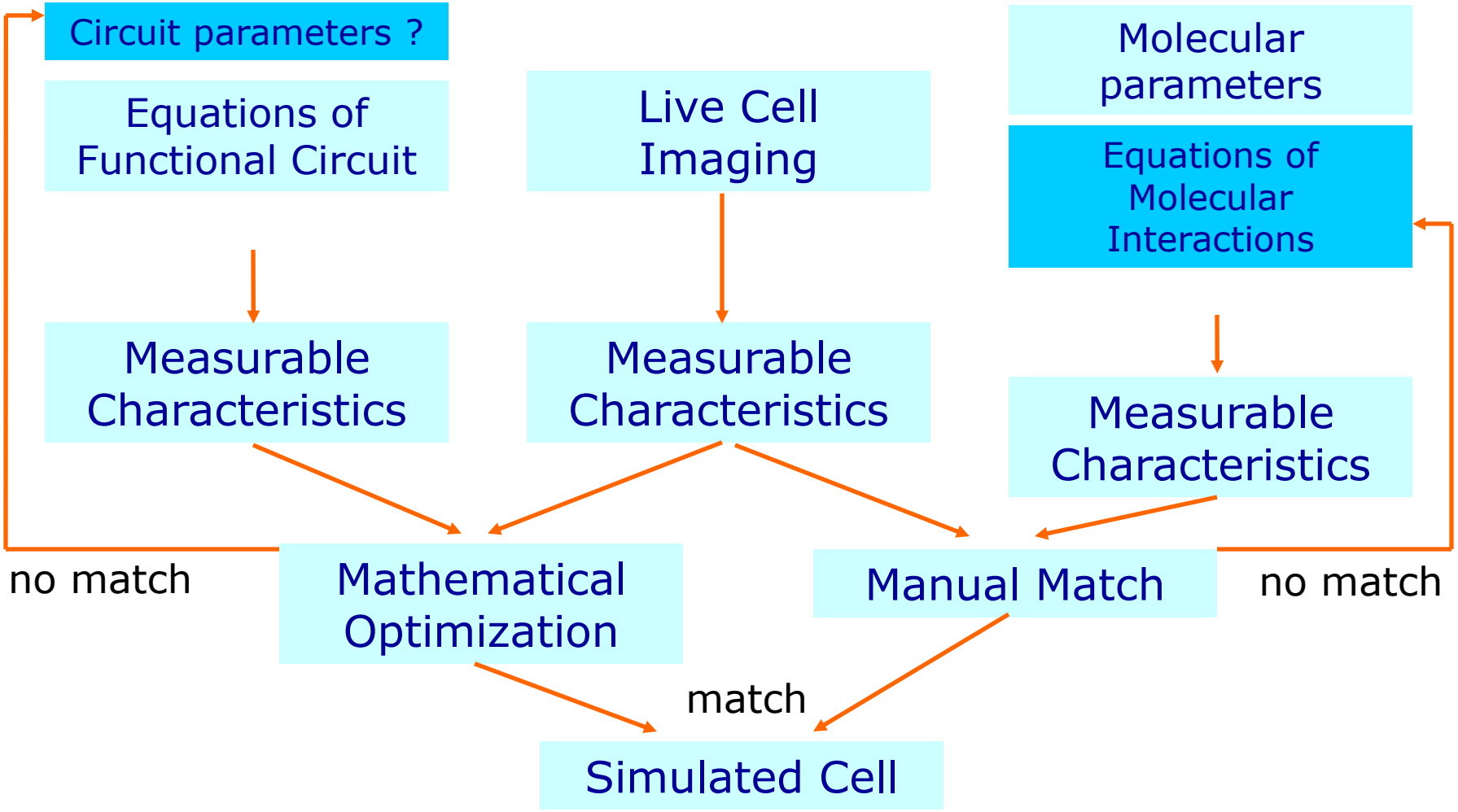


Original Signal Noise

From “Seeing” to “Understanding”

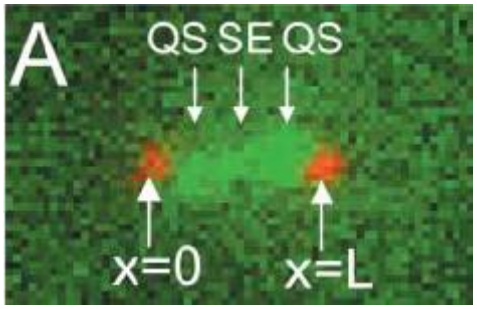
- What Biologists Can See with Light Microscopy -
  - Spatial and temporal details of chemical and physical properties encoded by optical sensors
- What Biologists Need for Understanding -
  - The rules of interactions that lead to biological functions
- Images Represent Manifestations or Consequences 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

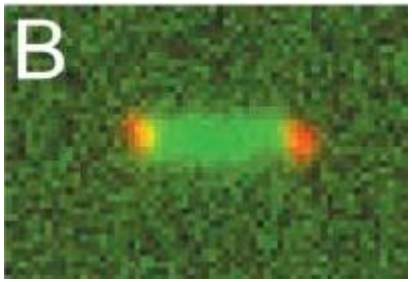


Modeling Microscope Images

Based on Biological Mechanisms and Instrumental Characteristics



Experimental images of microtubules in yeast spindle



Simulated images

Sprague et al (2003) Biophys. J. 84:3529

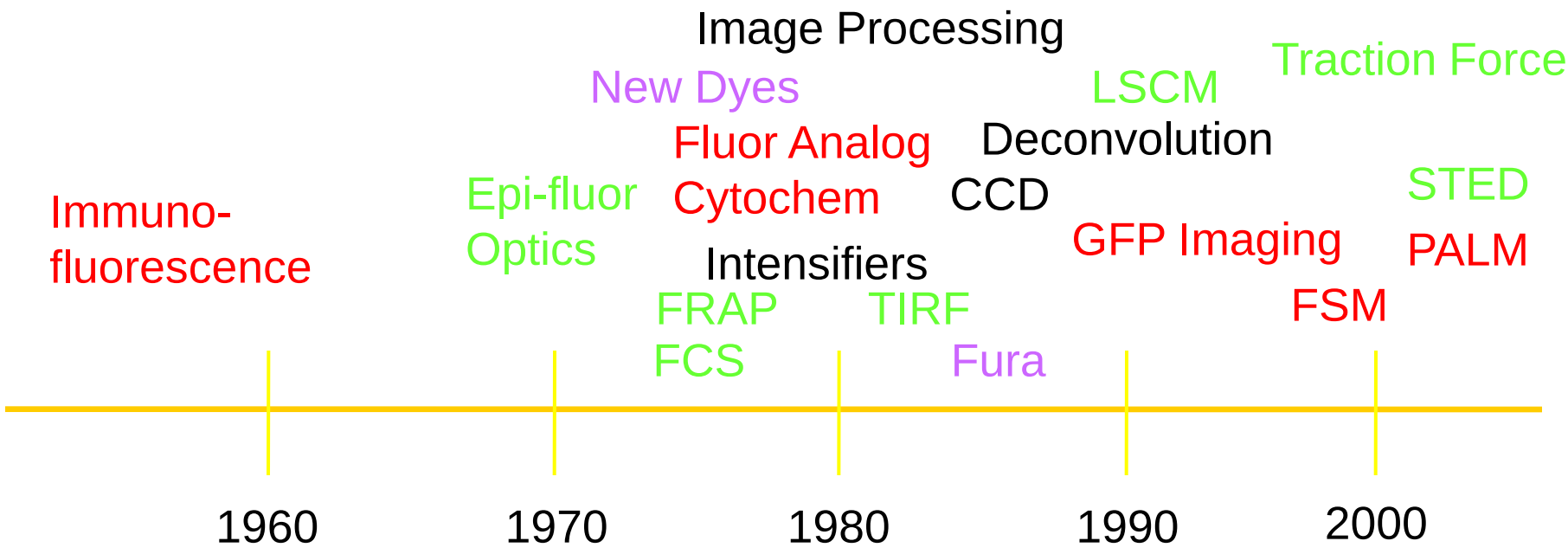
Observed distribution of microtubules

Predicted distribution of microtubules

Proposed dynamics of microtubules

PSF and camera noise

Fluorescence Microscopy Timeline



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.