Live cell microscopy

1. Why do live cell microscopy?

Why Do Live Cell Imaging?

Before

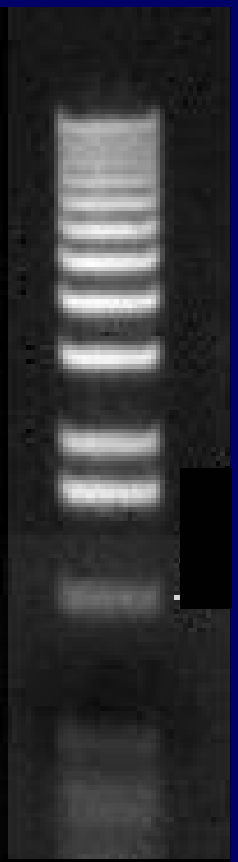
After

What are the Rules?

- Dave Piston, Vanderbilt

Why Do Live Cell Imaging?

Or we grind up the sample and run a gel



Defensive Backs
Receivers
Running Backs
Linebackers

Linemen

Nose Tackles

Now what do we know about the rules?

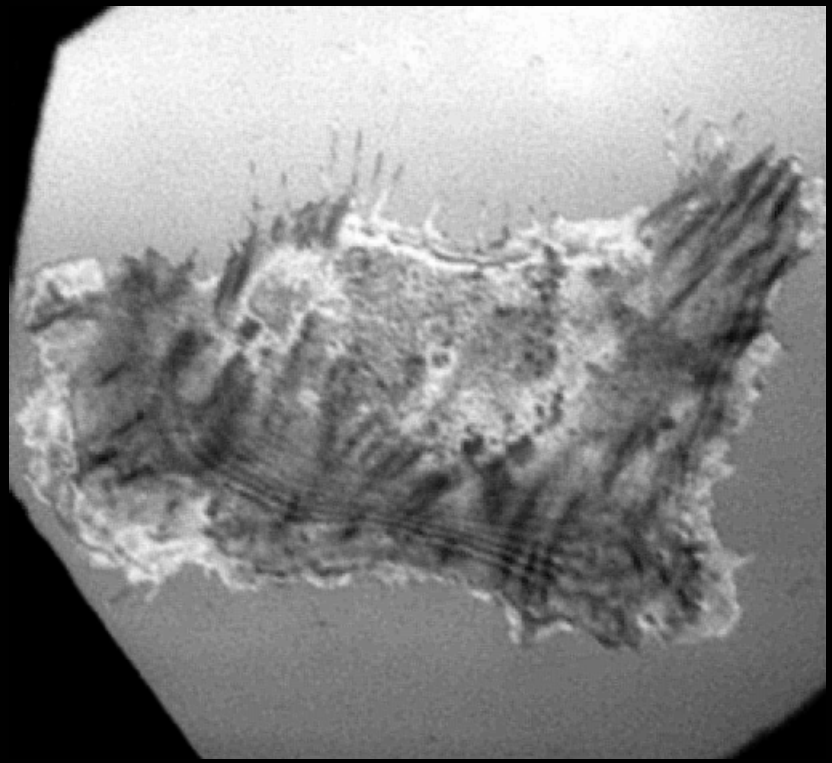
- Dave Piston, Vanderbilt

Why Do Live Cell Imaging?

If you want to learn the rules, you have to watch

- Dave Piston, Vanderbilt

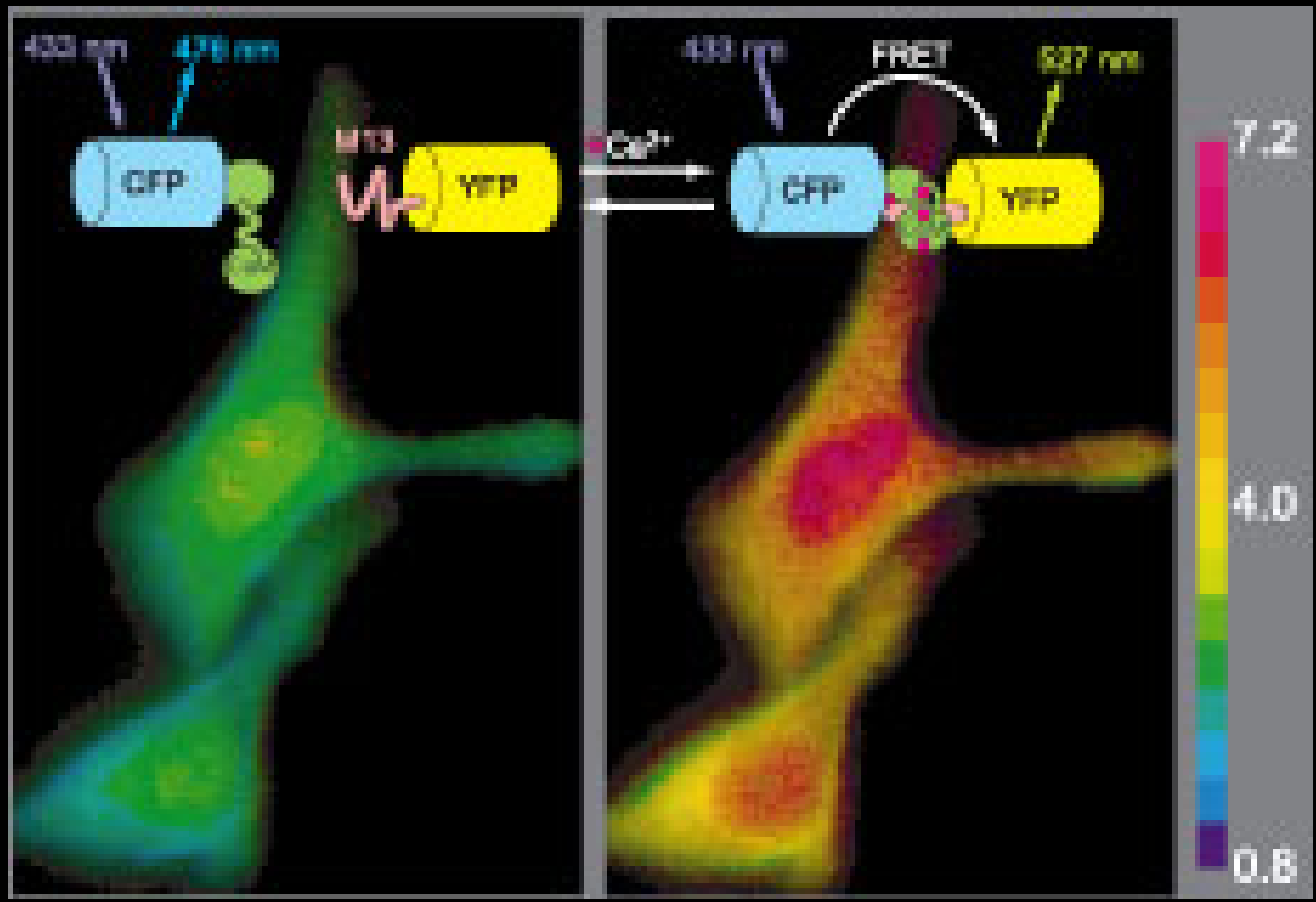
Live cell microscopy – imaging cell dynamics



Focal adhesion dynamics imaged by reflection interference contrast

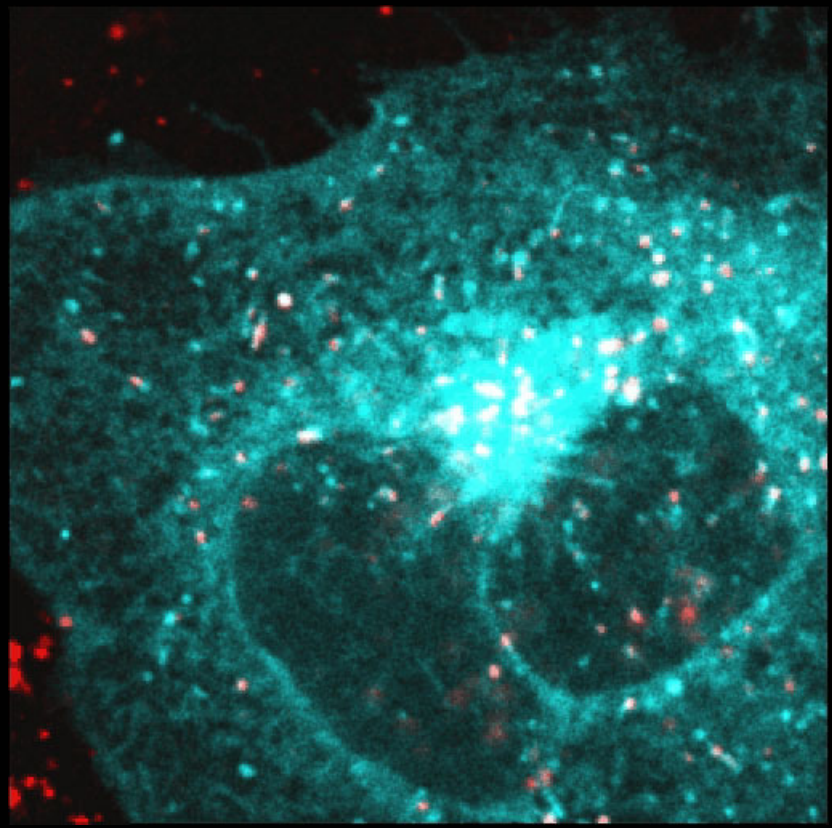
- Marysse Bailly, Einstein

Live cell microscopy – imaging cell physiology



Tsien and Miyawaki, 1998

Live cell microscopy – poorly fixed probes



Live cell microscopy

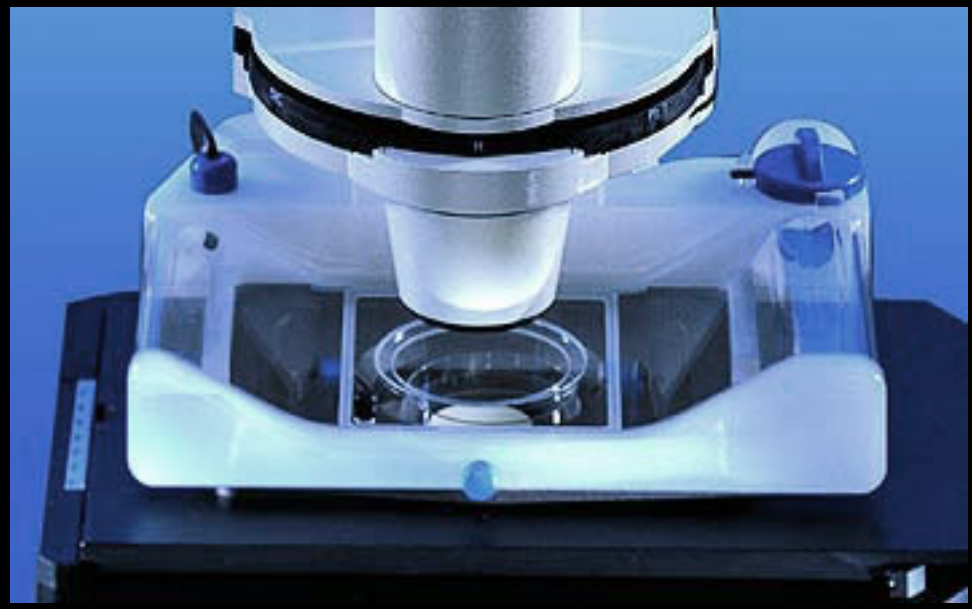
1. Why do live cell microscopy?
2. Maintaining living cells on a microscope stage.

Culturing live cells on the microscope stage

- pH control (gassing your cells)
- medium manipulations - perfusion
- temperature control
- where did the cells go? - focus drift

Commercial stage heaters

Comprehensive Heaters



Comprehensive Heaters

Heated Chamber Includes Stage, Objective Lenses Can Control CO_2, etc.

Camera (cooled) Stays Outside Heated Chamber

Comprehensive Heaters

ARRR . . .

Heated Chamber Includes Stage, Objective Lenses Can Control CO_2, etc.

Camera (cooled) Stays Outside Heated Chamber

Costs more than Ken Spring's house

Live cell microscopy

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Live cell microscopy

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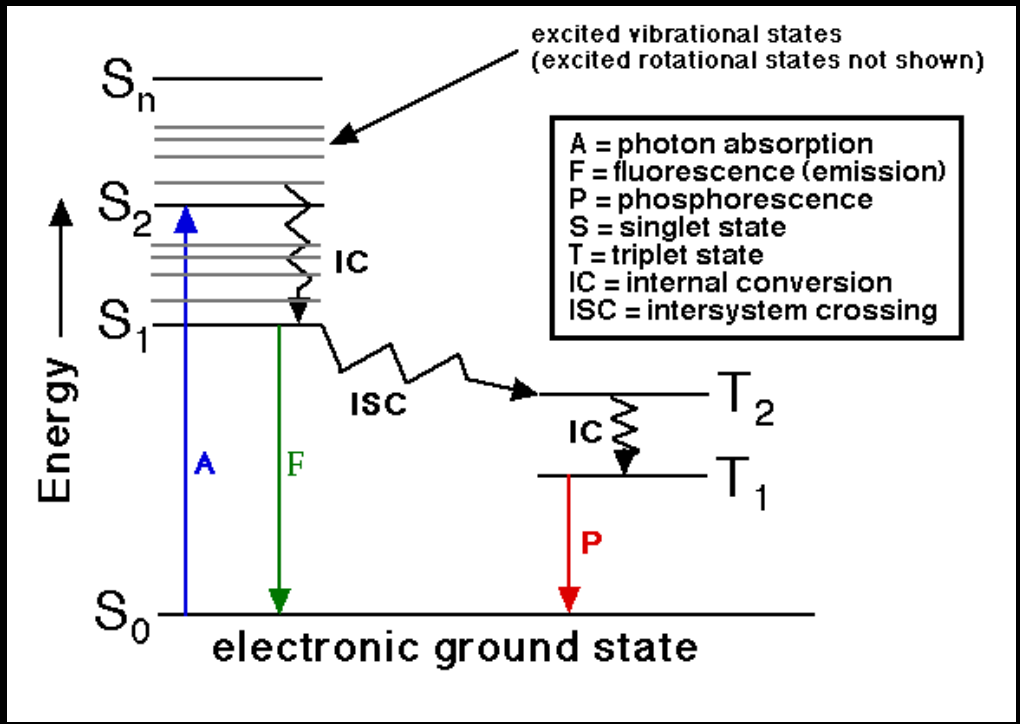
EVERYTHING IS THAT MUCH HARDER.

Limits to fluorescence microscopy

- Limited number of photons

Fluorescence runs out - photobleaching always accompanies fluorescence excitation



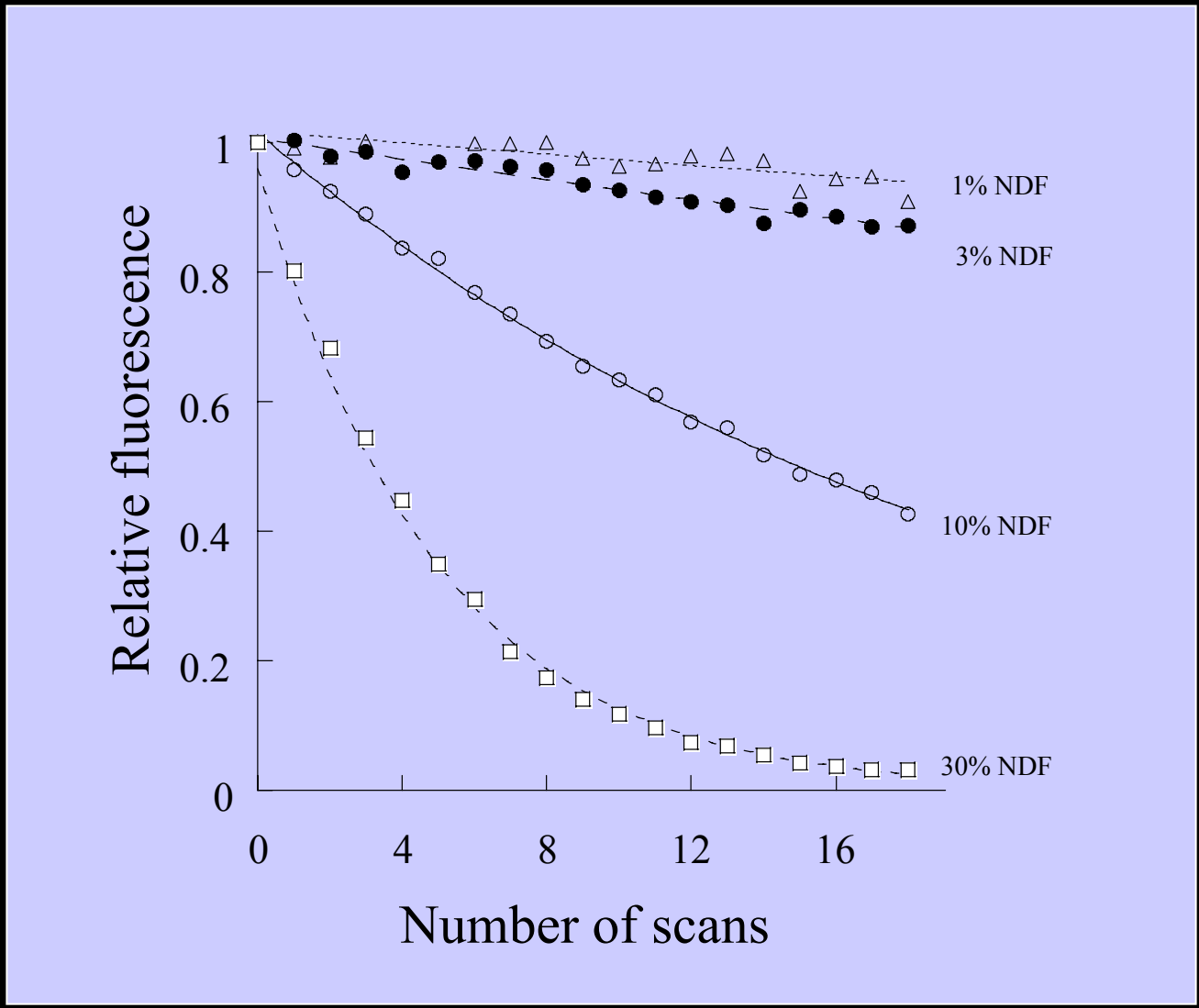
- For every photon absorbed, there is a fixed probability of either fluorescence emission or photobleaching.

- The total number of fluorescence photons emitted is given by the ratio of the quantum efficiency of fluorescence to the quantum efficiency of photobleaching or Q_e/Q_b

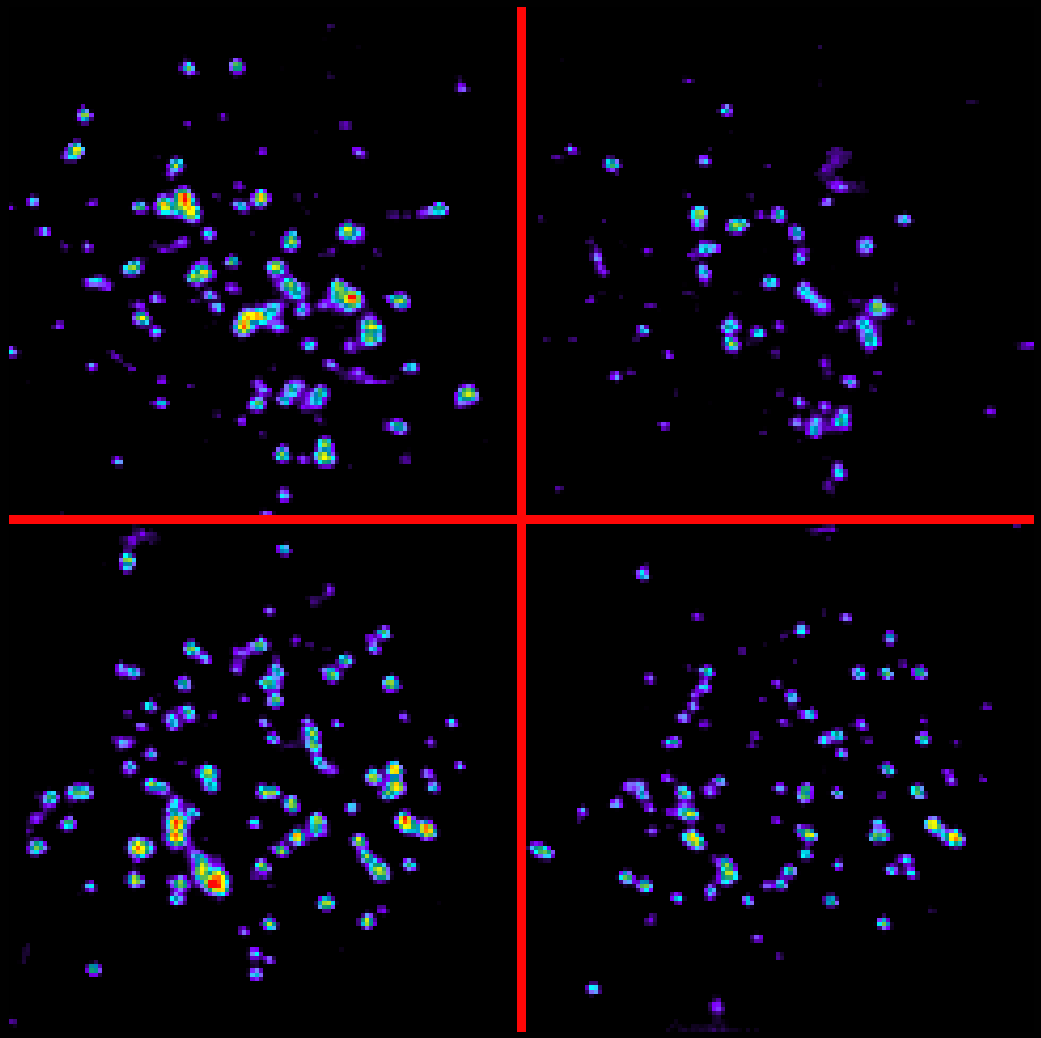
- For fluorescein this works out to around 30,000 photons.

- Since we collect ~1% of the photons - "every photon is sacred"

Photobleaching of diI-LDL



Oxyrase inhibits photobleaching of DiI-LDL in living cells (a little)



- with oxyrase

- control

Initial

After 150 scans

Limits to fluorescence microscopy

- Limited number of photons
- Limited flux of photons

Limits to photon flux – Excitation Saturation

- The rate of emission is dependent upon the time the molecule remains within the excitation state (the excited state lifetime τ_f)
- Optical saturation occurs when the rate of excitation exceeds the reciprocal of τ_f
- In a scanned image of 512 x 768 pixels (400,000 pixels) if scanned in 1 second requires a dwell time per pixel of 2×10^{-6} sec.
- Molecules that remain in the excitation beam for extended periods have higher probability of interstate crossings and thus phosphorescence
- Usually, increasing dye concentration can be the most effective means of increasing signal when energy is not the limiting factor (ie laser based confocal systems)

Limits to fluorescence microscopy

- Limited number of photons
- Limited flux of photons
- Limited resolution

Nyquist sampling

The minimum sampling frequency required to capture an event is twice the maximum frequency of the event.

In order to reproduce the resolution of the microscope, the detector must sample at twice the resolution of the optical system. At least.

Nyquist sampling

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Sampling at higher spatial resolution will reduce sensitivity as each pixel will collect fewer pixels.

Resolution versus signal-to-noise ratio

1. Say you have a point source in your sample, from which you can collect 40 photons.

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4. If you give up Nyquist sampling, you can collect 40 photons per pixel, and increase the signal-to-noise ratio.

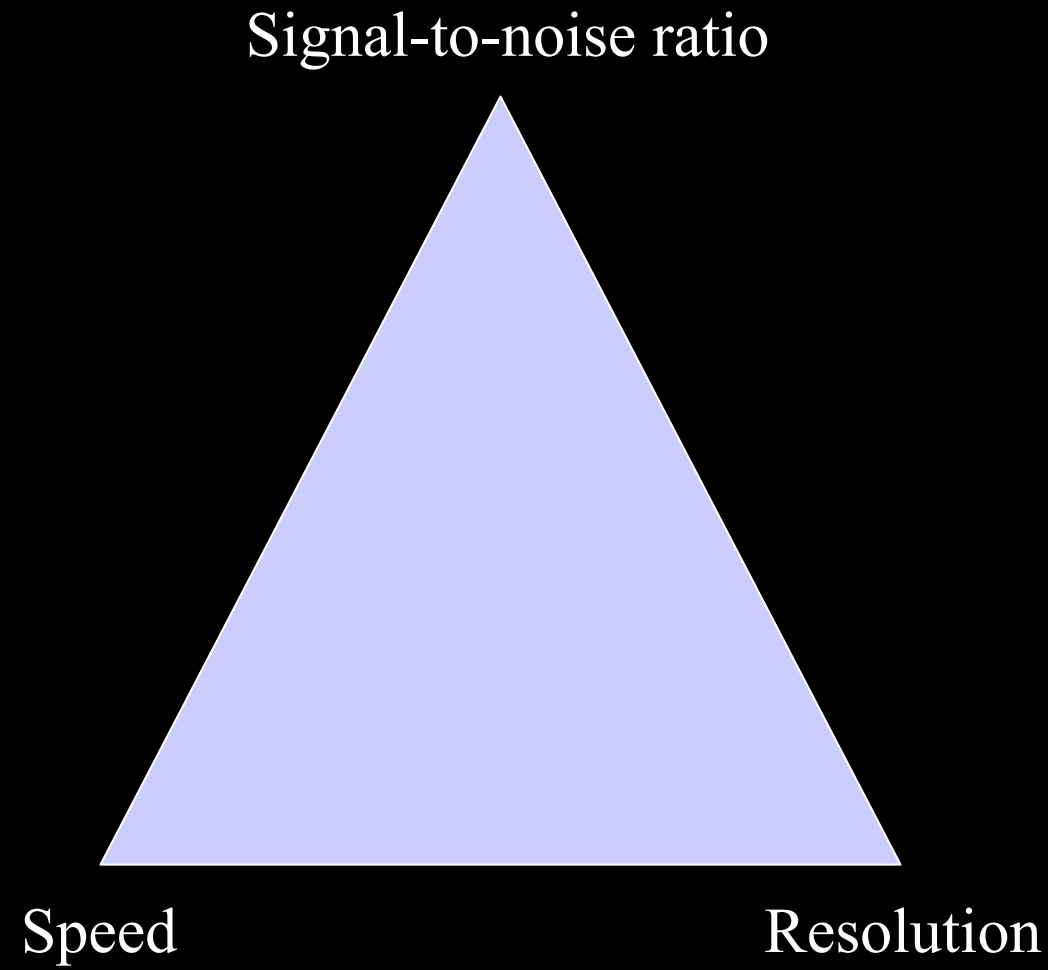
Live cell microscopy

So, fluorescence microscopy is limited by the number of photons that can be collected from a minimal volume.

The problem is aggravated in live cell microscopy, since cells are dynamic, they move, they move things, they change their constitution. Quickly.

So now you have to trade resolution, signal-to-noise and speed off of one another.

Live cell microscopy –
"The eternal triangle of compromise"



Live cell microscopy –
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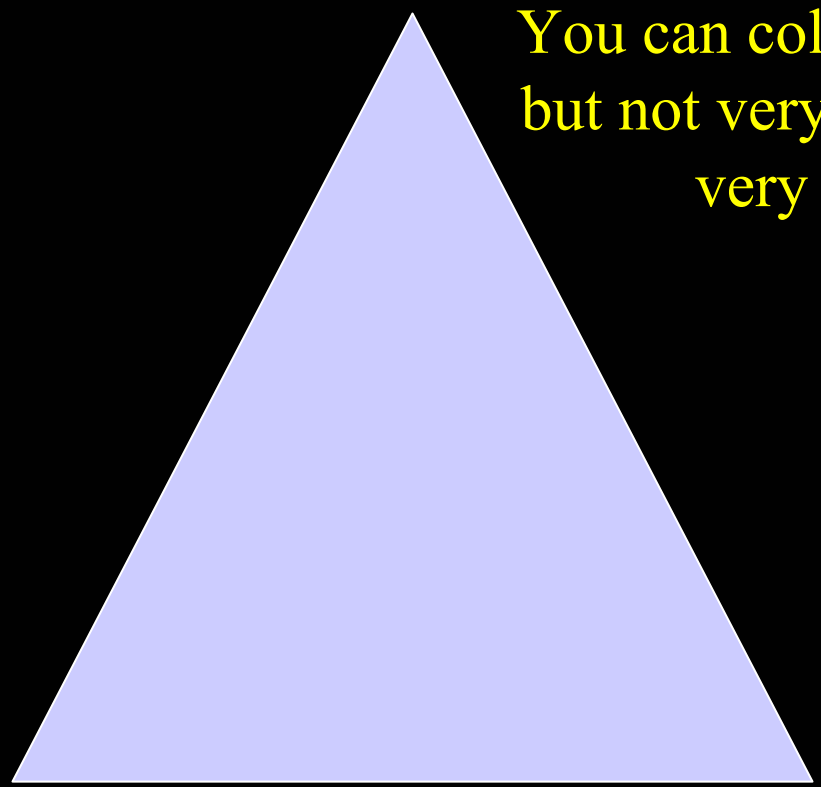

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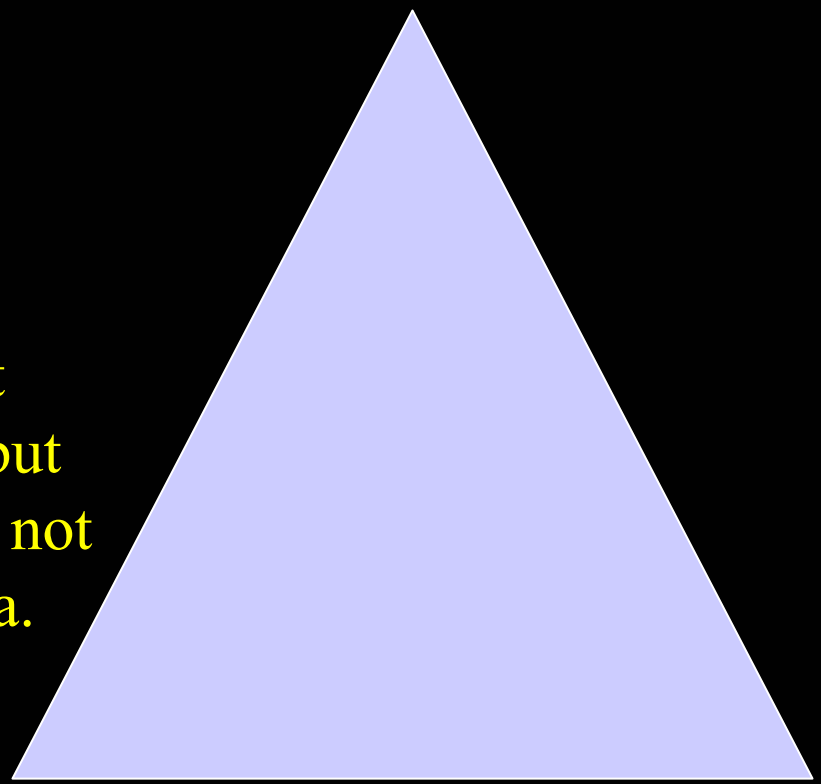
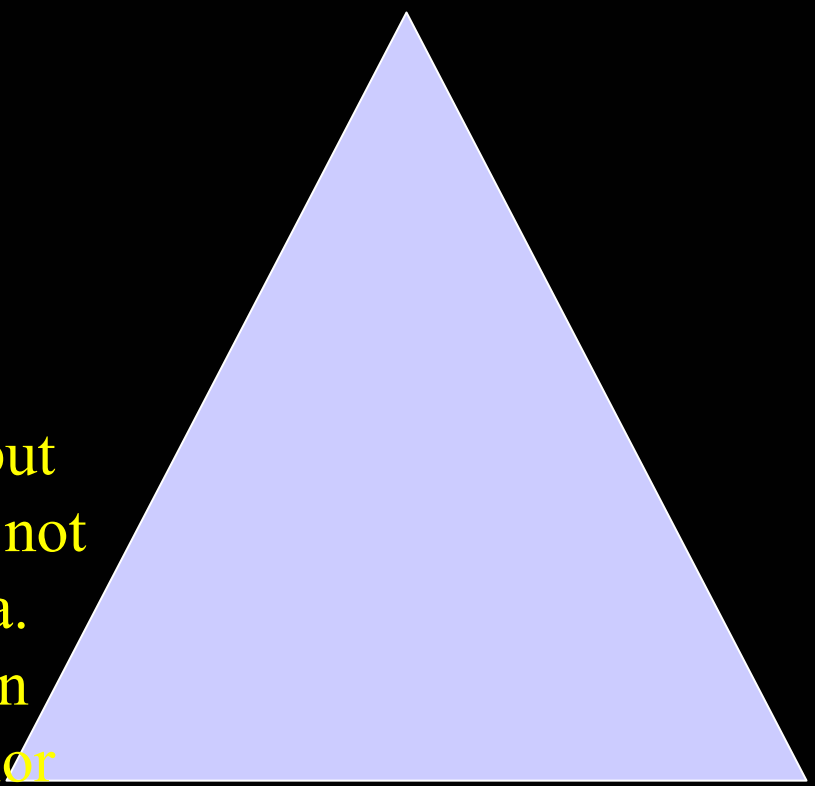


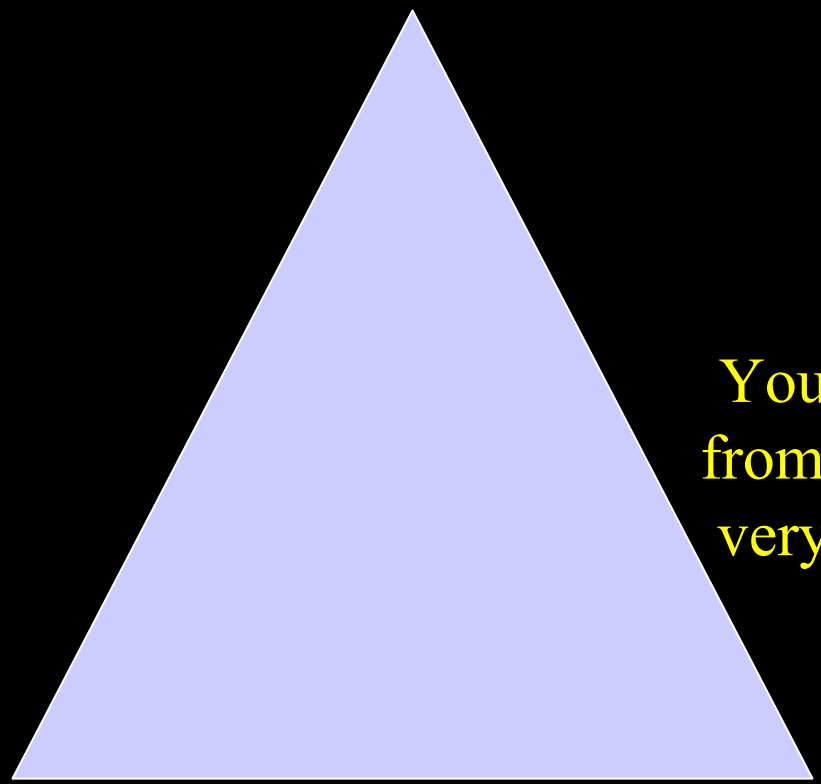
You can collect photons rapidly, but not very many and not from a small area.

Live cell microscopy –
"The eternal triangle of compromise"



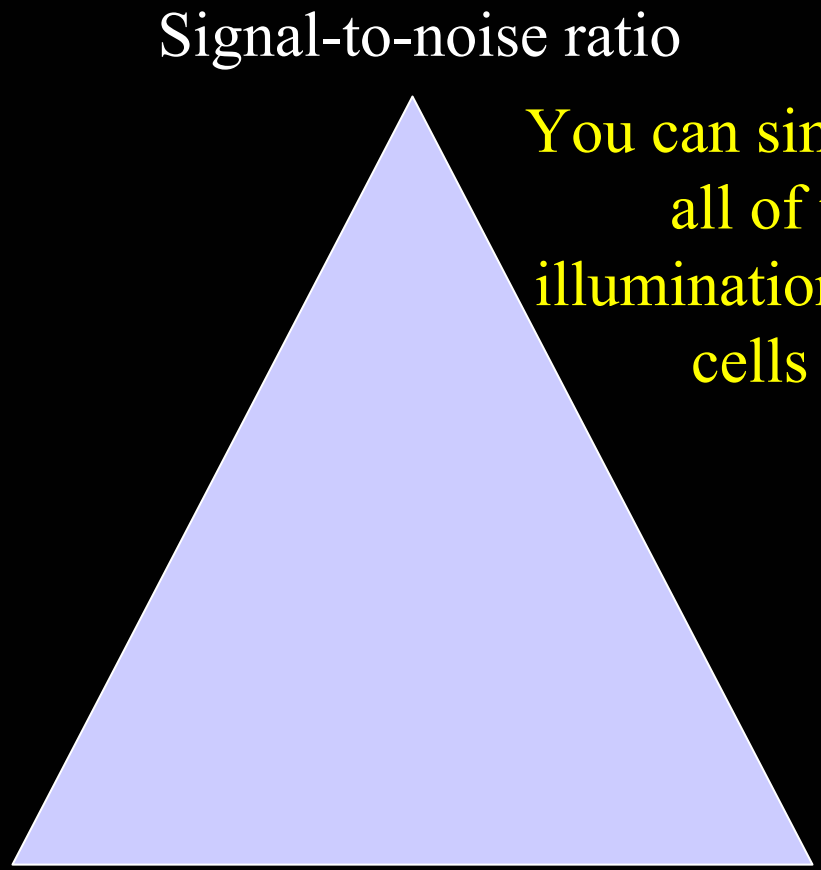
You can collect photons rapidly, but not very many and not from a small area. And maybe not in more than one color

Live cell microscopy –
"The eternal triangle of compromise"



You can collect photons from a small area, but not very many, and not very fast.

Live cell microscopy –
"The eternal triangle of compromise"



You can simultaneously improve all of these with more illumination, but you'll kill your cells and your fluor.

Phototoxicity

Phototoxicity is the absolute limiting factor in live cell imaging

- Excitation of fluorescent molecules in the presence of oxygen leads to fluorochrome bleaching and free radical generation.
- Free radicals kill cells.
- The interaction of light with cells > heat
- Heat kills cells.

If individual images are well separated temporally this effect can be minimized.

How do you recognize photodamage when imaging dynamics in living cells?

Compare your imaged cells to their non-imaged neighbors.

Coping with photodamage when imaging dynamics in living cells

1. Optimize the light collection efficiency of your system.
2. Minimize stray light.
3. Accept the lowest possible signal-to-noise needed for your analyses.
4. Optimize the sampling frequency according to the time scale of your dynamics.
5. Collect multiple fields, using computer-controlled stages.

Computer-driven stage controllers

Marzhauser, Inc.

Live cell microscopy

1. Why do live cell microscopy?
2. Maintaining living cells on a microscope stage.
3. Considerations for imaging living cells.
4. Fluorescence labeling of living cells.

Fluorescent labeling of cells

- Immunofluorescence
- Protein-binding toxins
- DNA intercalating dyes
- DNA hybridization
- Permeant dyes
- Membrane partitioning dyes
- Endocytosis of fluorescent molecules
- Microinjection of fluorescent molecules
- Expression of fluorescent chimeras

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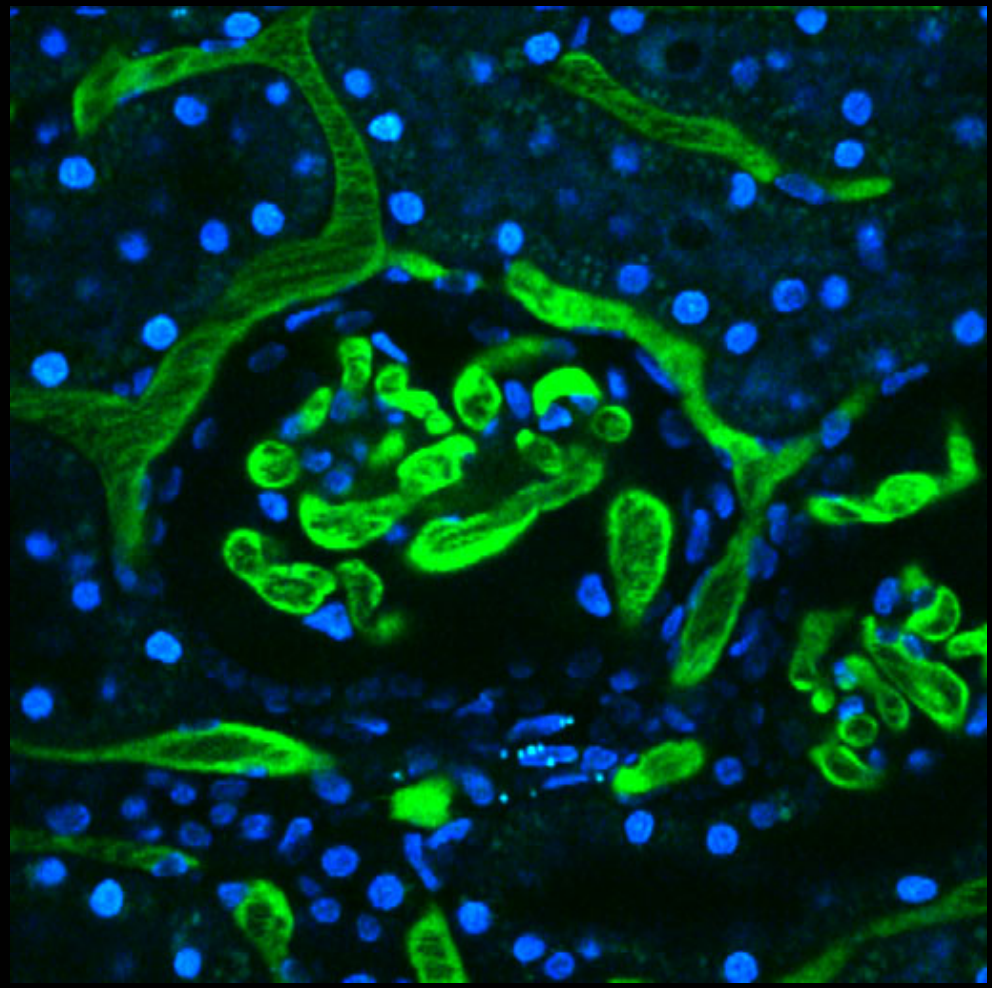
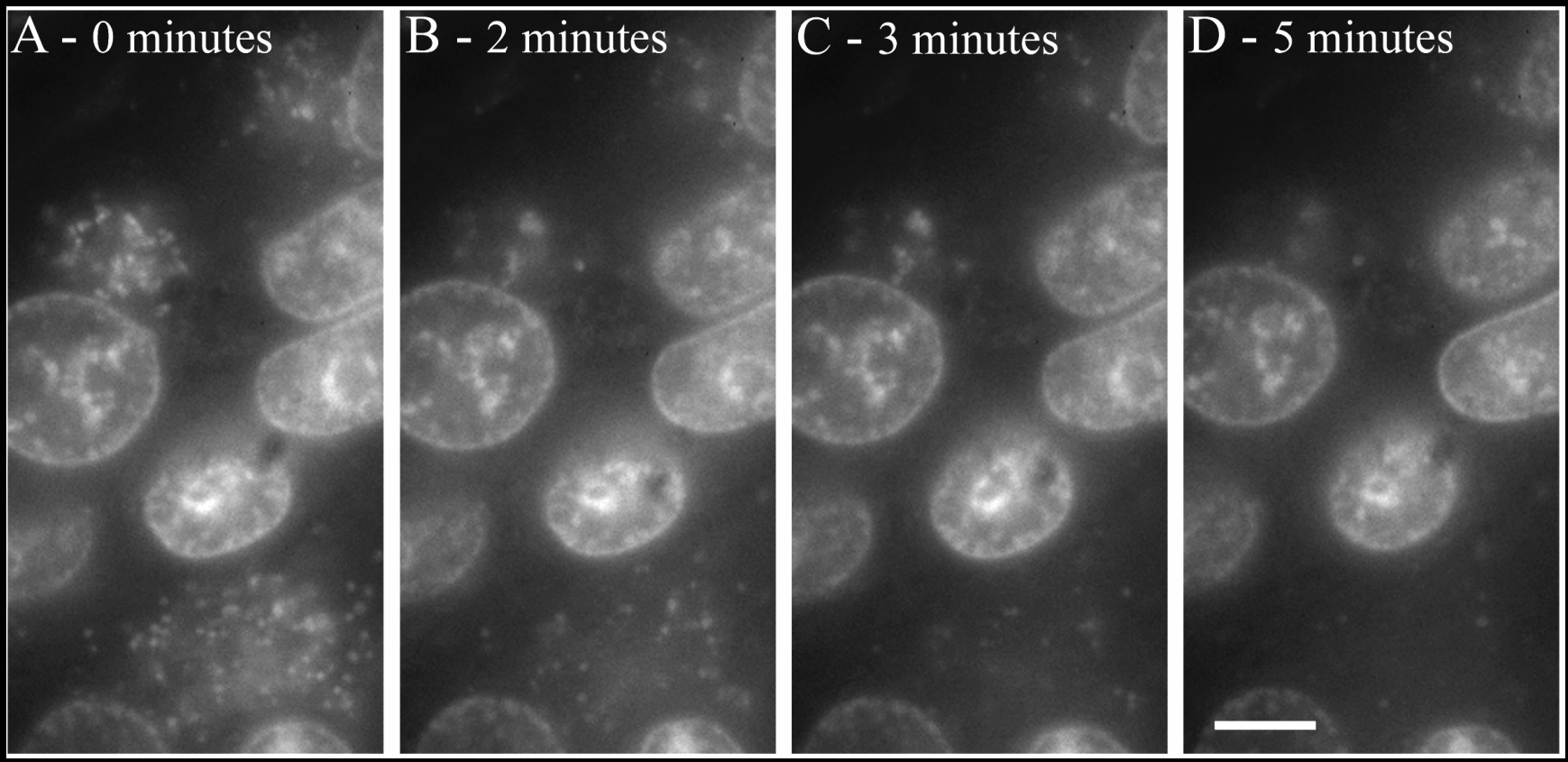
DNA intercalating dyes – Hoechst-labeled nuclei in a living rat



Permeant probes – pH-sensitive lysosomal accumulation of fluorescent doxorubicin



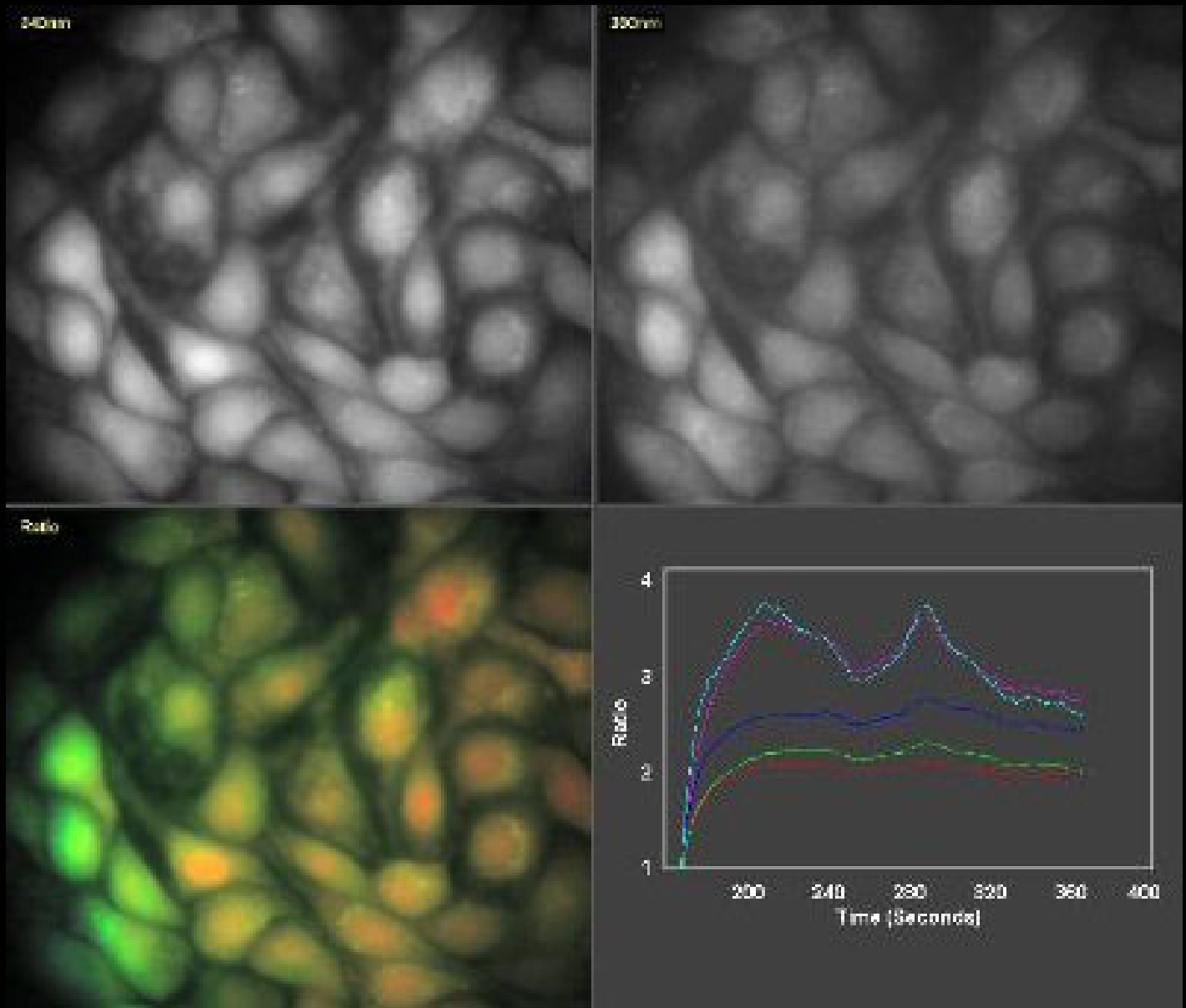
Time series following addition of methylamine

Permeant probes –
Loading cells with acetoxymethyl esters

340 nm

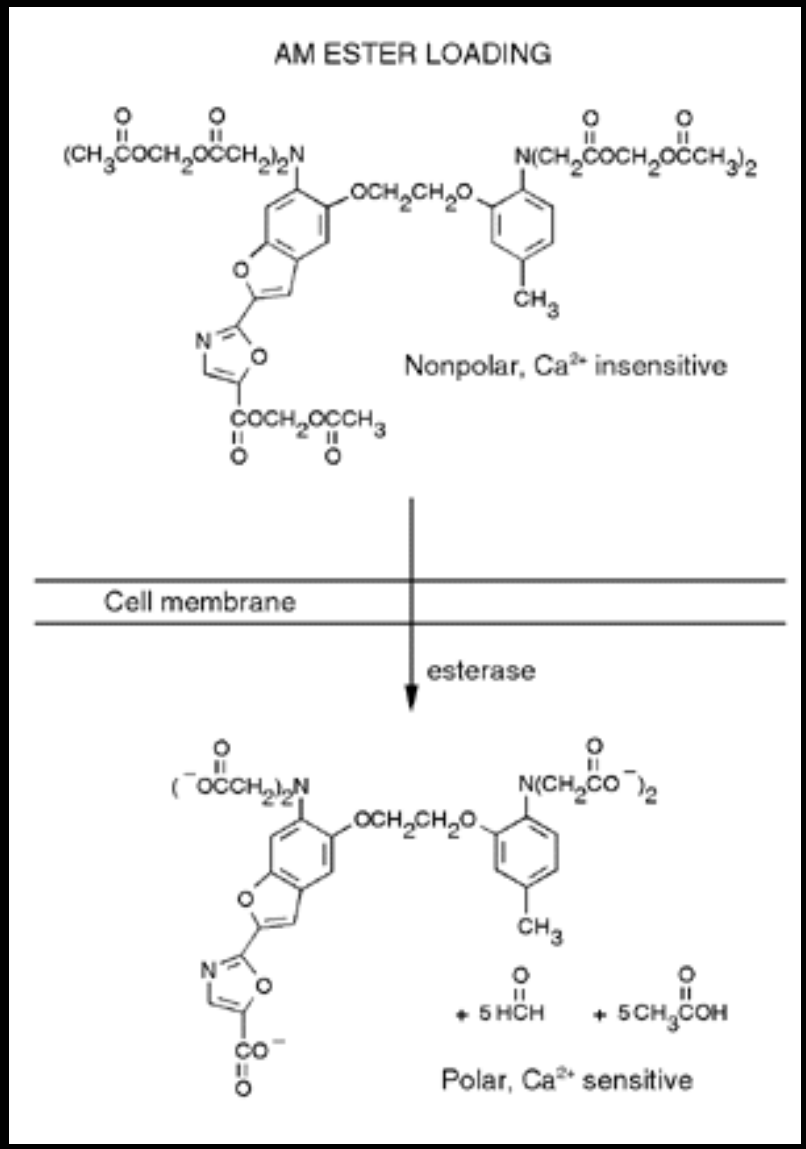
380 nm

340/380



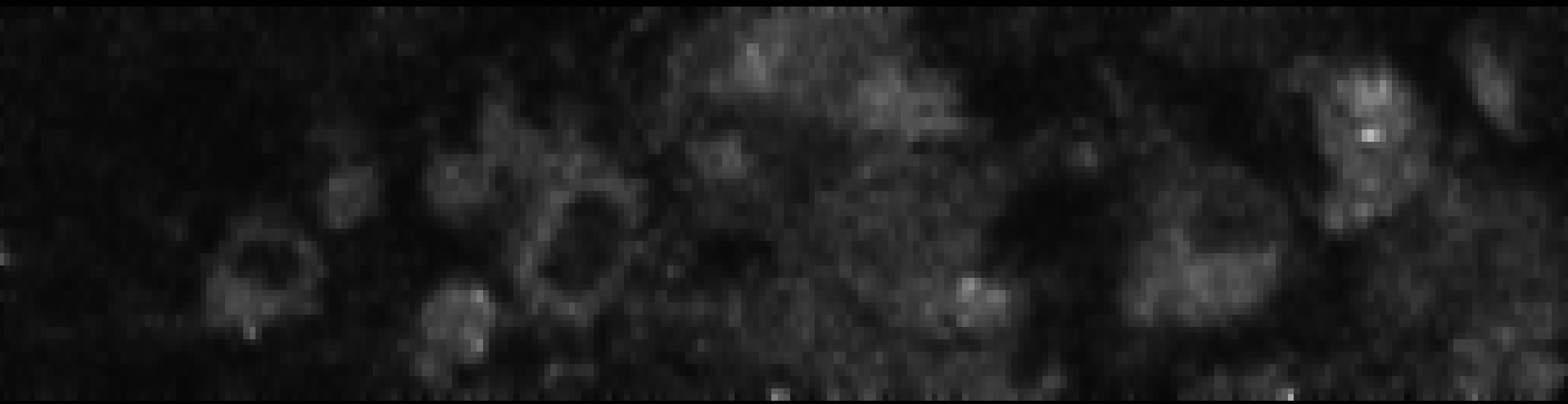
- Universal Imaging

Permeant probes – Loading cells with acetoxymethyl esters



- Molecular Probes, Inc

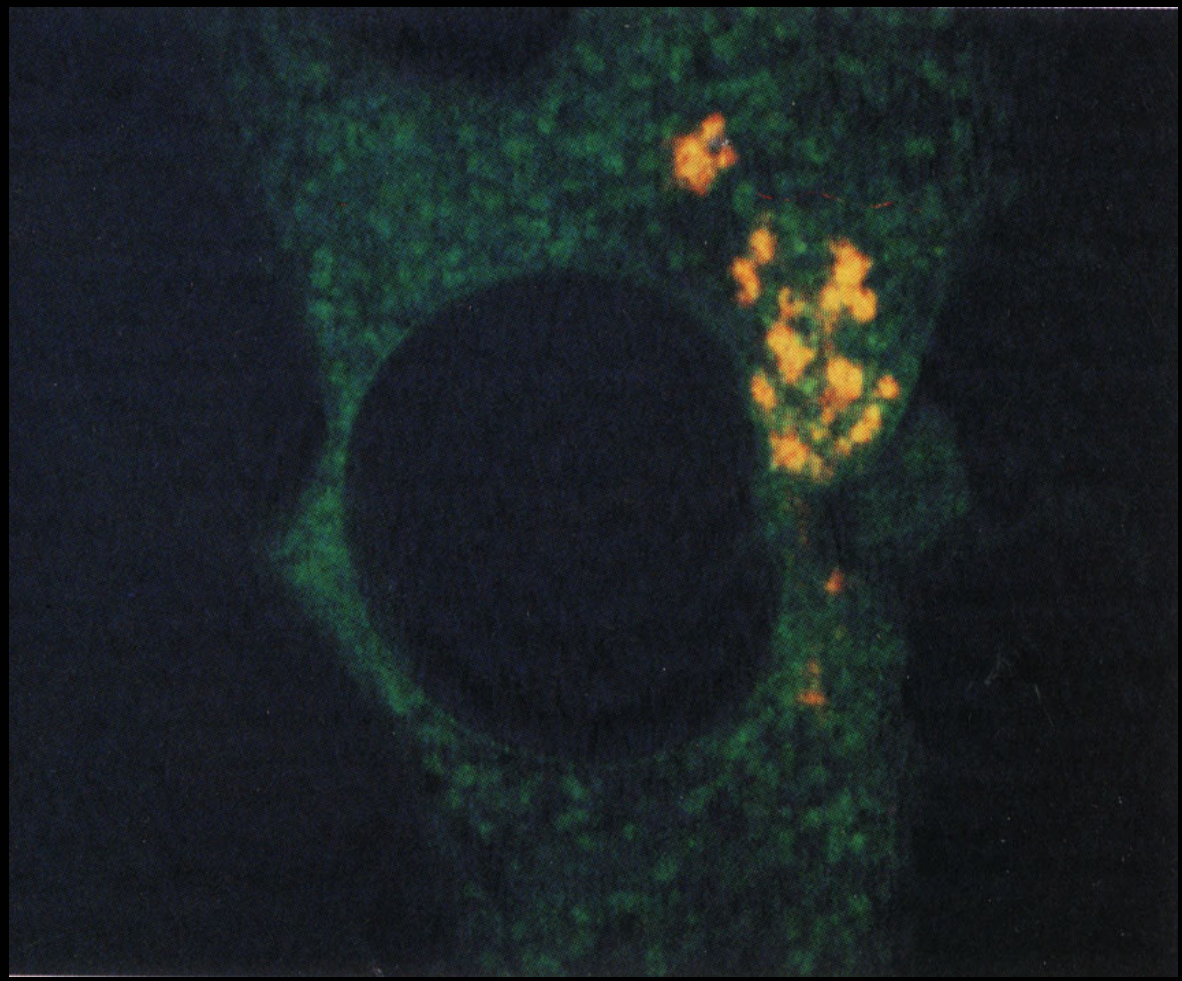
Permeant probes –
Loading cells with acetoxymethyl esters



Fluo-3 imaging of spontaneous calcium spikes in heart cells

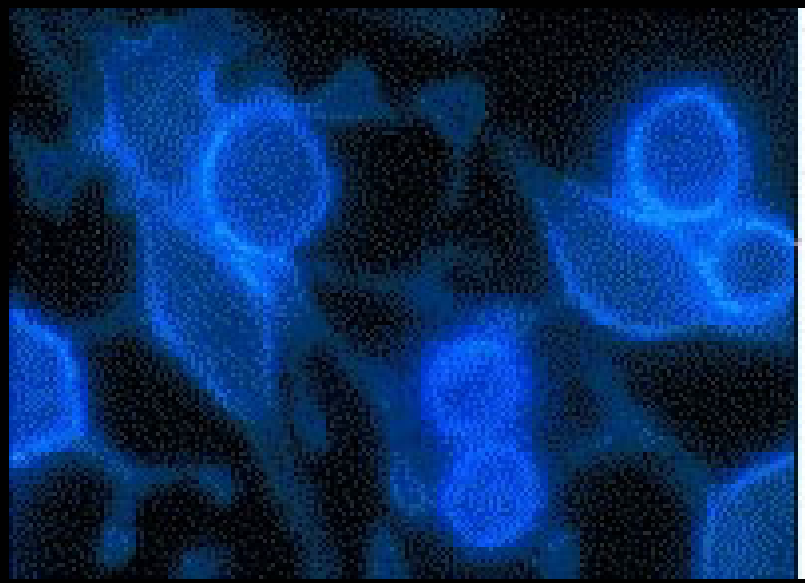
Mark Wagner

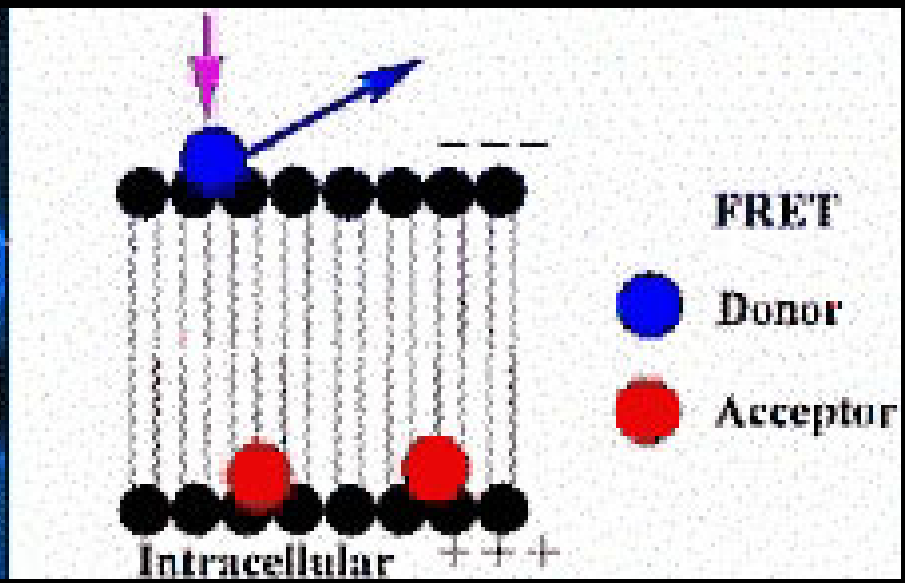
Membrane partitioning probes –
Bodipy-ceramide in the Golgi apparatus of living CHO cells



- Satyajit Mayor

Membrane partitioning probes –
Detecting changes in membrane polarity with FRET

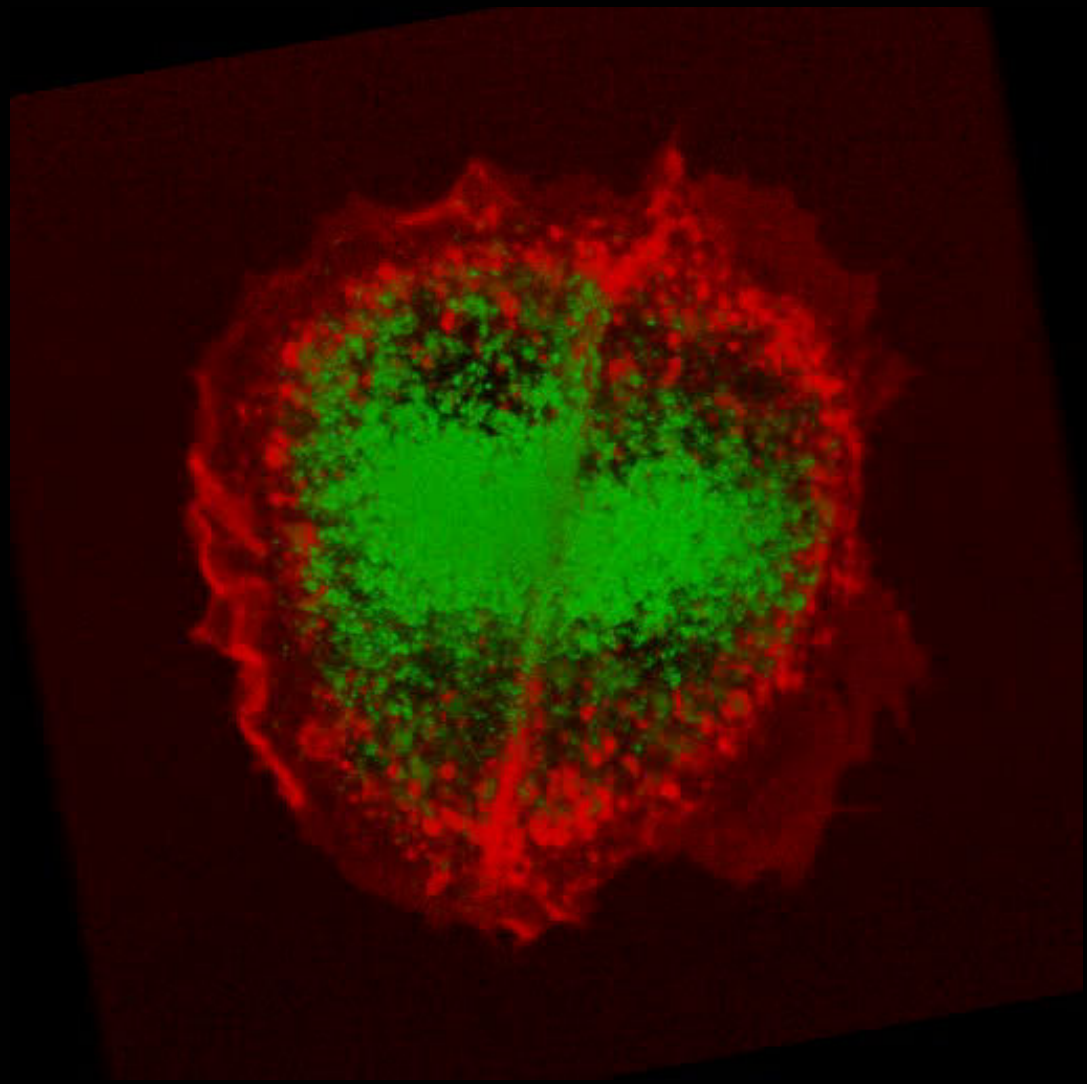




Coumarin labeled phospholipid – immobile in outer leaflet
Oxonol – charged, mobile in membrane

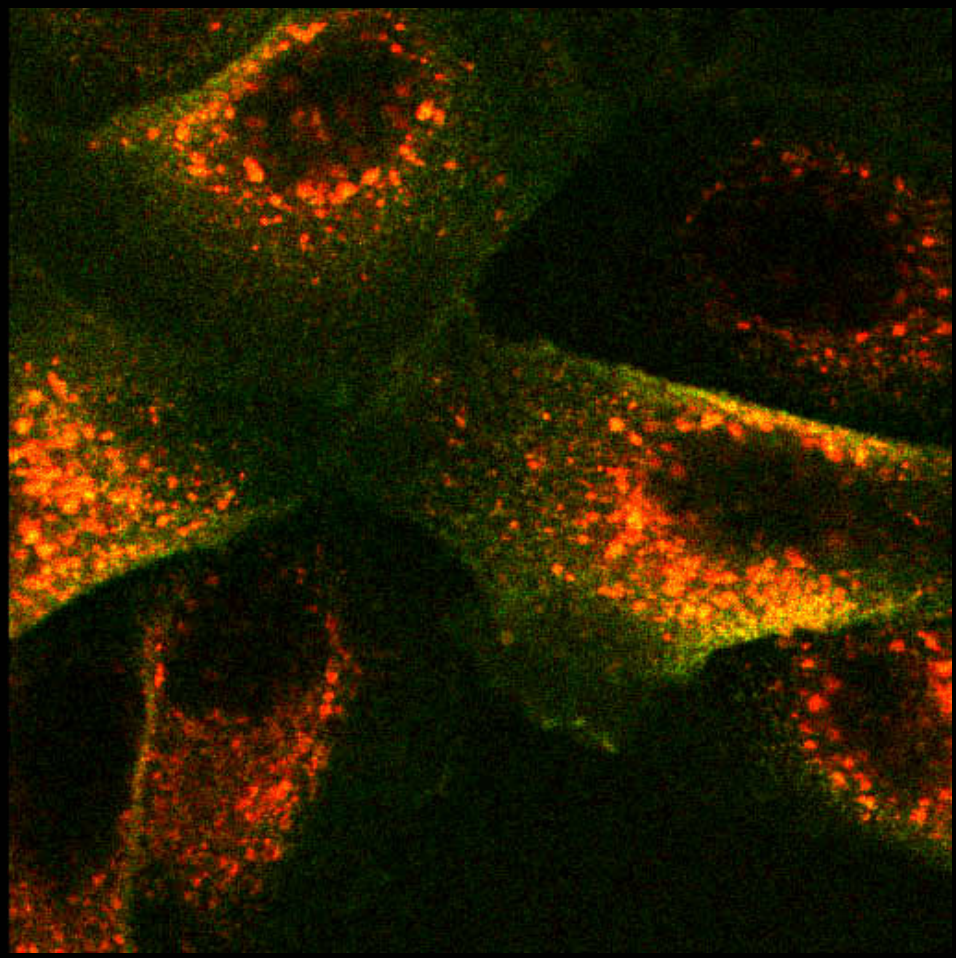
Gonzalez et al., 1997

Endocytosis of fluorescent probes by living cells



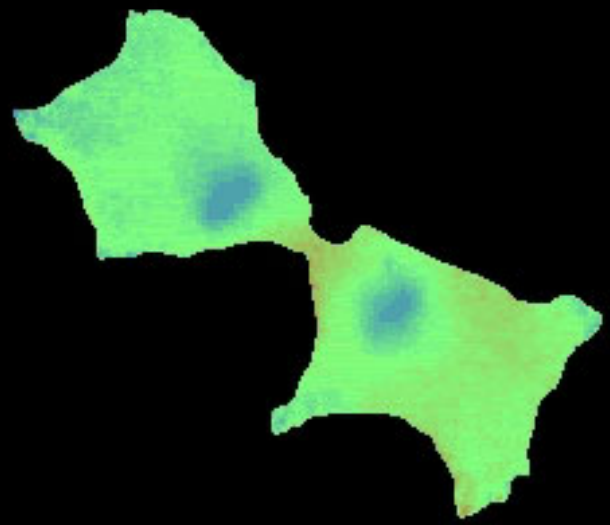
Internalized transferrin and GFP-Rab25 in living MDCK cells

Endocytosis of fluorescent probes by living cells



Optical sectioning of confocal allows distinction of extracellular (neutral) FR-Tf from endosomal (acidic) FR-Tf

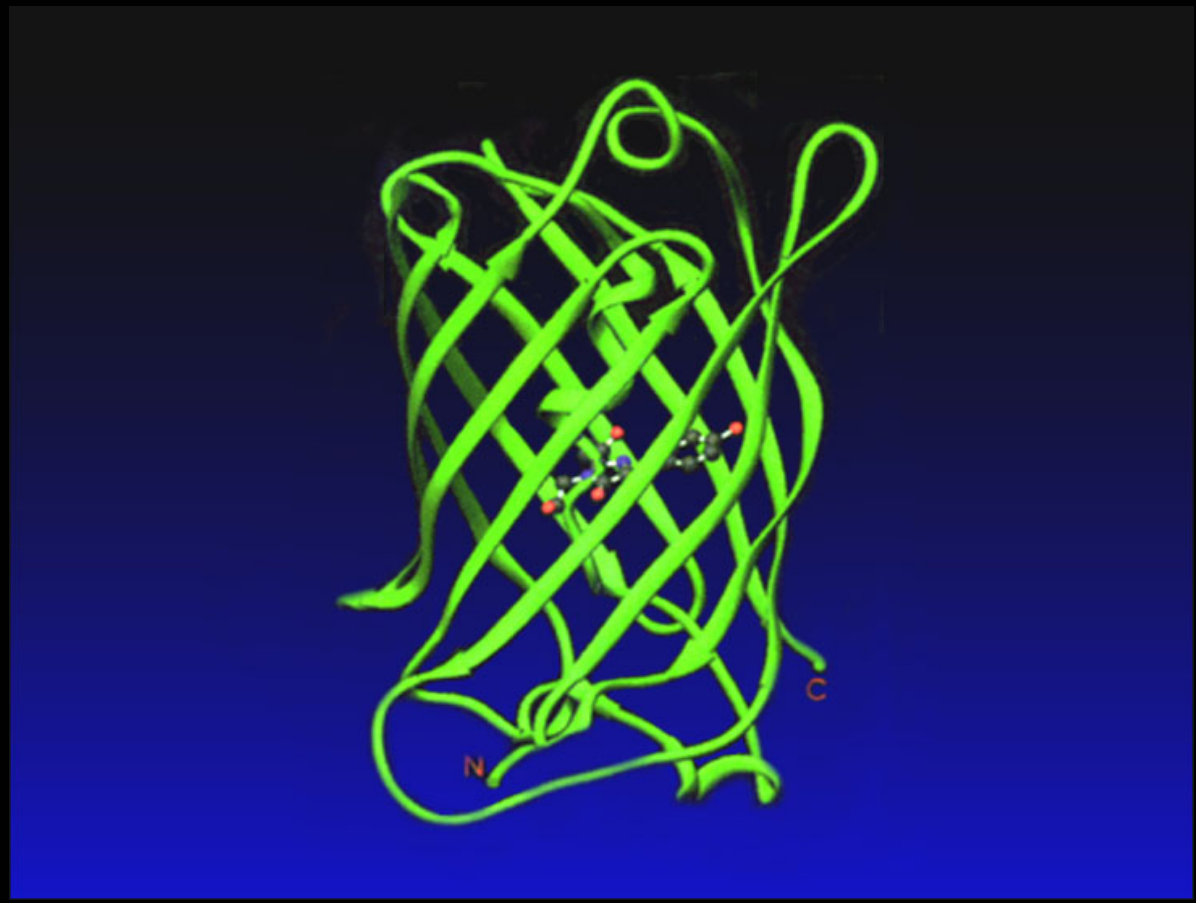
Microinjection of fluorescent probes



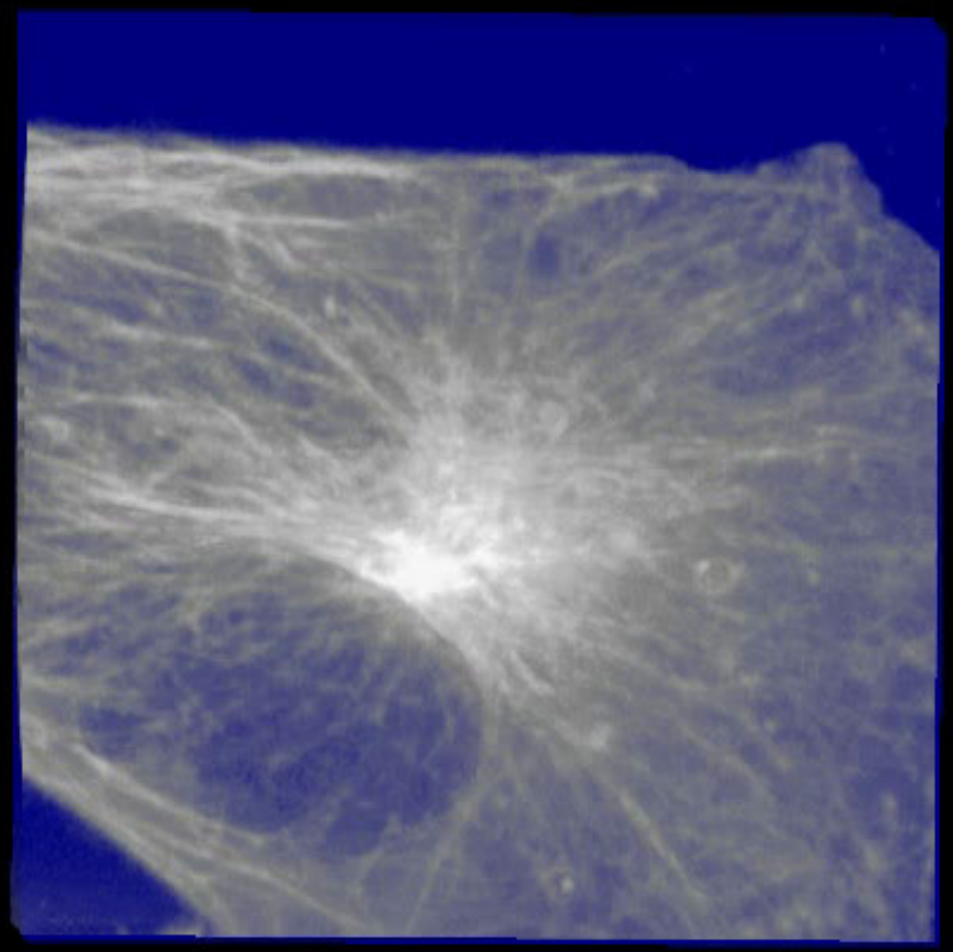
Ratio of rhodamine myosin II to cy2dextran

DeBiasio et al., 1996. MBC (Lance Taylor lab)

Expression of fluorescent protein chimeras – Green Fluorescent Protein



Expression of fluorescent protein chimeras – expression of GFP-tubulin in living CHO cells



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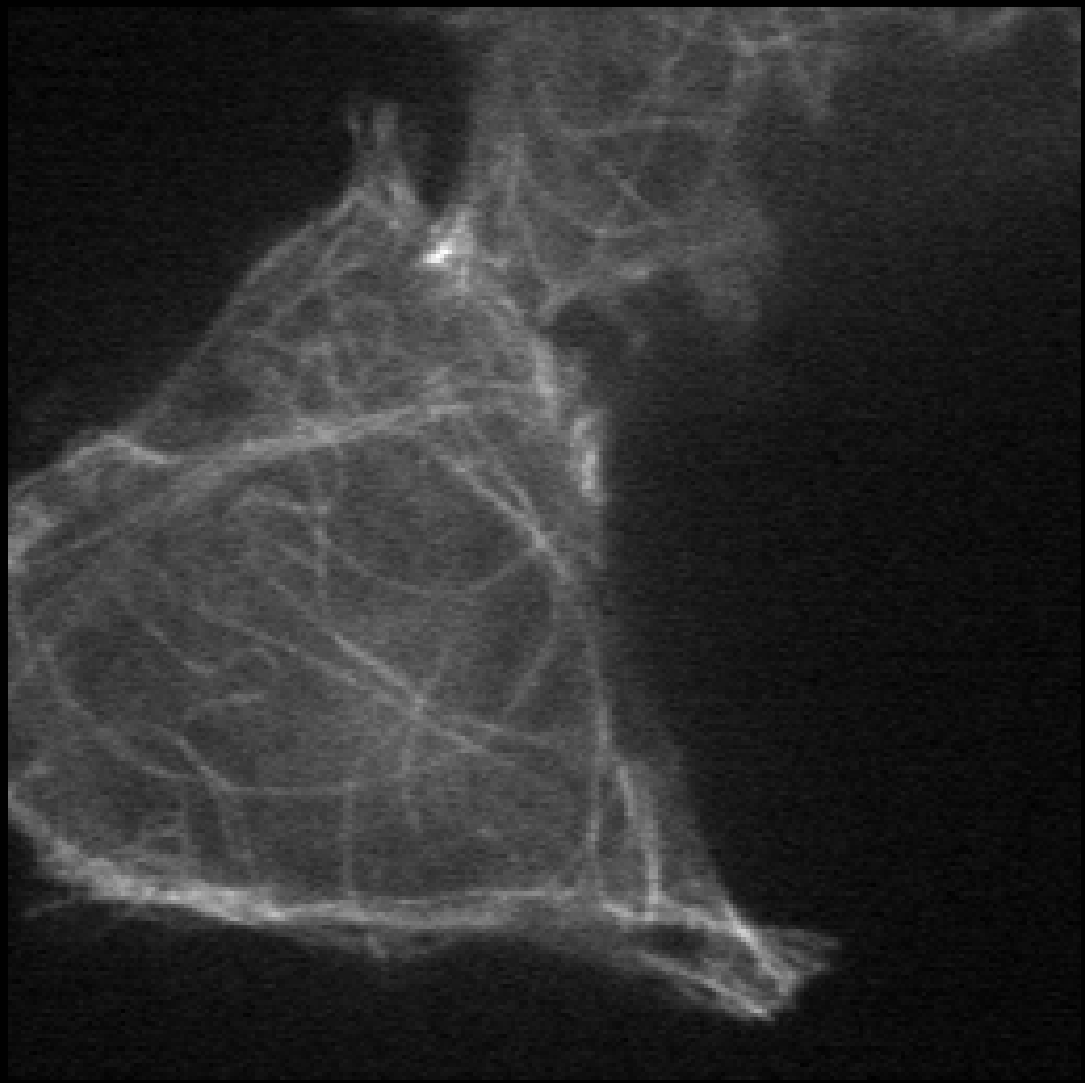

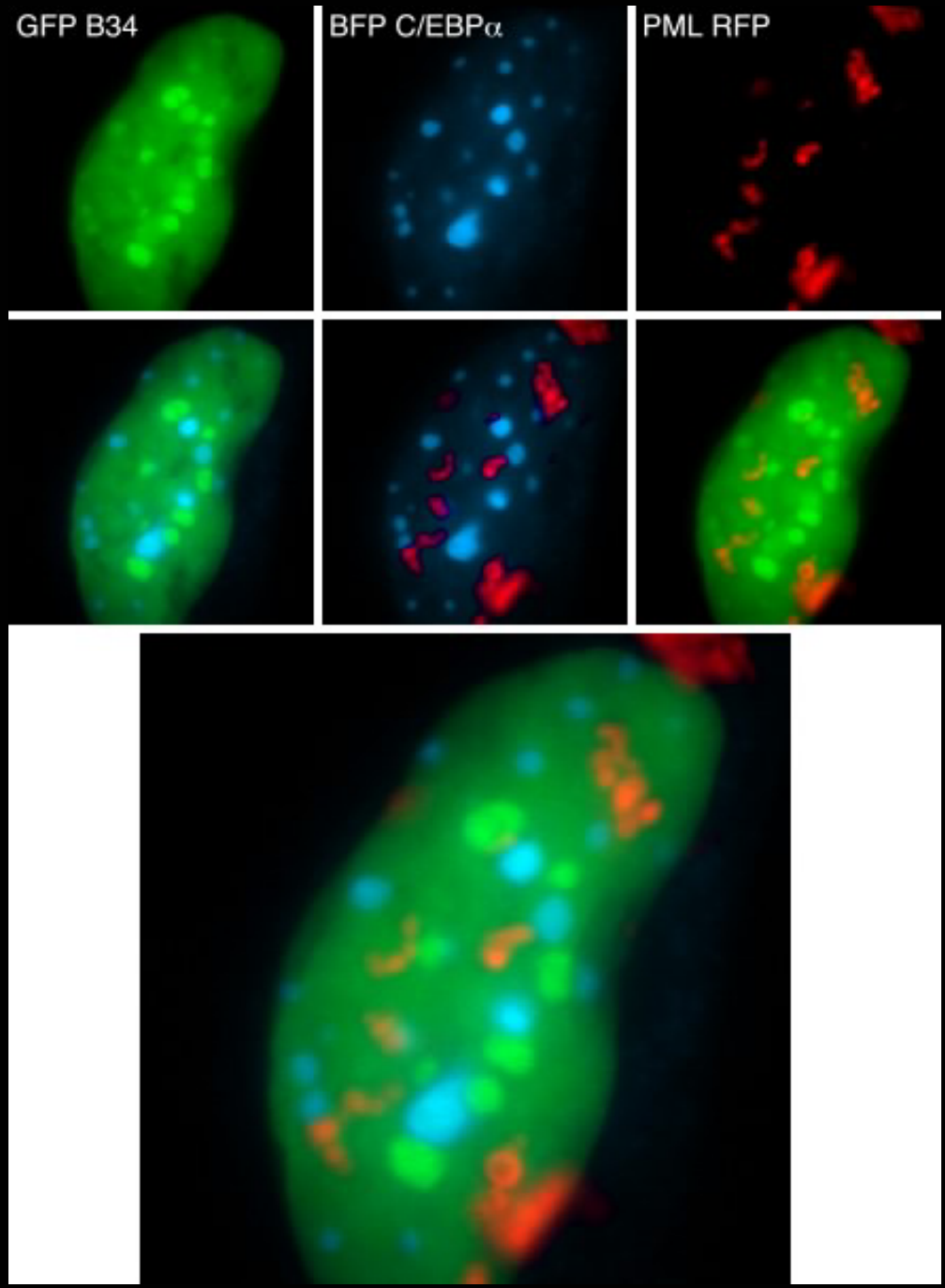

Three Color "GFP" Imaging

BFP
GFP
RFP

Rich Day
Univ. of Virginia

Live cell microscopy

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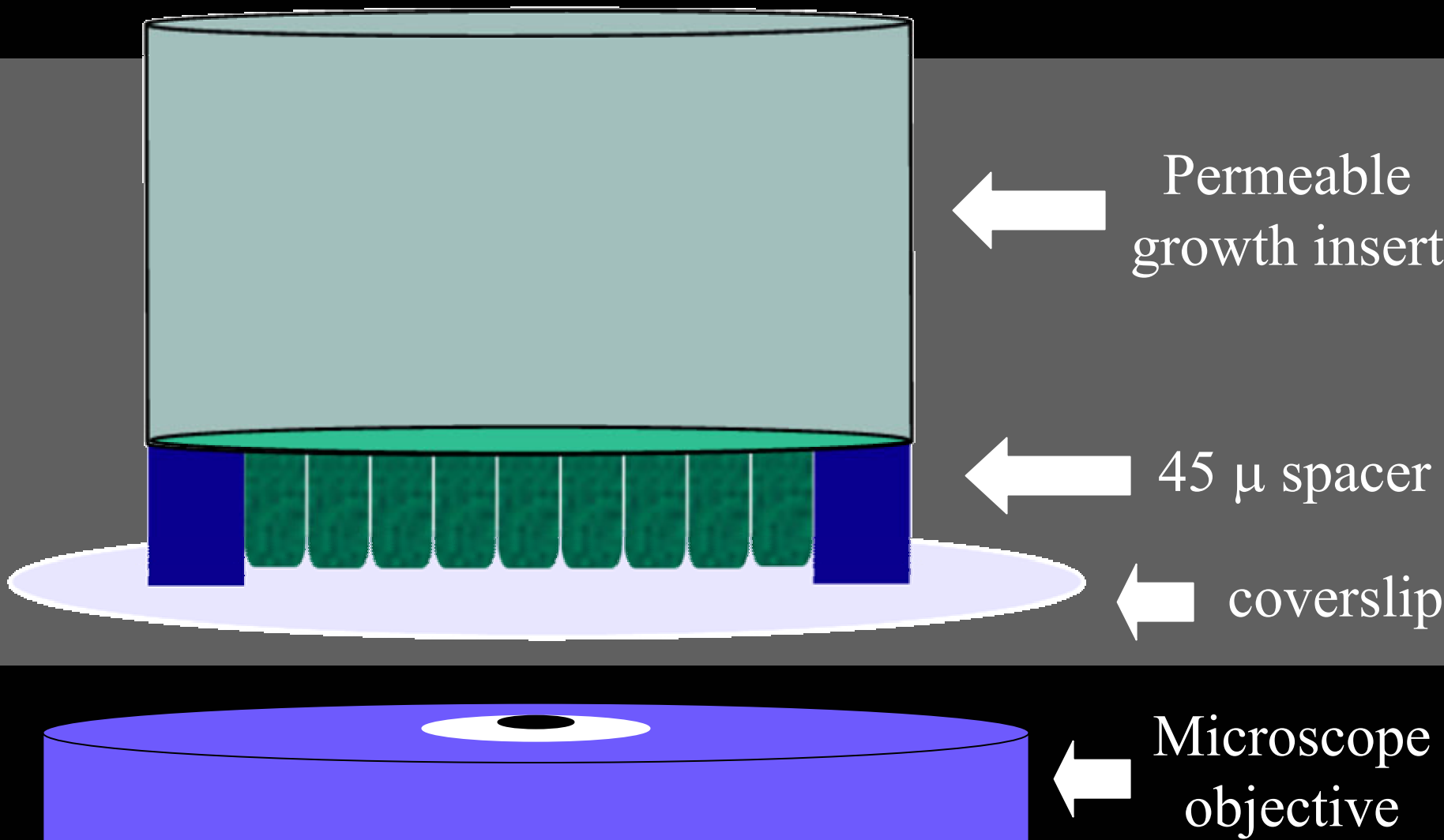
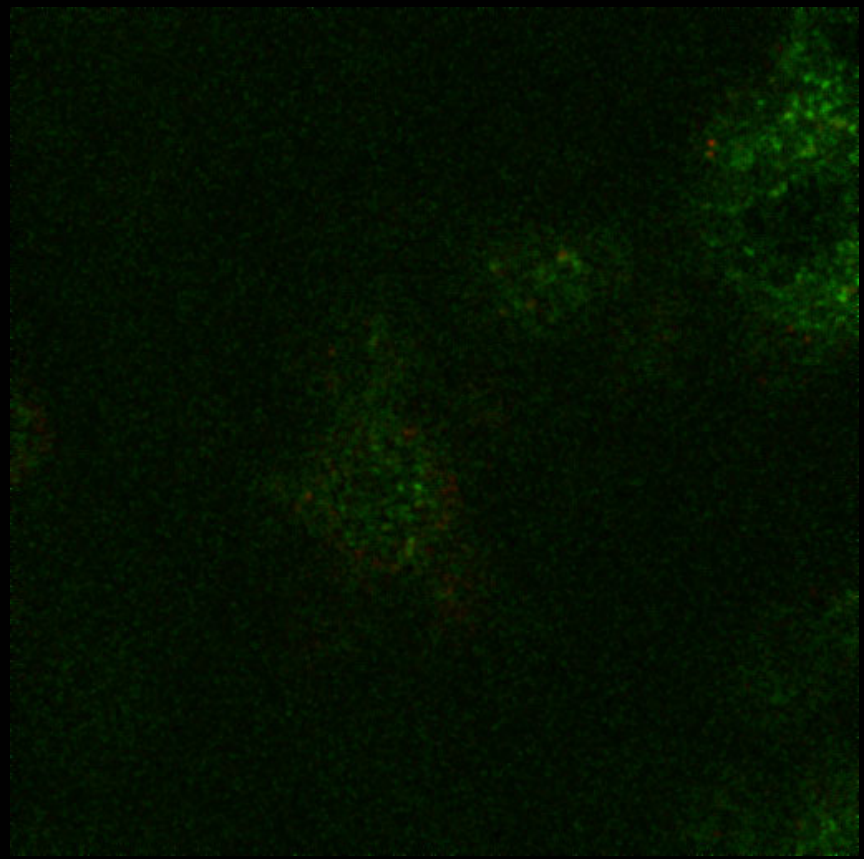
2. Maintaining living cells on a microscope stage.
3. Considerations for imaging living cells.
4. Fluorescence labeling of living cells.
5. Imaging polarized cells on permeable filters.

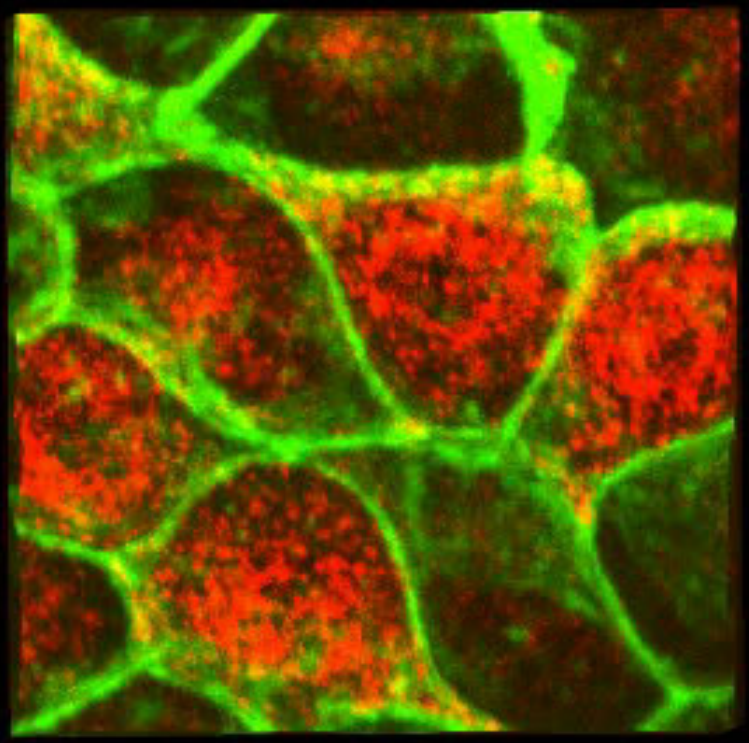
Approach – Fluorescence microscopy of cells incubated with fluorescent ligands



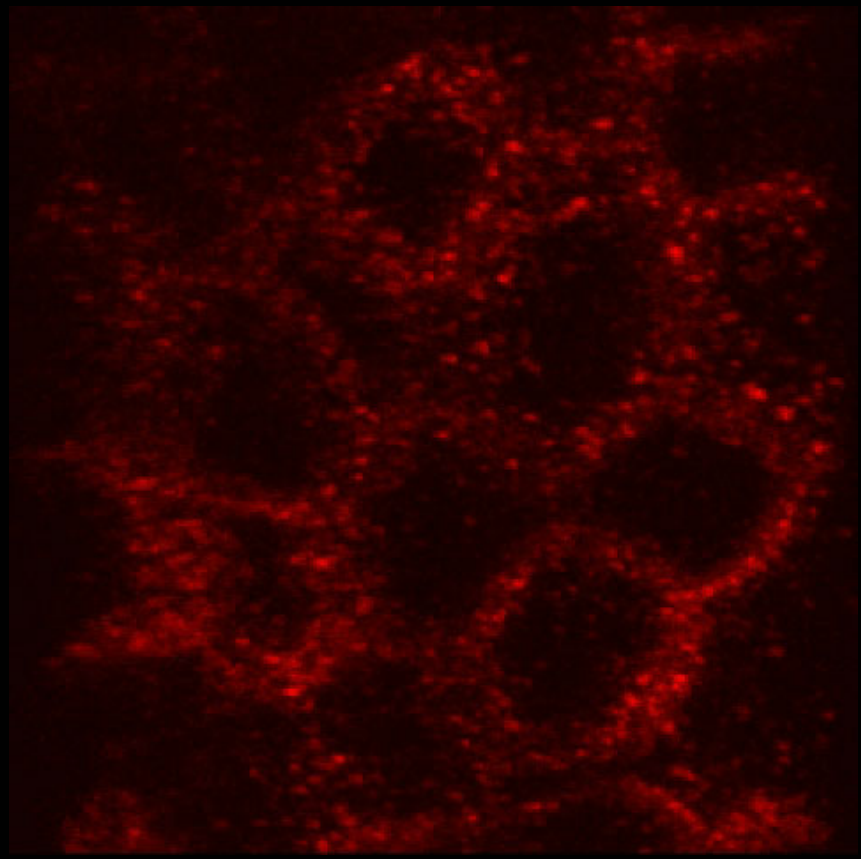
Serial optical sections of LDL and transferrin in endosomes of polarized epithelial cells



3D microscopy of polarized MDCK cells – internalized IgA and anti-cadherin

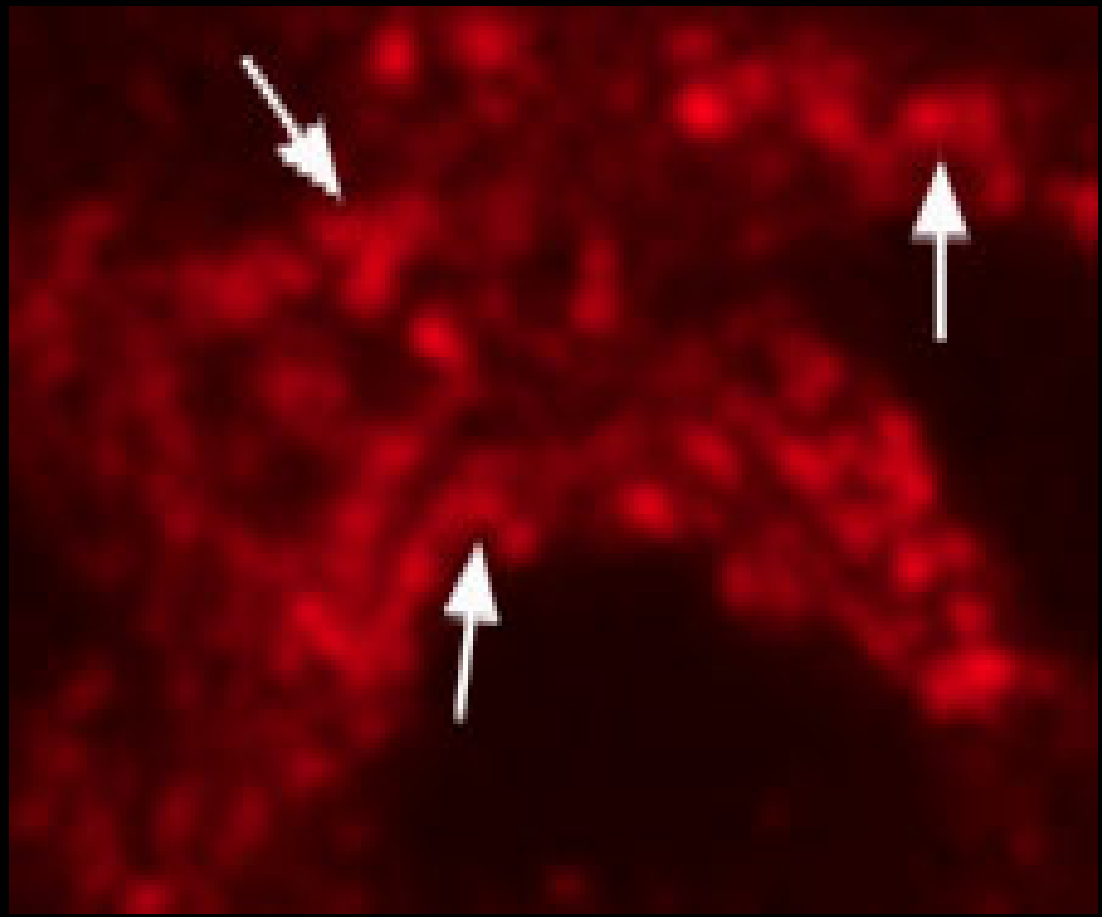


Transferrin endocytosis in polarized MDCK cells



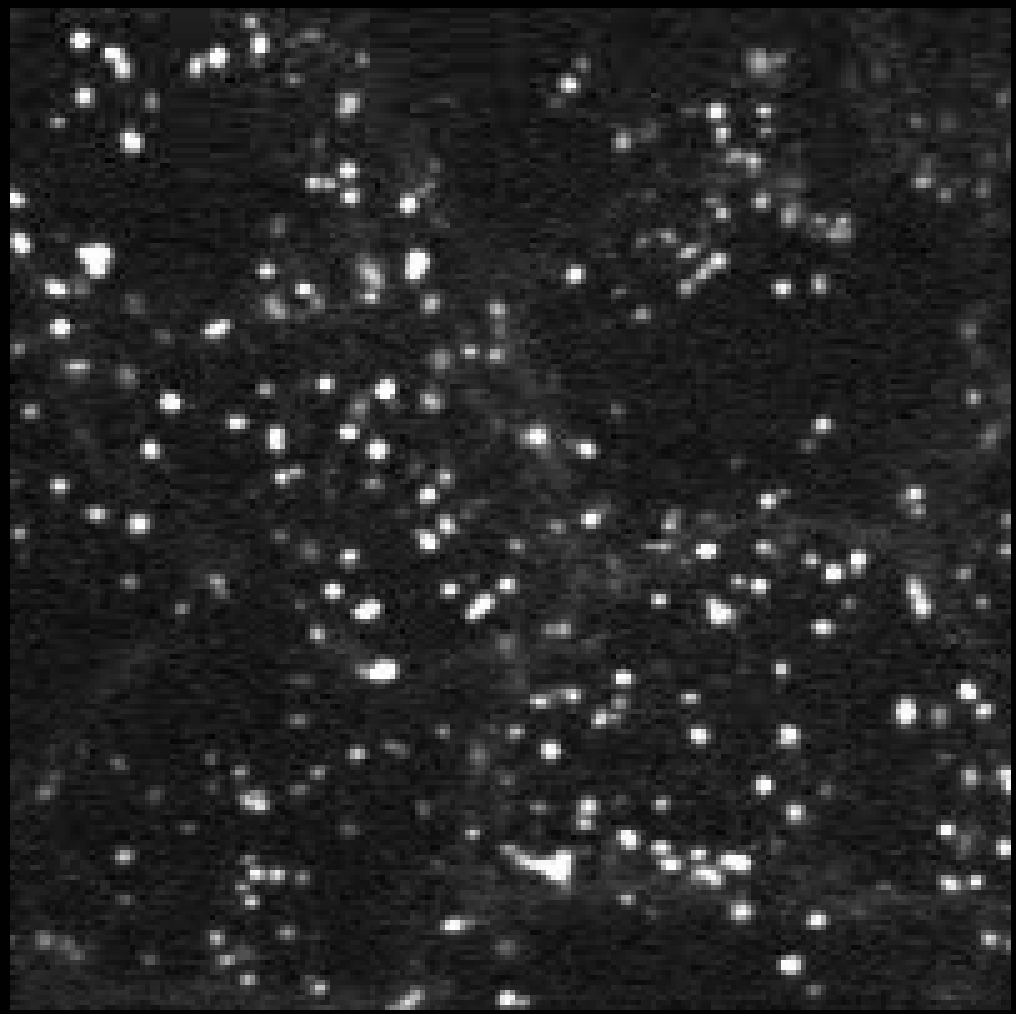
After 2 minutes exposure
to fluorescent transferrin

Live cell microscopy of polarized MDCK cells on permeable supports

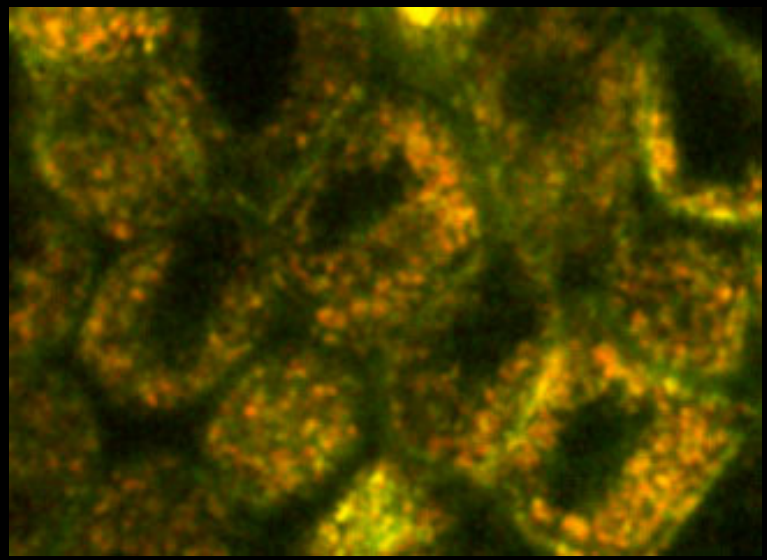


Apical IgA (green) and basal
Tf (red) - medial plane

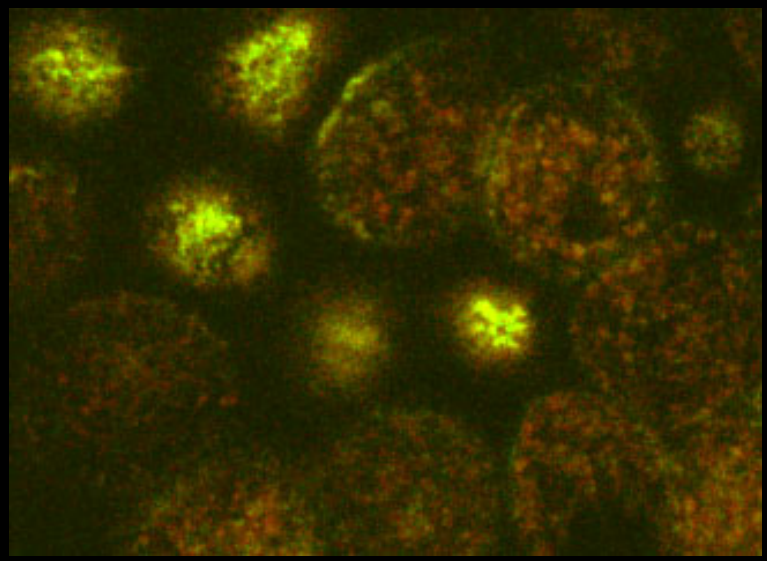
Live cell microscopy of polarized MDCK cells on permeable supports



Live cell microscopy of polarized MDCK cells on permeable supports



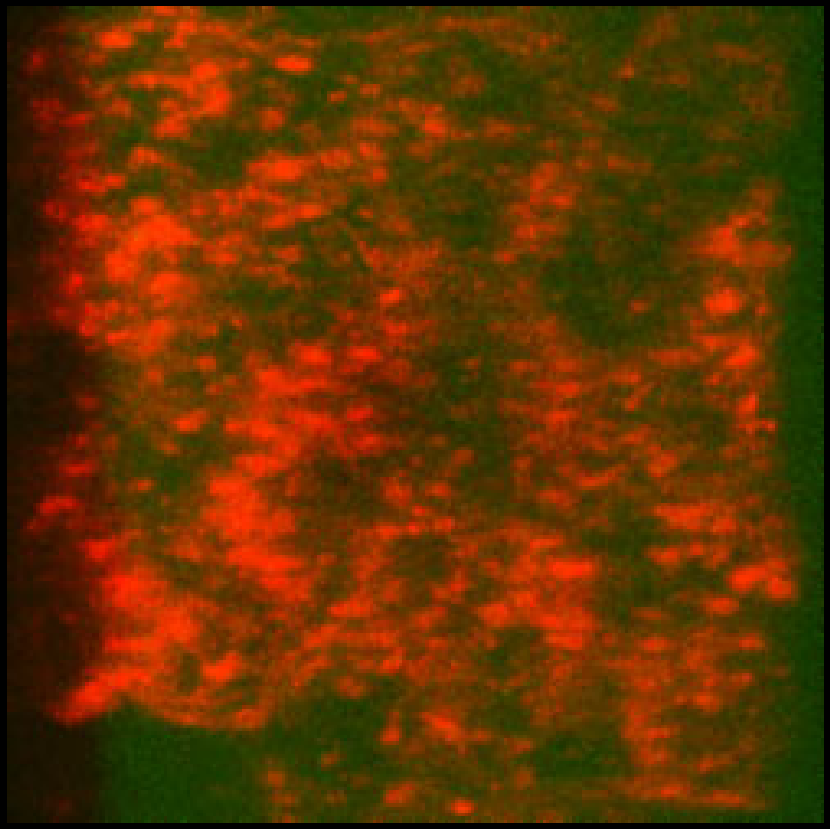
Medial plane



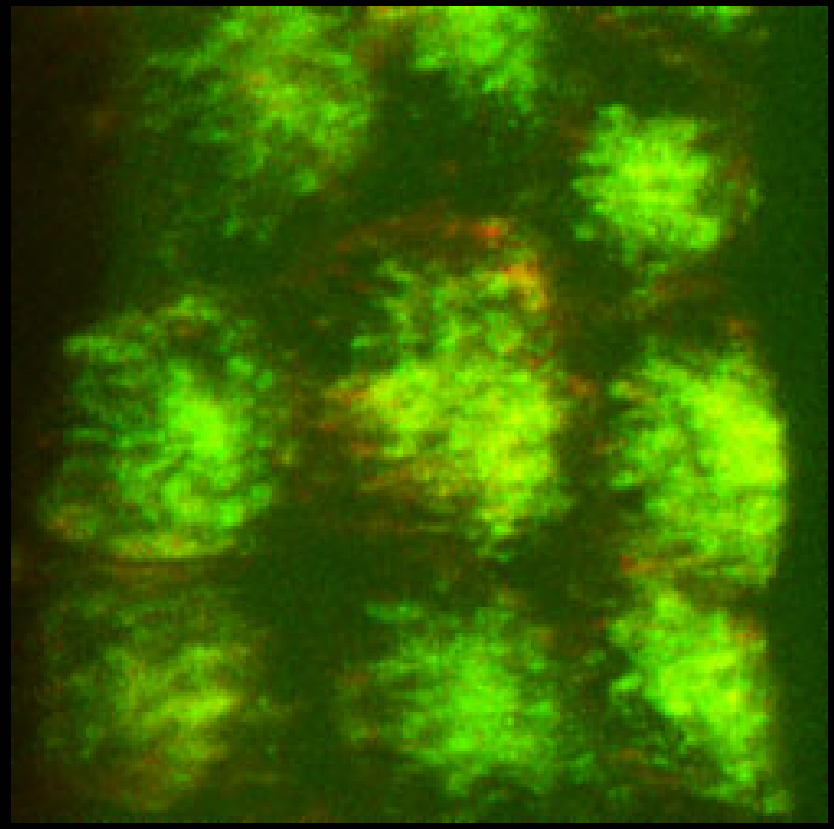
High plane

- Cells labeled with pH-sensitive IgA conjugated to both fluorescein and rhodamine
- Medial endosomes are acidic (red), but AREs are relatively alkaline (green)

Live cell microscopy of polarized MDCK cells on permeable supports

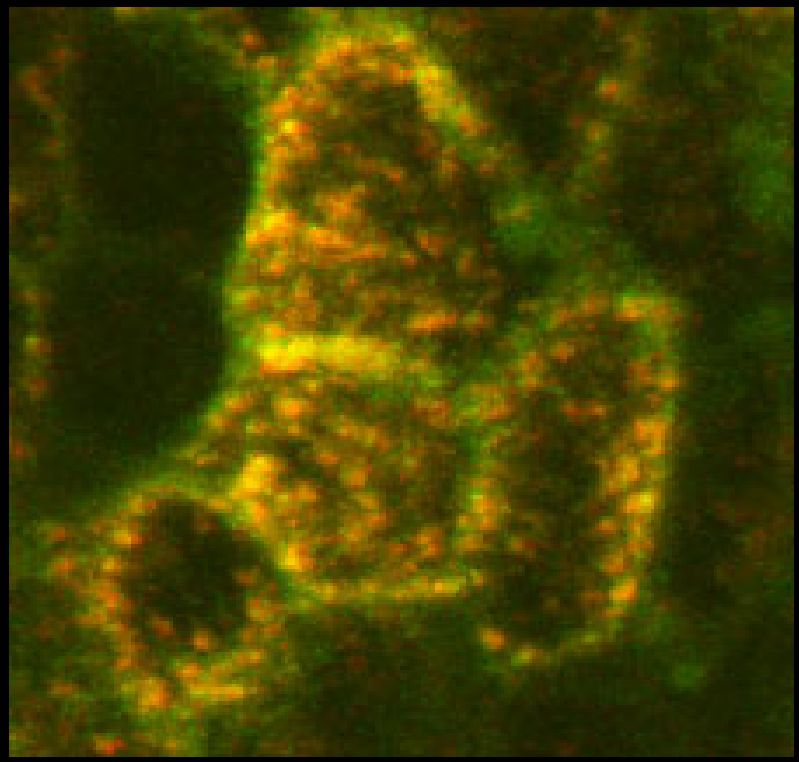


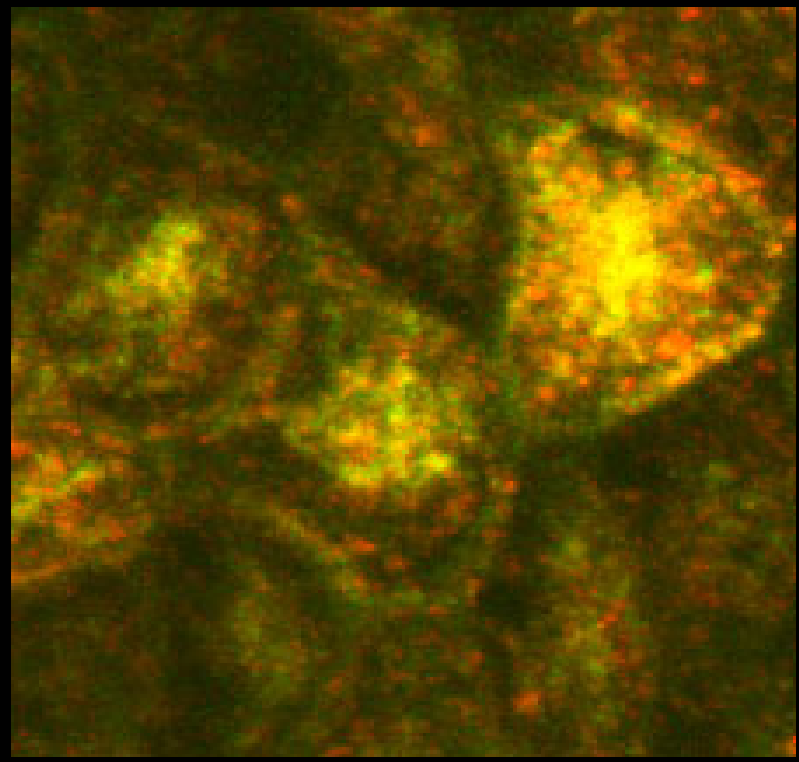
30 min. in red basolateral Tf and green apical Tf



10 μM BFA for 15 min., then 30 min. with basolateral and apical Tf

Live cell microscopy of polarized MDCK cells on permeable supports



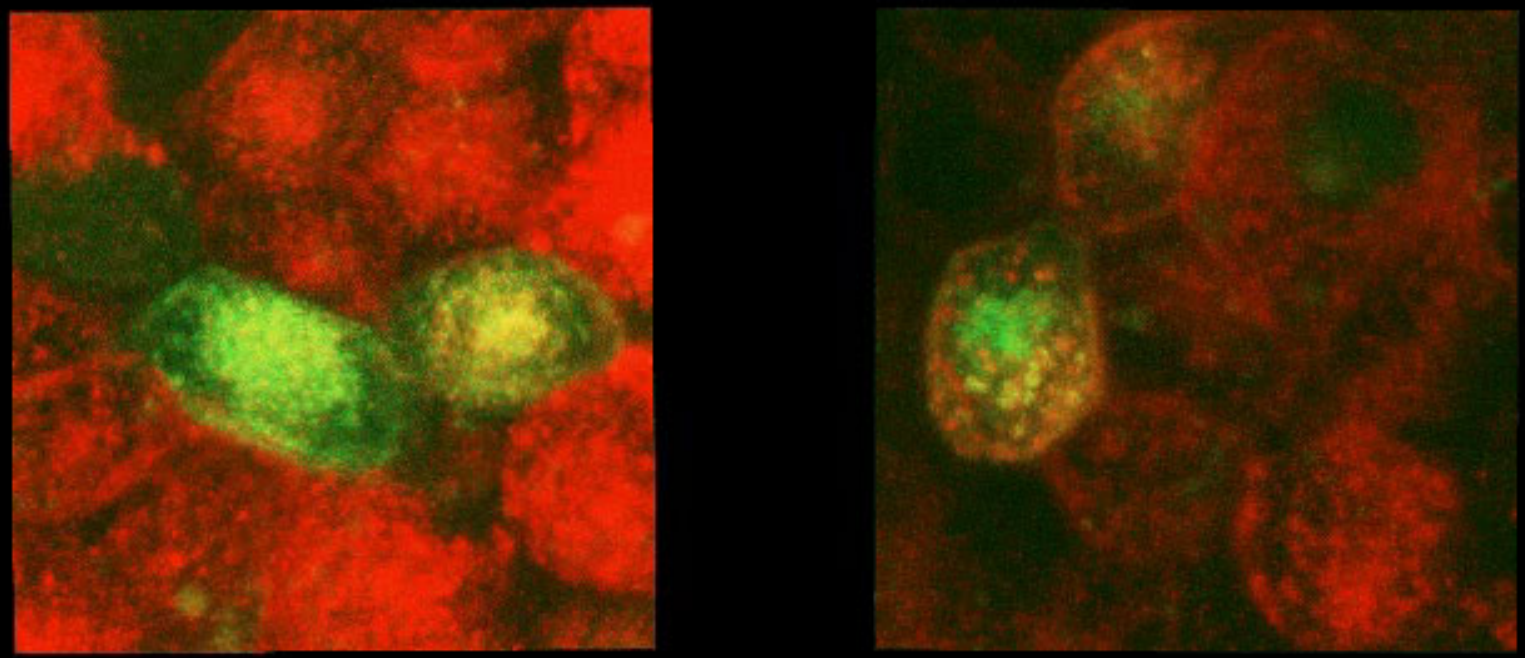


control

10 μM BFA

Living cells in basolateral F-R-transferrin

Live cell microscopy of polarized MDCK cells on permeable supports



Endotubin, an apical endosome marker, colocalizes completely with apically internalized IgA, and in common endosomes with basolaterally internalized transferrin

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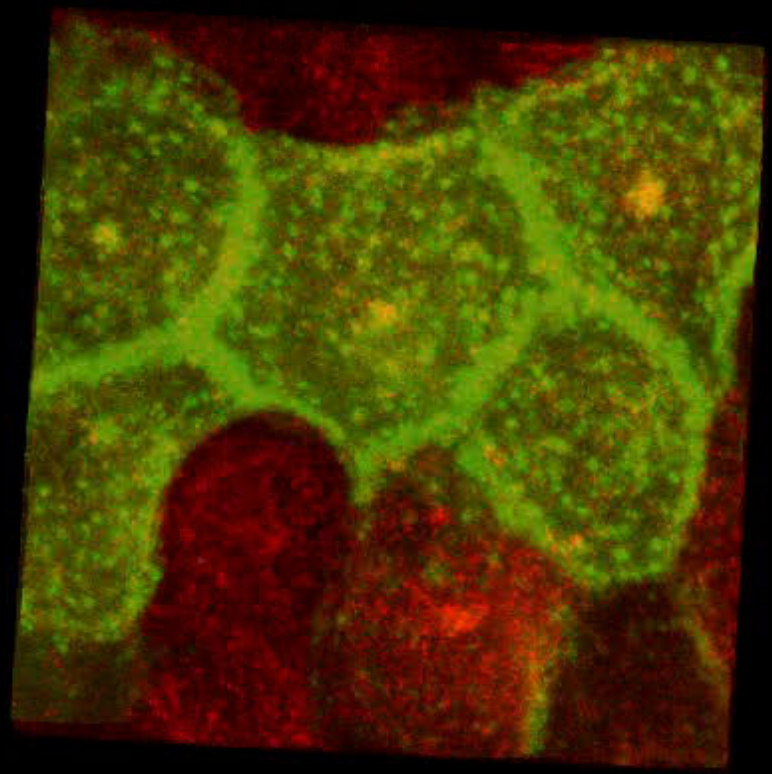
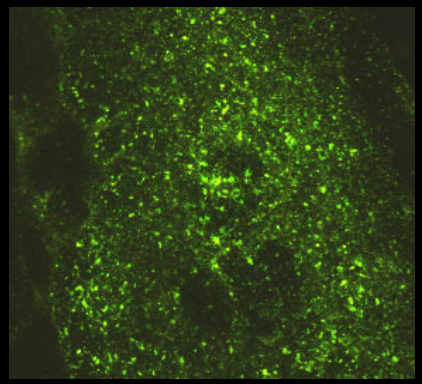


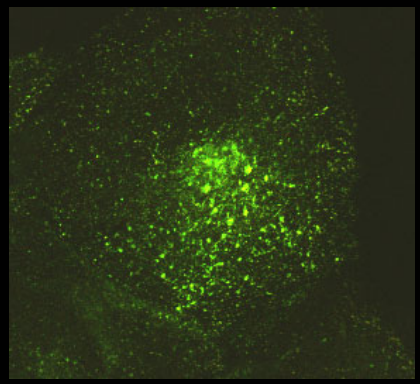
Rme-1 and IgA

Imaging living cells on filters – use a water immersion objective

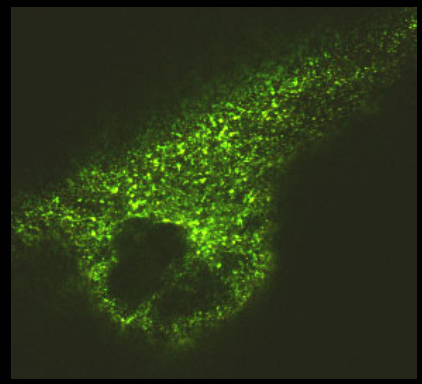
60X water immersion



0 microns

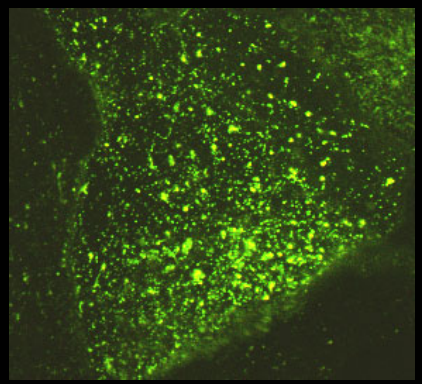


50 microns

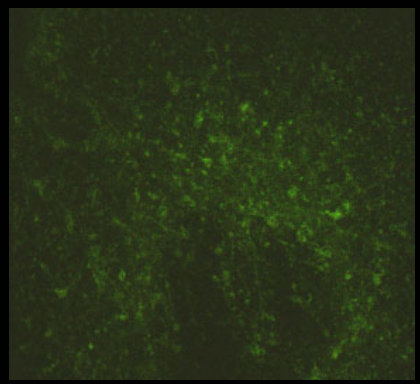


60 microns

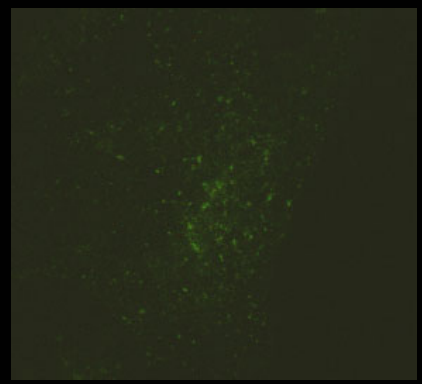
100X Oil immersion



0 microns



40 microns



50 microns

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- Inverted.

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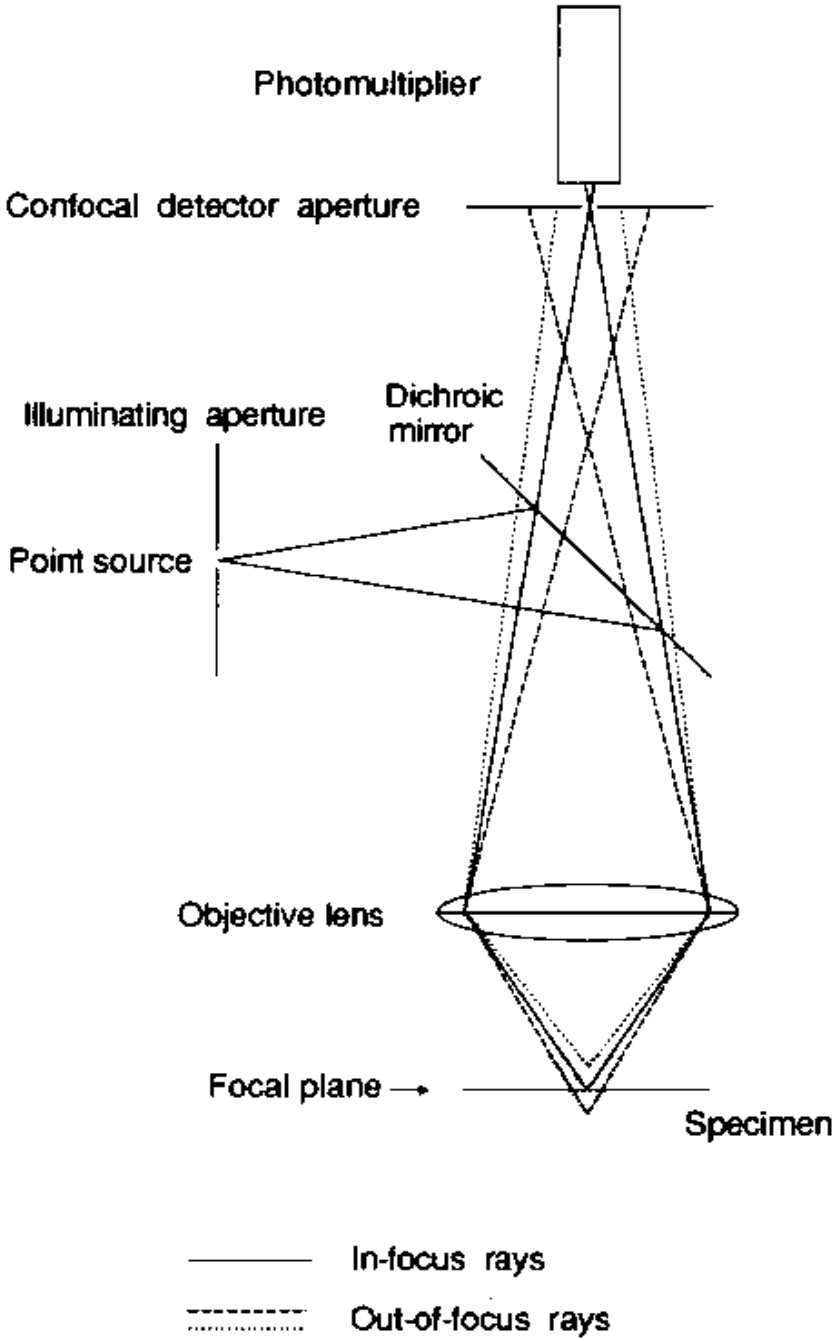

Spinning disk systems

Pinhole crosstalk

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2. Multicolor images must be collected sequentially.
3. Single mode fiber optic – non-uniform illumination.

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