Michael Cammer, MAT, MFA
Director of Light Microscopy & Image Analysis
Analytical Imaging Facility
Albert Einstein College of Medicine
Bronx, NY

The Shared Microscope Resource: A Central Spot for Research

Why Shared Resources?

- **Cost Effective**
  Economy of Scale – Cost Sharing
  Competitive for R01 & Center Grant Support
  Competitive for SIGs
- **Technical Expertise Centralized**
- **Cross Fertilization of Ideas**

THE FASEB JOURNAL 1999;13:595-601

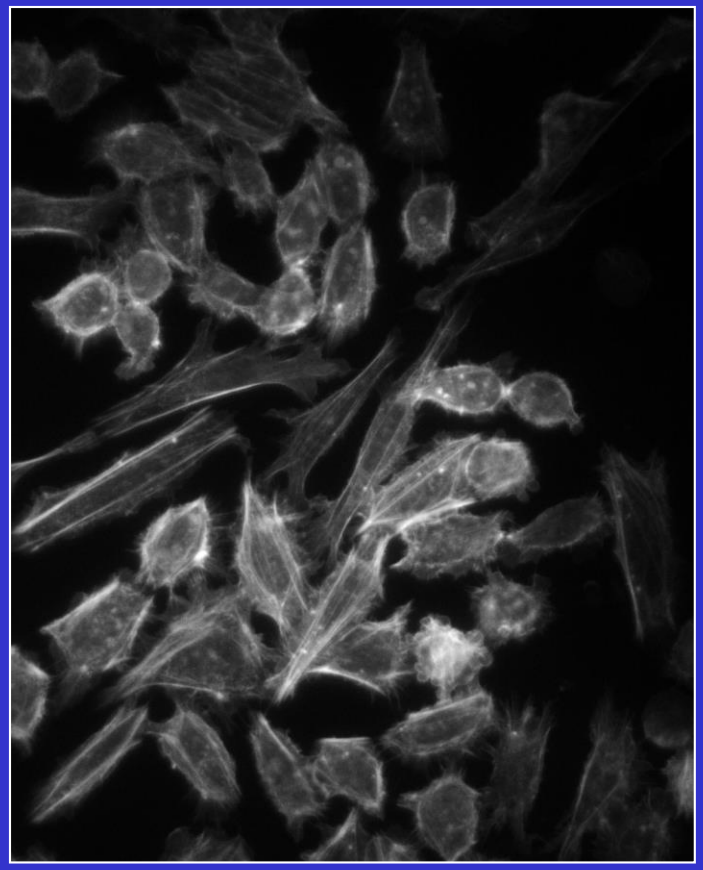
Research Technologies: Fulfilling the Promise

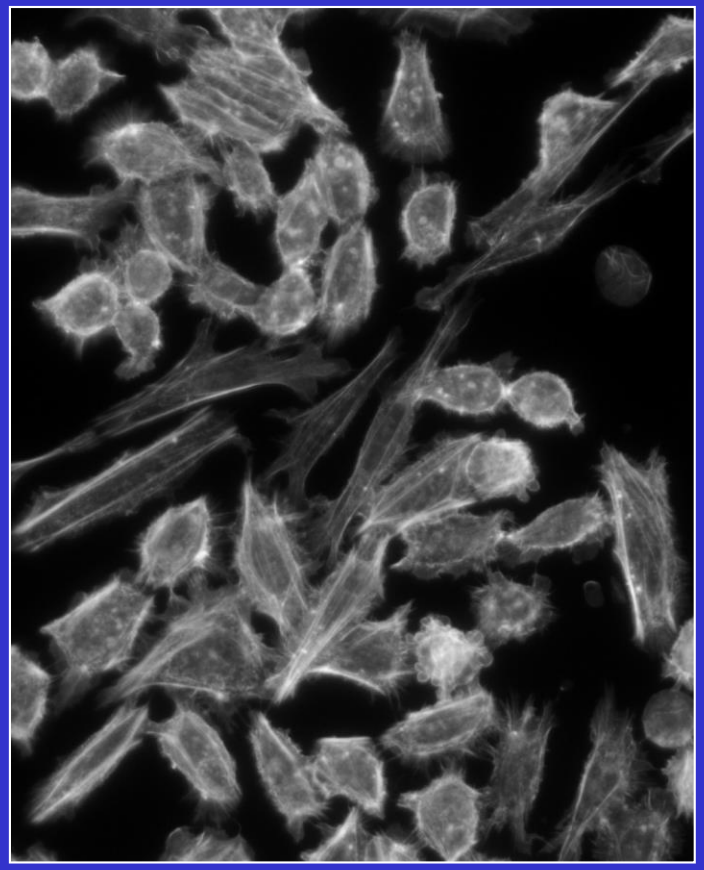
RUTH HOGUE ANGELETTI,*,[2] LYNDA F. BONEWALD,† KAREN DE JONGH,‡ RONALD NIECE,§ JOHN RUSH,†† AND JOHN STULTS‖

Expert Staff

- Maintain Instruments
- Train in Proper Instrument Use
- Plan Experiments
- Analyze Data
- Central Point of Knowledge of Microscopy at Institution
- Identify Needs for Technology Upgrades or Development
- Crosstraining to cover on vacations/sickdays/meetings and during other appointments
- Work as team – Complement Expertises
- Business Functions – Billing and Fundraising

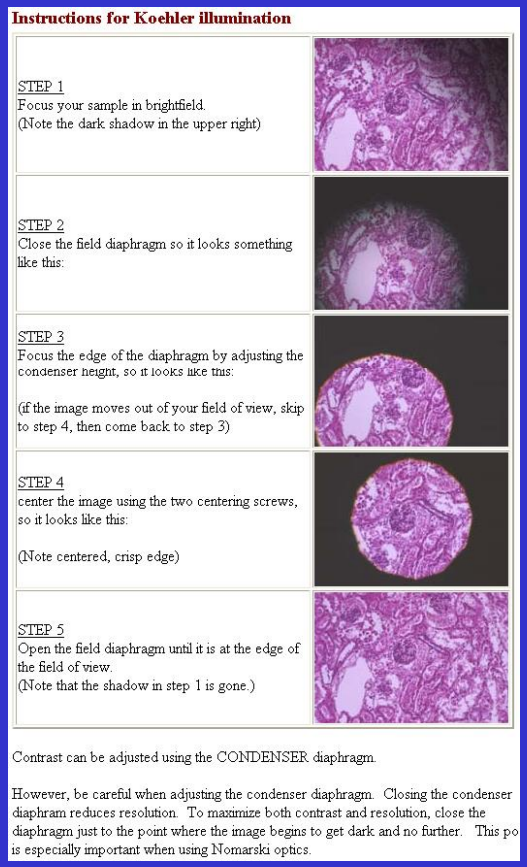
When A User Turns On An Instrument, It Works Correctly





Example of Training

Every user is taught to set up Koehler illumination



Instructions for Koehler illumination

STEP 1
Focus your sample in brightfield.
(Note the dark shadow in the upper right)

STEP 2
Close the field diaphragm so it looks something like this:

STEP 3
Focus the edge of the diaphragm by adjusting the condenser height, so it looks like this:

(if the image moves out of your field of view, skip to step 4, then come back to step 3)

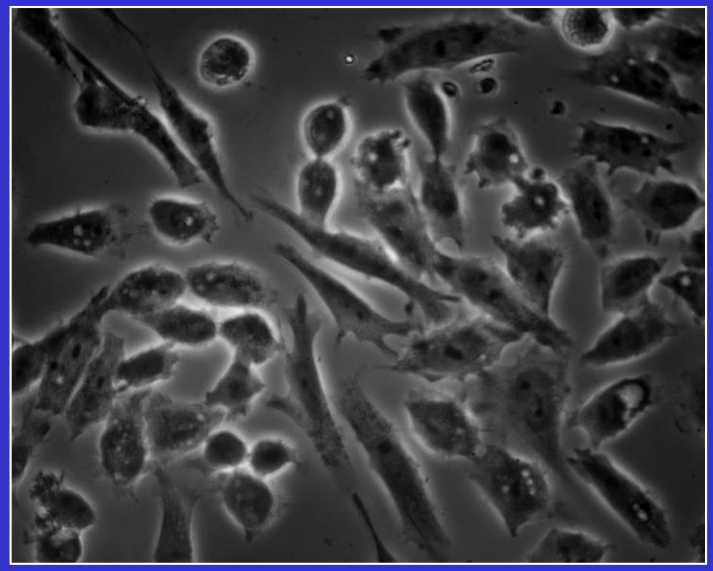
STEP 4
center the image using the two centering screws, so it looks like this:

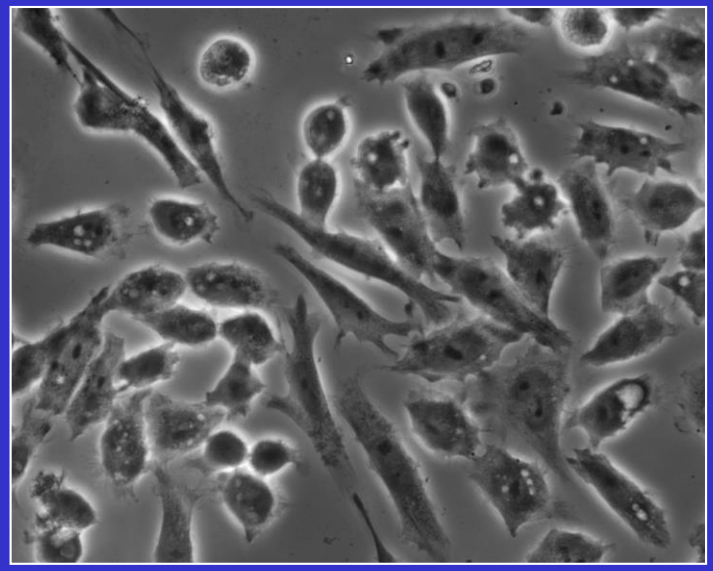
(Note centered, crisp edge)

STEP 5
Open the field diaphragm until it is at the edge of the field of view.
(Note that the shadow in step 1 is gone.)

Contrast can be adjusted using the CONDENSER diaphragm.

However, be careful when adjusting the condenser diaphragm. Closing the condenser diaphram reduces resolution. To maximize both contrast and resolution, close the diaphragm just to the point where the image begins to get dark and no further. This po is especially important when using Nomarski optics.





Example of Training Combined With Maintained Instrumentation

In a training session, a new user can go from this…

GETTING STARTED:

1. **Sign in log book.** If you do not sign in, you may no longer be able to use the facility. You have been duly warned.
2. Turn on mercury lamp.

On switch from O to |. Pause. Press ignition button. Wait until lamp is on before proceeding.

3. Turn on computer or log in with your user name and with your password.
4. On big power unit to left of table

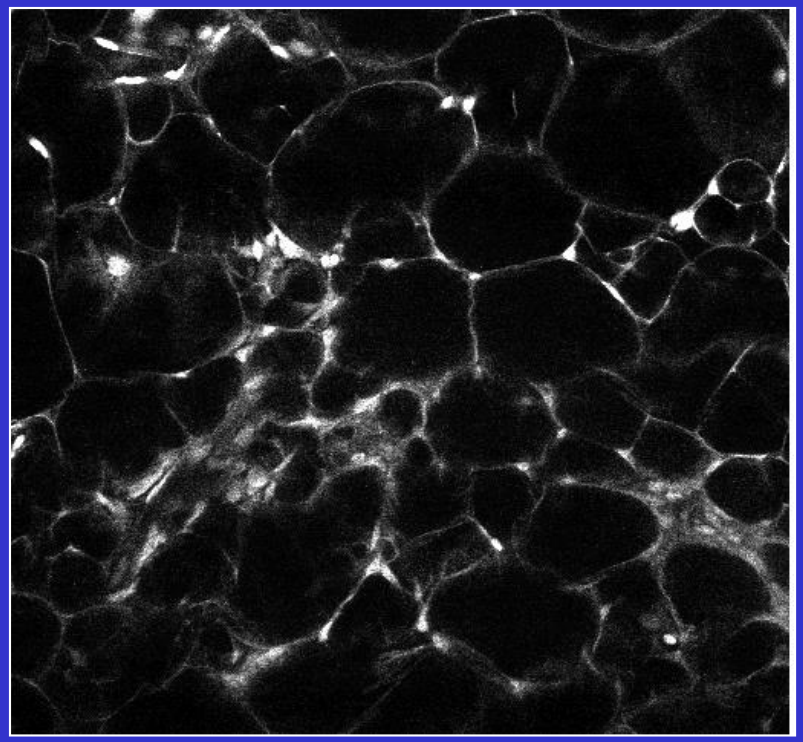
A. Turn on power switch marked "1" (this is the main power switch)
B. Push button marked "2" (this starts up the Kr/Ar laser for excitation of FITC and rhodamine)
C. Push button marked "3" (this starts up the red laser for excitation of Cy5 or for some transmited methods)

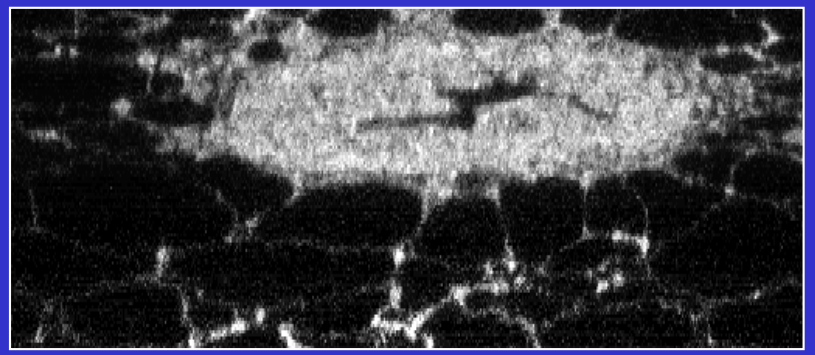
5. Wait for green light on front of scan head to turn on and wait for beeping to stop.

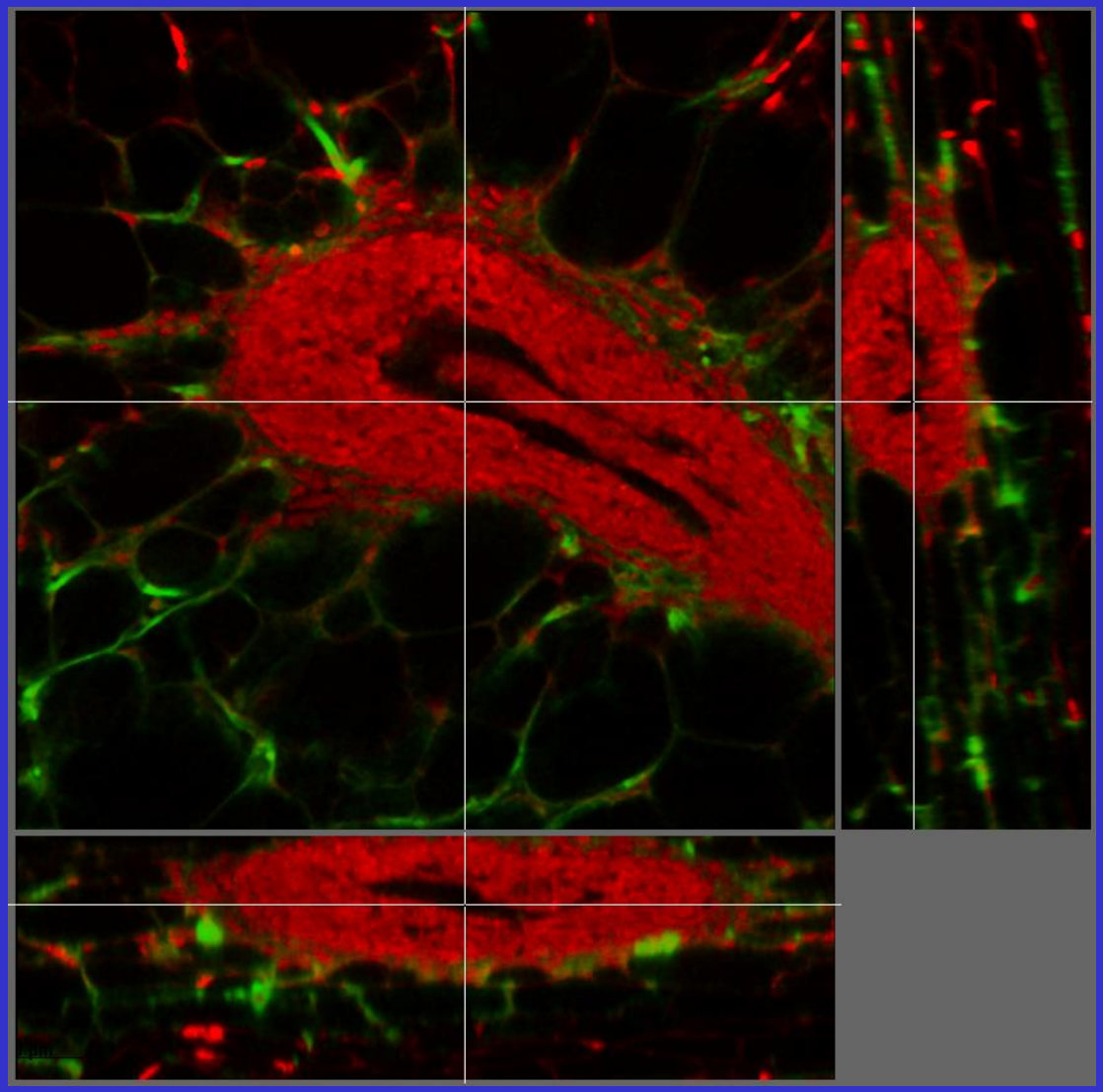
SS

6. Run LaserSharp 2000 software.

7. Login with your user name and password.
8. Choose a method. This is in the Methods menu.
9. Set objective to proper magnification in the control panel toolbox. This must be done every time you change methods.
10. Find an image by eye on the microscope.
11. File]New Experiment or <ctrl>N to open a new imaging window.
12. Any time you want to save an image as a TIF or BMP file, right click on the image with the mouse and choose Export. *More explanation here.*
13. To save a time lapse series or a Z series, simply click on the X in the upper







Help Plan Experiments

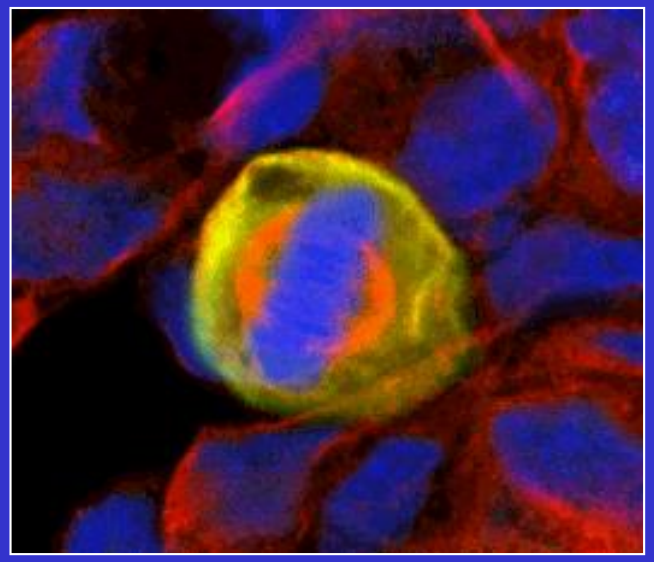
I'm studying a receptor and need to see where it is in the cell before and after treatment with a drug…

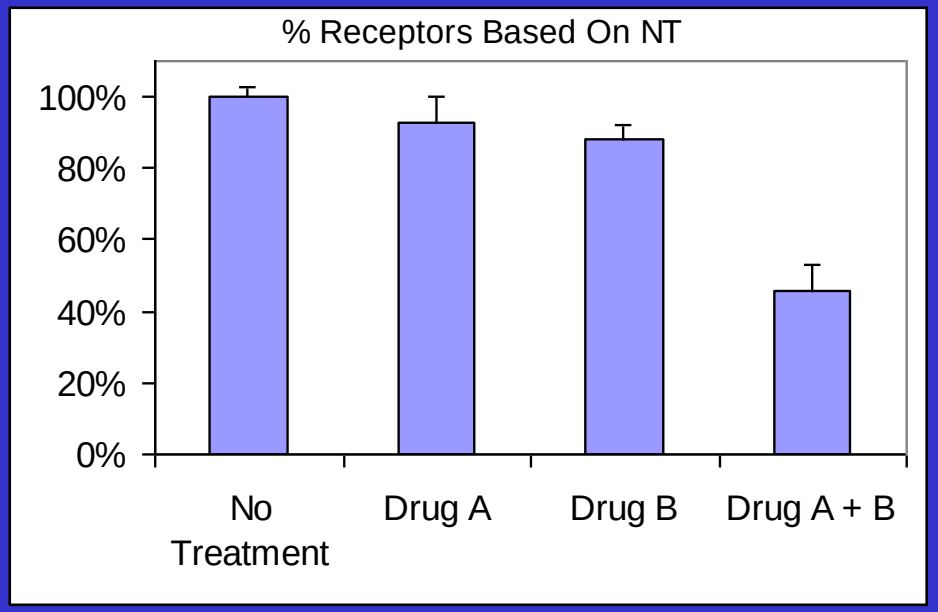
According to biochemical data, the kinetics of polymerization is 7 minutes after stimulation, but we need to see it in the cell as it happens…

I need to use the confocal.

After treatment, the tumors appear to be smaller, but we need to quantify this. Also, the cells in the surrounding tissue seem to be more spiky. Is there a way to quantify and correlate this too?

Analyze Data

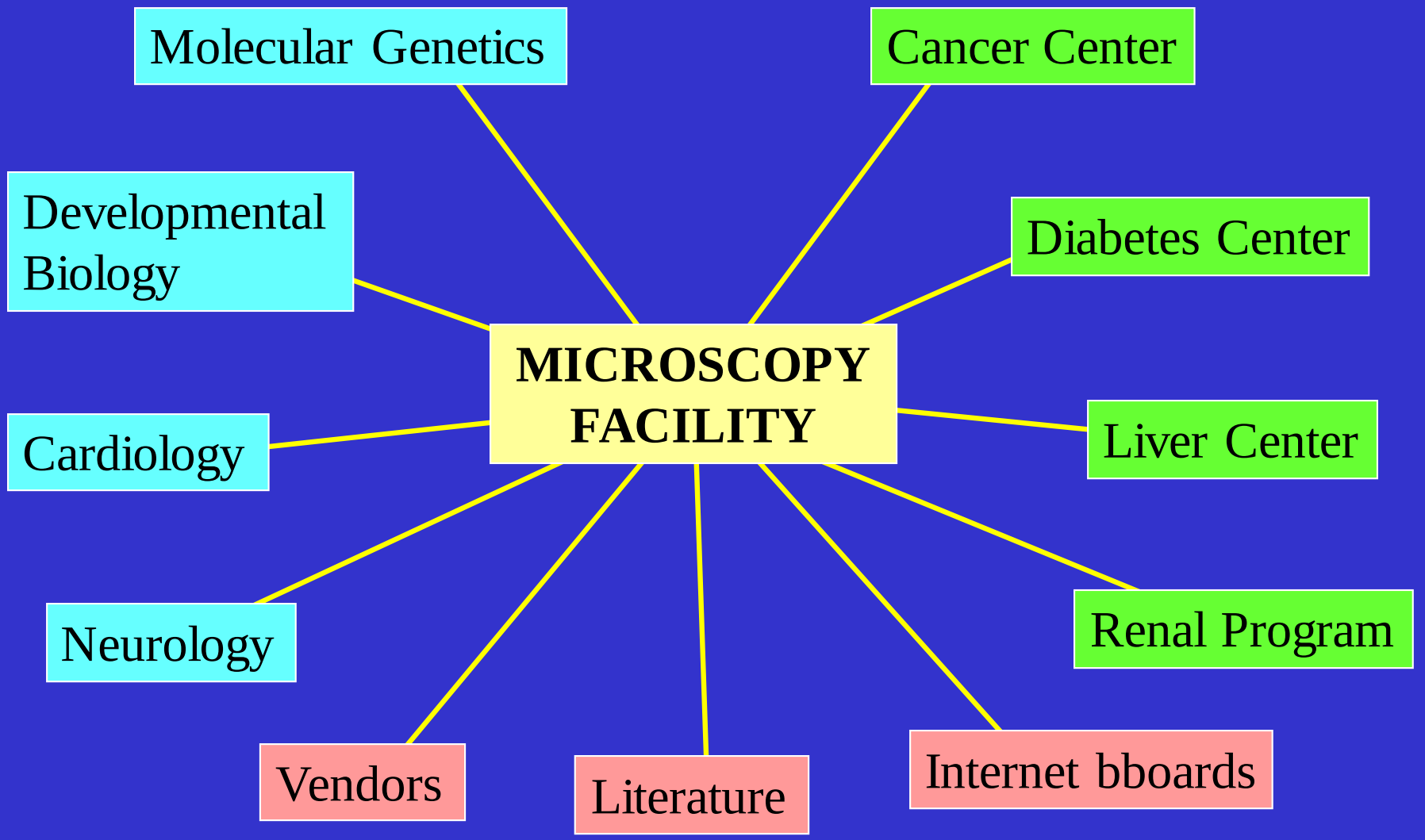




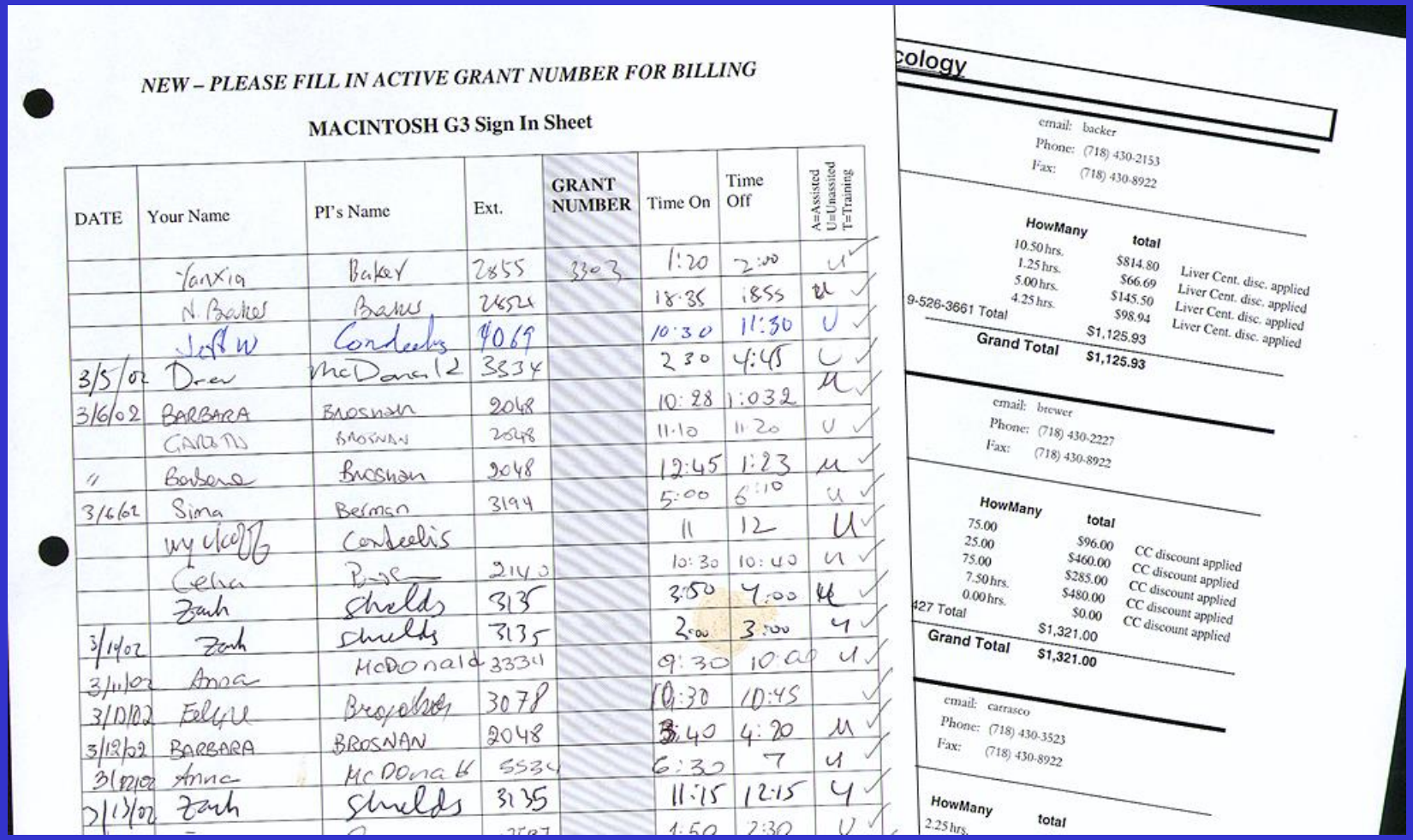
Investigators may not be cell biologists.

Only the metaphase cell expresses the protein of interest.

Centralized Knowledge



Logging Usage



NEW – PLEASE FILL IN ACTIVE GRANT NUMBER FOR BILLING

MACINTOSH G3 Sign In Sheet

DATE	Your Name	PI's Name	Ext.	GRANT NUMBER	Time On	Time Off	A=Assisted U=Unassisted T=Training
	Yanxia	Baker	2855	3303	1:20	2:00	U
	N. Baker	Baker	2652		18:35	1855	U
	Jeff W	Condeelis	4069		10:30	11:30	U
3/5/02	Drew	McDonald	3334		2 30	4:45	U
3/6/02	BARBARA	BROSNAN	2048		10: 28	1:032	U
	GARETH	BROSNAN	2048		11:10	11 20	U
"	Barbara	Brosnan	2048		12:45	1:23	U
3/6/02	Sima	Berman	3194		5:00	6:10	U
	Wyckoff	Condeelis			11	12	U
	Celia	Backer	2140		10: 30	10: 40	U
	Zach	Shields	3135		3:50	4:00	U
3/14/02	Zach	Shields	3135		2:00	3:00	U
3/11/02	Anna	McDonald	3334		9: 30	10:00	U
3/11/02	Felix	Bruzelius	3078		10:30	10:45	
3/12/02	BARBARA	BROSNAN	2048		3:40	4:20	U
3/12/02	Anna	McDonald	5534		6:30	7	U
2/13/02	Zach	Shields	3135		11:15	12:15	U
					1:50	2:30	U

cology

email: backer
Phone: (718) 430-2153
Fax: (718) 430-8922

	HowMany	total	
	10.50 hrs.	$814.80	Liver Cent. disc. applied
	1.25 hrs.	$66.69	Liver Cent. disc. applied
	5.00 hrs.	$145.50	Liver Cent. disc. applied
9-526-3661 Total	4.25 hrs.	$98.94	Liver Cent. disc. applied
		$1,125.93	
Grand Total		$1,125.93	

email: brewer
Phone: (718) 430-2227
Fax: (718) 430-8922

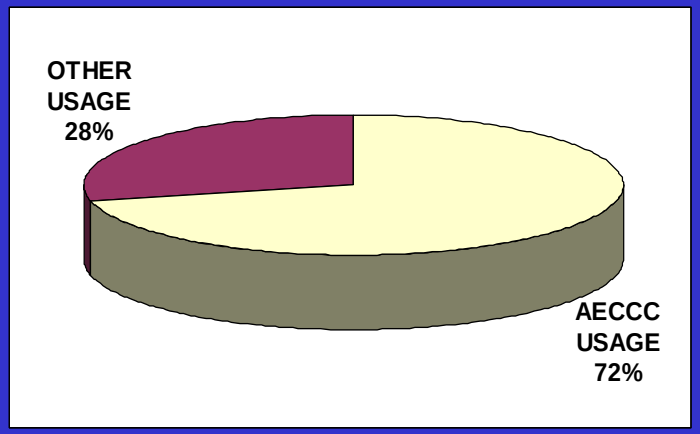
	HowMany	total	
	75.00	$96.00	CC discount applied
	25.00	$460.00	CC discount applied
	75.00	$285.00	CC discount applied
	7.50 hrs.	$480.00	CC discount applied
427 Total	0.00 hrs.	$0.00	CC discount applied
		$1,321.00	
Grand Total		$1,321.00	

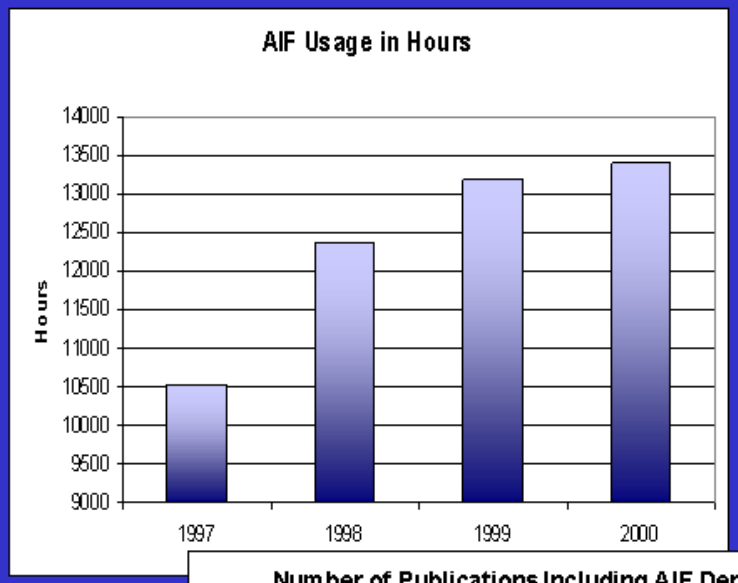
email: carrasco
Phone: (718) 430-3523
Fax: (718) 430-8922

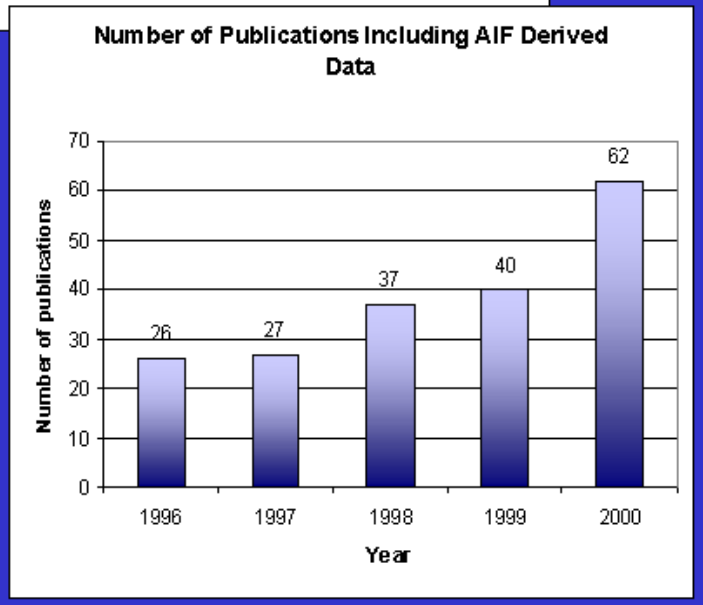
HowMany	total
2.25 hrs.	

Granting Agencies Require Usage Statistics

ANALYTICAL IMAGING FACILITY		
Hourly usage calendar year 2001		
	hours	percent
AECCC USAGE	11379	71.5%
ALL USAGE	15913	100.0%

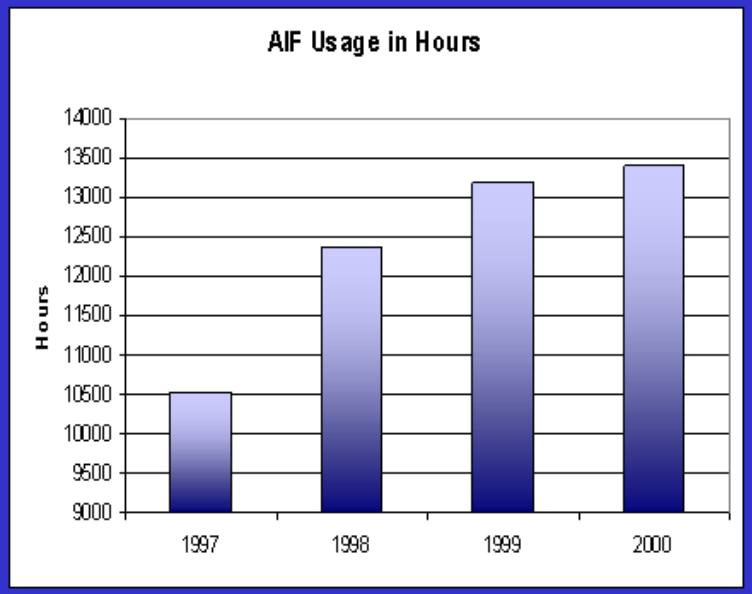


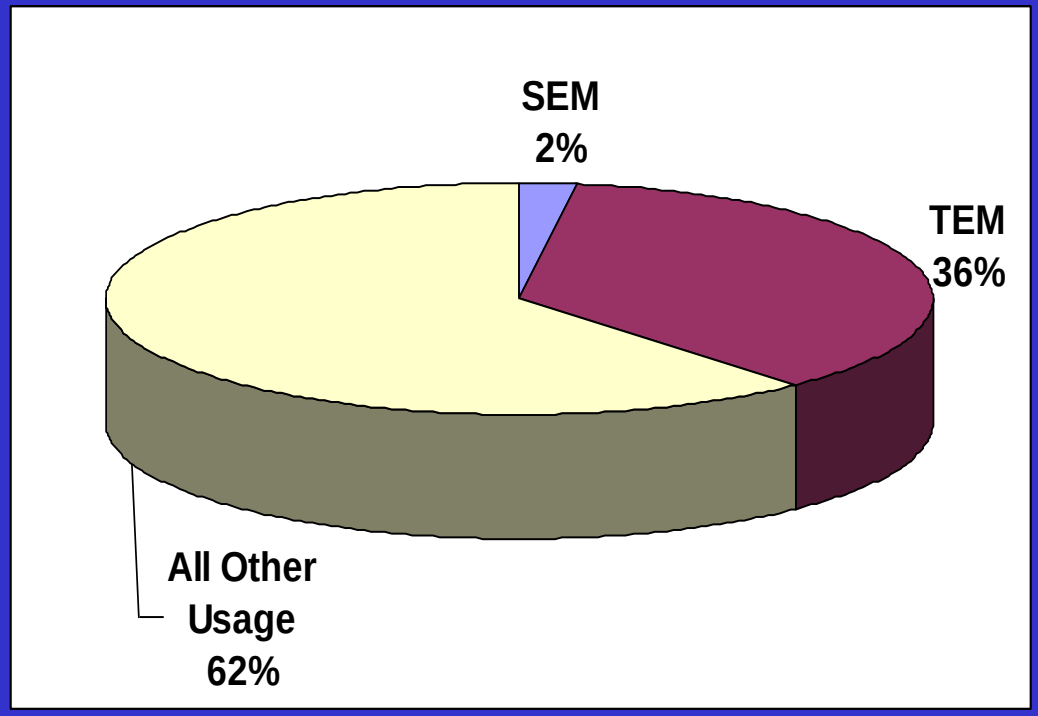




In 2001 the AIF served 171 labs with investigators from at least 21 departments…

Usage Statistics Contribute to Internal Budget Decisions





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- Work as team – Complement Expertises
- Business Functions – Billing and Fundraising

Director

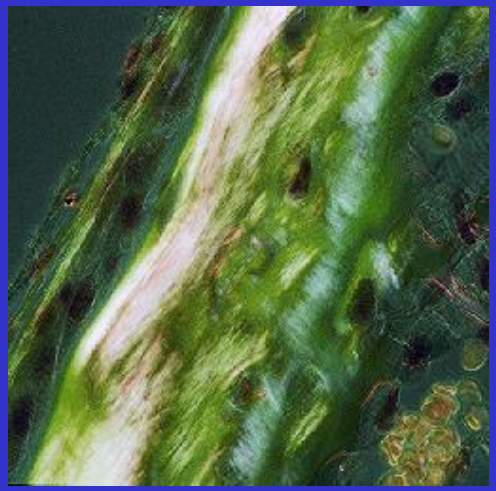
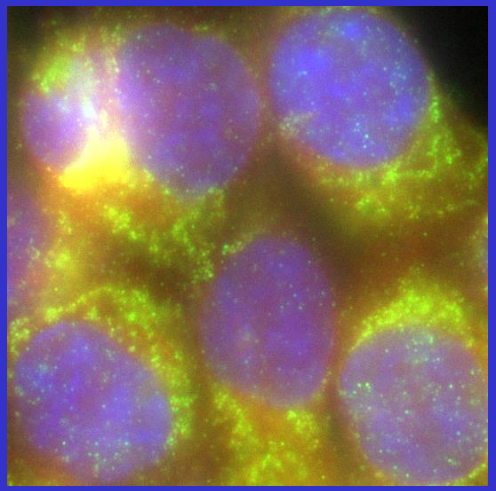
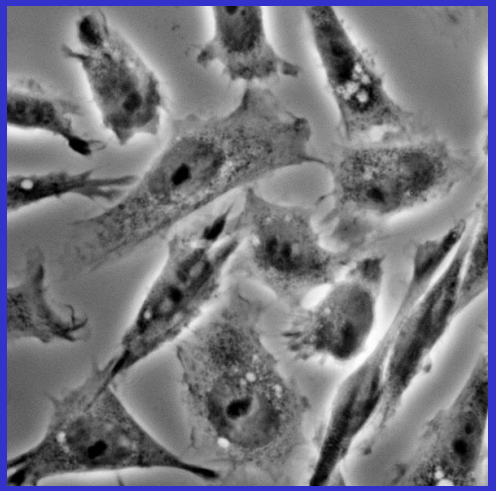
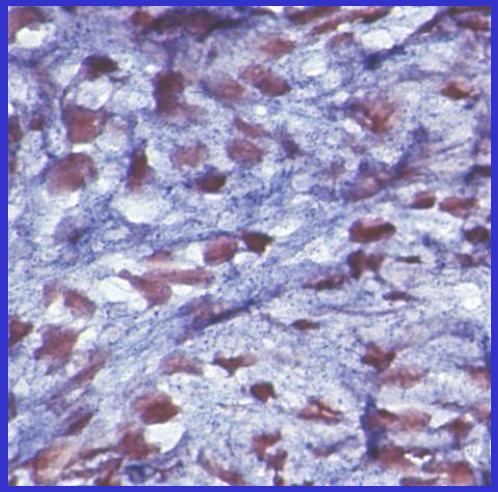
- Supervise Staff
- Control Quality
- Keep users in line
- Multi-Task
- Manage Time
- Teach Remedial Math, Histology, Cell Biology
- Know when to SAY NO!

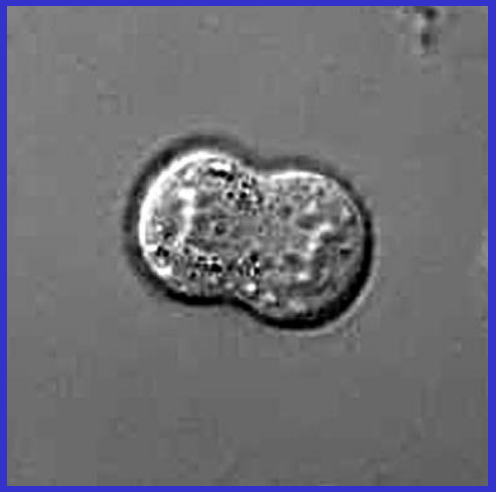
Imaging Facility Techniques

Provide imaging by light and analysis of structures on the order of 10^{-2} m to 10^{-7} m.

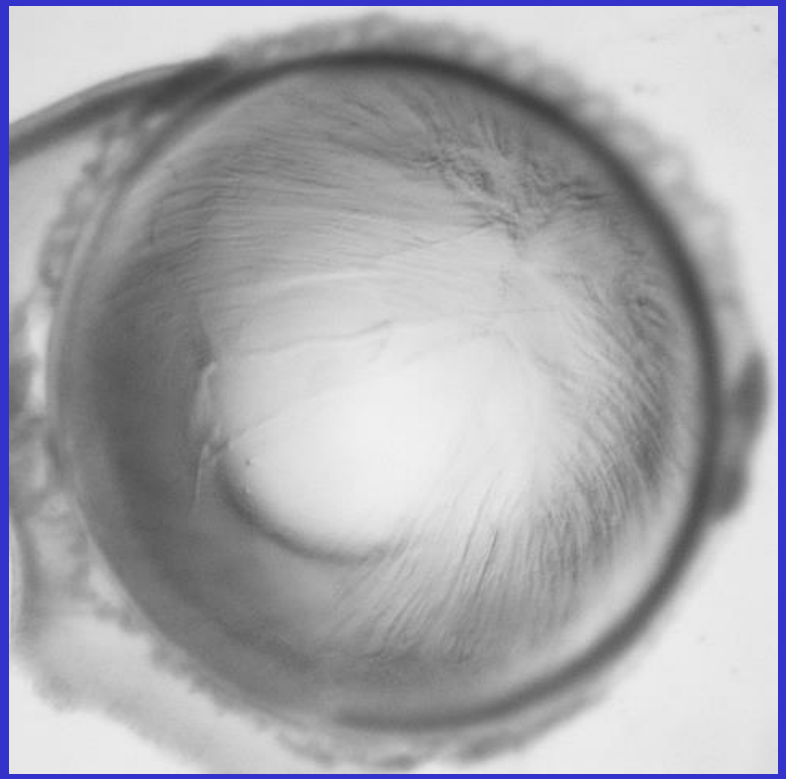
This is standard macro imaging down to high resolution light microscopy.

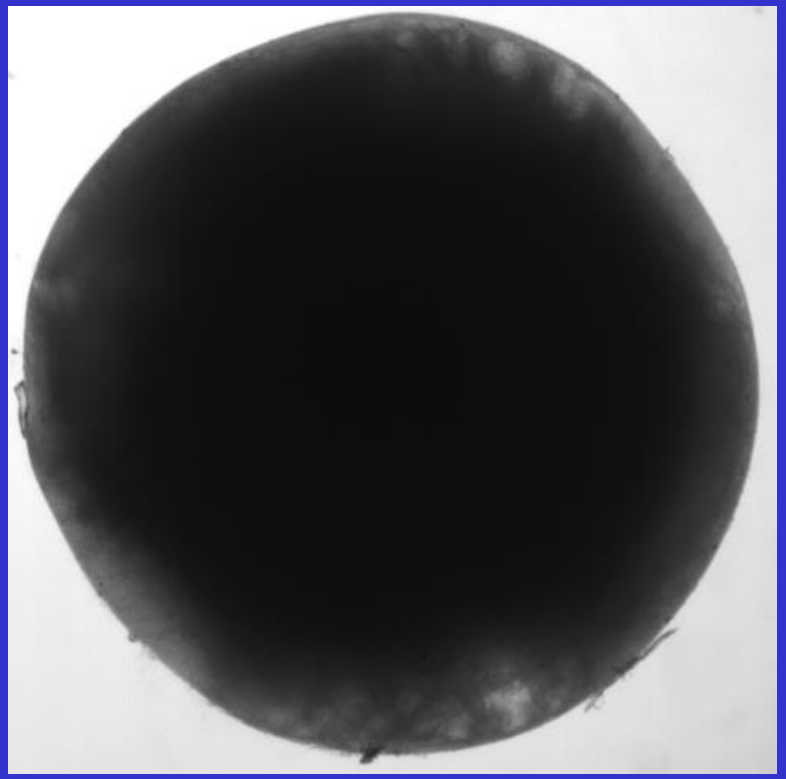
Routine Light Microscopy



Simple Phenotype Analysis

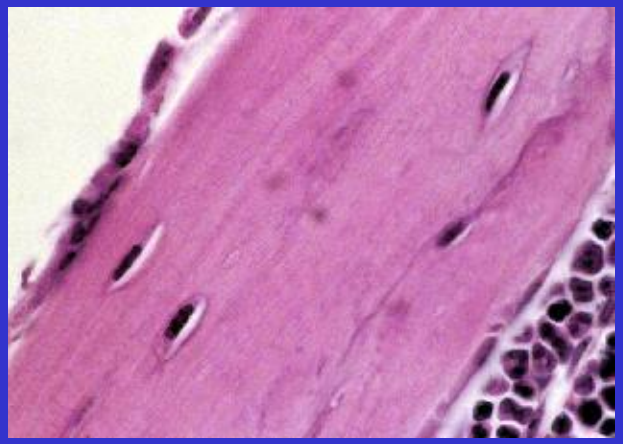


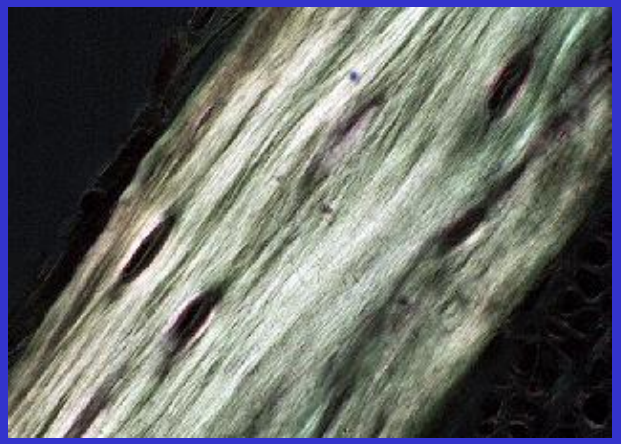


Because the mouse eyes are themselves lenses, optical tricks were necessary to image the eyes even with simple brightfield optics.

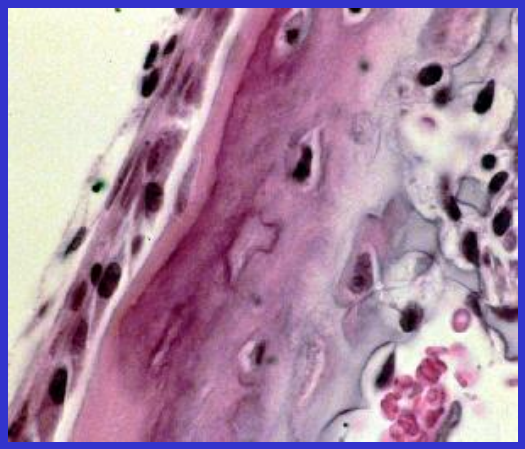
Simple Phenotype Analysis

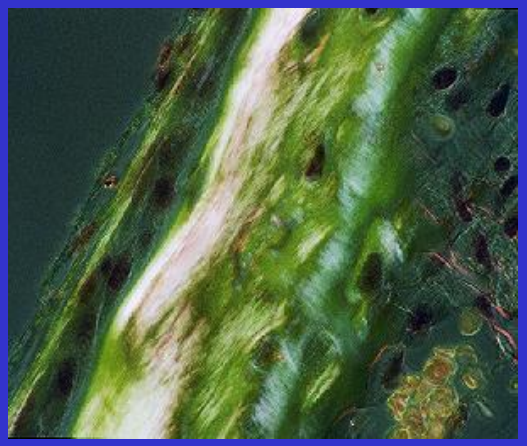
WT





MUT

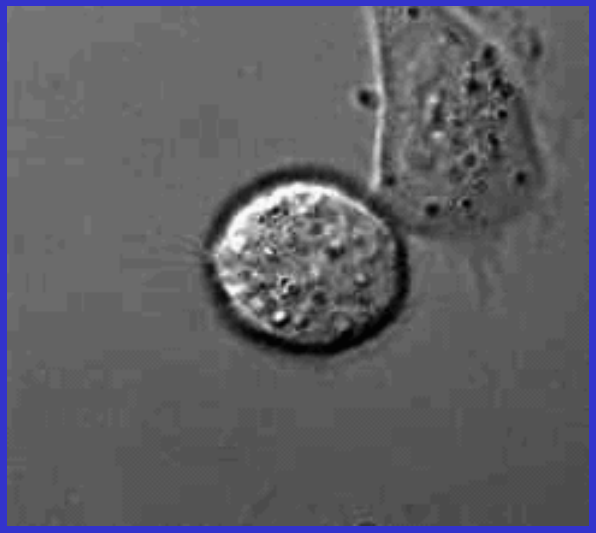




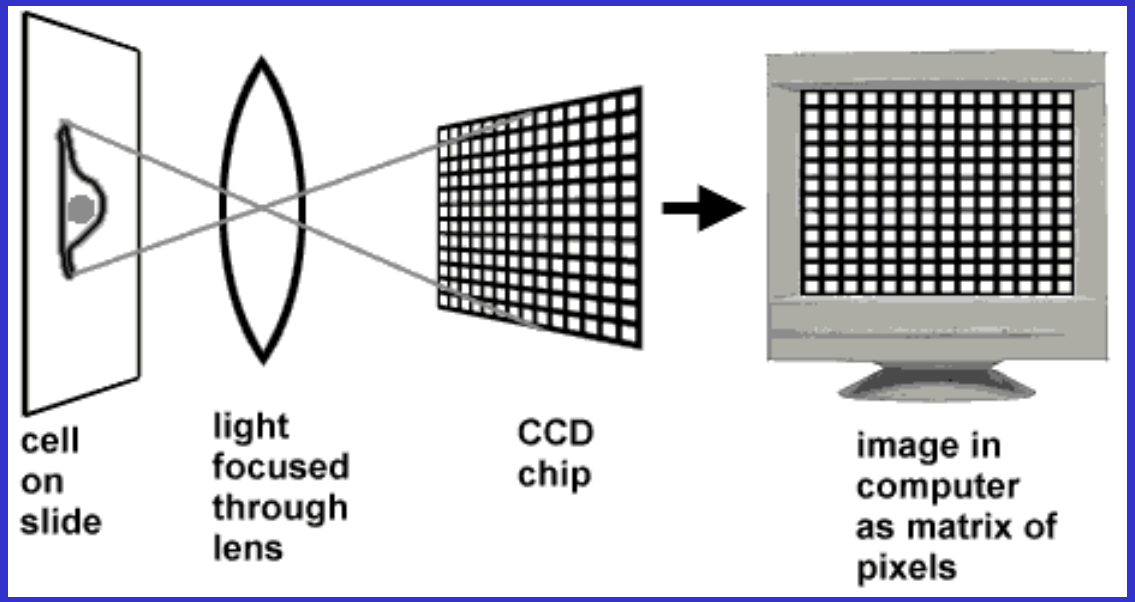
Cortical bone area of femurs from 2-week-old mice were shown, slides were stained with H & E

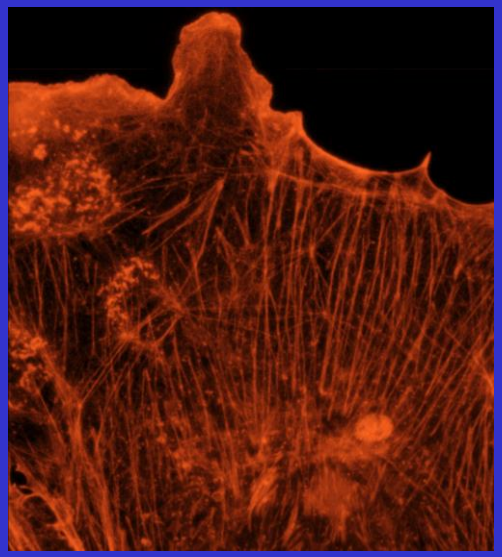
Pictured by Dr. Xu-Ming Dai in Dr. E. Richard Stanley's laboratory at AECOM

Live Cell Imaging



Next Level Of Sophistication: Cooled CCDs

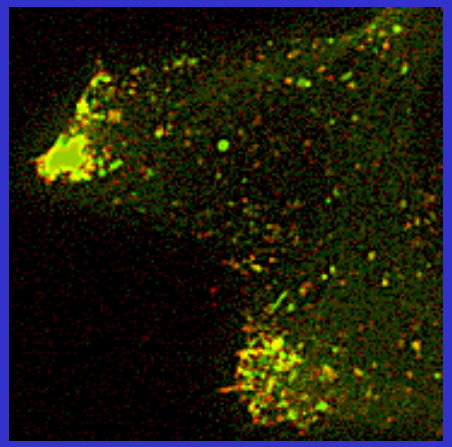




CCD Benefits & Drawbacks

Benefits

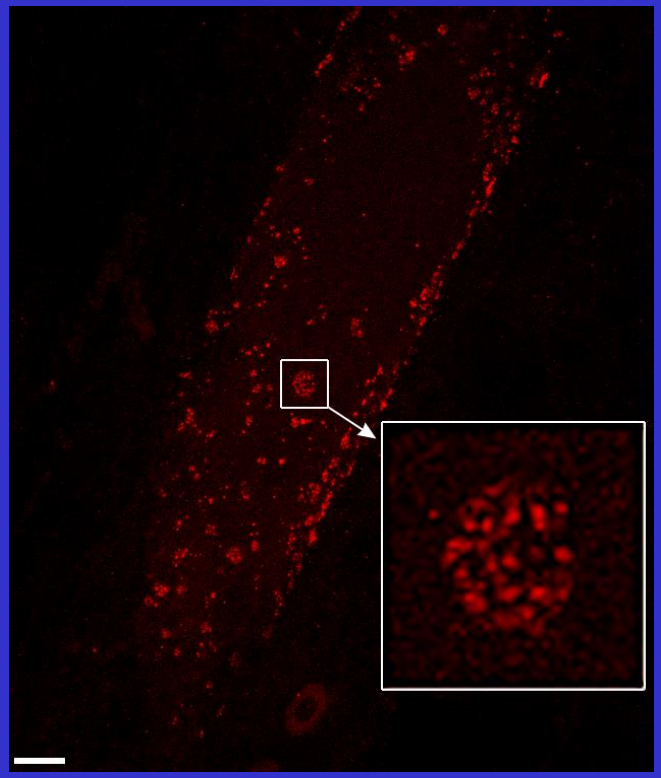
- Widefield exposes entire field simultaneously (time resolved)
- Fast & low noise
- Wide dynamic range
- Linear (allows quantification)
- Deconvolution to fix out of focus images

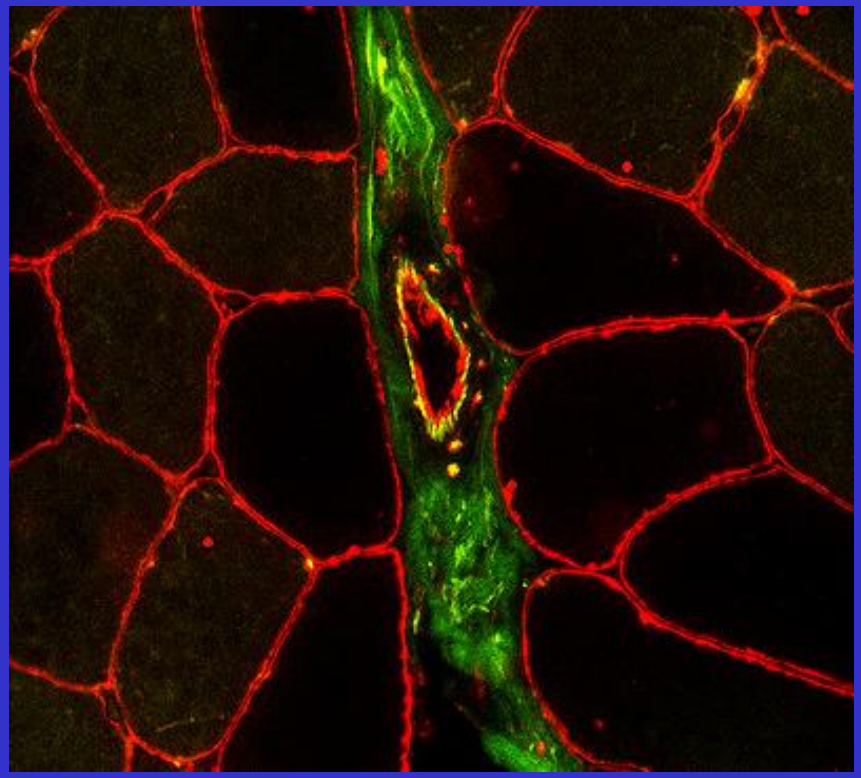


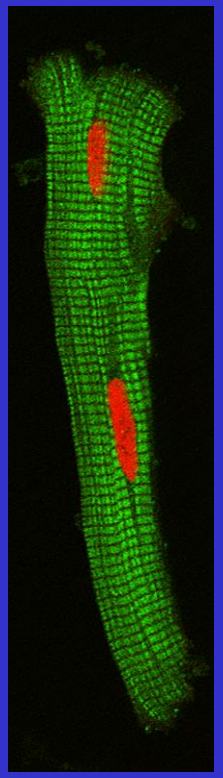
Drawbacks

- Deconvolution (postprocessing time & only on thin low-scattering samples)
- Sequential imaging of multiple probes (now fixed)

Confocal

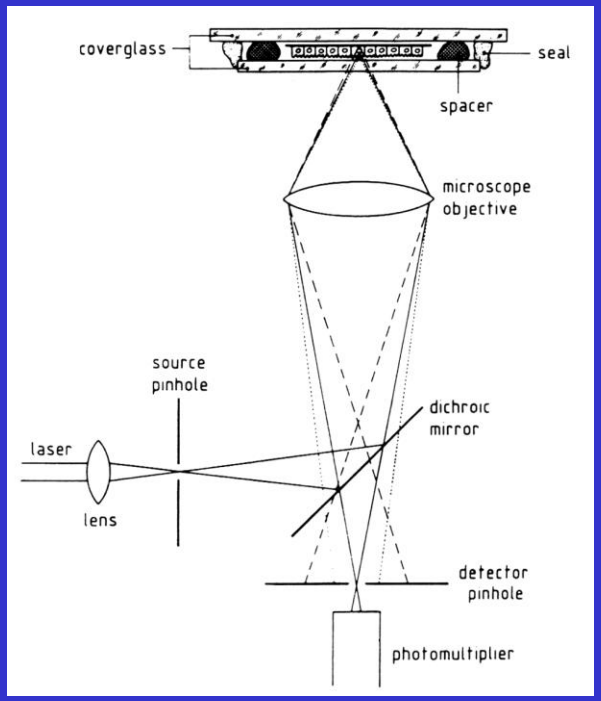


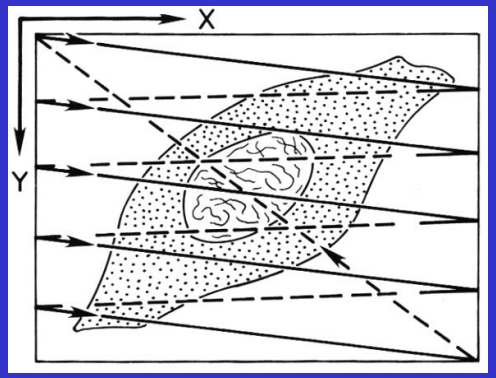


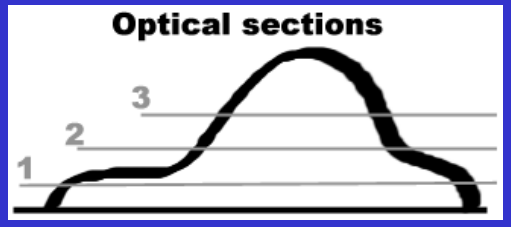


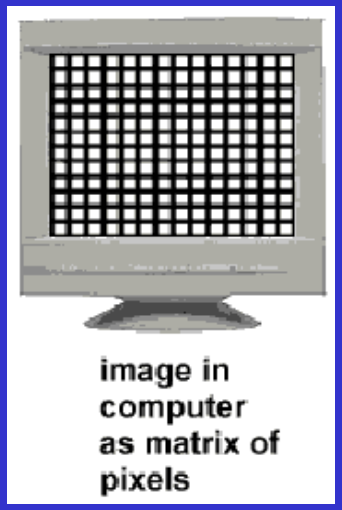
Thin Optical Sectioning

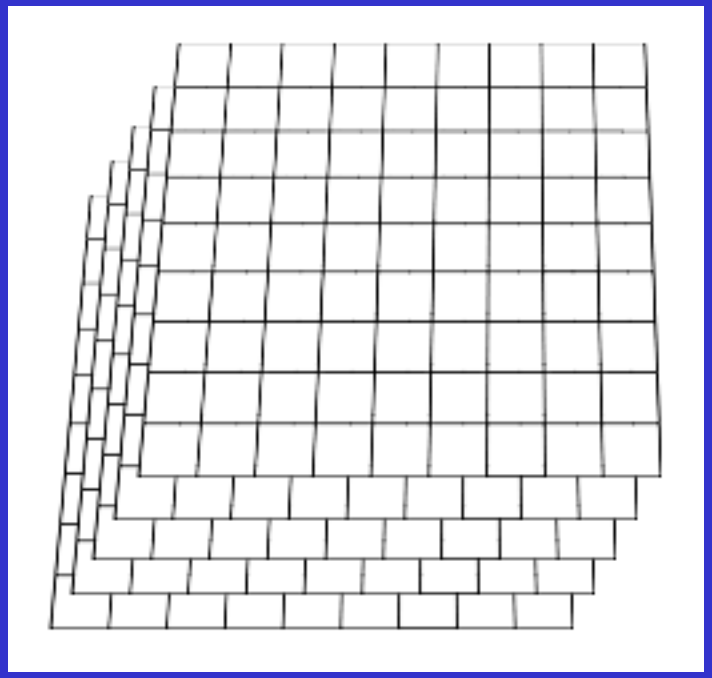
From a point to a 2D matrix to a 3D matrix (to true 4D)



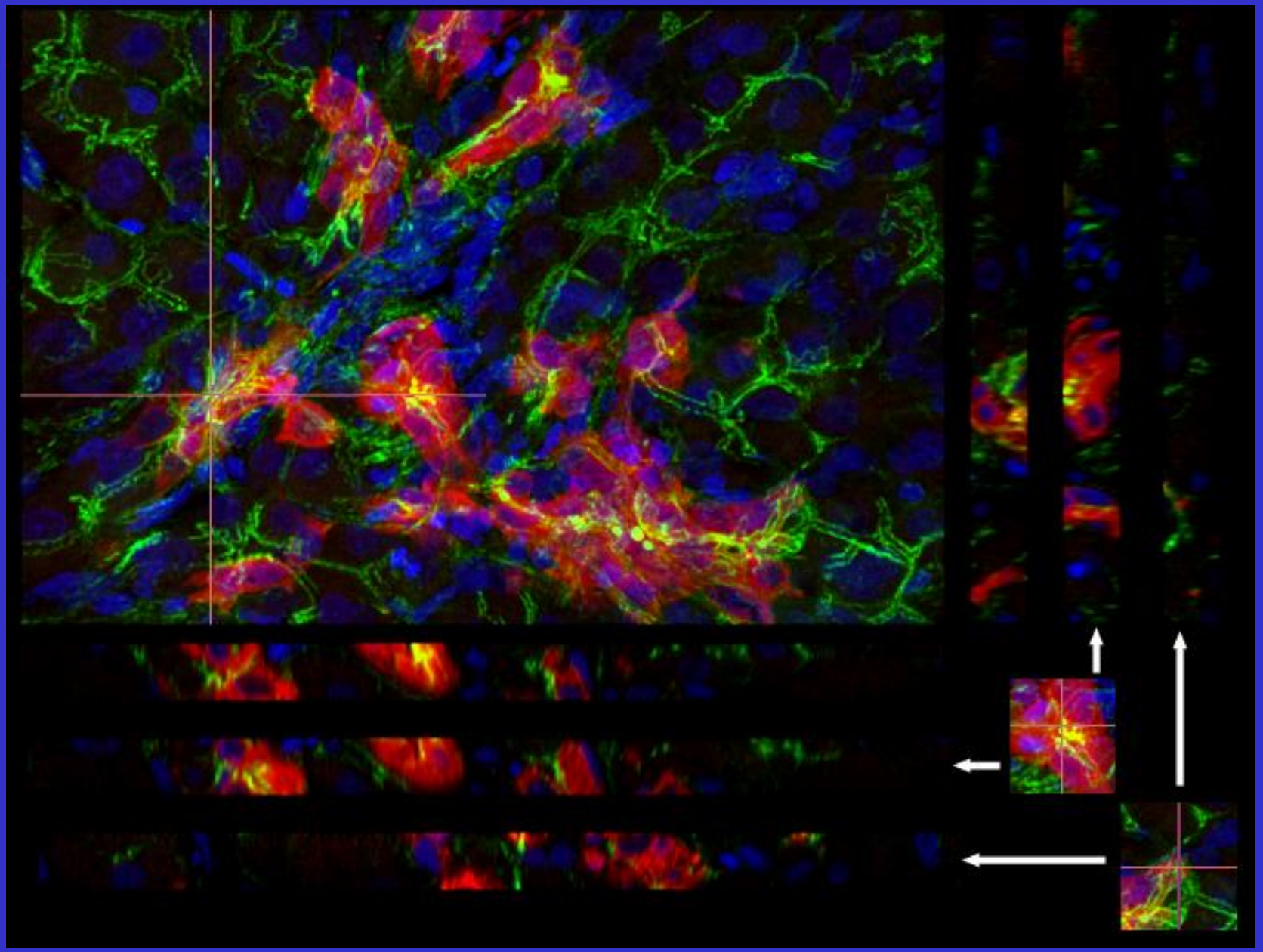


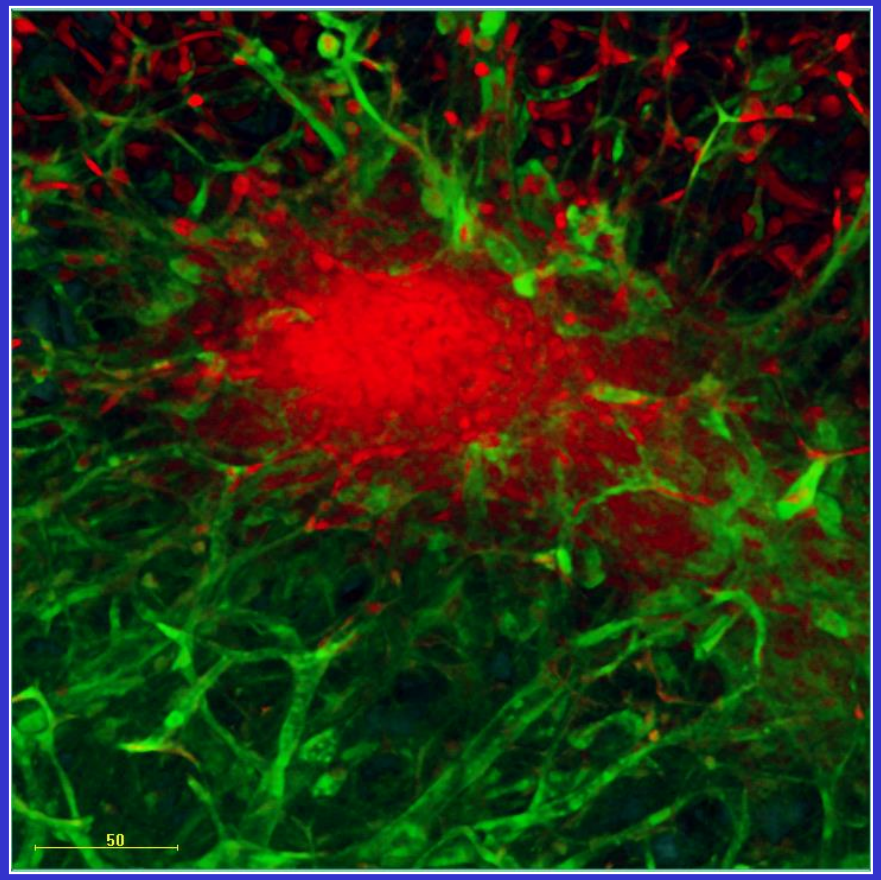


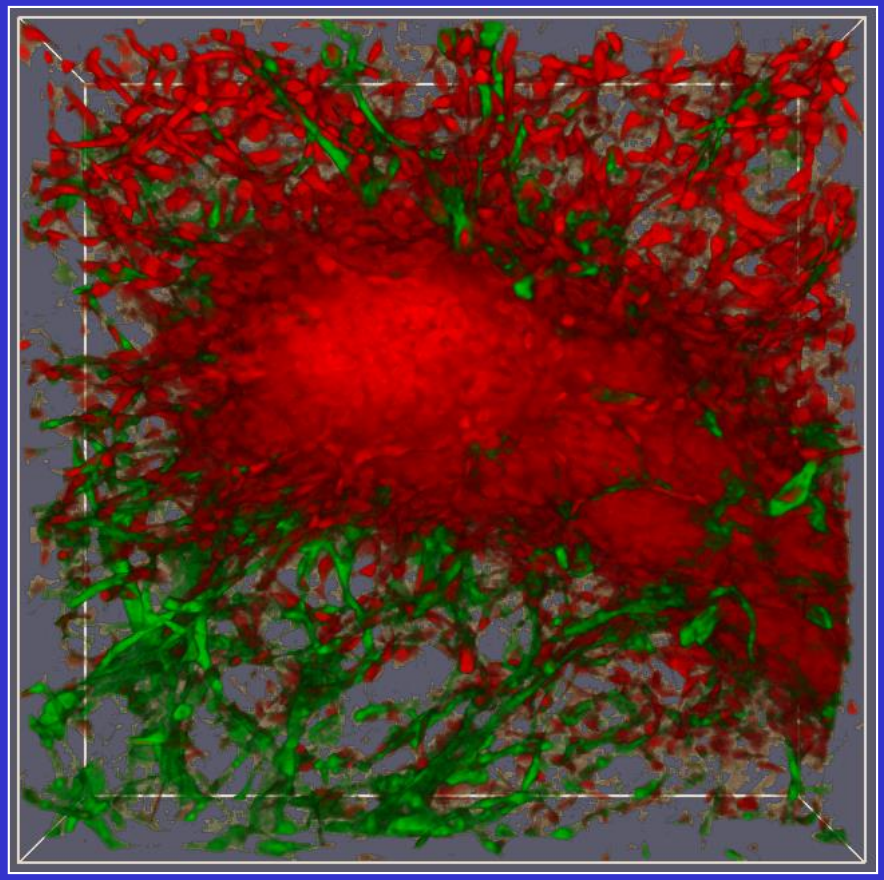




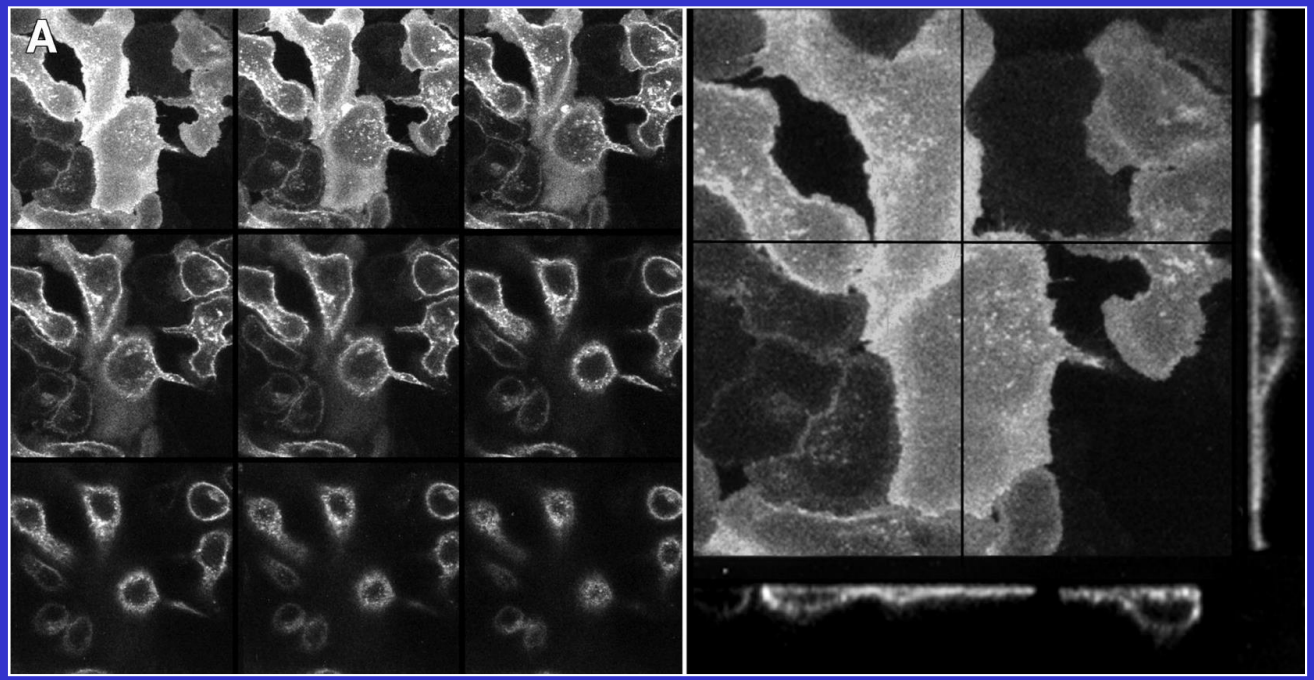
3D in tissue

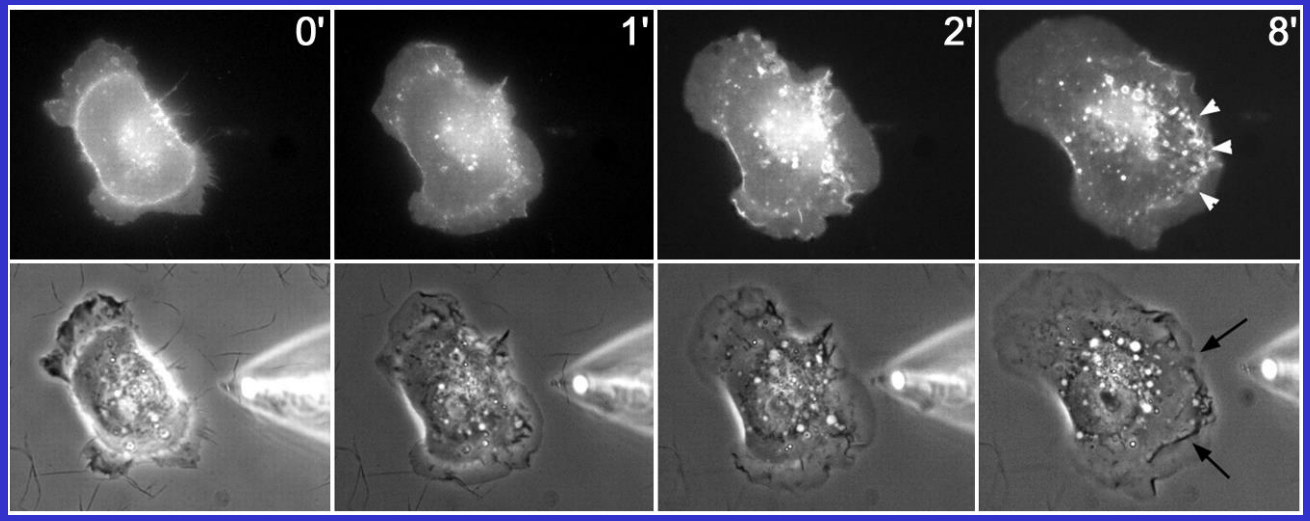



50



Time-Lapse 3D Confocal

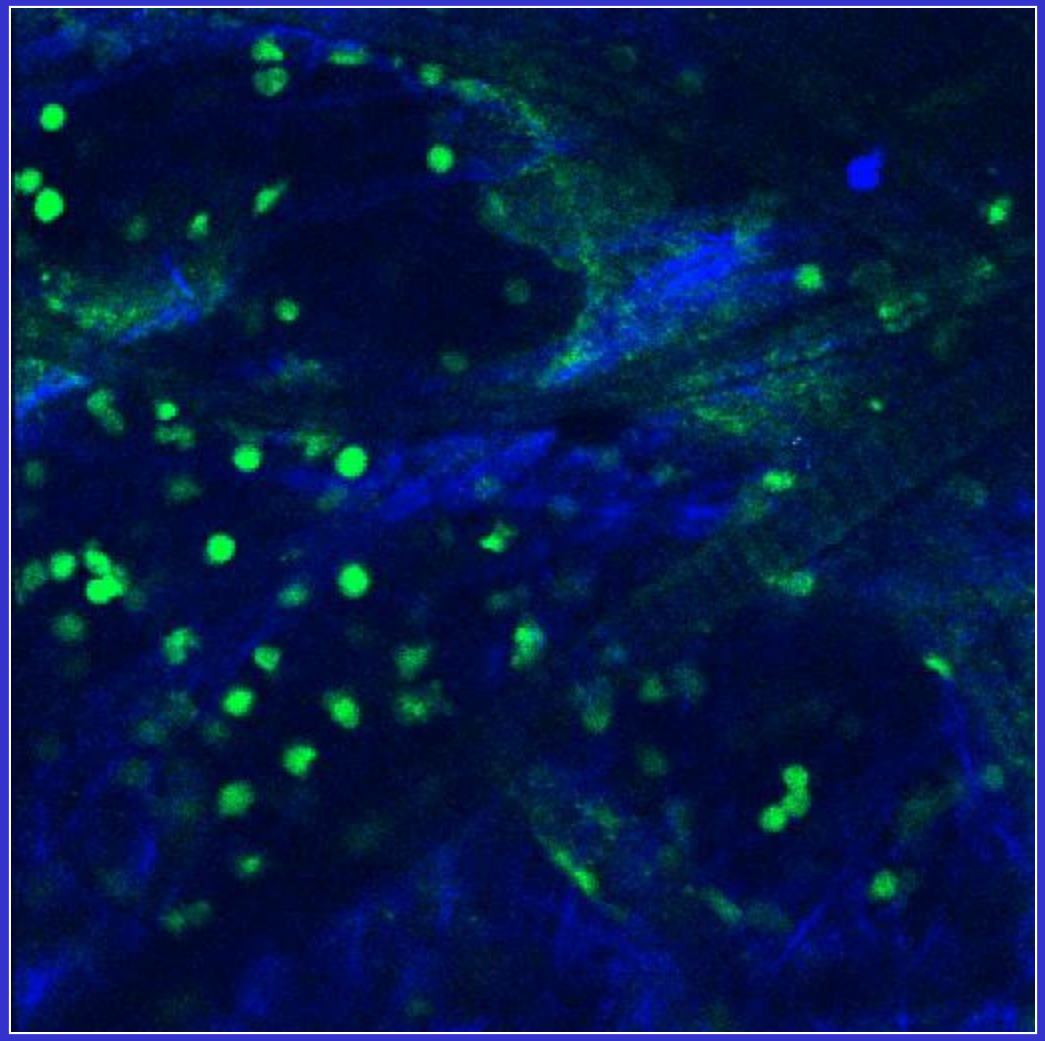




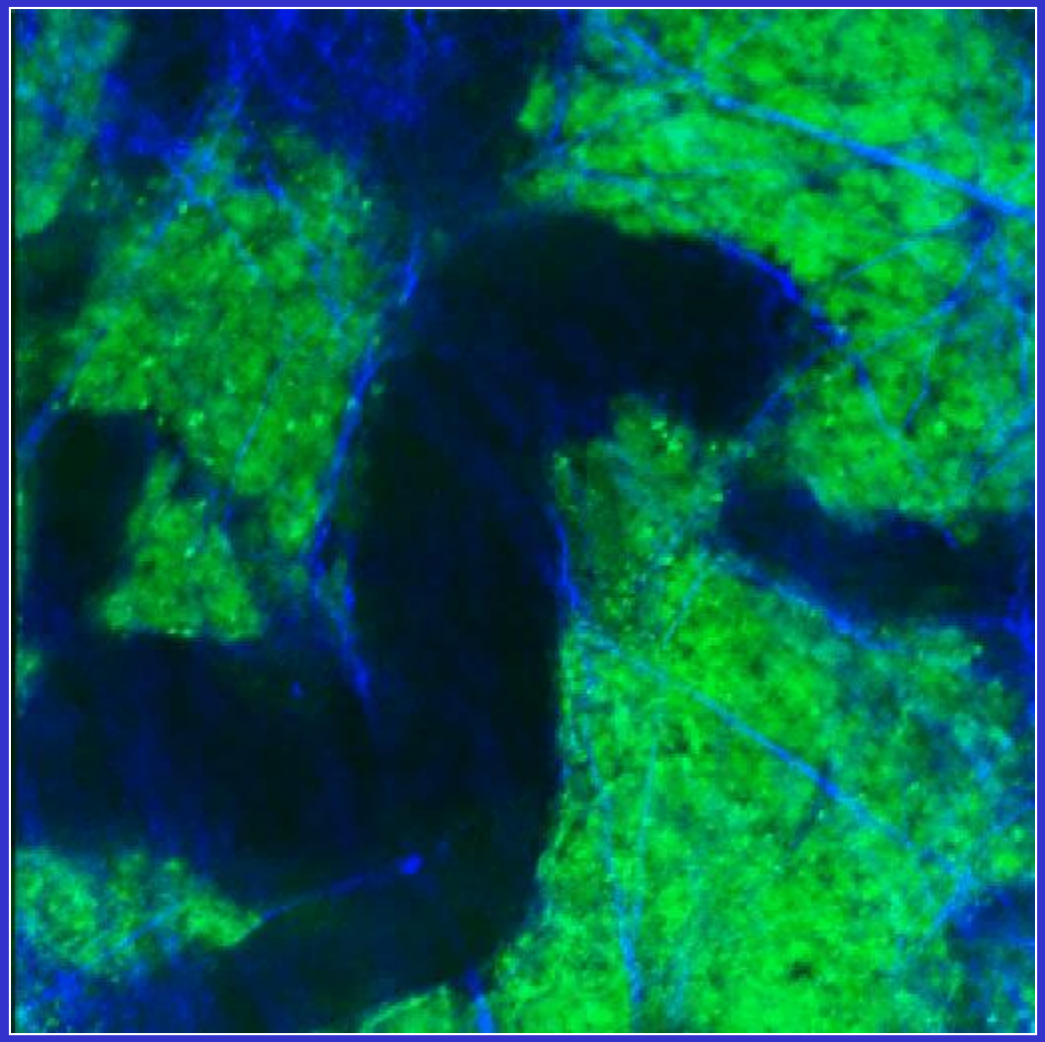
Live Imaging Problems With Confocal

- Single photon excitation scatters & is absorbed
- Emitted photons scatter & miss pinhole
- Bleaching & photodamage in entire cone of illumination

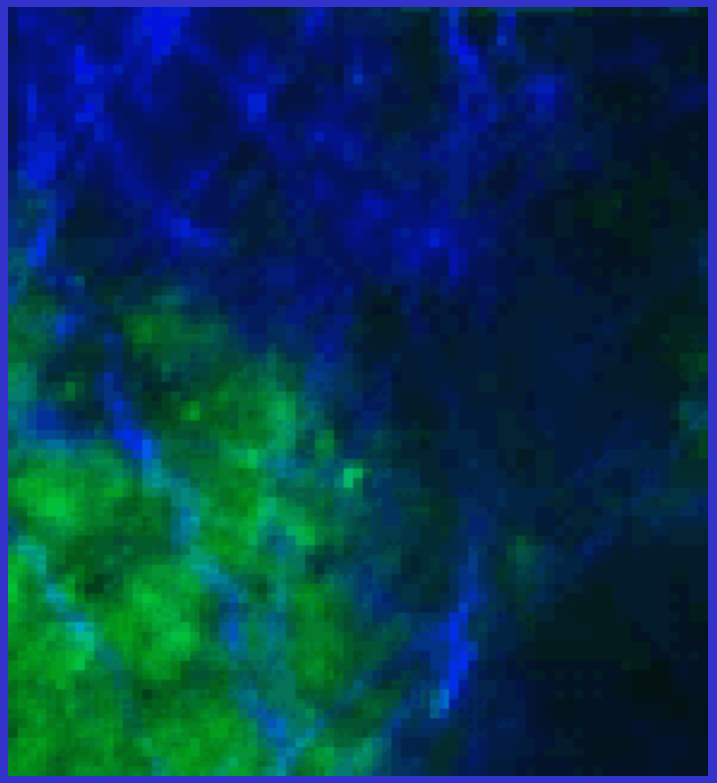
Multiphoton



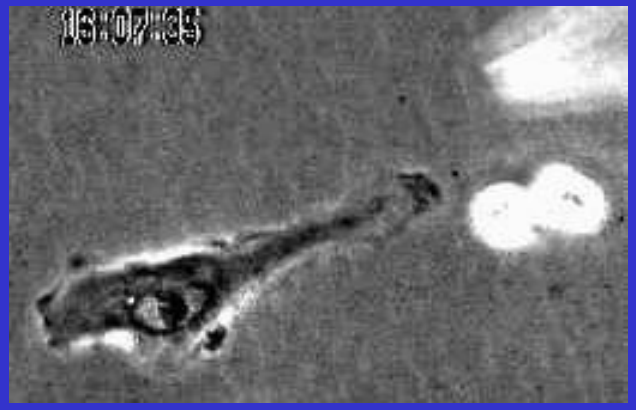
Multiphoton



Multiphoton

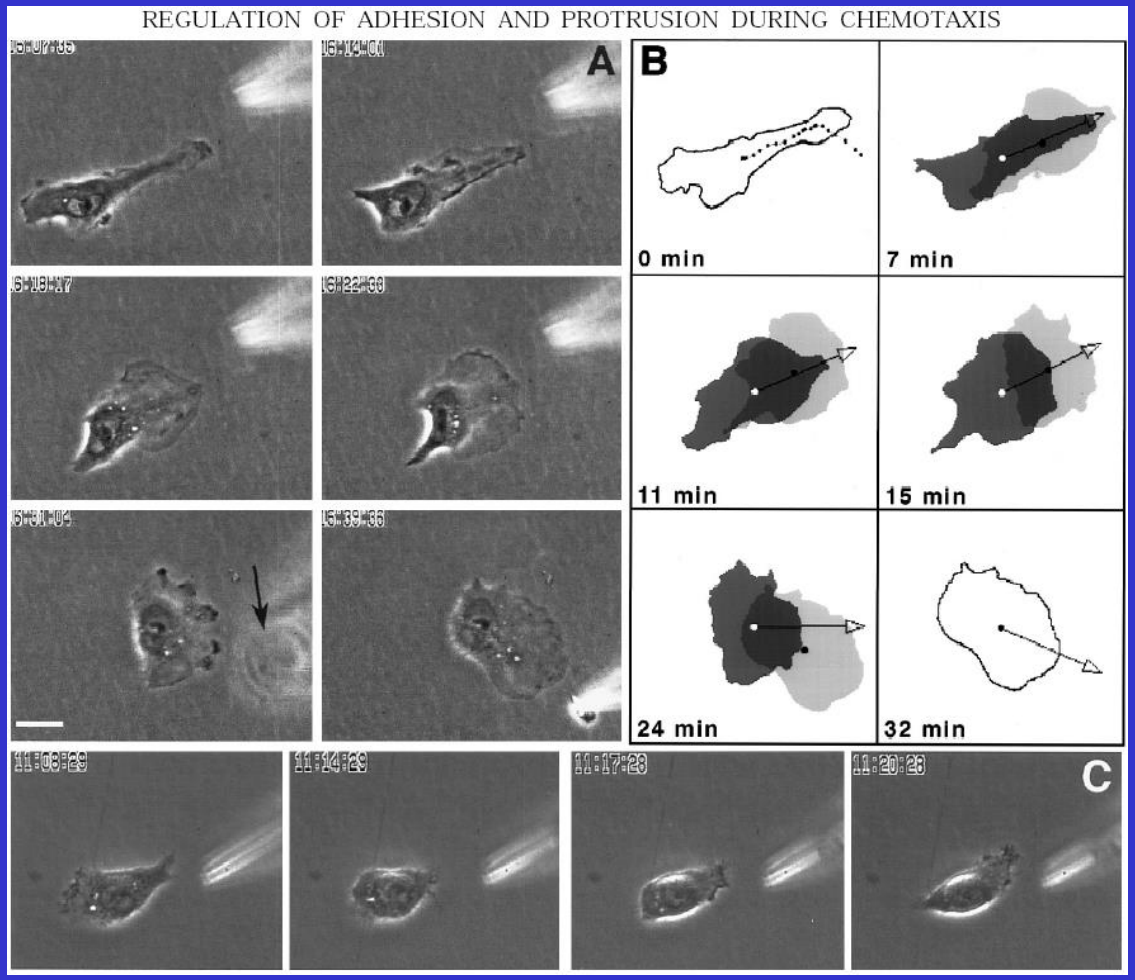


Quantification of Movement

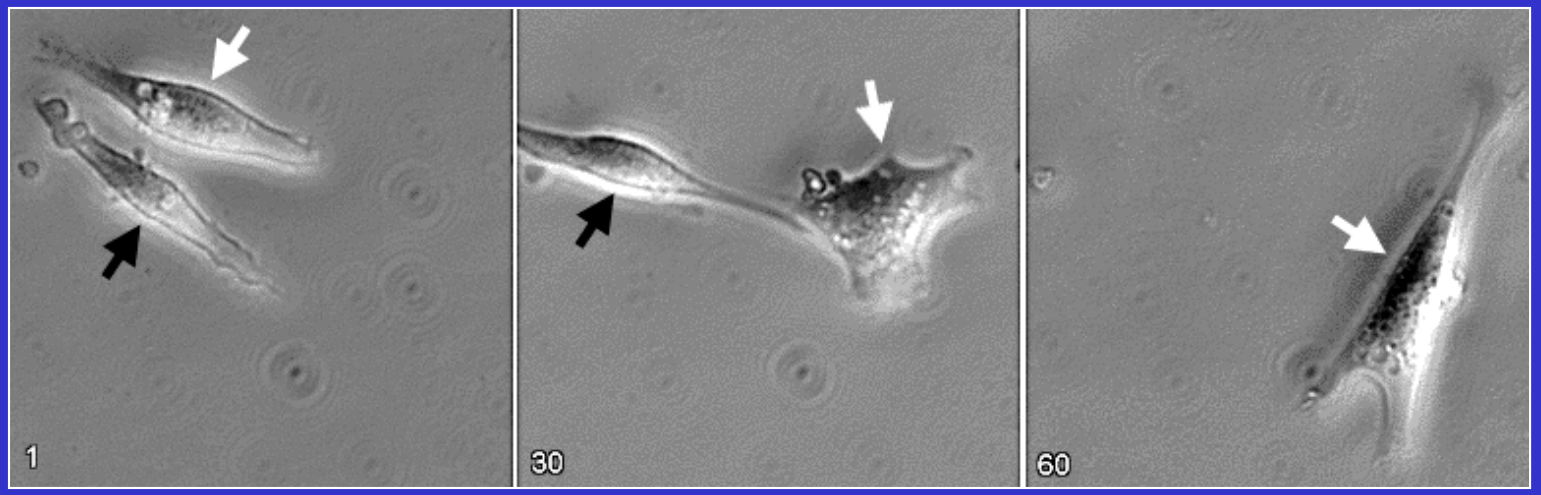


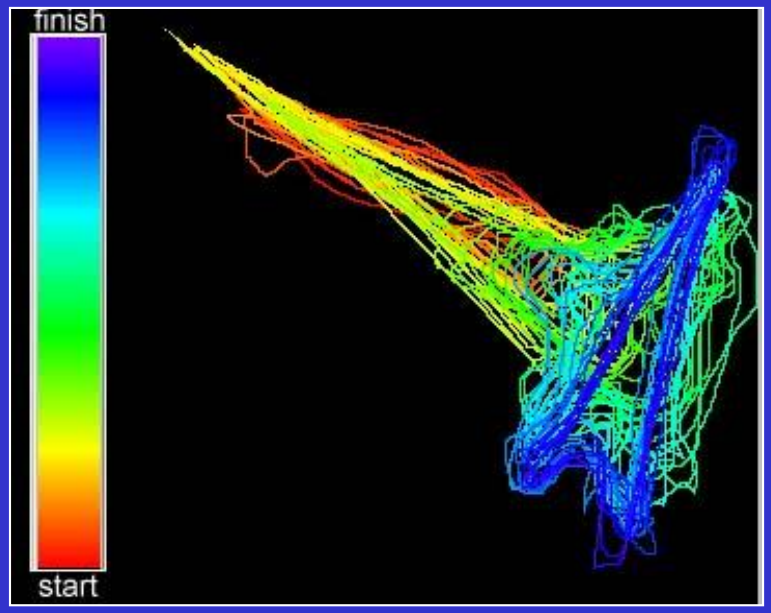
Typical Parameters:

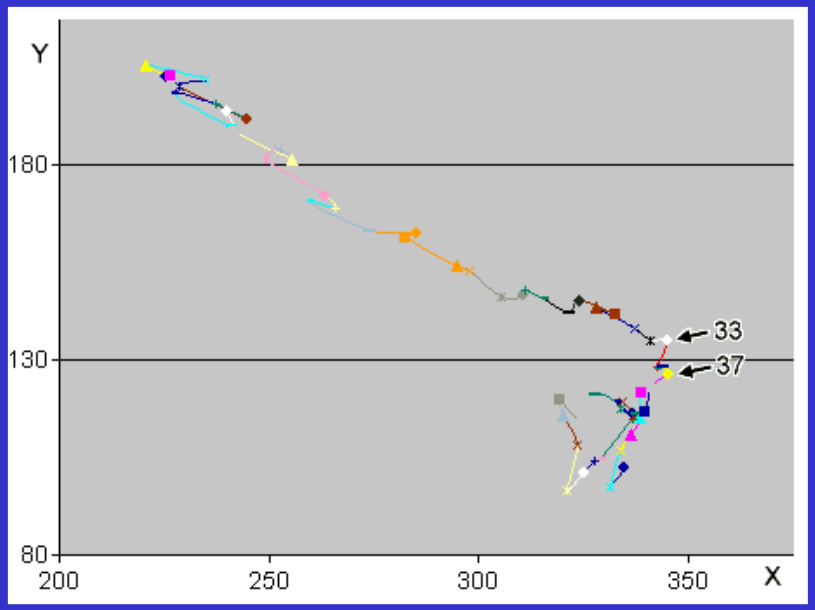
- Velocity
- Persistence
- Area Change
- Shape Change



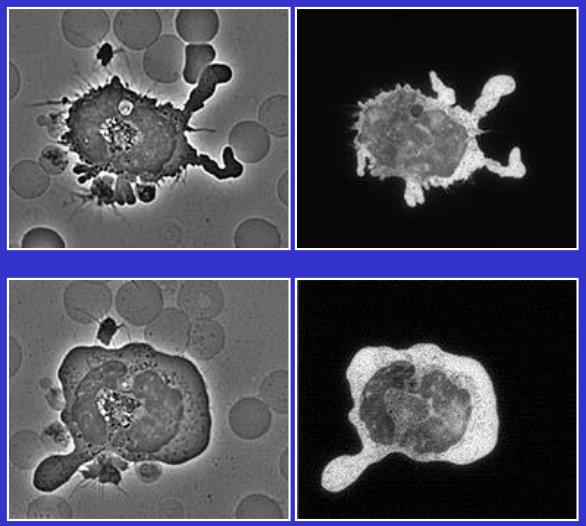
Time Lapse for Morphometrics

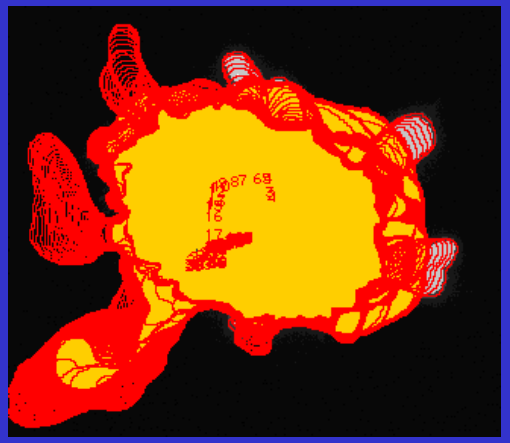


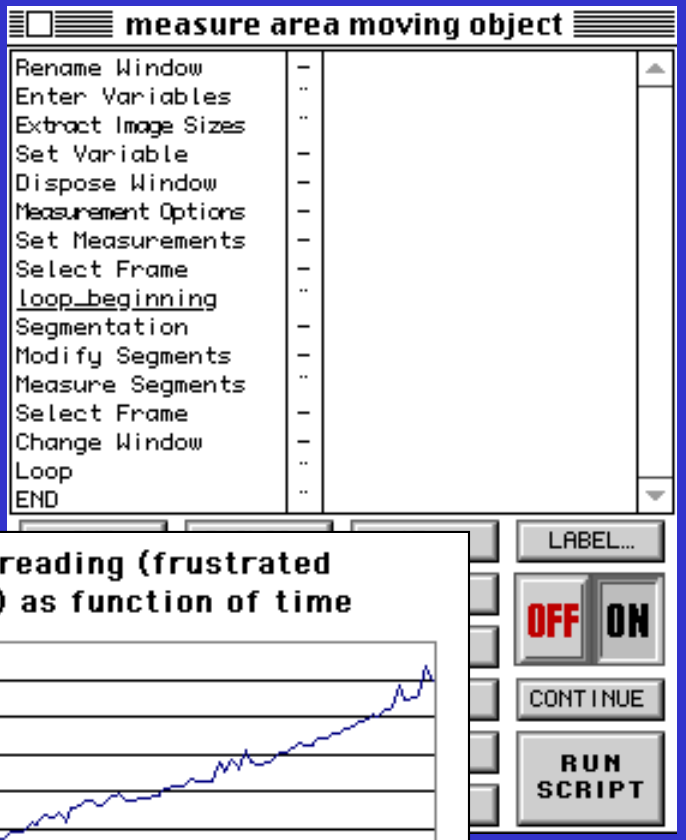


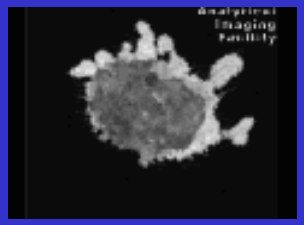


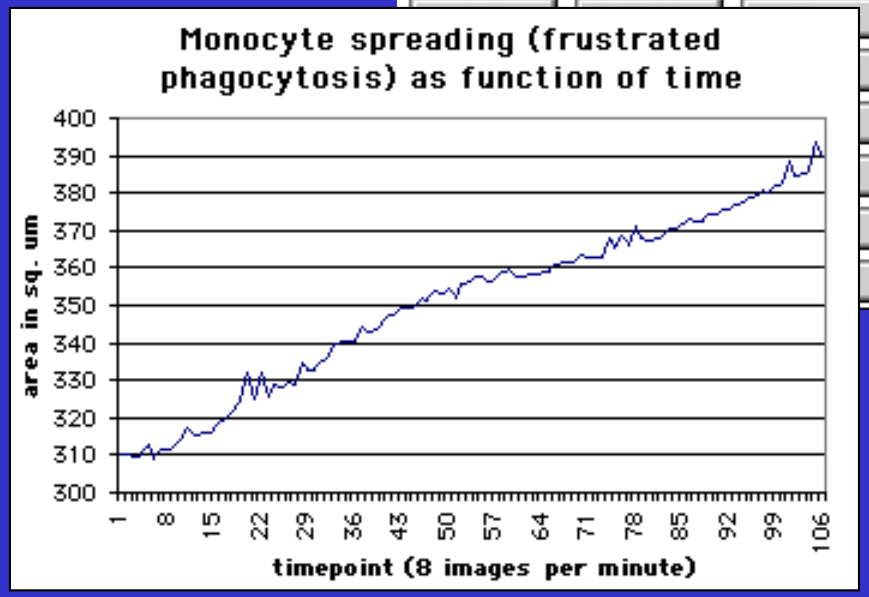
Semi-Automated Measurement of Motility



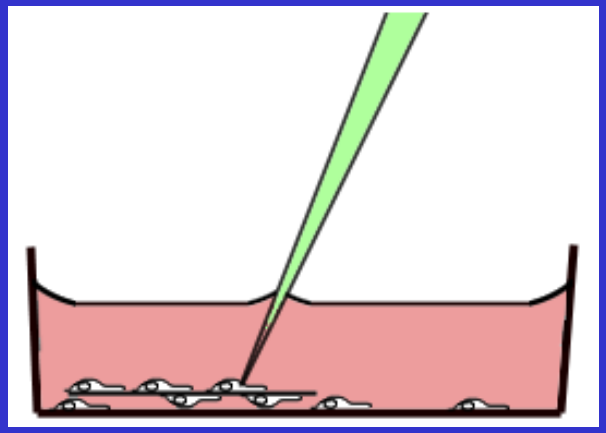


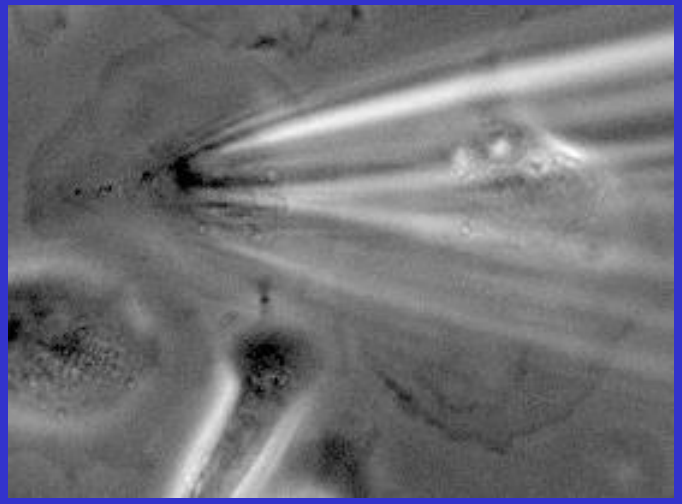


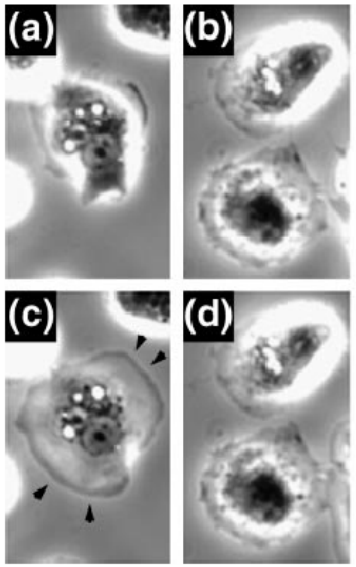


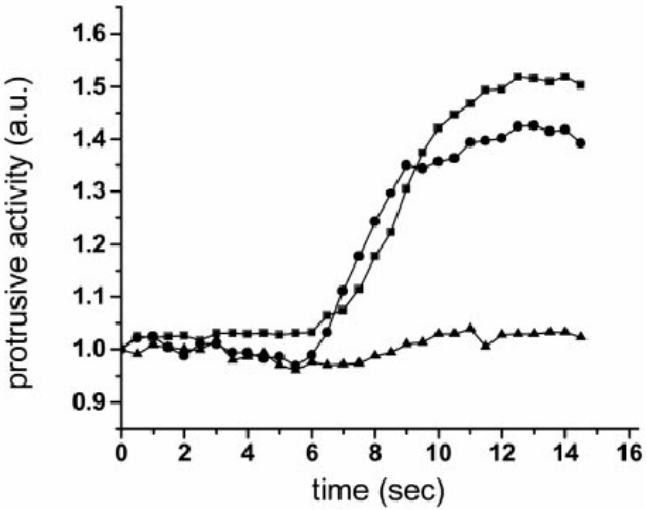


Microinjection







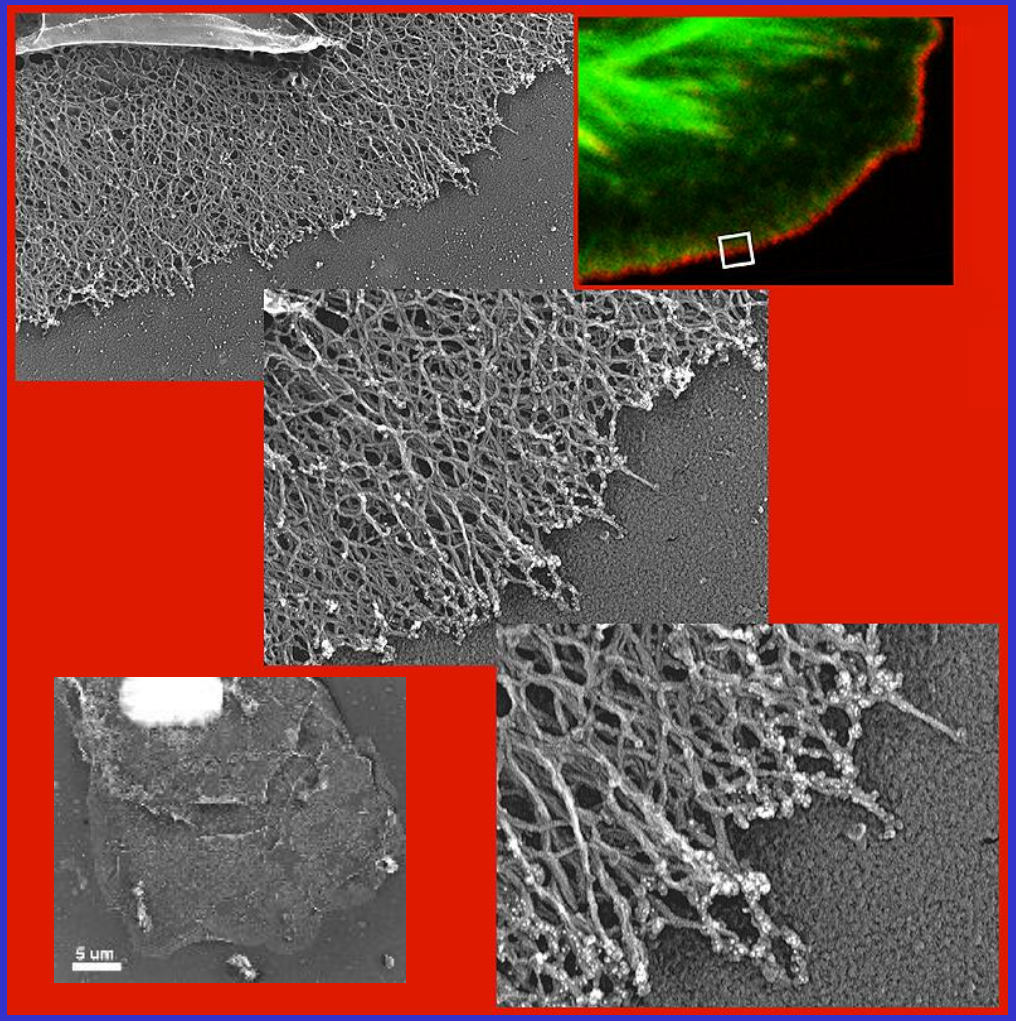


Inhibition of lamellipod extension after microinjection of AE360 anti-p34 antibodies. Left panel; **(a,c)** morphology of control noninjected cells, and **(b,d)** cells microinjected with AE360 anti-p34 antibodies immediately (a, b) before and (c,d) 3 min after EGF stimulation. Lamellipod extension is shown on control cells (arrowheads). Right panel; quantitation of the protrusive activity after EGF stimulation in cells 30–45 min after antibody microinjection, measured as described previously [3, 21]. Triangles, AE360 anti-p34 antibodies (n = 14); squares, nonimmune IgGs (n = 75); and diamonds, control mock-injected cells (n = 17). SEM < 5%.

The F-actin side binding activity of the Arp2/3 complex is essential for actin nucleation and lamellipod extension

Maryse Bailly*‡‖, Ilia Ichetovkin*‡, Wayne Grant*, Noureddine Zebda*, Laura M. Machesky†, Jeffrey E. Segall* and John Condeelis*

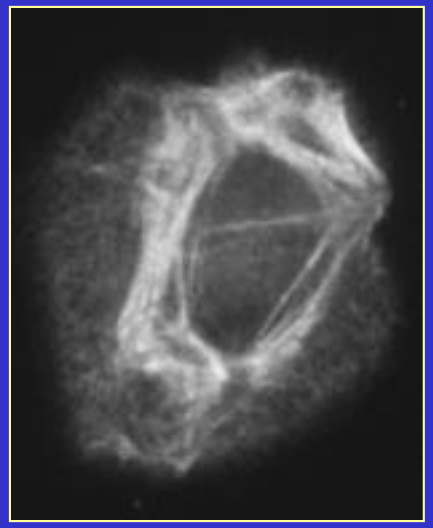
Correlation of Techniques



Each Image Acquisition & Analysis Package Has Scripting Capability

The Following Slides Show An Example Of Semi-Automated Analysis Combined With Training Of Very Simple Quantification of Concentration or Total Mass of a Chemical Species

Quantification of mass and concentration in cells by fluorescent microscopy and computer analysis



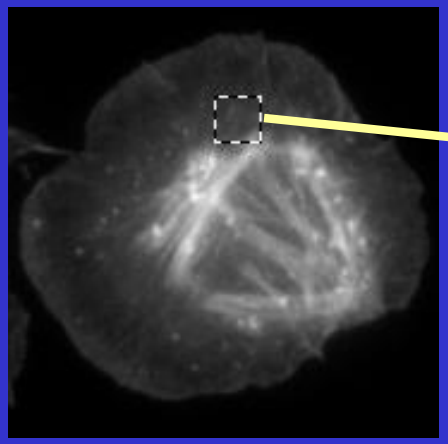
= ???

There are two types of measurements we get from intensity information.

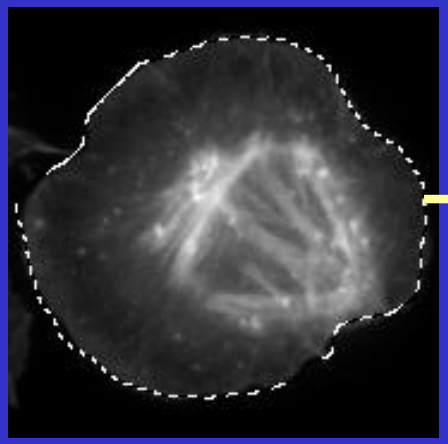
1. Mass (total amount of a species in a given area or volume)
2. Concentration (amount of mass per unit area or volume)

Mass

Mass = integrated intensity = sum of all pixel values in a given area



238,088

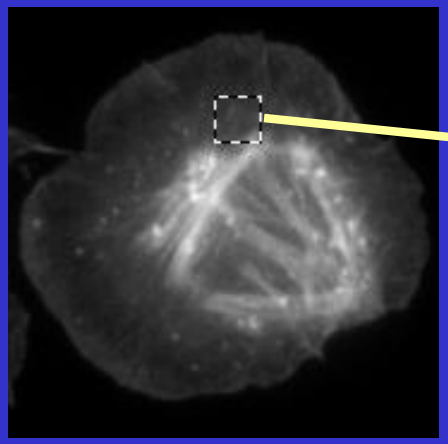


11,852,009

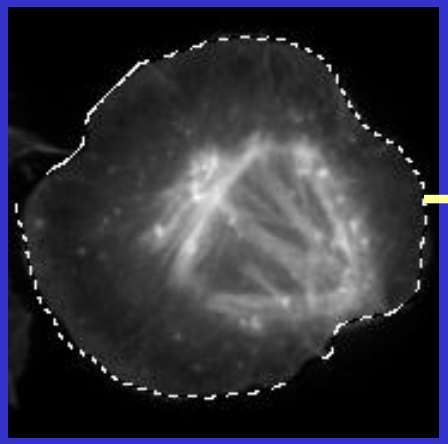
DO NOT use the Integrated Intensity command in NIH-Image!!

Concentration

Concentration = mass per unit area (# of pixels) = mean mass



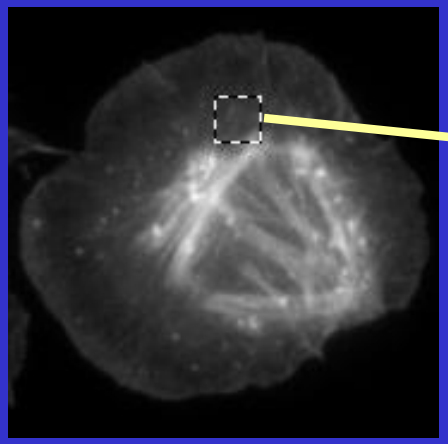
595.22



625.04

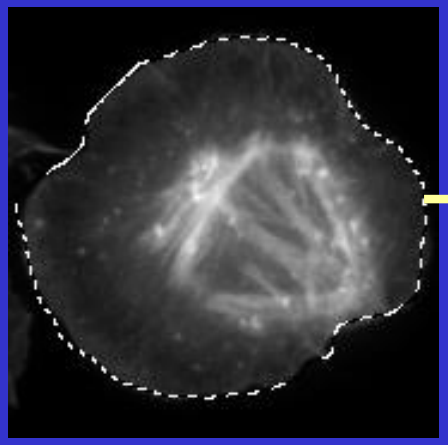
Concentration

Concentration = mass per unit area (# of pixels) = average mass



238,088 / 400 =

595.22



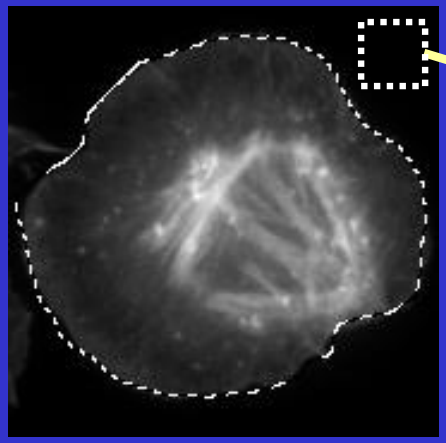
11,852,009 / 18962 =

625.04

Whoops, we skipped a step!

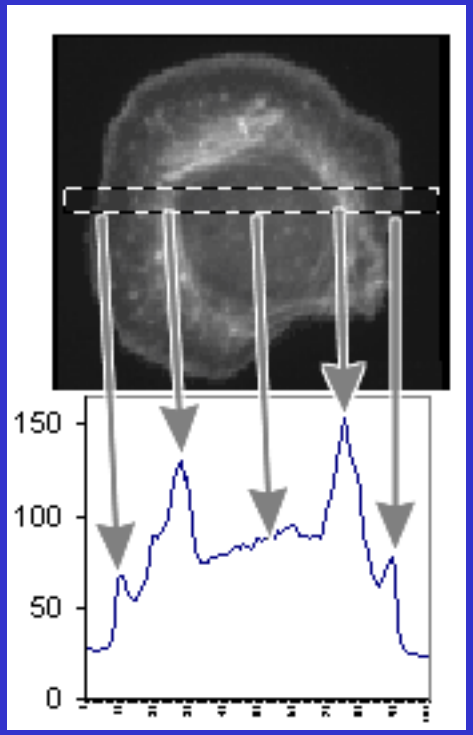
The previous measurements assumed an ideal situation where everything that was non-cell was pure black or zero.

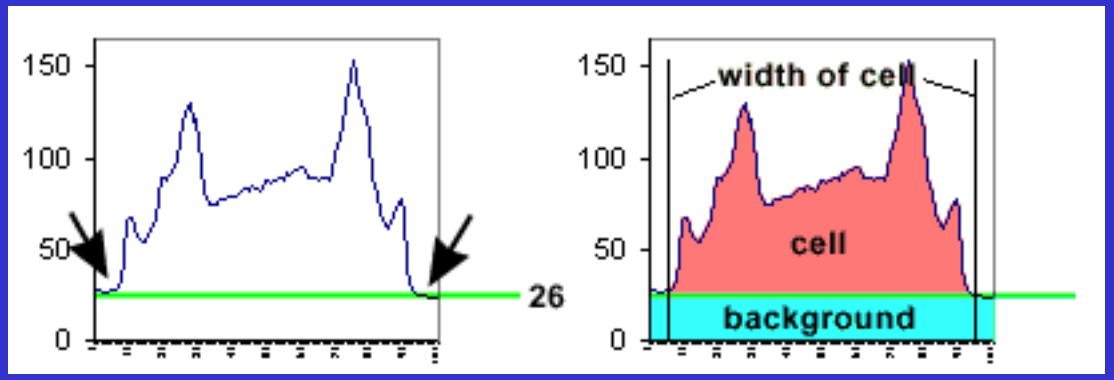
But when we collect images, this isn't true!



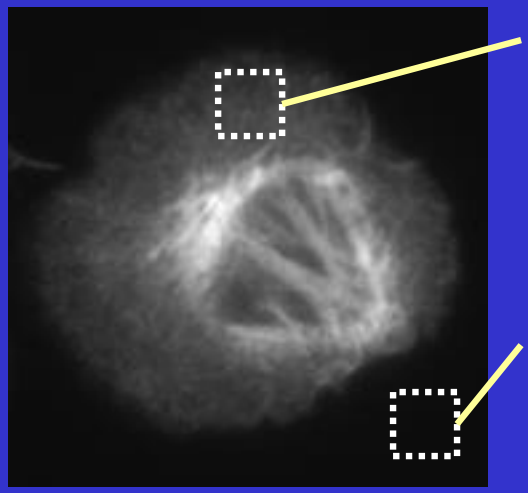
440 +/- 10

Area under the curve





Subtract the background

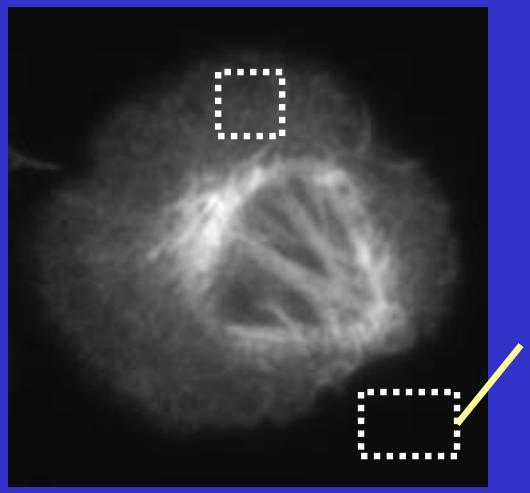


Let's say that this box is 10 X 10 pixels.
Let's say its integrated intensity = 100,000.

Let's say that this box is 10 X 10 pixels.
Let's say its integrated intensity = 44,000.

The mass of the box within the cell is really
100,000 – 44,000 = 56,000

Subtract the background



Because the background area may vary in size, it it simpler to always do the math based on the concentration.

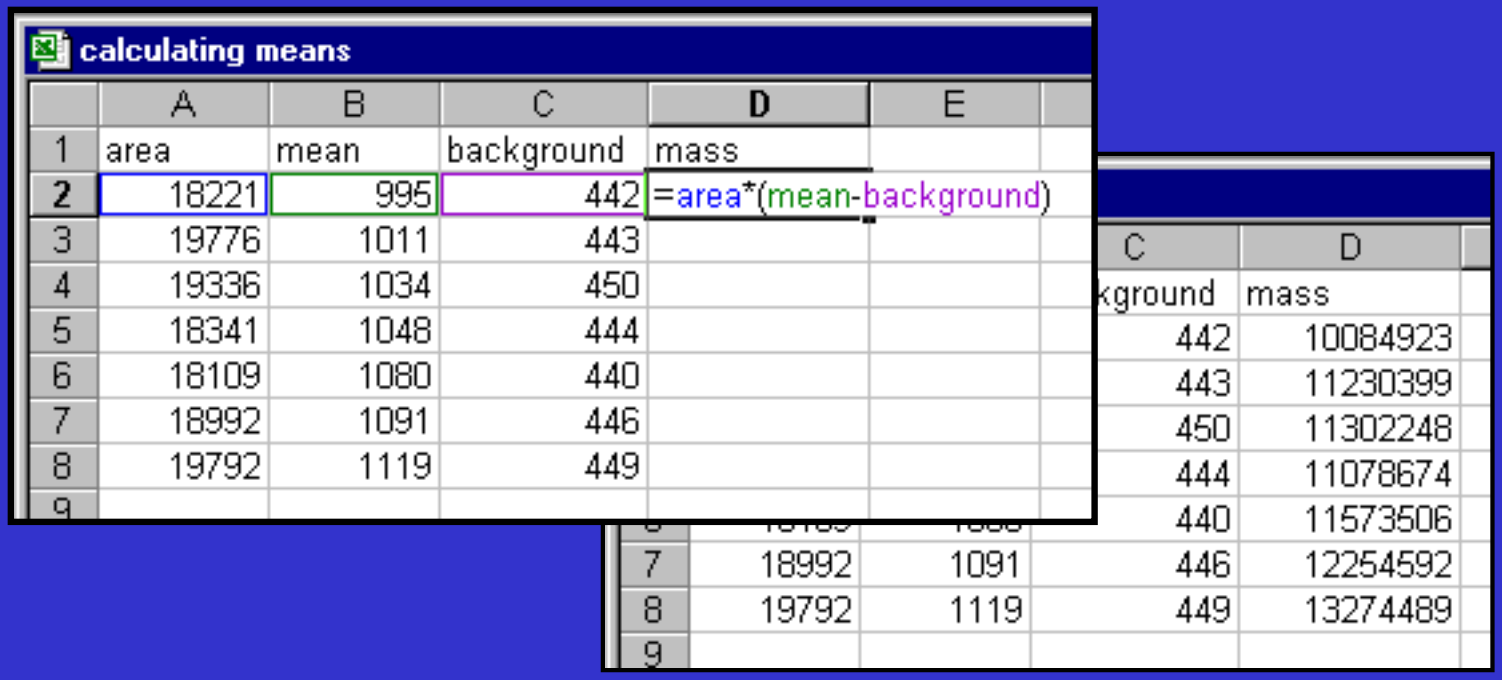
The size or shape of this box doesn't matter. All we care about is that its Mass / area = 440.

NIH-Image doesn't give an integrated intensity measurement, but it gives a good mean (or average), therefore…

More simple math operators…

mass = cell_area * (cell_mean – background)

which is simple to calculate in Excel.



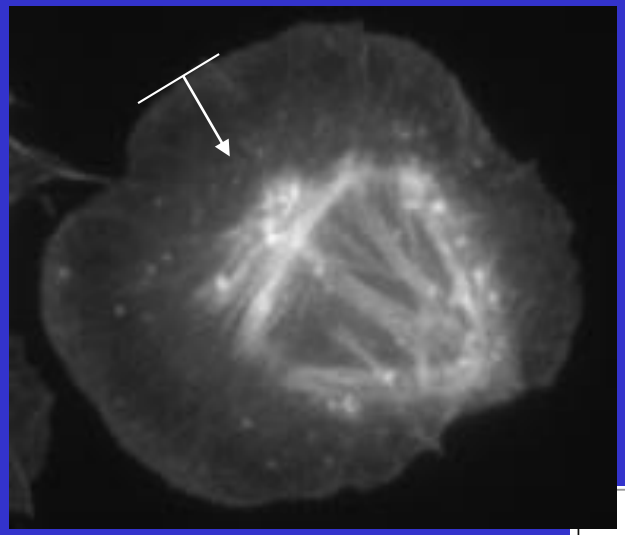
Macros

What is a macro or script?
A short computer program which operates within an application.

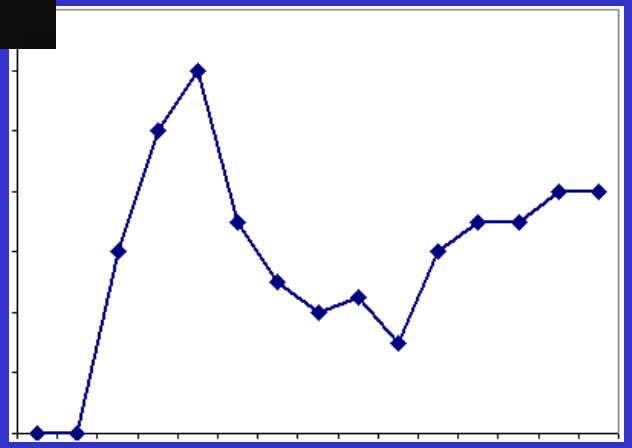
Why use a macro?
One keystroke or click executes many commands. This saves you time and helps maintain quality control. And much of the math gets done for you without Excel.

But the intensity measurements look really simple to do, even with Excel, so why bother?
You need to do repetitions on particular compartments or on multiple probes. Also, quality control and prevention of Repetitive Stress Disorder.

An example of why you need macros

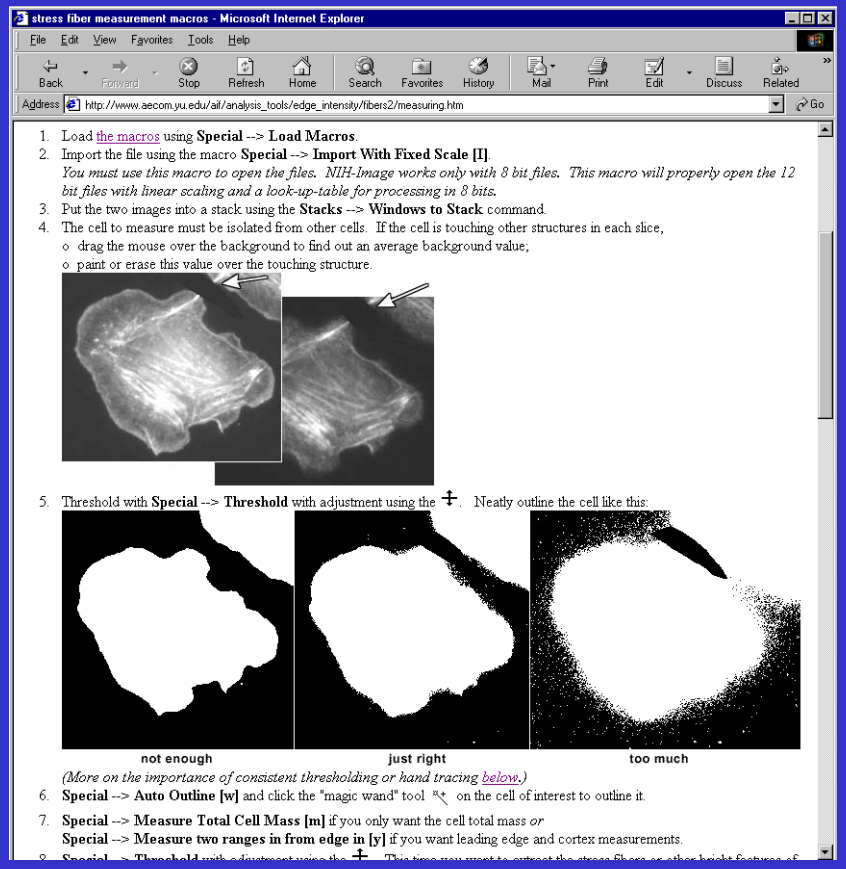


Measuring concentration of a protein as a function of distance from the leading edge.

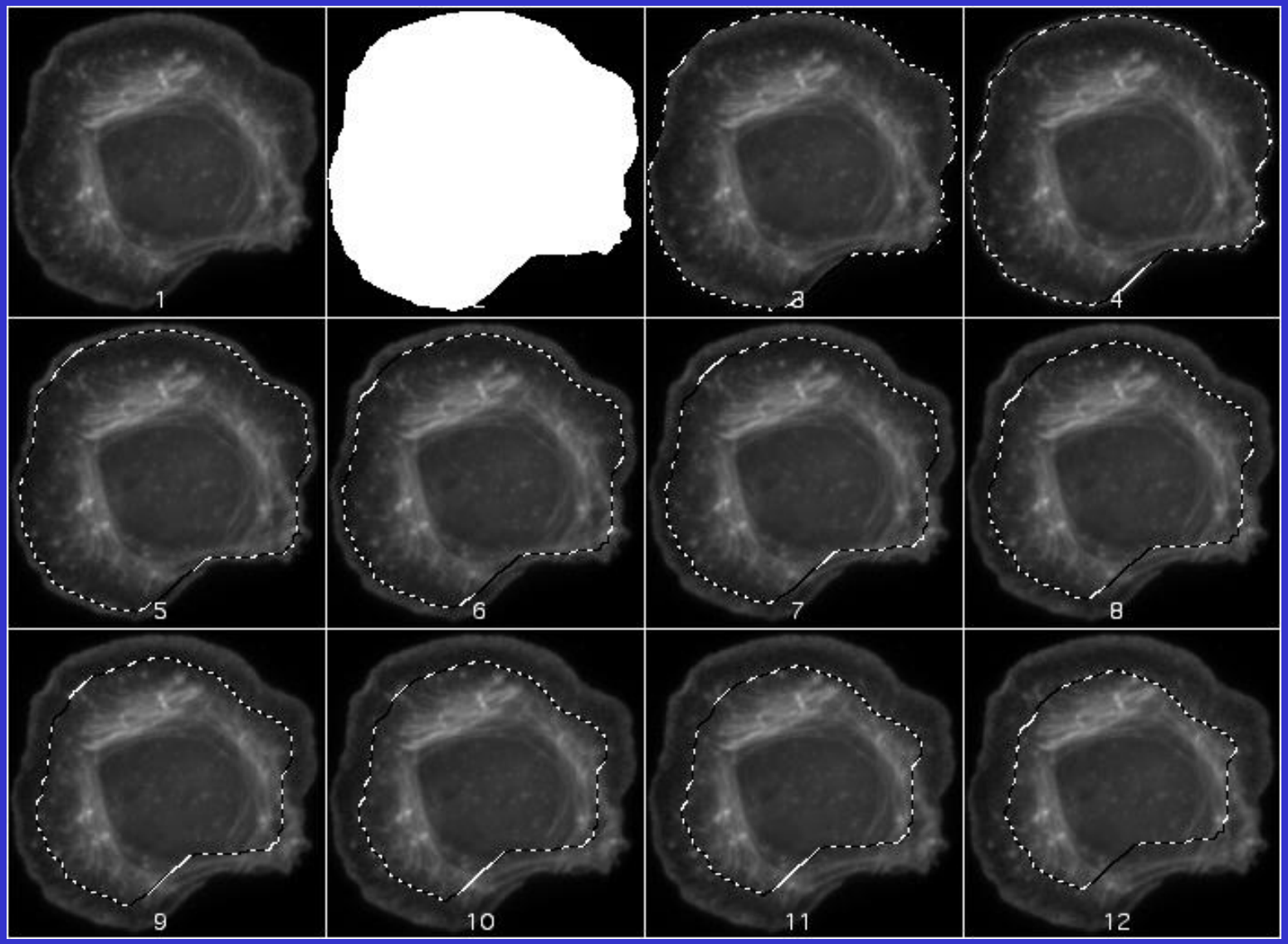


Where to find macros

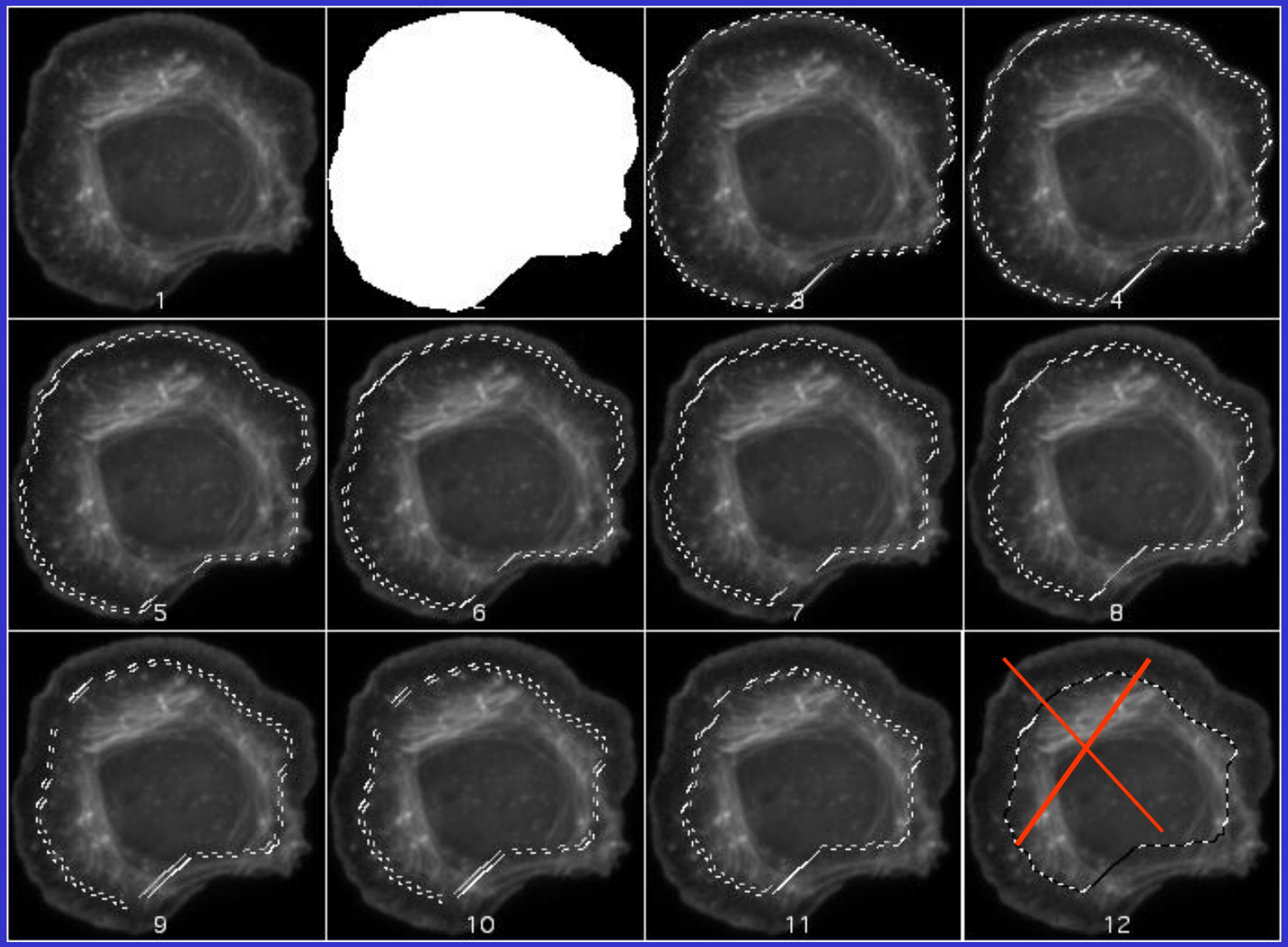
http://www.aecom.yu.edu/aif/analysis_tools/intensity.htm



An example



The “Annulus”



Results calculated for you

results	slice 1 of stack	slice 2 of stack
total mass	7399409.5000	4596954.0000
leading edge mass	345670	49508
cortext mass	622947.2500	217867.5938
background	459.2374	418.9246
total cell area	25417	25417
leading edge area	3372	3372
cortex area	2985	2985
total mass fibers	5716333.5000	4151482.2500
percent mass leading edge	0.0467	0.0108
percent mass cortext	0.0842	0.0474
percent mass fibers	0.7725	0.9031

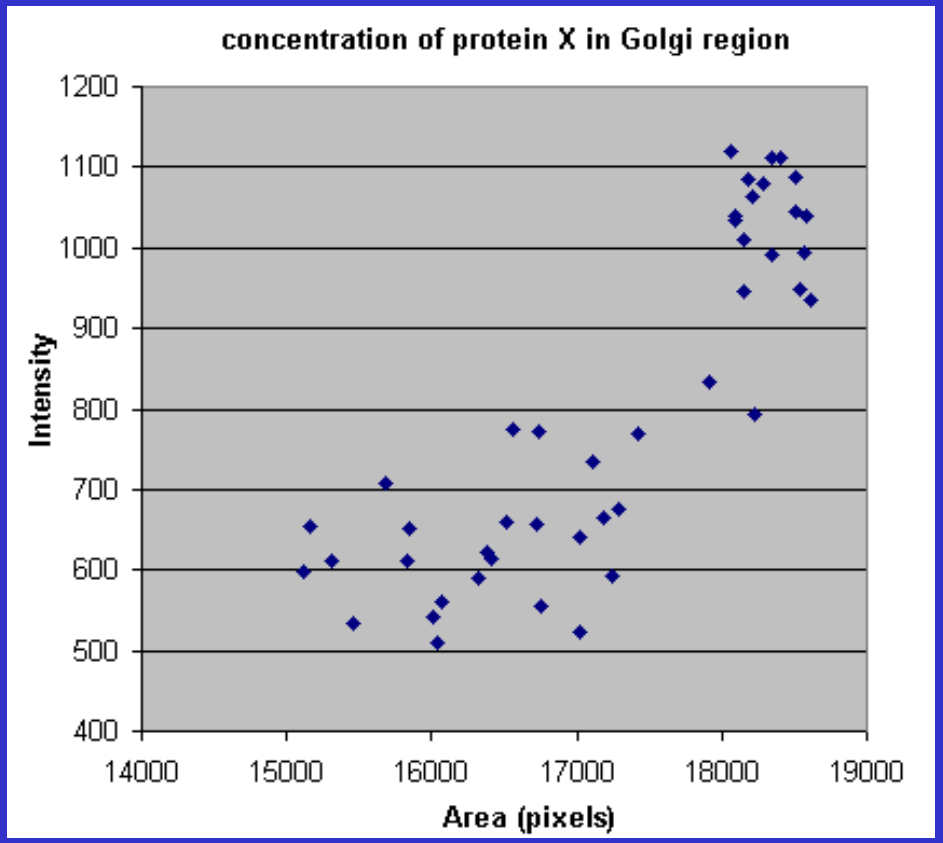
	A	B	C
1	results	slice 1 of stack	slice 2 of stack
2	total mass	7399409.5	4596954
3	leading edge mass	345670	49508
4	cortext mass	622947.25	217867.5938
5	background	459.2374	418.9246
6	total cell area	25417	25417
7	leading edge area	3372	3372
8	cortex area	2985	2985
9	total mass fibers	5716333.5	4151482.25
10	percent mass leading edge	0.0467	0.0108
11	percent mass cortext	0.0842	0.0474
12	percent mass fibers	0.7725	0.9031
13			

Correlating intensity measurements with other parameters

Mass & concentration measurements of whole cells or sub-structures can be grouped or classified by:

1. Cell area
2. Cell perimeter (a ratio of area to perimeter is a rough quality of roundness)
3. Cell polarity
4. Uptake of an external structure (e.g. bead ingestion)
5. Other morphological features

Ways to show these data

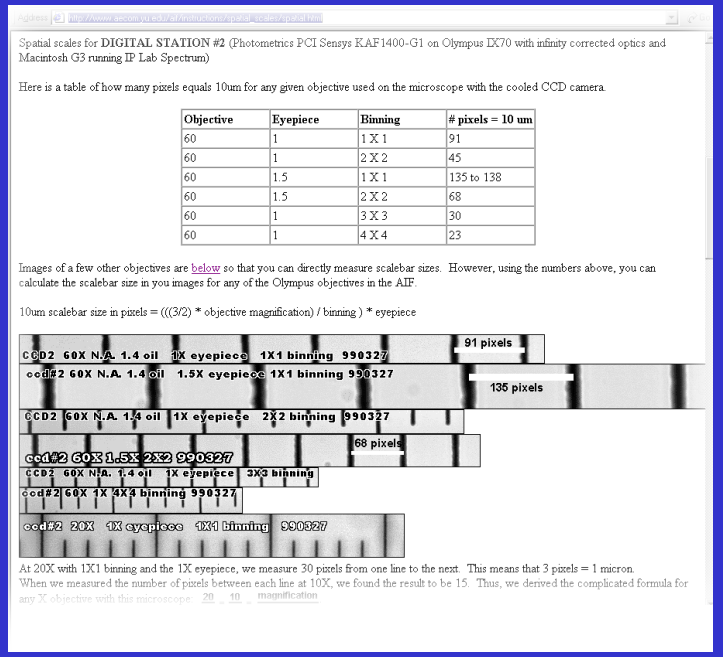


There is a correlation between higher concentration in the Golgi region and increased cell spreading.

Notes on intensity values.

	CCD# 1,2,3	Uncaging	confocal	NIH-Image	15 bit camera
White	4095	4095 or 65535	255	0 or indexed	32767
Black	0	0	0	255 or indexed	0

Converting pixels to µm



Spatial scales for **DIGITAL STATION #2** (Photometrics PCI Sensys KAF1400-G1 on Olympus IX70 with infinity corrected optics and Macintosh G3 running IP Lab Spectrum)

Here is a table of how many pixels equals 10um for any given objective used on the microscope with the cooled CCD camera.

Objective	Eyepiece	Binning	# pixels = 10 um
60	1	1 X 1	91
60	1	2 X 2	45
60	1.5	1 X 1	135 to 138
60	1.5	2 X 2	68
60	1	3 X 3	30
60	1	4 X 4	23

Images of a few other objectives are below so that you can directly measure scalebar sizes. However, using the numbers above, you can calculate the scalebar size in you images for any of the Olympus objectives in the AIF.

10um scalebar size in pixels = (((3/2) * objective magnification) / binning) * eyepiece

At 20X with 1X1 binning and the 1X eyepiece, we measure 30 pixels from one line to the next. This means that 3 pixels = 1 micron. When we measured the number of pixels between each line at 10X, we found the result to be 15. Thus, we derived the complicated formula for any X objective with this microscope: 20 ÷ 10 = magnification

CCD#1 or CCD#2

Objective	eyepiece	binning	typical approximate image size	pixel size
60X	1X	1 X 1	1024 X 1024 pixels	0.11 um
60X	1X	2 X 2	512 X 512 pixels	0.22 um
60X	1.5X	1 X 1	1024 X 1024 pixels	0.073 um
60X	1.5X	2 X 2	512 X 512 pixels	0.147 um

Interchangeable Jargon

Mass = integrated intensity = sum of pixels

Concentration = mass / area = mean intensity = average intensity

Background = mean value of that stuff around the cell which really isn't black

Not discussed today

- Justification of 2D imaging for our 3D cell measurements
- Requirements of light microscopy for getting images that can be analyzed
- How these methods can be used for other applications, such as automatic measuring of neurite outgrowth by density or by absolute count or for charting the tides

Resources

http://www.aecom.yu.edu/aif/

Manuals & tips links to

http://www.aecom.yu.edu/aif/instructions/manuals.htm

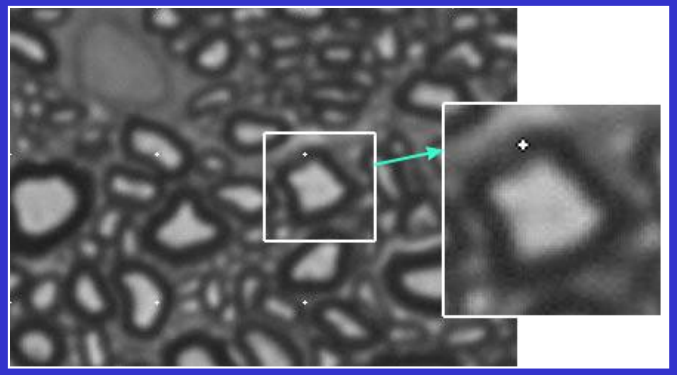
Analysis Tools links to

http://www.aecom.yu.edu/aif/analysis_tools/intensity.htm

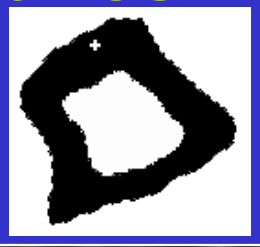
I wrote the macros. Make an appointment to talk to me.
Leave a note or email to make an appointment if I'm busy.

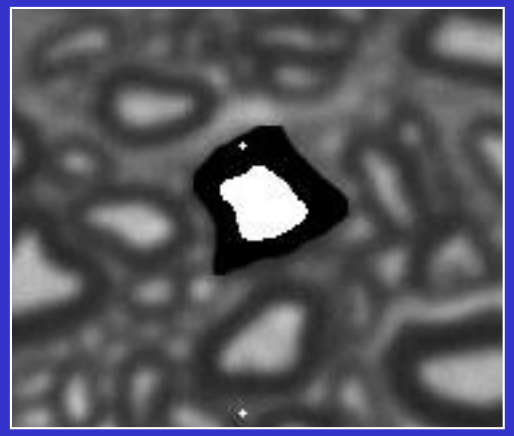
Another Example of Simple Scripts For Quantification Customized For A Researcher

Semi-Automated Measurements of Axons with Myelin Sheathes



```
MACRO 'Measure a traced axon [m]';
VAR
left, top, width, height : integer;
original : real;
axon, myelin : integer;
BEGIN
GetROI(left, top, width, height);
IF width = 0 THEN Exit;
original := PIDnumber;
Measure;
ChangeValues(0,0,rMean[rCount]);
AutoThreshold;
MakeBinary;
Measure;
axon := Histogram[0];
myelin := Histogram[255];
SelectWindow('AM_results');
Writeln(axon, chr(9), myelin);
SelectPIC(original);
KillROI;
END;
```





axons	myelin
259	796
670	1128
502	1407
468	1358
177	543
413	1003
885	1874
485	1054
320	811

Development

New Directions of the Resource Are Driven By Research Needs. Development Leads to More Sophisticated Service.

What a Facility Can Provide:

All the Routine Service and Support *Plus*…

- Customization of instrumentation to meet needs of researchers
- Development

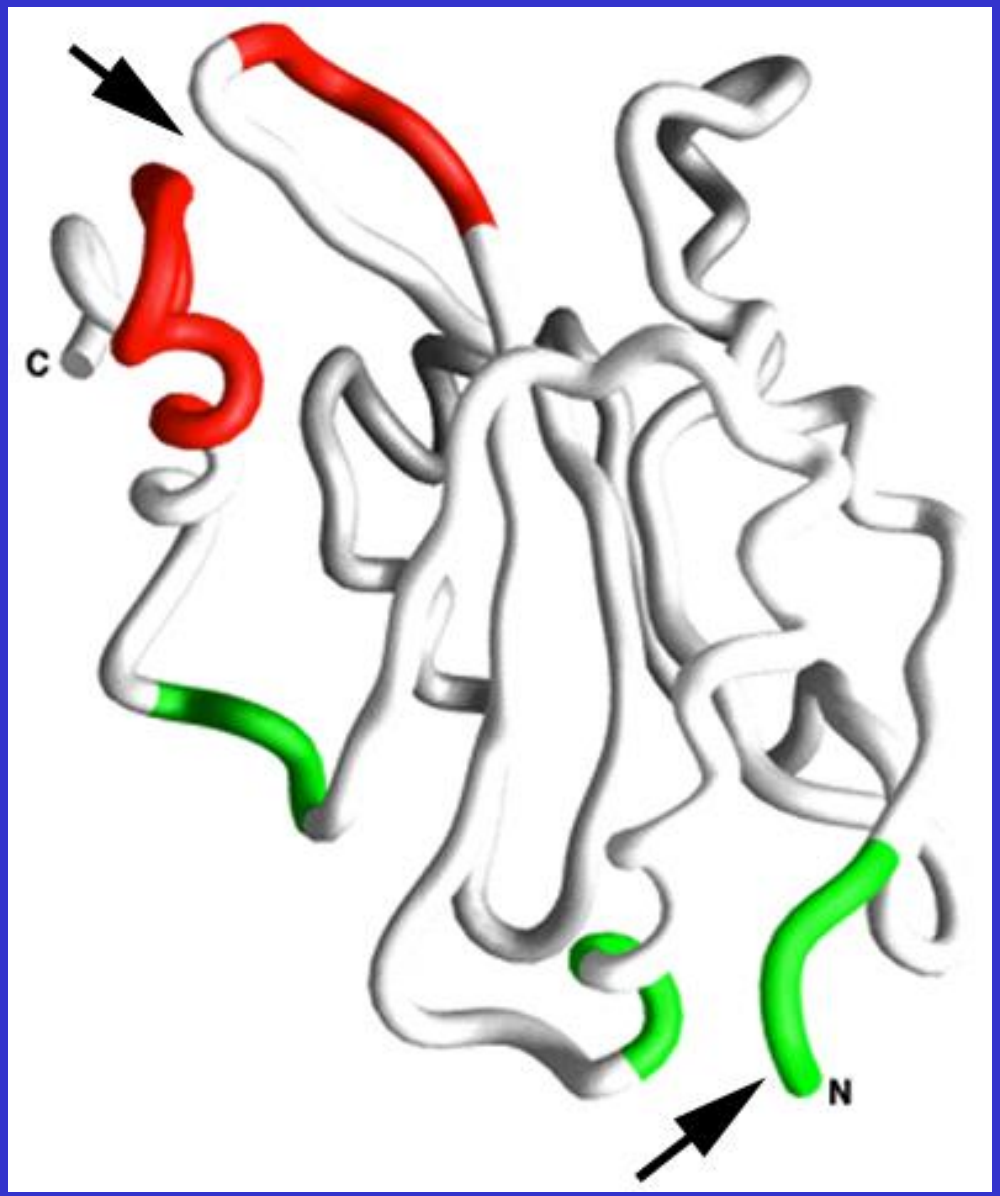
Biochemical Kinetics: The Cell as a Cuvette

Example project for customization and development.

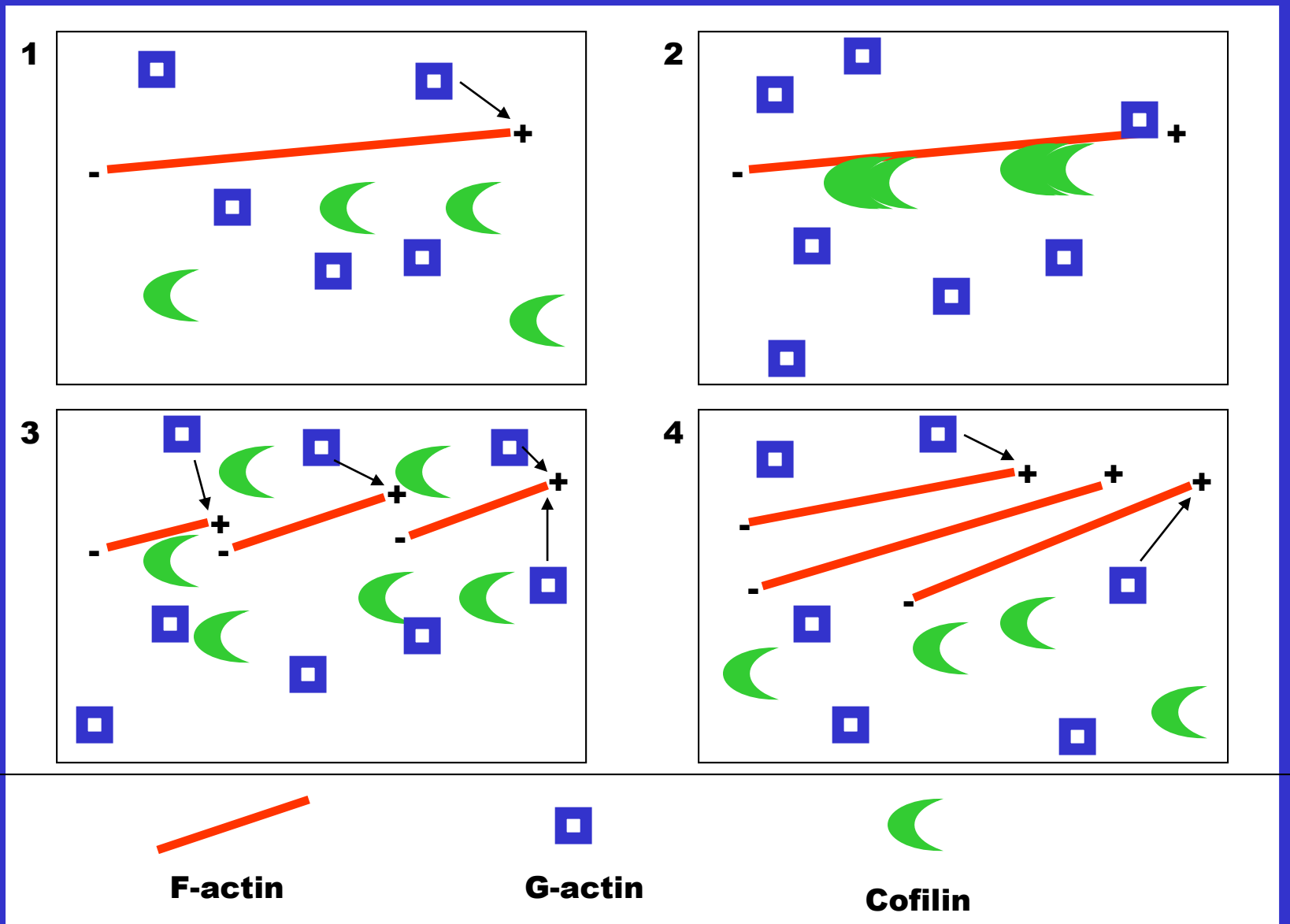
Cofilin

- Necessary for F-actin severing
- Phosphorylation regulates
- Binds to F- and GDP G-actin

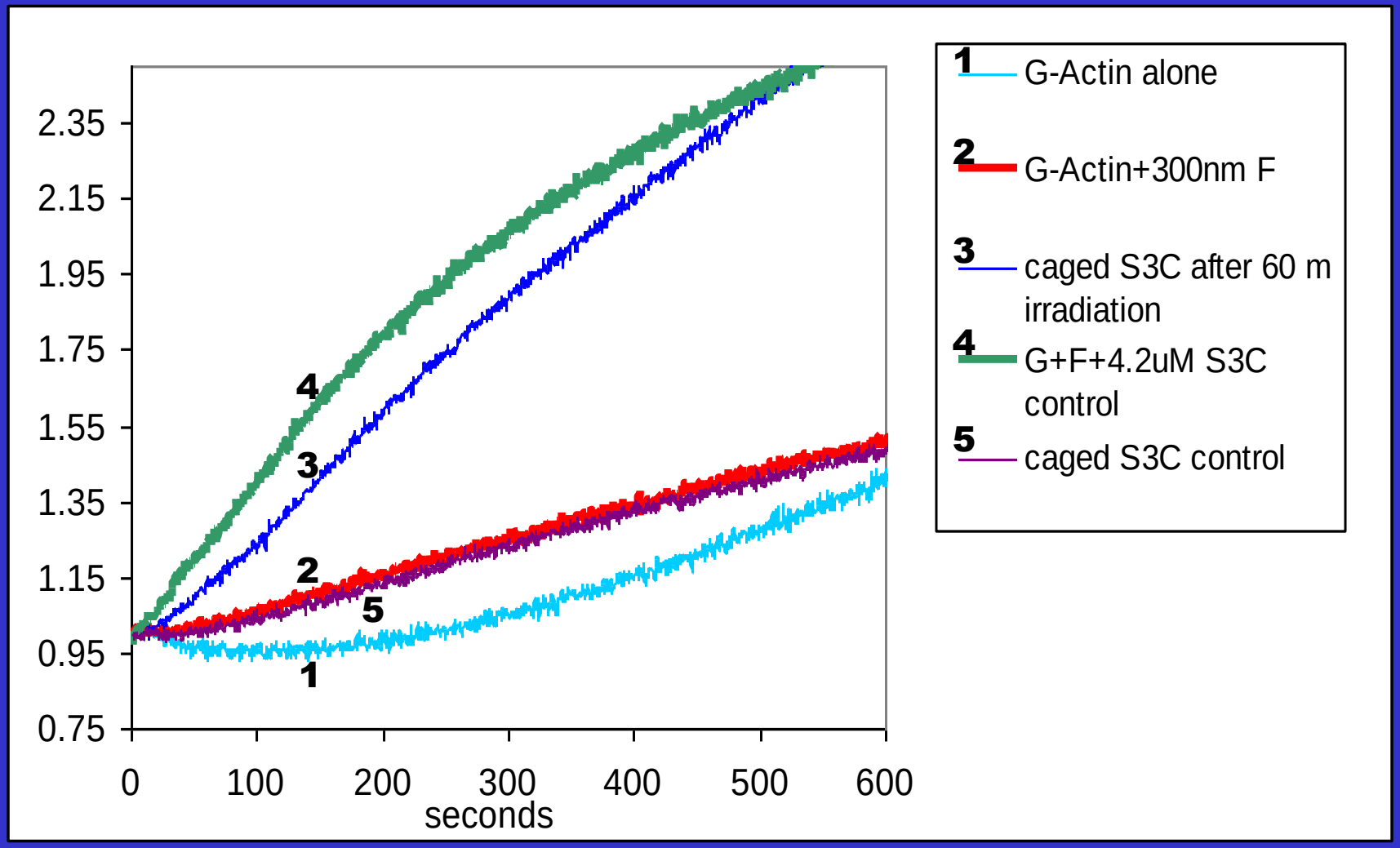
Caged at cysteine at S3 position



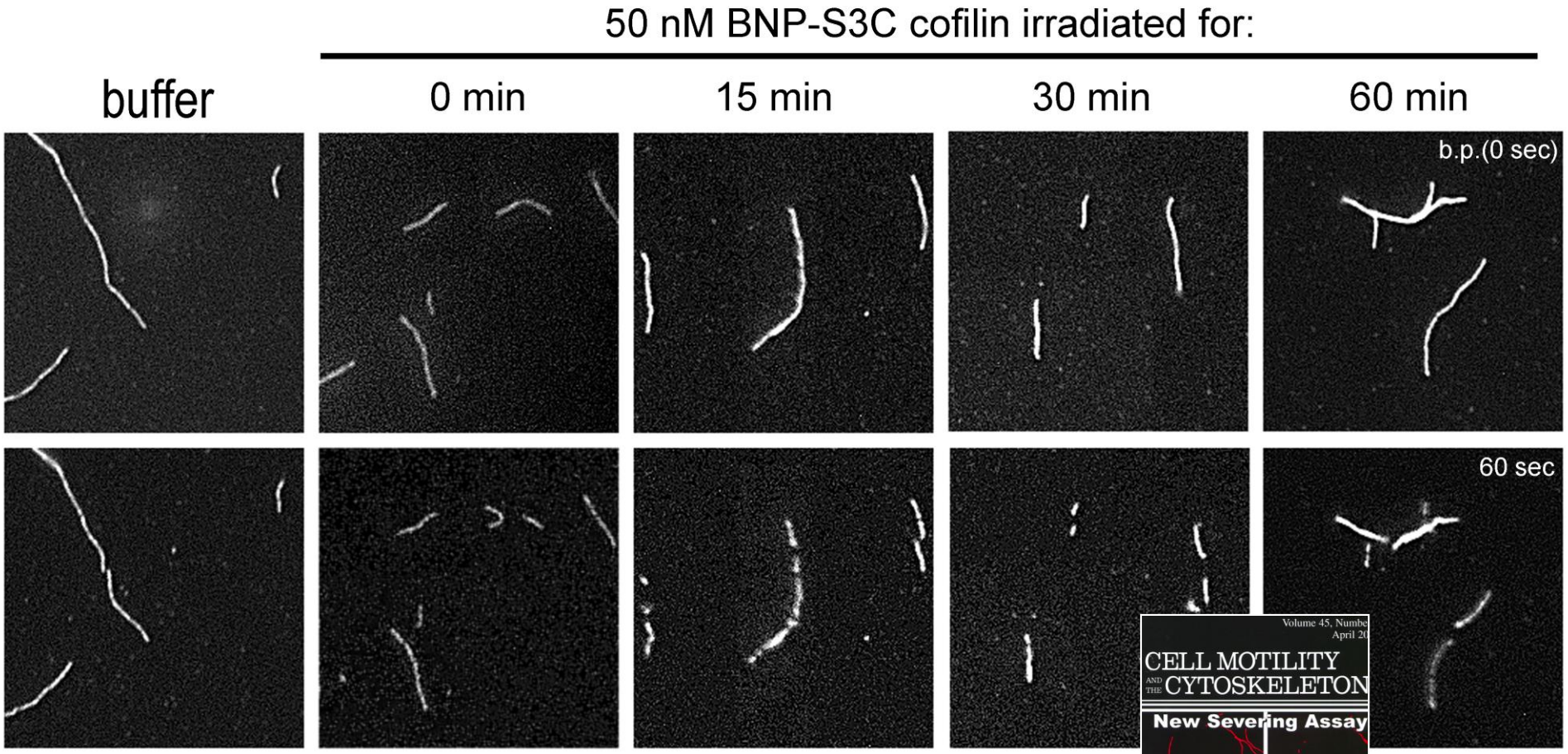
Cofilin increases F-actin nuceation



Uncaged S3C cofilin increases F-actin nuceation



Actin filaments are severed by uncaged S3C



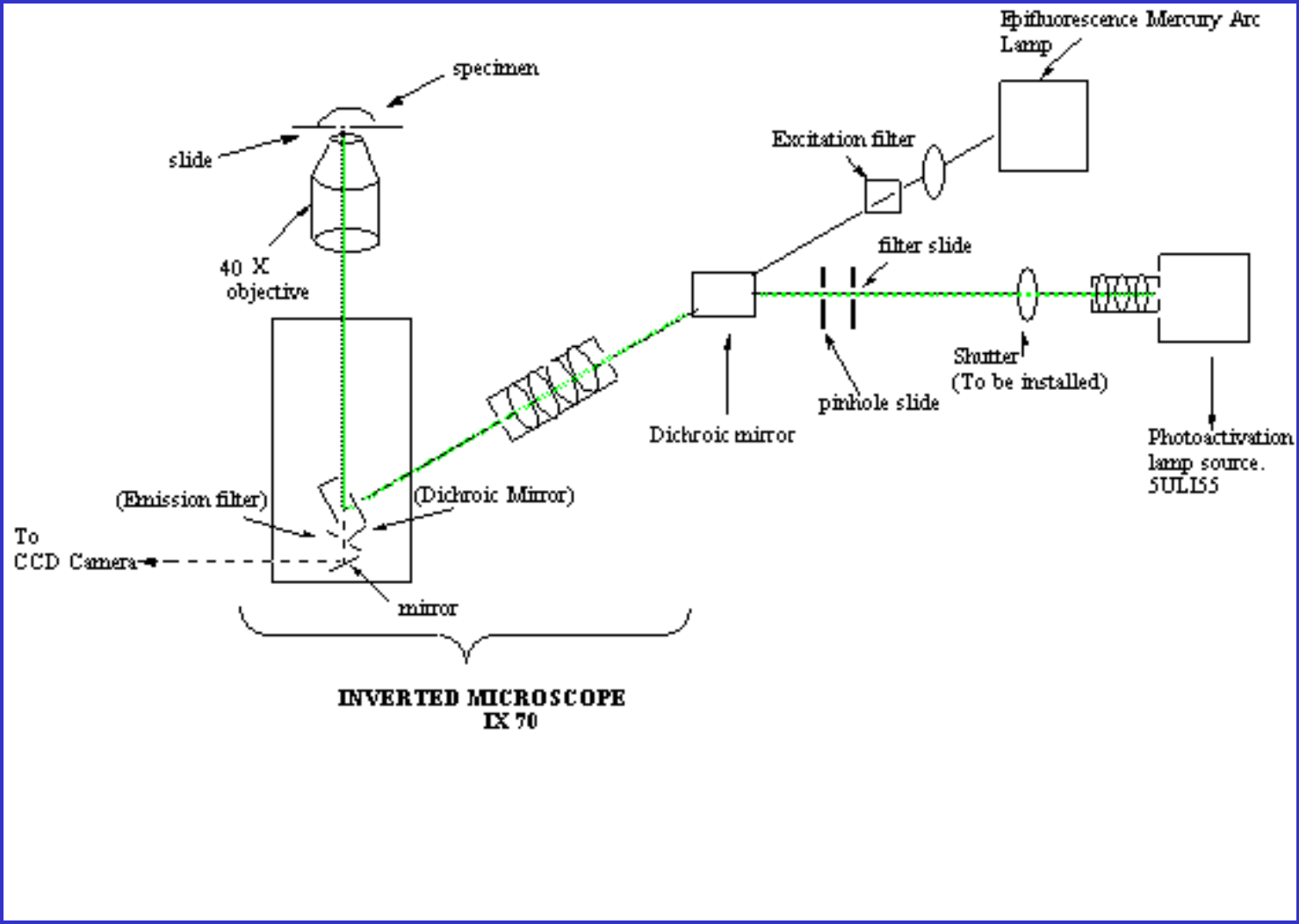
Technical Hurdles

- Experiments in cofilin expressing cells will have a high background due to endogenous cofilin
- Cofilin cannot be knocked-out; essential for viability
- Need alternate strategy

Caged Cofilin

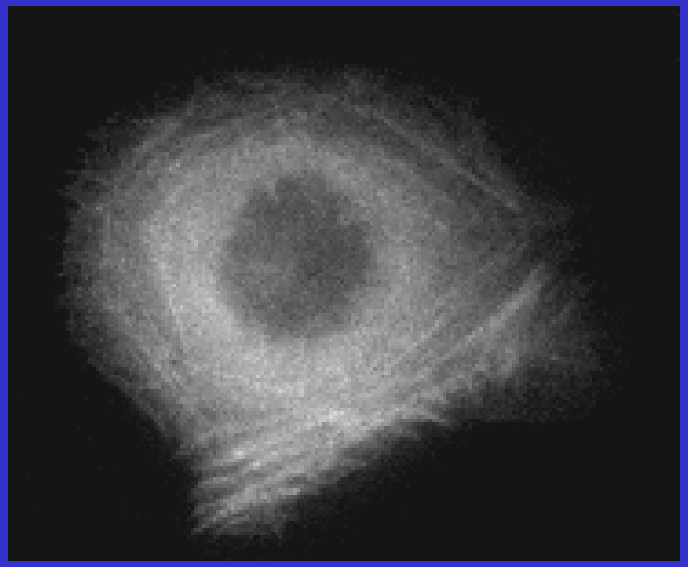
1. Serine 3 replaced with alanine or cysteine to prevent phosphorylation and shutdown
2. Microinject a plasmid to make cell overexpress LIM kinase to phosphorylate endogenous cofilin and shut down activity (activity can be regained by microinjecting S3A or S3C cofilin)
3. Microinject caged S3C
4. Uncage
5. Observe

Light path for uncaging through inverted microscope

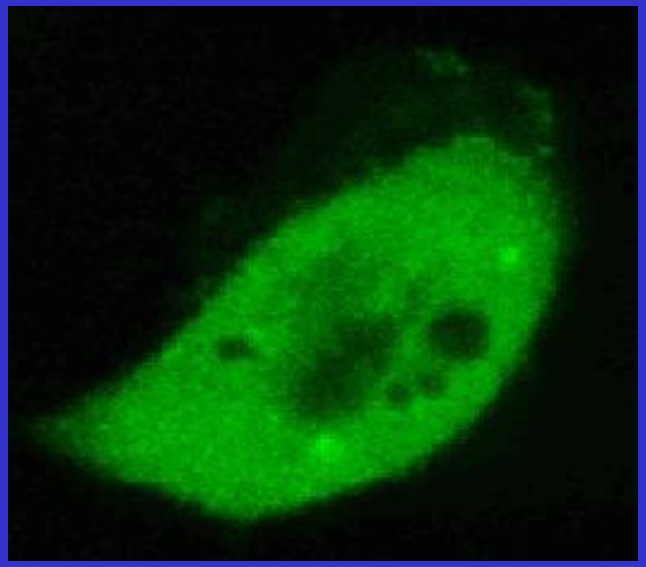


During Upshift Lamellipod is 500 nm thick

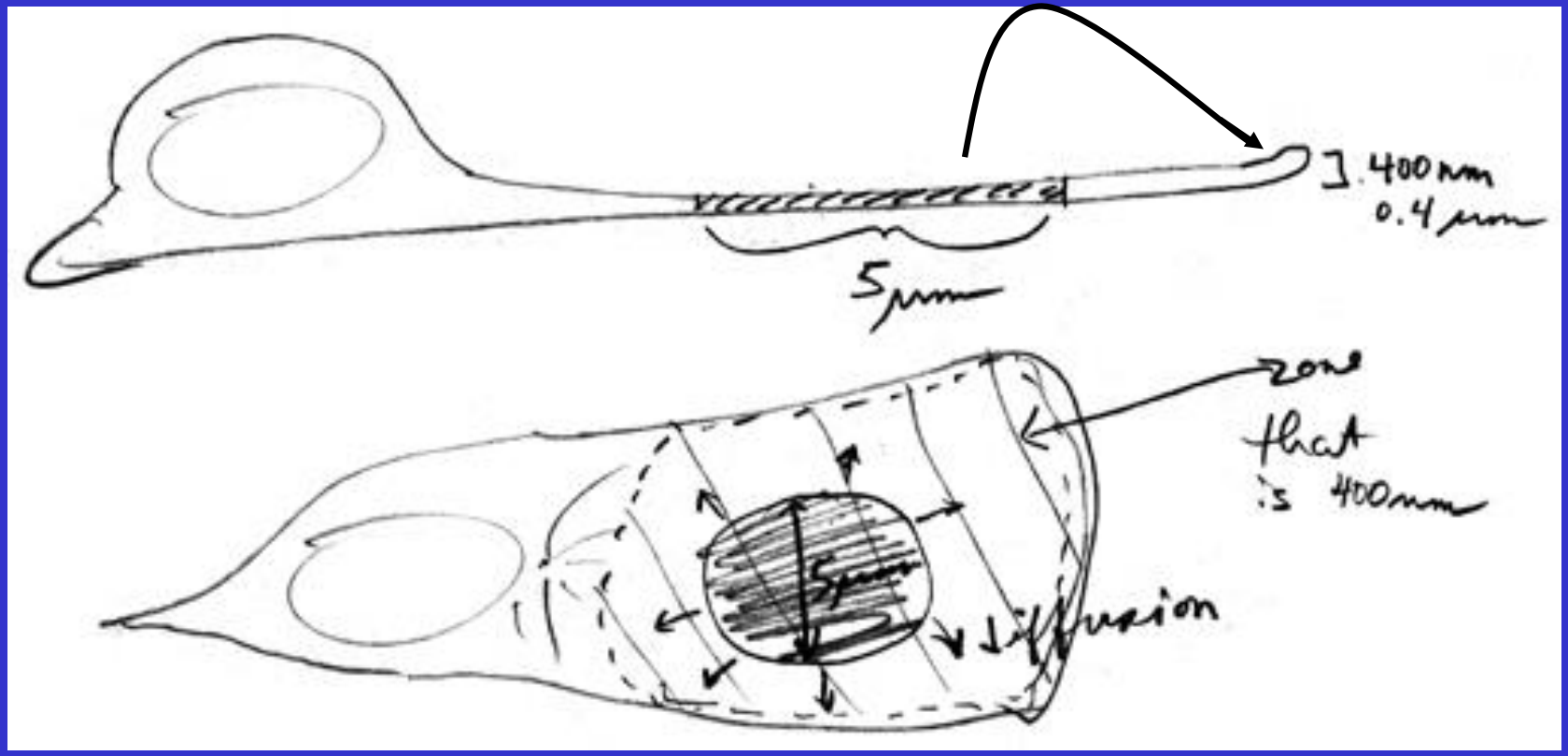
Watch Specific Proteins During Physiologic Events



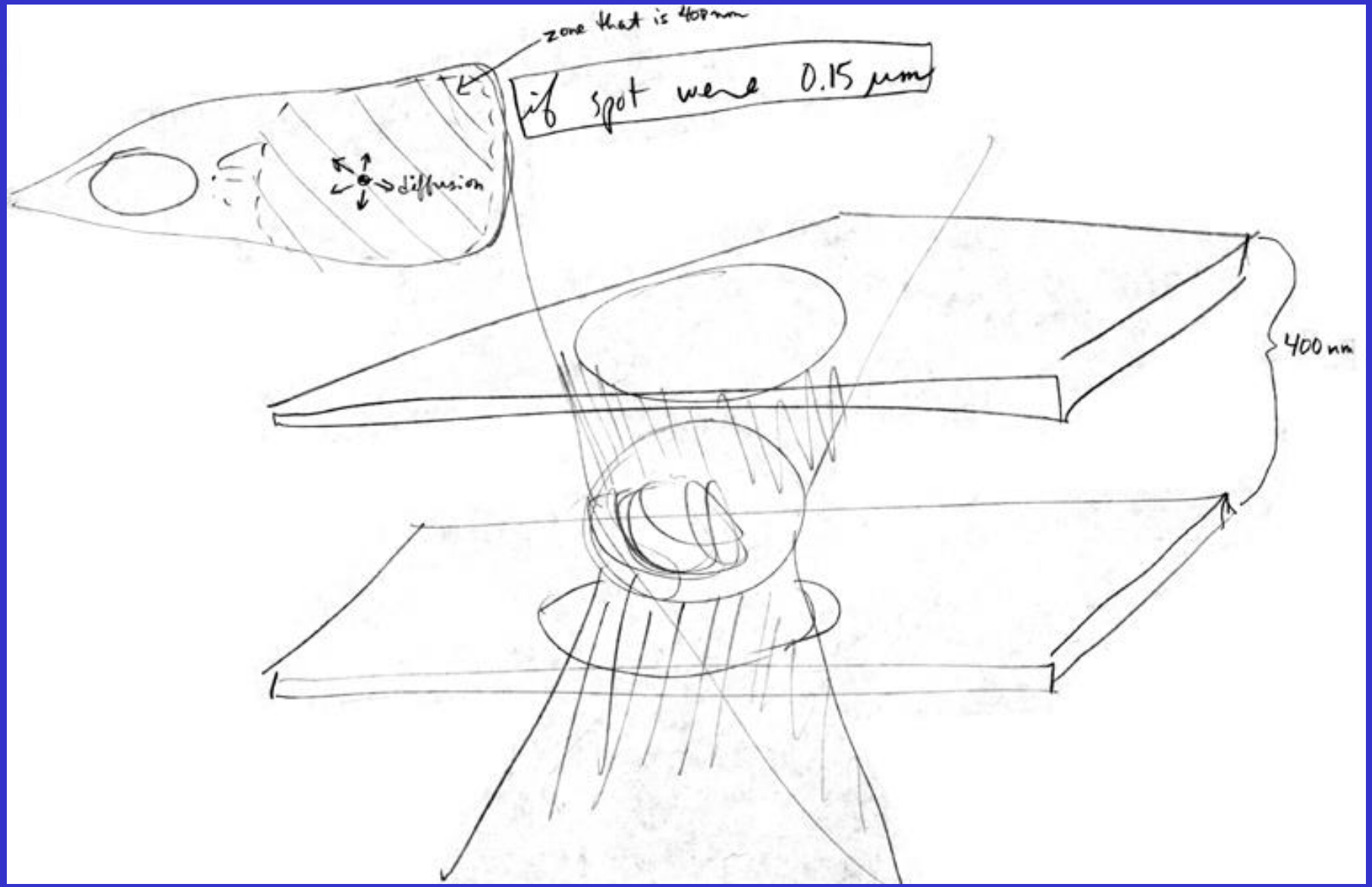
Watch Specific Proteins During Physiologic Events



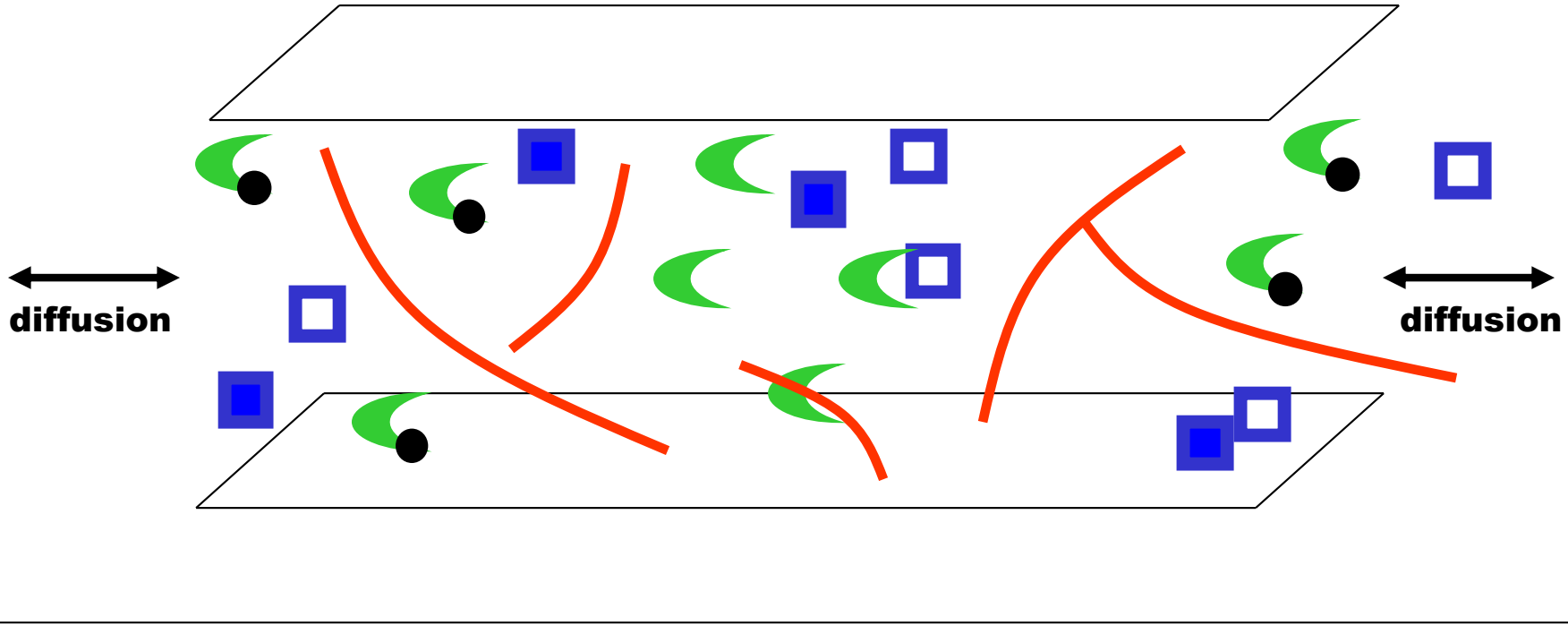
Uncaging in 5um spot



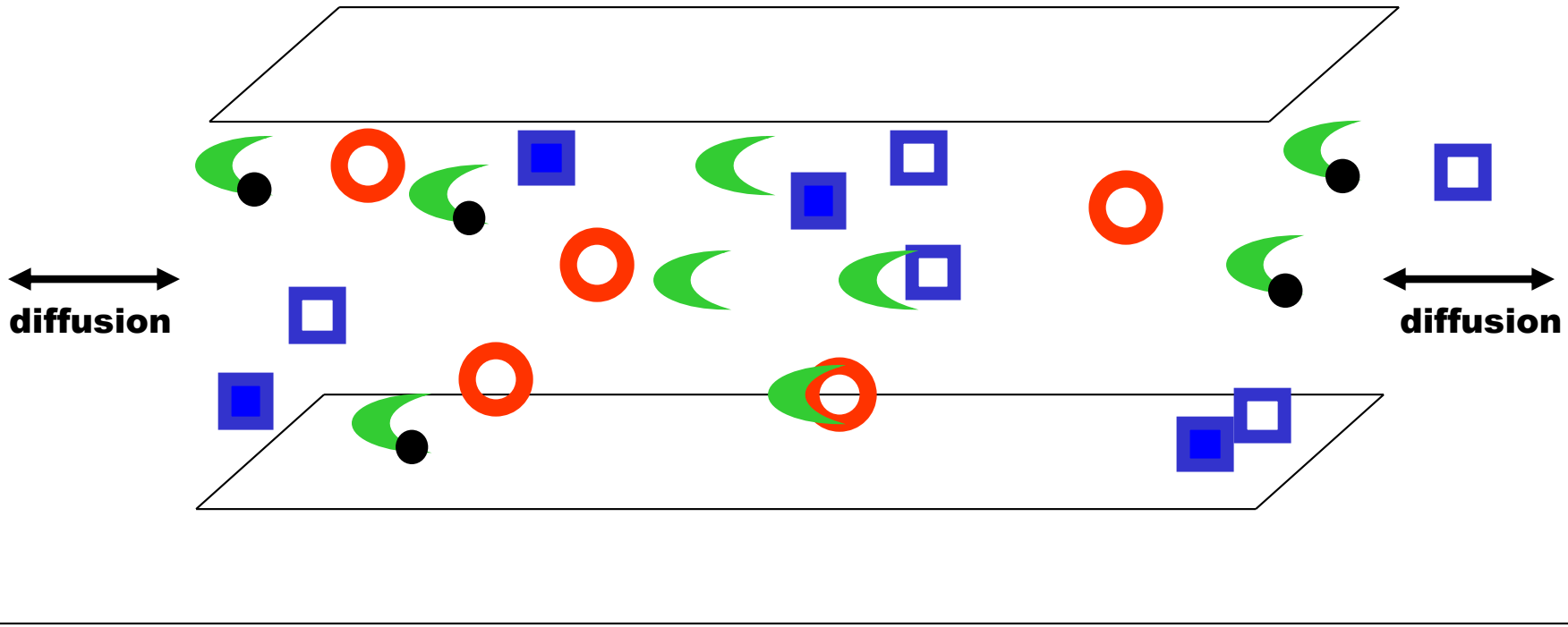
Uncaging in Diffraction Limited Spot



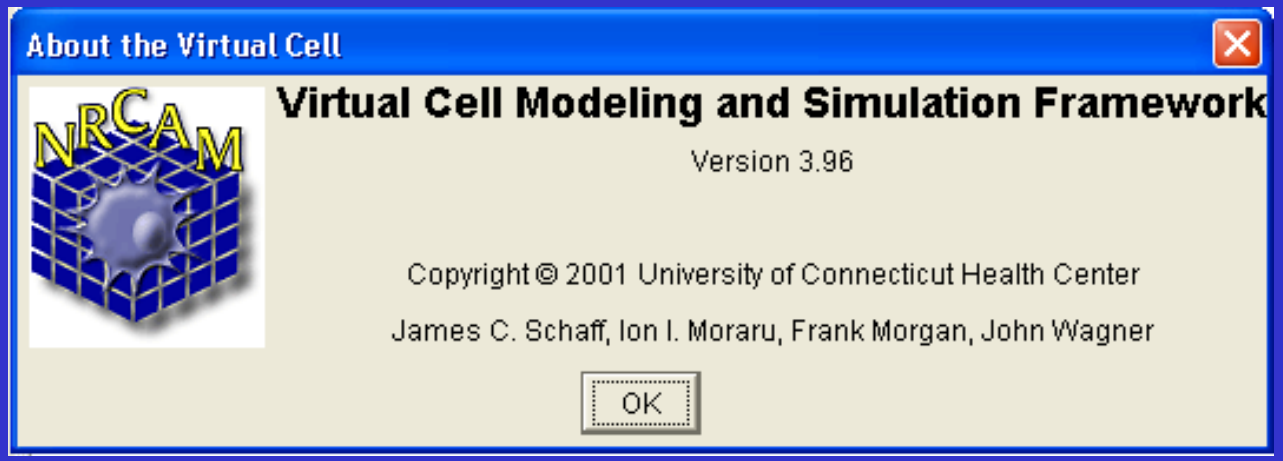
The Model


diffusion
diffusion
F-actin
(anchored:
rate of diffusion 0)
ADP
ATP
G-actin
Caged
Uncaged
Cofilin

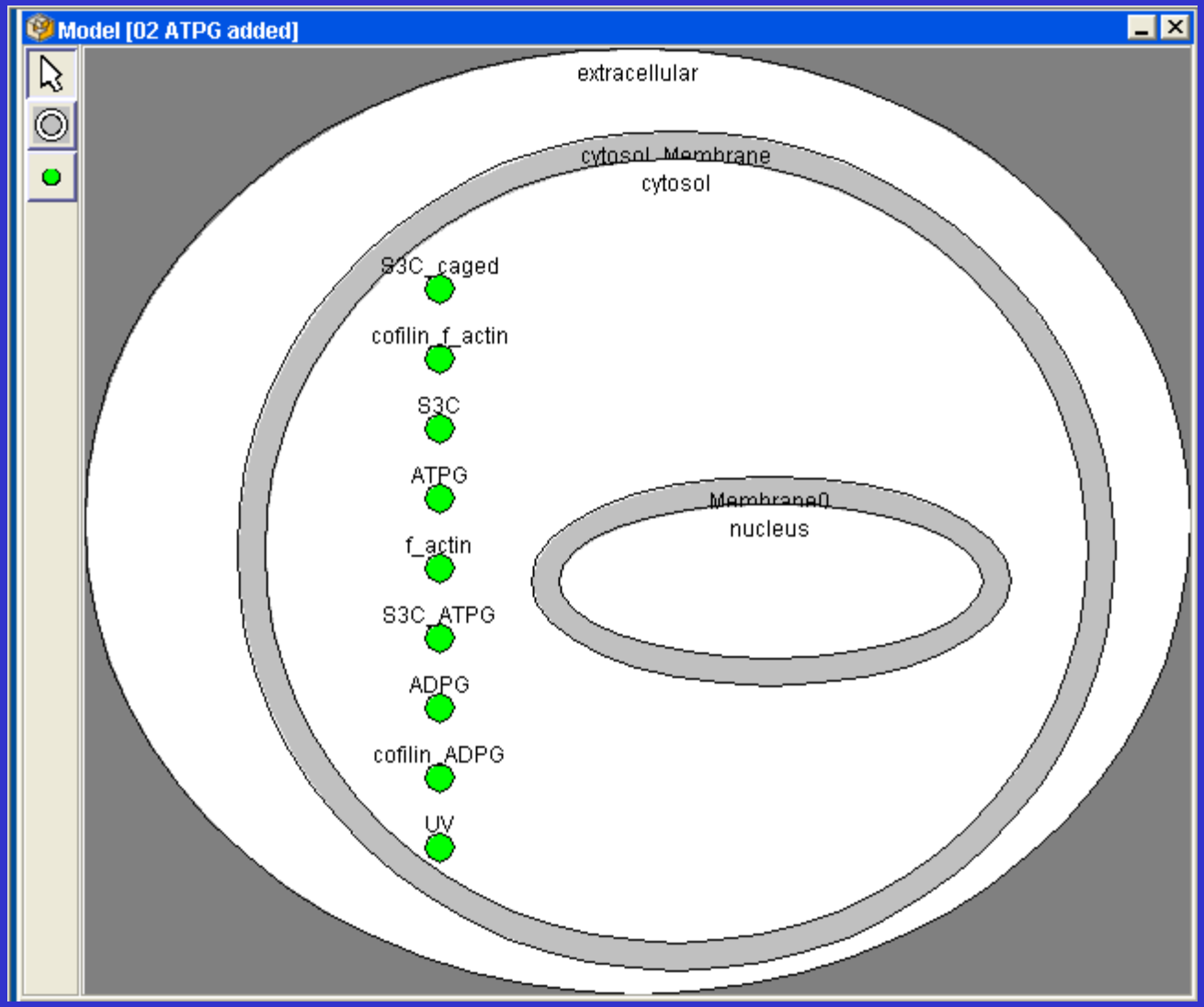
The Model


diffusion
diffusion
F-actin
ADP
ATP
Caged
Uncaged
(anchored:
rate of diffusion 0)
G-actin
Cofilin

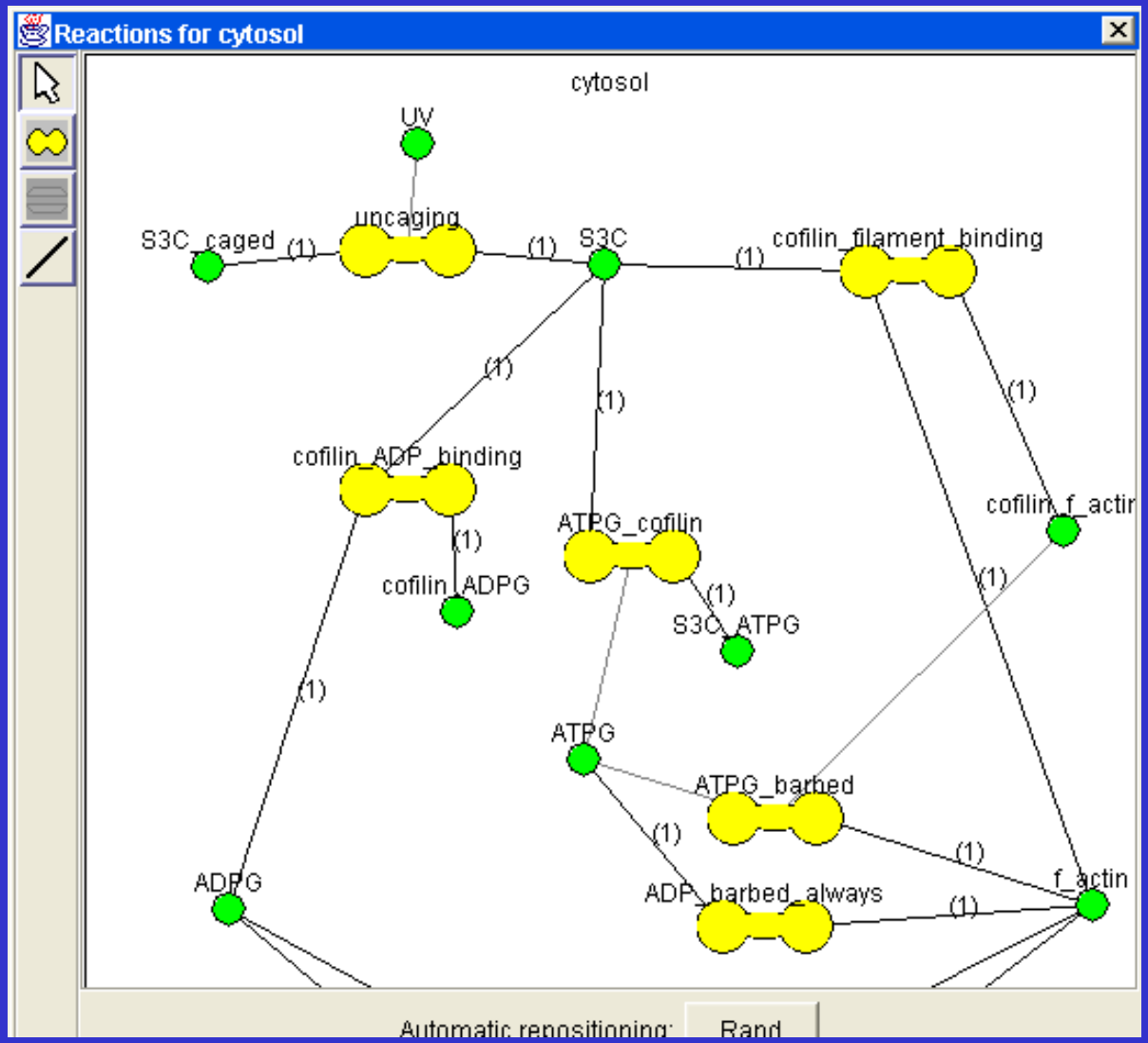
Integration of Imaging and Modeling



Define Species Within a Structure



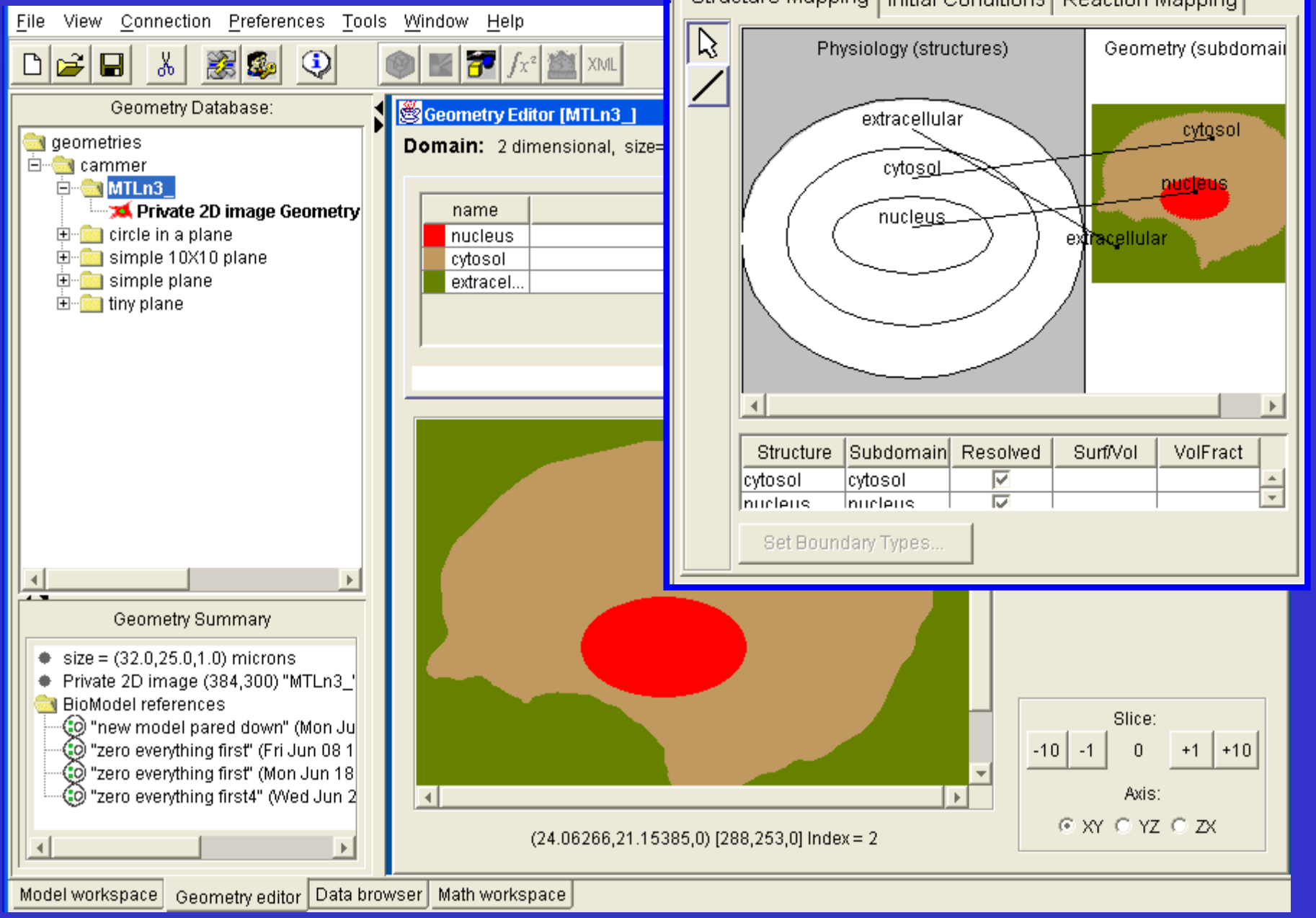
The Reactions Are Mapped Graphically



Math [Application: small spot in cell]

Equations | VCMDL

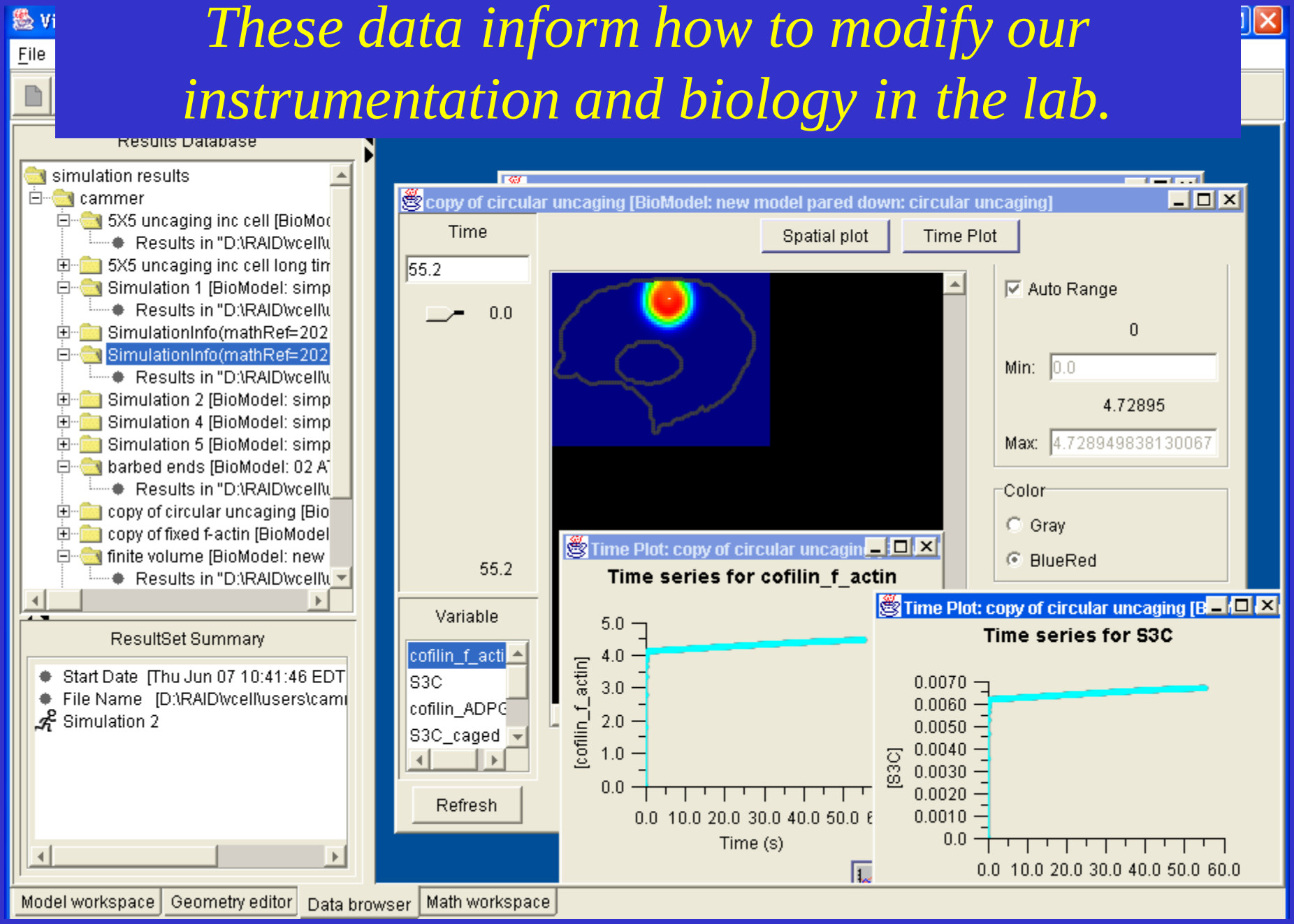
```
        OdeEquation f_actin {
                Rate ( - ((130.0 * f_actin * S3C) - (39.0 * cofilin_f_actin * (S3C > 0.0))) + (kpolymerization * ATPG * cofilin_f_actin) + (11.6 * ADPG / numfilaments) - (1.4 * (S3C > 0.0) * f_actin / numfilaments) + (1.3 * ADPG / numfilaments2) - (0.8 * (S3C > 0.0) * f_actin / numfilaments2));
                Initial         f_actin_init;
        }
        PdeEquation S3C_ATPG {
                Rate            ((KonS3C * S3C * ATPG) - (KoffS3C * S3C_ATPG));
                Diffusion       4.0;
                Initial         ATPG_cofilin_init;
        }
        PdeEquation ADPG {
                Rate            ( - ((1.3 * ADPG / numfilaments2) - (0.8 * (S3C > 0.0) * f_actin / numfilaments2)) - ((11.6 * ADPG / numfilaments) - (1.4 * (S3C > 0.0) * f_actin / numfilaments)) - ((155.0 * ADPG * S3C) - (16.0 * cofilin_ADPG)));
                Diffusion       4.0;
                Initial         ADPG_init;
        }
        PdeEquation S3C_caged {
                Rate             - (UV * (t >= starting) * (t <= ending) * Vmax * S3C_caged);
                Diffusion       4.0;
                Initial         S3C_caged_init;
        }
        PdeEquation cofilin_ADPG {
                Rate            ((155.0 * ADPG * S3C) - (16.0 * cofilin_ADPG));
                Diffusion       2.0;
                Initial         cofilin_ADPG_init;
        }
        PdeEquation S3C {
```


File View Connection Preferences Tools Window Help
Geometry Database:
geometries
cammer
MTLn3_
Private 2D image Geometry
circle in a plane
simple 10X10 plane
simple plane
tiny plane
Geometry Summary
size = (32.0,25.0,1.0) microns
Private 2D image (384,300) "MTLn3_'
BioModel references
"new model pared down" (Mon Ju
"zero everything first" (Fri Jun 08 1
"zero everything first" (Mon Jun 18
"zero everything first4" (Wed Jun 2
Geometry Editor [MTLn3_]
Domain: 2 dimensional, size=
name
nucleus
cytosol
extracel...
(24.06266,21.15385,0) [288,253,0] Index = 2
Slice:
-10 -1 0 +1 +10
Axis:
XY YZ ZX
Structure Mapping
Initial Conditions
Reaction Mapping
Physiology (structures)
Geometry (subdomai
extracellular
cytosol
nucleus
extracellular
cytosol
nucleus
Structure
Subdomain
Resolved
Surf/Vol
VolFract
cytosol
cytosol
Set Boundary Types...
Model workspace
Geometry editor
Data browser
Math workspace

Simulation Movie

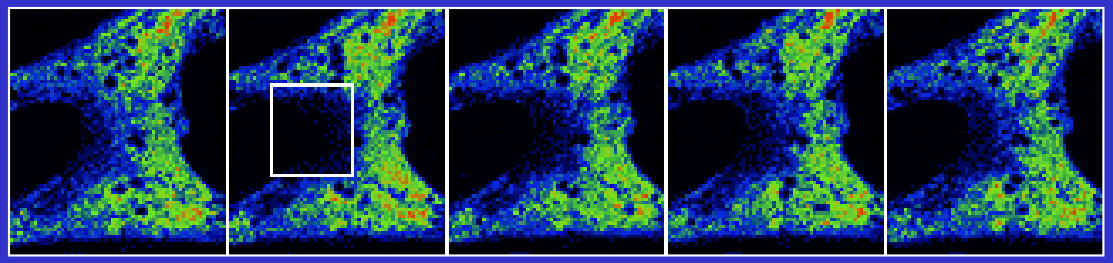
Simulation of uncaging for 1 s and the following 9 s of recovery in a thin flat lamellipod of a cell approximately 30 um wide. The caged S3C is quickly depleted and diffusion of caged S3C into the depleted volume is shown. Red represents high concentration and blue represents low concentration.

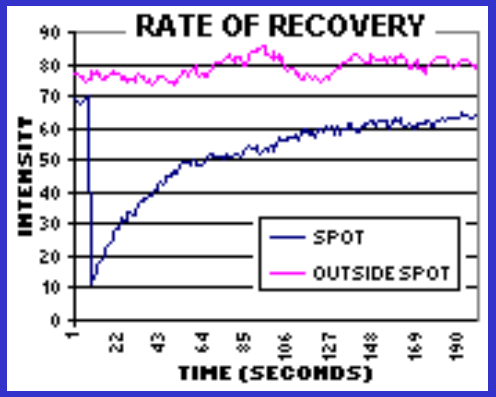
These data inform how to modify our instrumentation and biology in the lab.



One Technique Applies to Another

Expertise in one technique provides expertise in others.





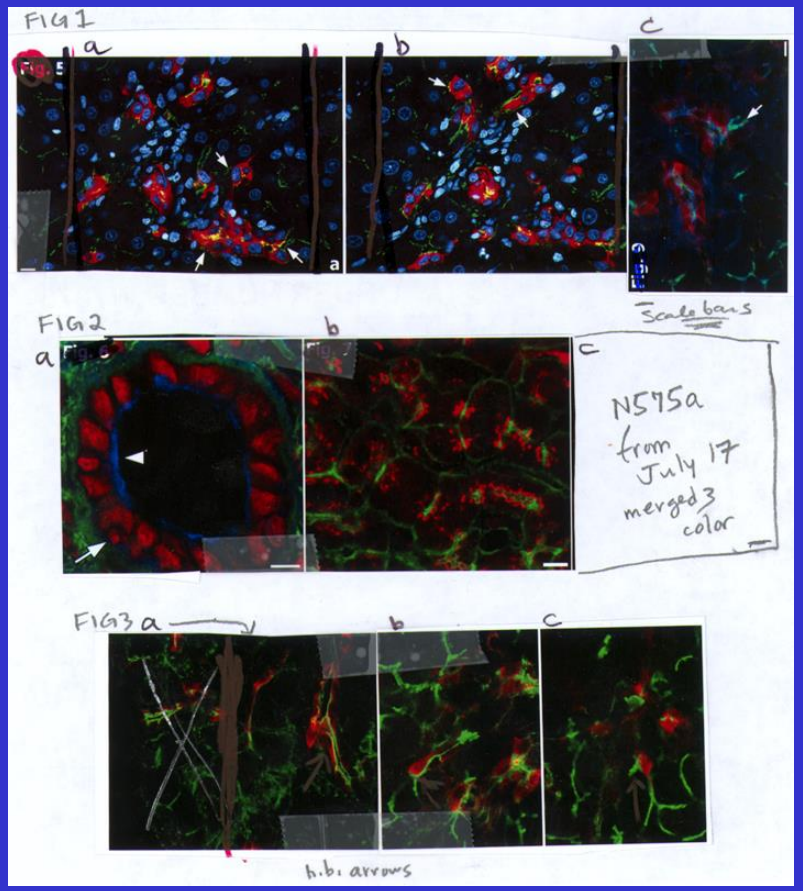
Biological data fed into simulations to calculate mechanism of transport, e.g. diffusion vs. motors

Developed Techniques for Single Investigators Become Available For Everybody

And, Finally…

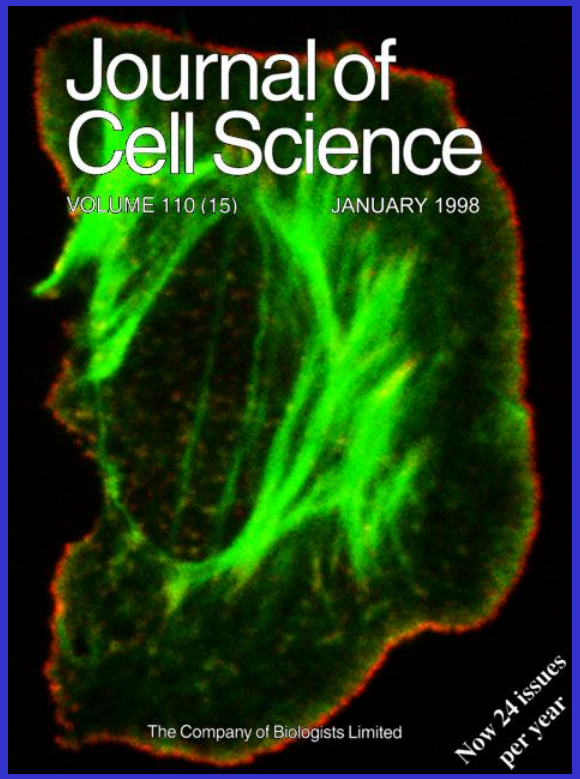
The scientist who tells the story in the best way wins.

- What are DPI and PPI?
- What are RGB and CMYK?
- How do I make the figures 17.8 cm wide for the journal?



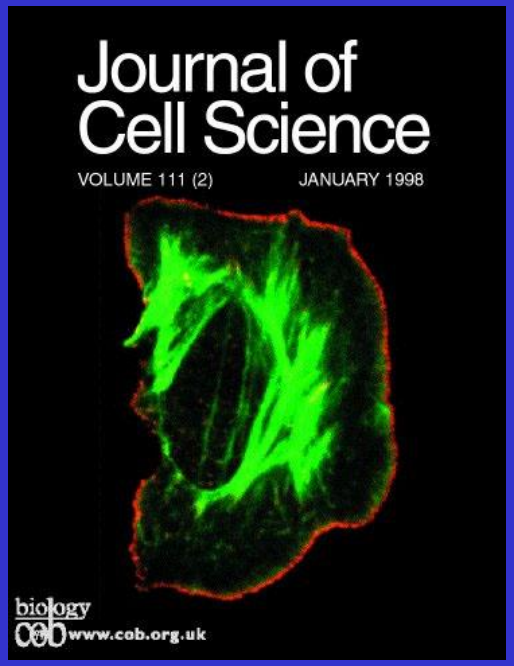
Make clear and simple final figures that are aesthetically pleasing.

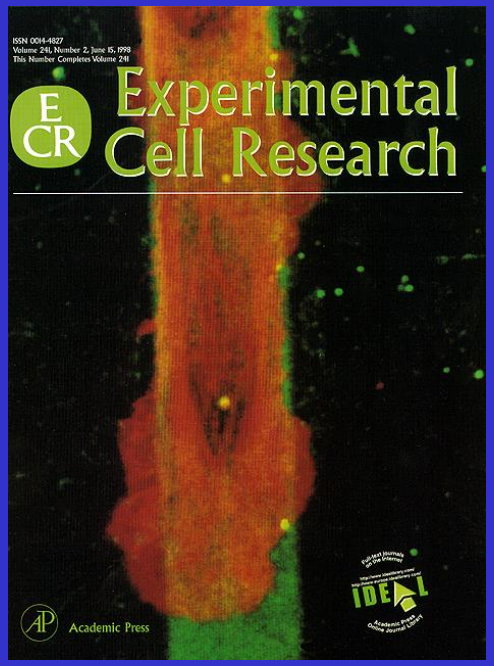
Command Visibility

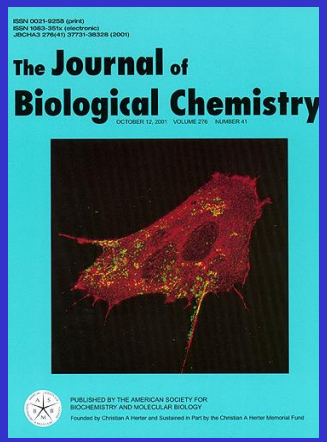


Proposals

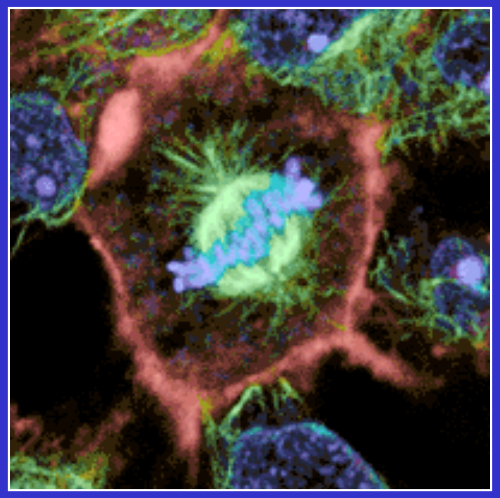
Be Published

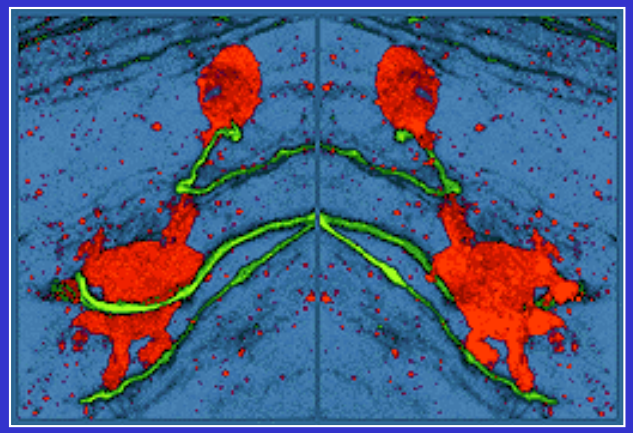


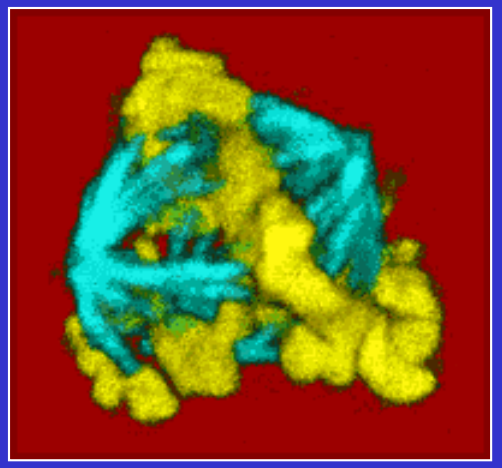


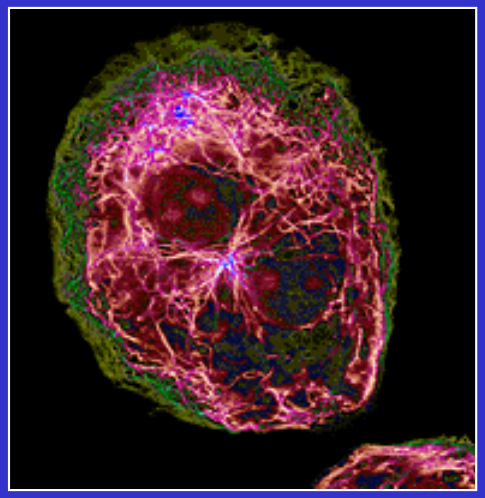


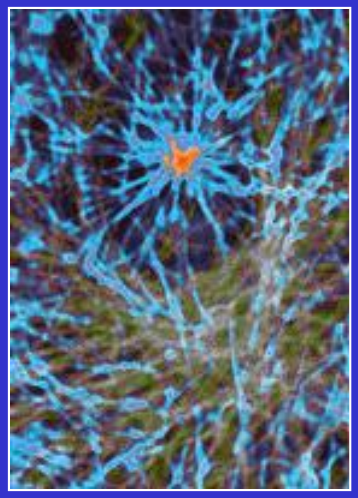
Microscopy is an Art

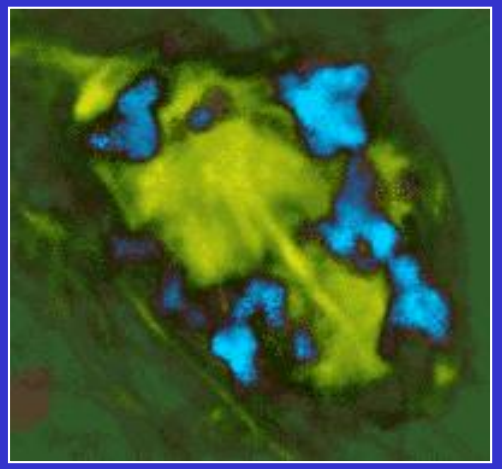












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Dr. John Condeelis
Dr. Peter Satir

The AIF Users:

PAPERS

1. Albanese, C, D'Amico, M., Reutens, A.T., Fu, M, Watanabe, G., Lee, R.J, Kitsis, R.N., Henglein, B., Avantaggiati, M., Somasundaram, K, Thimmapaya, B, and. Pestell, R.G. (1999) Activation of the cyclin D1 gene by the E1A-associated protein p300 through AP-1 inhibits cellular apoptosis. *J Biol Chem.* 274 (48):34186-34195.
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4. Austin, C.D. and Shields, D. (1996) Formation of nascent secretory vesicles from the *trans*-Golgi network of endocrine cells is inhibited by tyrosine kinase and phosphatase inhibitors. *J. Cell Bio.* 135(6):1471-1483.
5. Bailly M, Ichetovkin I, Grant W, Zebda N, Machesky LM, Segall JE, Condeelis J. (2001) The F-actin side binding activity of the Arp2/3 complex is essential for actin nucleation and lamellipod extension. *Curr Biol* 11(8):620-5.
6. Bailly, M., **Macaluso, F., Cammer, M.**, Chan, A., Segall, J.E. and Condeelis, J.S. (1999) Relationships between Arp2/3 complex and the barbed ends of actin filaments at the leading edge of carcinoma cells after EGF-stimulation. *J. Cell Biol.*, 145(2):331-345.
7. Bailly, M., Condeelis, J.S. and Segall, J.E. (1998) Chemoattractant-induced lamellipod extension. *Microscopy Research and Technique* 43:433-443. [Related movies.] [Other related movies.]
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