Live imaging microscopy

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Why Live imaging?

The understanding of complex and/or fast cellular dynamics is an important step for exploring biological processes. Therefore, today's life science research is increasingly focusing on dynamic processes like cell migration, morphological changes of cells, organs or whole animals and physiological (e.g. changes of intracellular ion composition) events in living specimens in real time.

One approach to address these challenging demands is to employ certain optical methods that are collectively called live-cell imaging. Live-cell imaging allows investigation of dynamical processes of living cells instead of giving a "snapshot" of a cell's current state – it turns snapshots into movies.

Live-cell imaging provides spatial and temporal information of dynamic events in single cells, cellular networks (*in situ*) or even whole organisms (in vivo).

General issues in live-cell imaging

Live-cell imaging generally is possible with cultured cell lines (e.g. HEK cells, HeLa cells), primary cell cultures (e.g. skin cells, neural cells), acute slice preparations (e.g. brain slices) or whole organs or organisms. A major task is to keep the cells in a healthy state during the experiments as the cells suffer from phototoxicity and are taken out of their “natural” environment.

KEEPING CELLS ALIVE

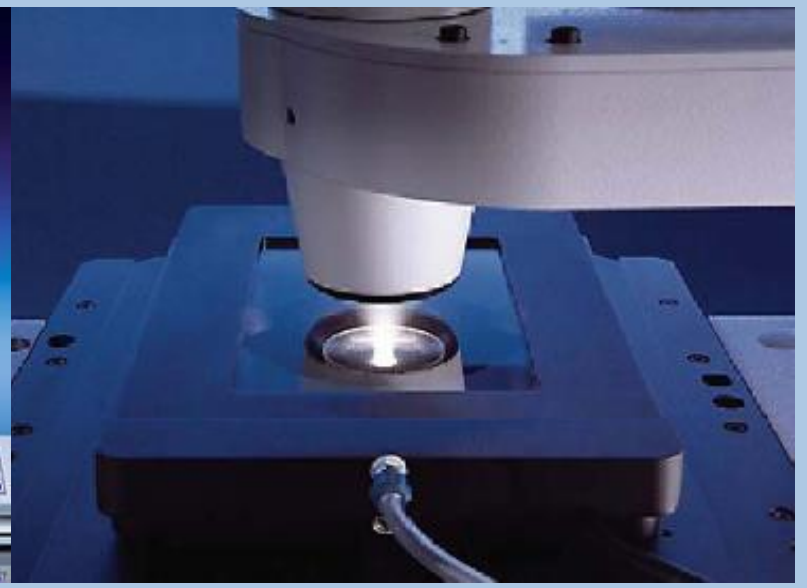
- Temperature (37°C)
- CO_2 (5%) and Ph
- Humidity

LIVE CELL IMAGING: INCUBATION

Cell Health Parameter	Short-Term Study	Longer-Term Study
pH	HEPES Buffer	CO_2 Incubator
Temperature	Stage Warmers	Objective Lens Warmers Cell chambers with integrated heating elements Enclose microscope within a heated box
Humidity	Open cell chambers	Tightly sealed cell chambers
Oxygenation	Large volume of media	Media changes during study Large media volume
Osmolarity	Sealed chamber	Enclose system within a humidified chamber

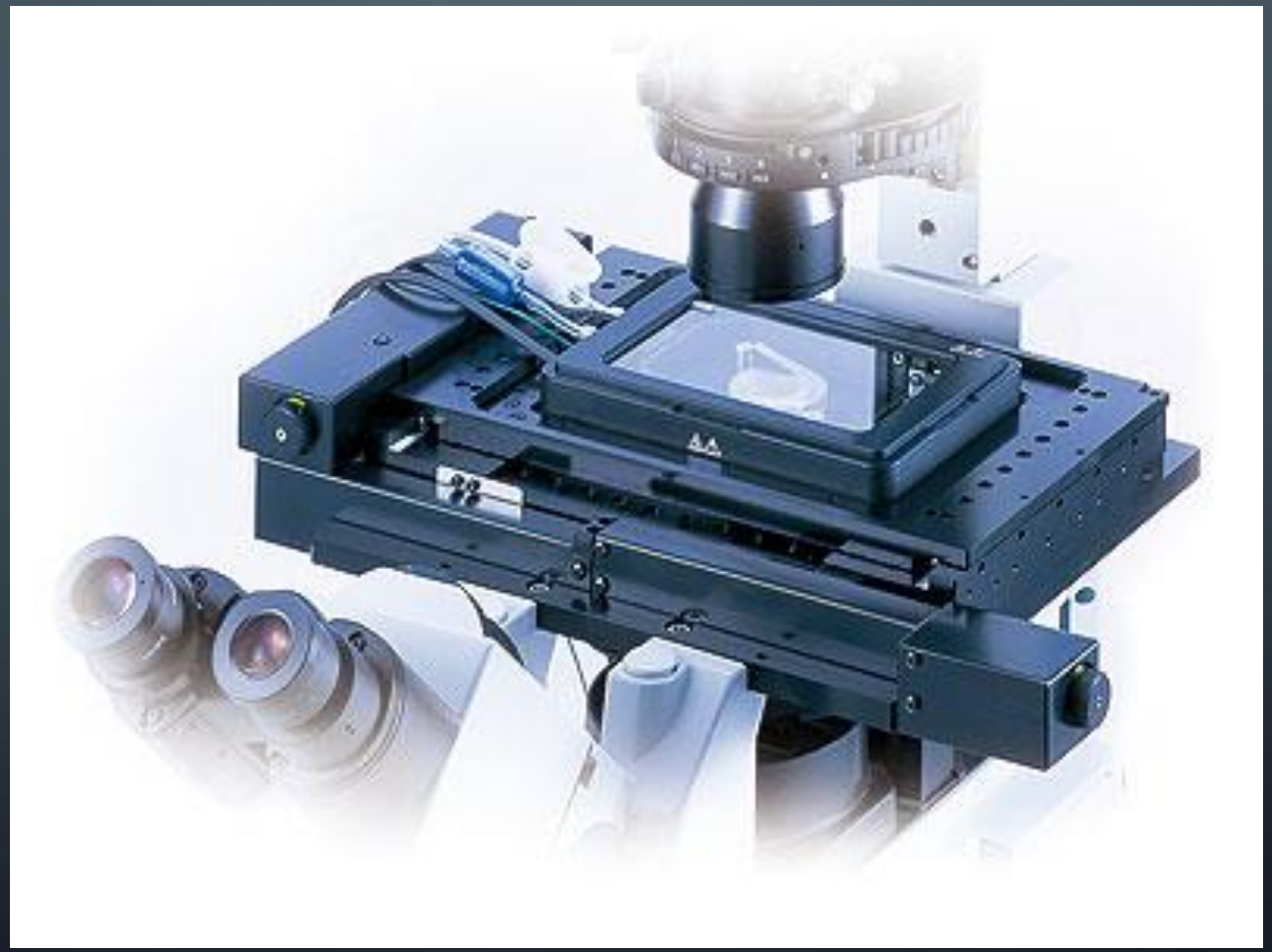
LIVE CELL IMAGING: INCUBATION

Incubation chambers can surround the microscope stand or just the cell dish. Both can maintain cells at the correct temperature for long periods of time.



INCUBATOR MICROSCOPE

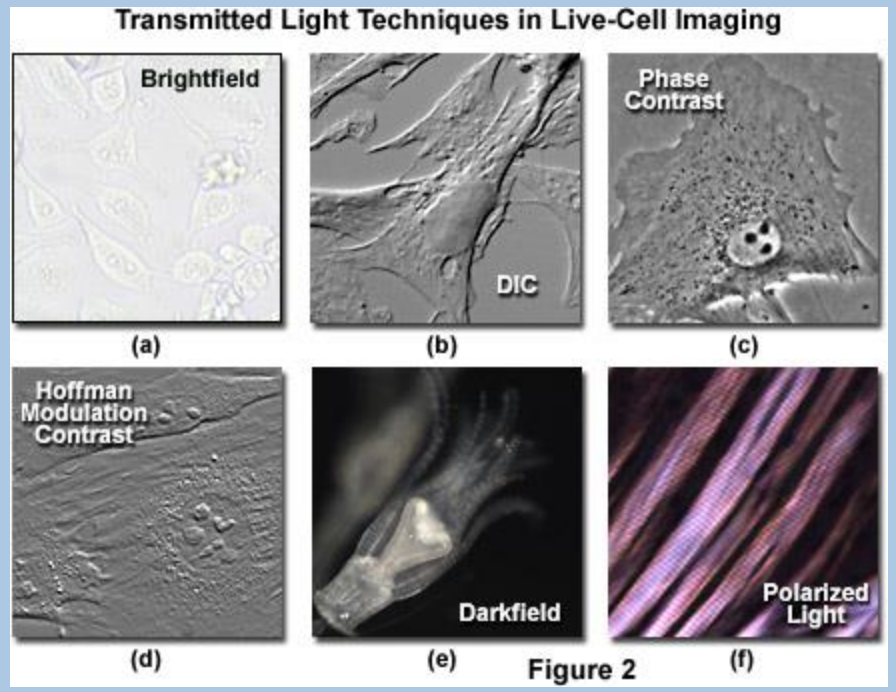
SMALL FORMAT CHAMBER



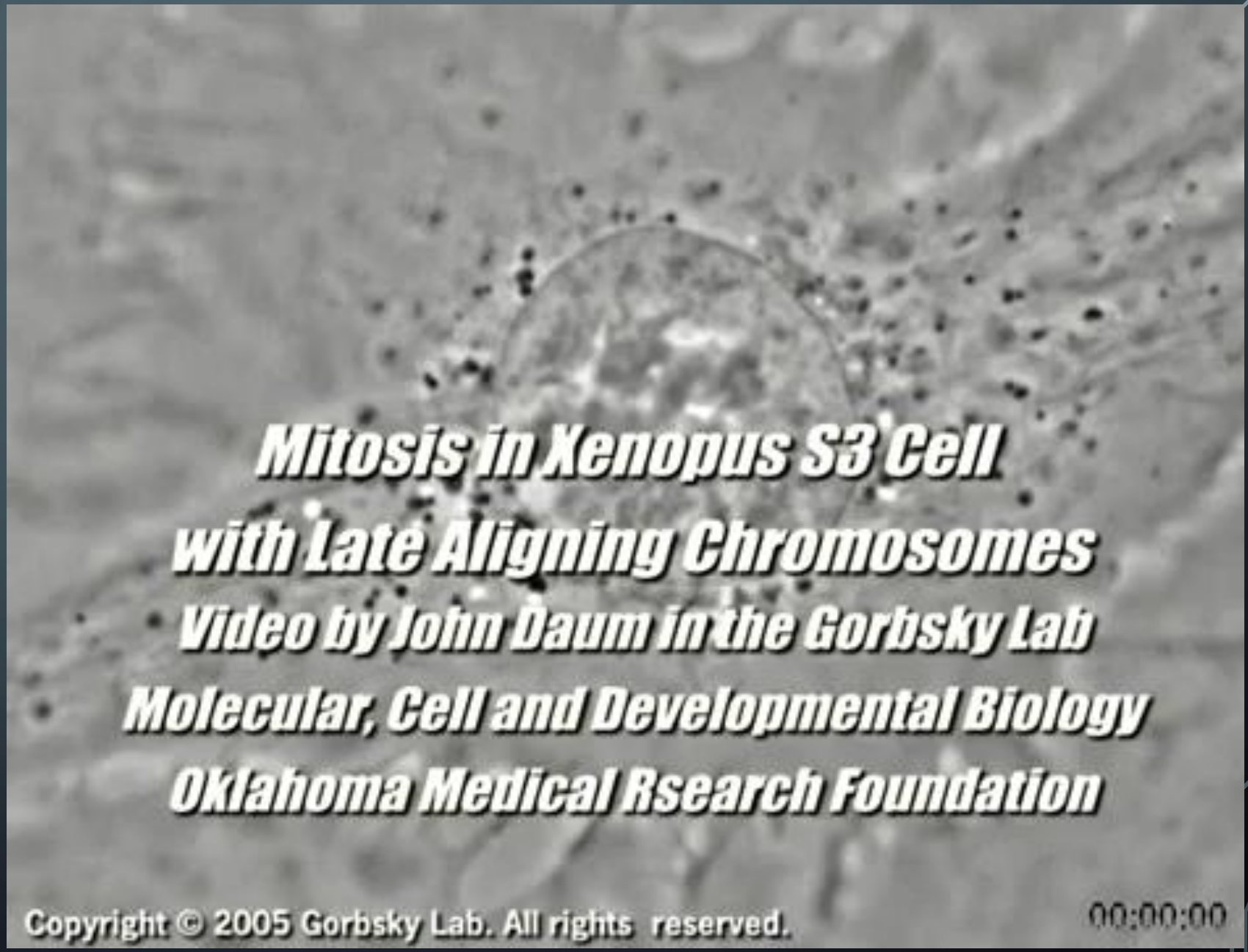
LIVE CELL IMAGING: LABELING CELLS

The most basic challenge is detecting clear, nearly invisible cells when using a microscope.

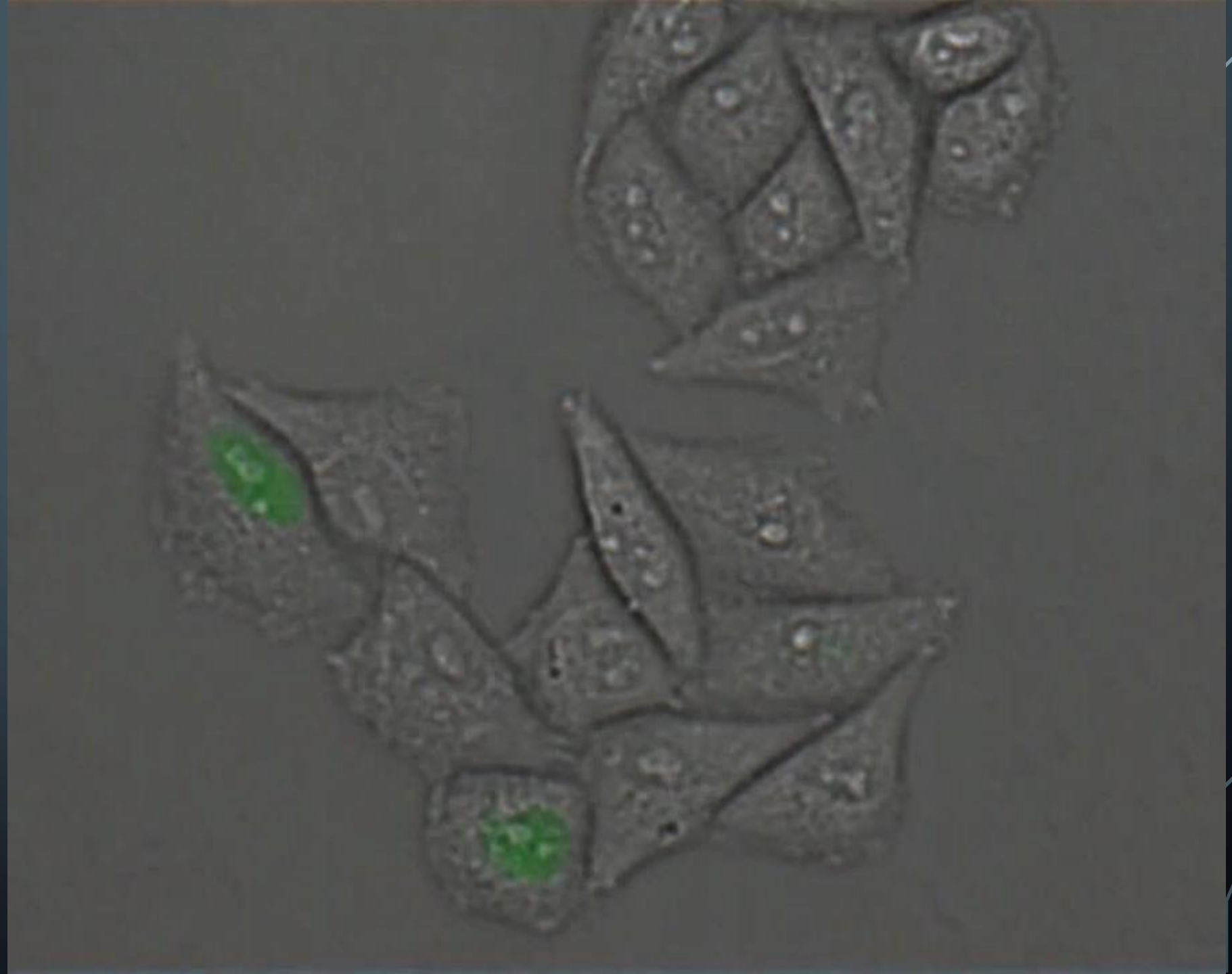
Optical Techniques to Enhance contrast:
Differential Interference Contrast (DIC)
Phase Contrast
Darkfield
Polarized Light
Fluorescence



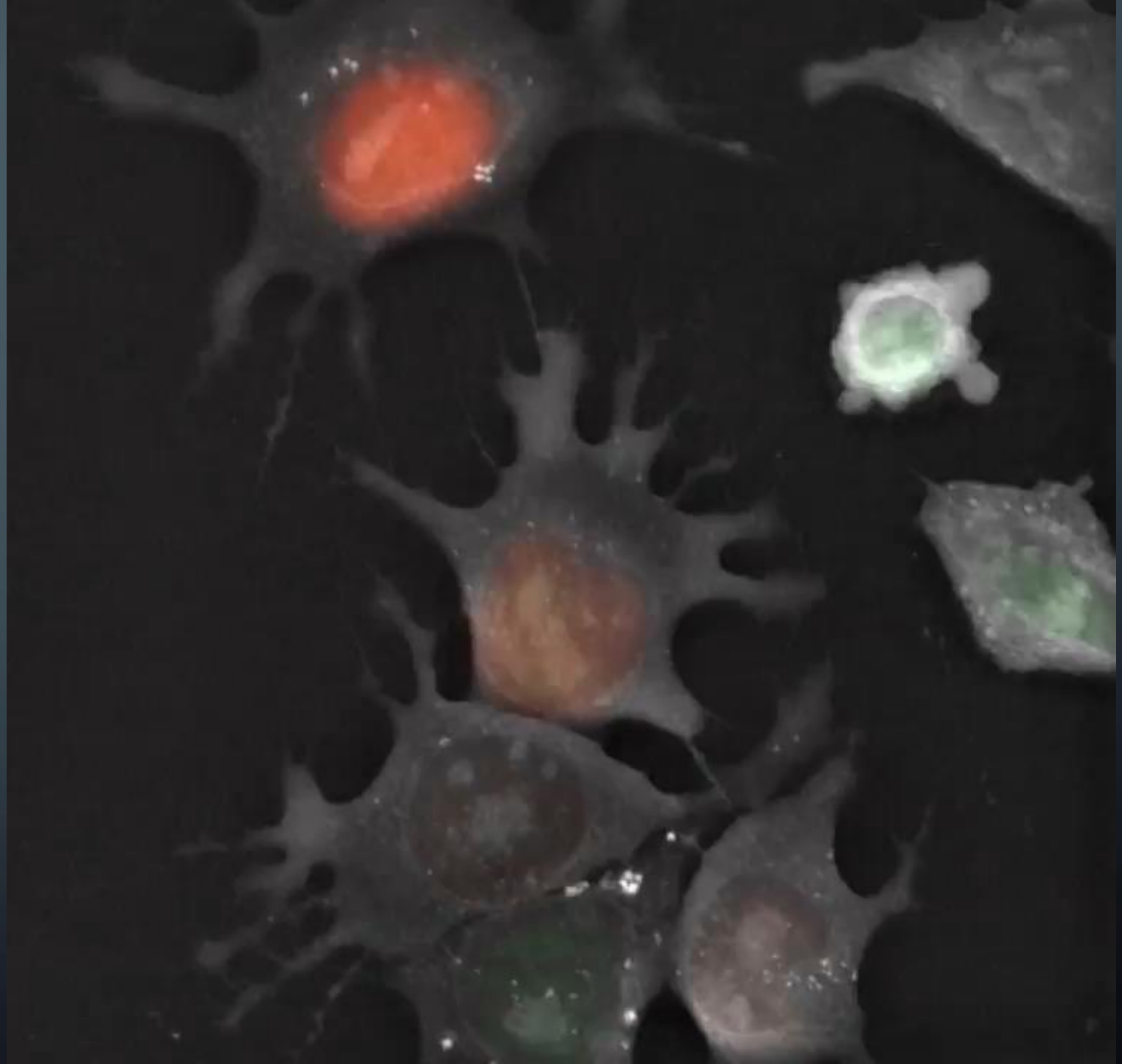
In vitro cells



In vitro cells

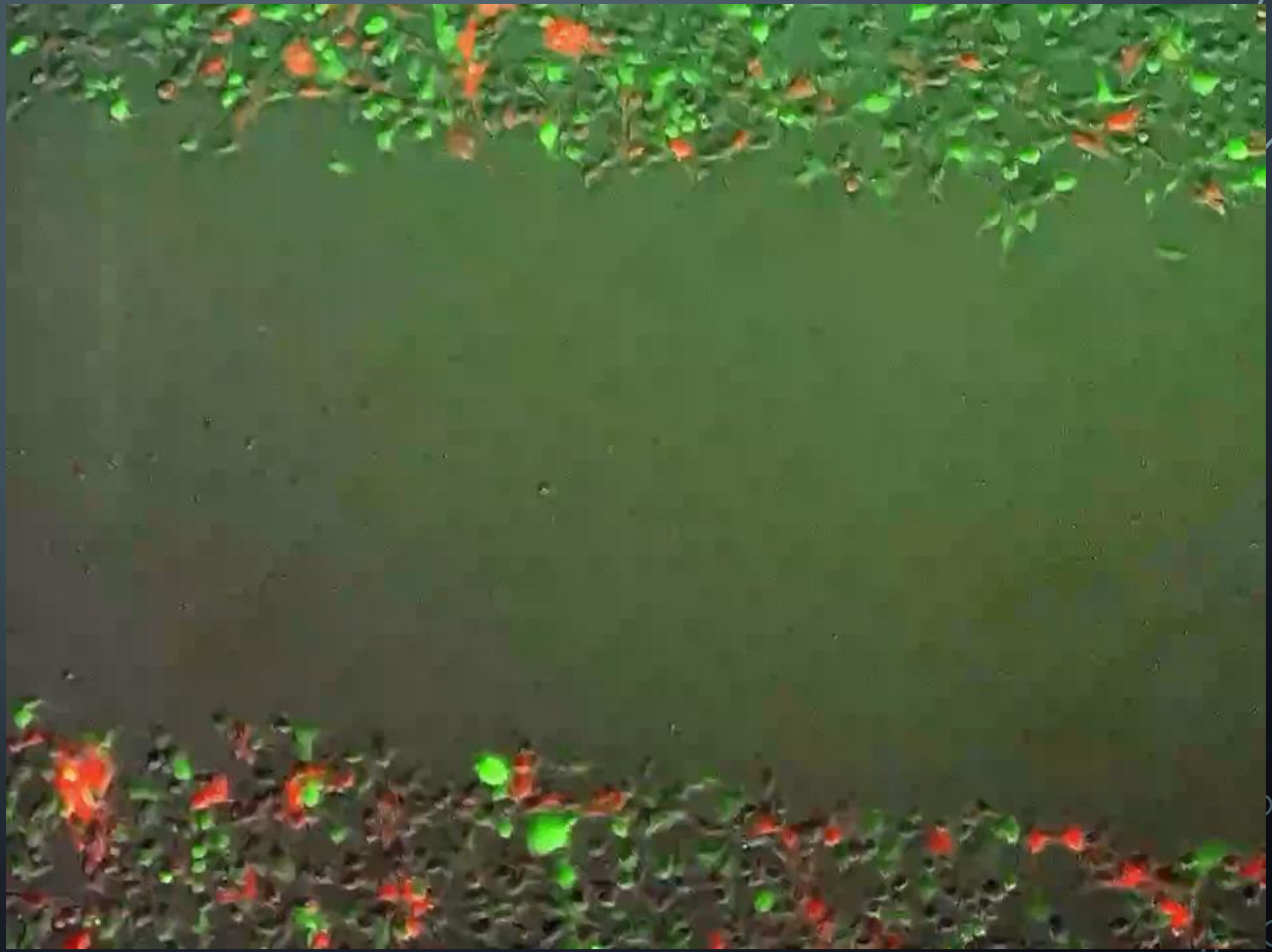


In vitro cells



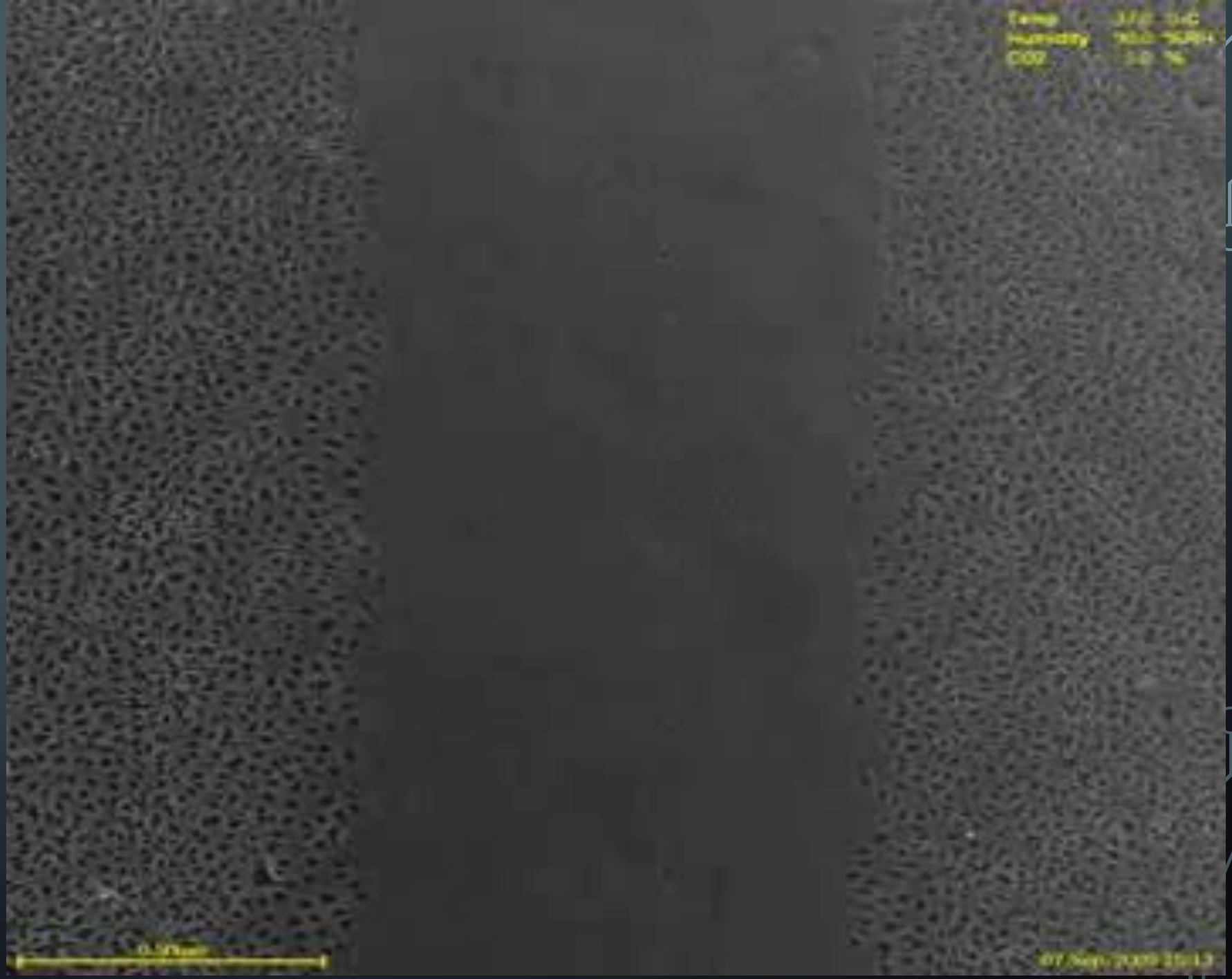
Collective Cell Migration

In vitro cells scratch assay



Collective Cell Migration

In vitro cells scratch assay



LIVE CELL IMAGING: GENTLE IMAGING

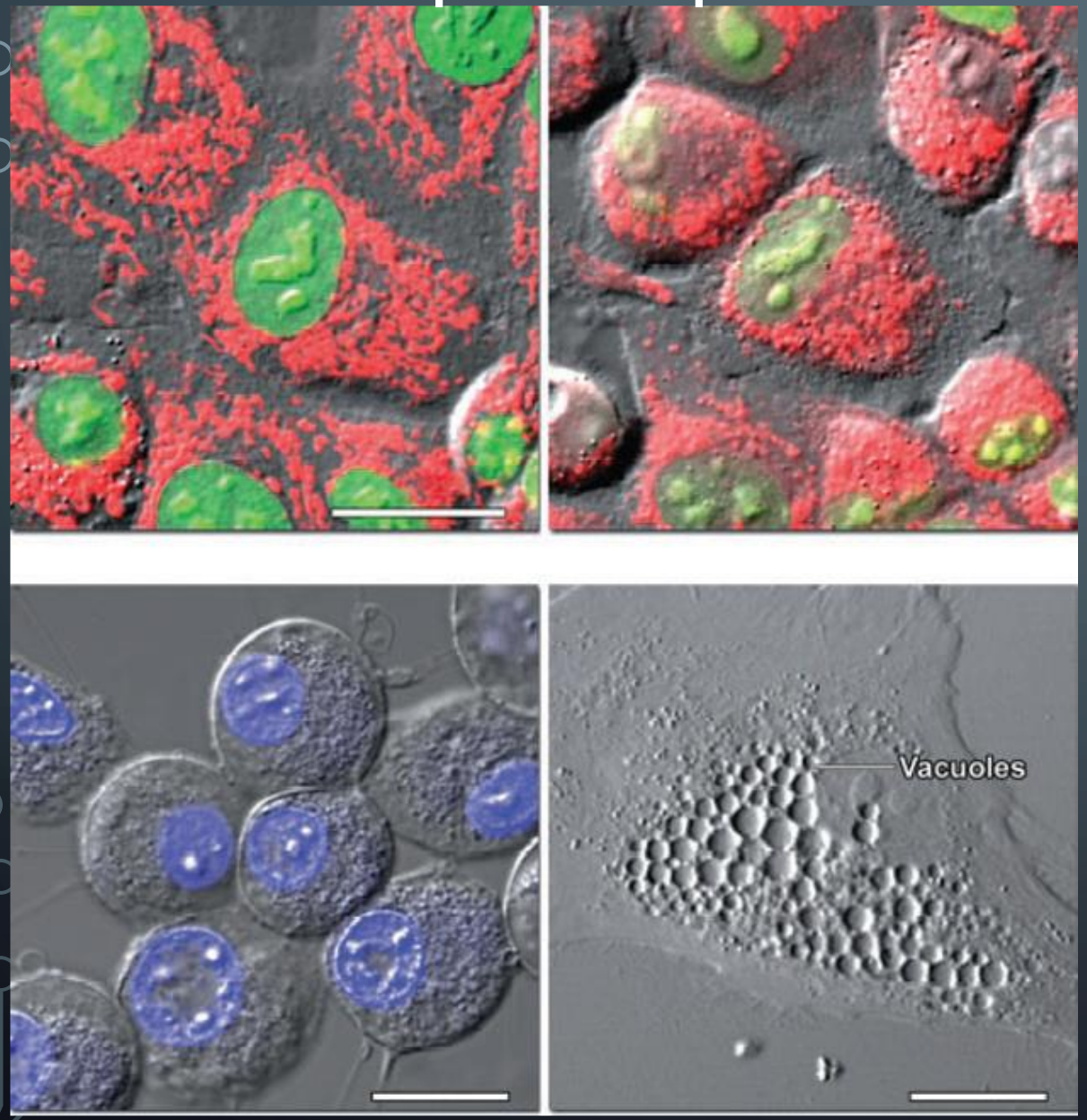
The **central** challenge in live cell imaging – Cells are inherently photosensitive!

Formation of free radicals by fluorophores only exacerbates the problem.

Free radical formation cannot be prevented, only managed by careful tuning of imaging parameters to reduce light exposure.

Live cell imaging requires “gentle imaging” - short exposures of low intensity Illumination to succeed.

Examples of phototoxicity

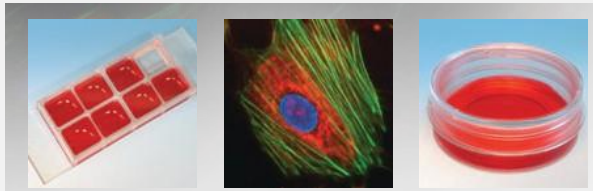


Phototoxicity during live-cell imaging. (a) (RK13) cells expressing EYFP fused to a nuclear localization signal (green nucleus) were treated with the synthetic dye MitoTrackerTM
(b) Same view field as panel a after time-lapse imaging for 2 hours at 15 second intervals.

(c) HeLa cells labeled with Hoechst 33342 imaged for 30 minutes at 10-second intervals Cells
have detached from the coverslip and are rounding.

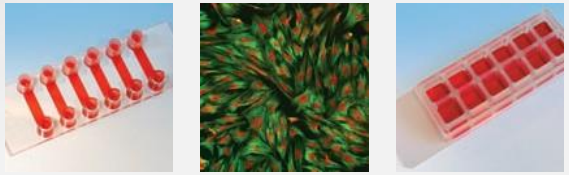
(d) Vacuole formation in a fibroblast cell after imaging for 8 hours at 30-second intervals using tungsten halogen illumination and DIC optics.

FLOW CELLS AND MICRO-FLUIDICS



Cell Microscopy

- Perfect cell growth
- High imaging quality

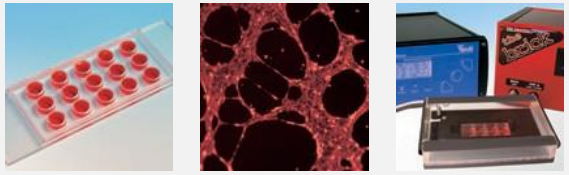


Immunofluorescence

- All-in-one chambers
- Minimum volume of 25 µl

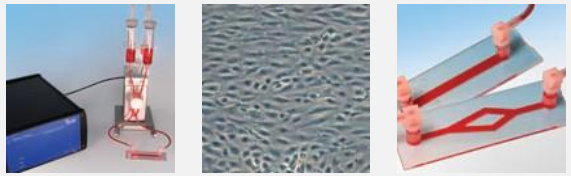
Chemotaxis Assays

- Adherent & non-adherent cells
- Stable, linear gradients



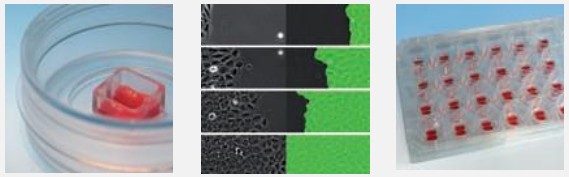
Angiogenesis Assays

- Tube formation & sprouting assays
- Video microscopy



Flow Assays

- Defined shear rates
- Adhesion assays



Wound Healing Assays

- Defined separated areas
- Highly reproducible

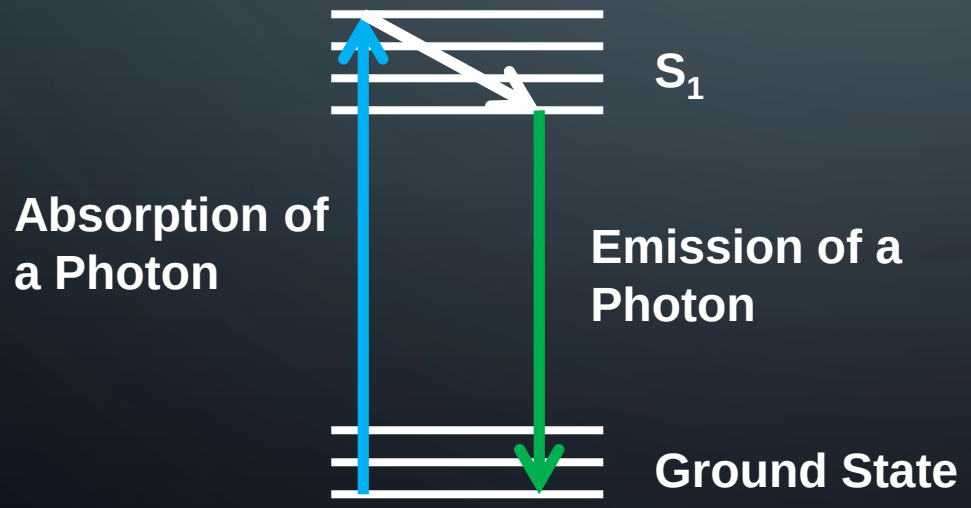
FLUORESCENCE LABELING OF LIVING CELLS

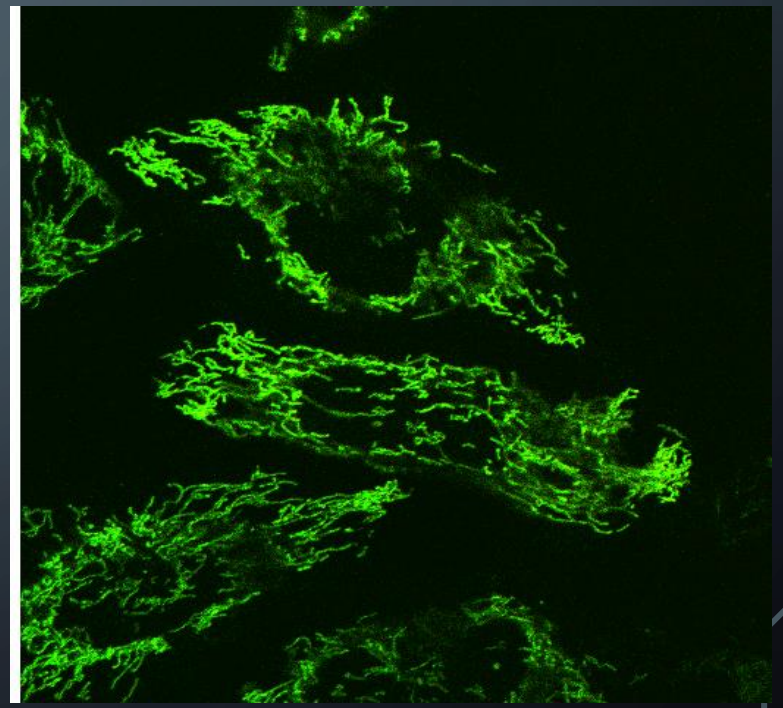
- Advantages of fluorescence labeling
- Categories of Fluorescence labels
- Fluorescent Proteins

WHY WE USE FLUORESCENCE

- Detect specific molecules (immunocytochemistry, in situ hybridization, fluorescent proteins)
- Must keep the specimen transparent.
- Detect multiple molecules at the same time

Jablonski Diagram





Courtesy of Dr. Yien Che Tsai, NCI

LIVE CELL IMAGING: LABELING CELLS

Technique	Example	Pros	Cons
Cell Permeable Dyes	MitoTracker®Red CMXRos DAPI BAPTA / FURA	•Simple •*In vivo* labeling	•Often toxic to cells
Fluorescent Proteins	EGFP	•Simple; *in vivo* •Well developed •Very specific labeling	•Large tag •Oligomerization
Direct labeling of Protein	FITC labeled actin	•Very specific labeling	•Highly purified protein required •Delivery into cell
Auto-labeling of protein fusions	HaloTag™ SnapTag™	•*In vivo* labeling	•Large tag •Limited colors
Labeling peptide tags	FlAsH and ReAsH	•Specific labeling •Small label •*In vivo* labeling	•Limited colors

LIVE CELL IMAGING: LABELING CELLS

Protein (Acronym)	Ex (nm)	Em (nm)	EC (x 10^{-3})	QY	Photostability	Quaternary Structure	Brightness (% of EGFP)
Green Fluorescent Proteins							
EGFP	488	507	56.0	0.60	++++	Monomer*	100
Emerald	487	509	57.5	0.68	+++	Monomer*	116

Protein (Acronym)	Ex (nm)	Em (nm)	EC (x 10^{-3})	QY	Photostability	Quaternary Structure	Brightness (% of EGFP)
mApple	568	592	75.0	0.49	+++	Monomer	109
mStrawberry	574	596	90.0	0.29	+	Monomer	78
AsRed2	576	592	56.0	0.05	+	Tetramer	8
mRFP1	584	607	50.0	0.25	++	Monomer	37
JRed	584	610	44.0	0.20	++	Dimer	26
mCherry	587	610	72.0	0.22	+++	Monomer	47

For live cell experiments, choose FPs with high photostability.

MINIMIZING DAMAGE WHILE GETTING GOOD IMAGES

- Light Gathering Power and Focus Maintenance
- Which immersion media
- Correct coverslip thickness
- Resolution vs Speed vs Sensitivity
- 3D Imaging

USING THE CORRECTION IMMERSION MEDIA

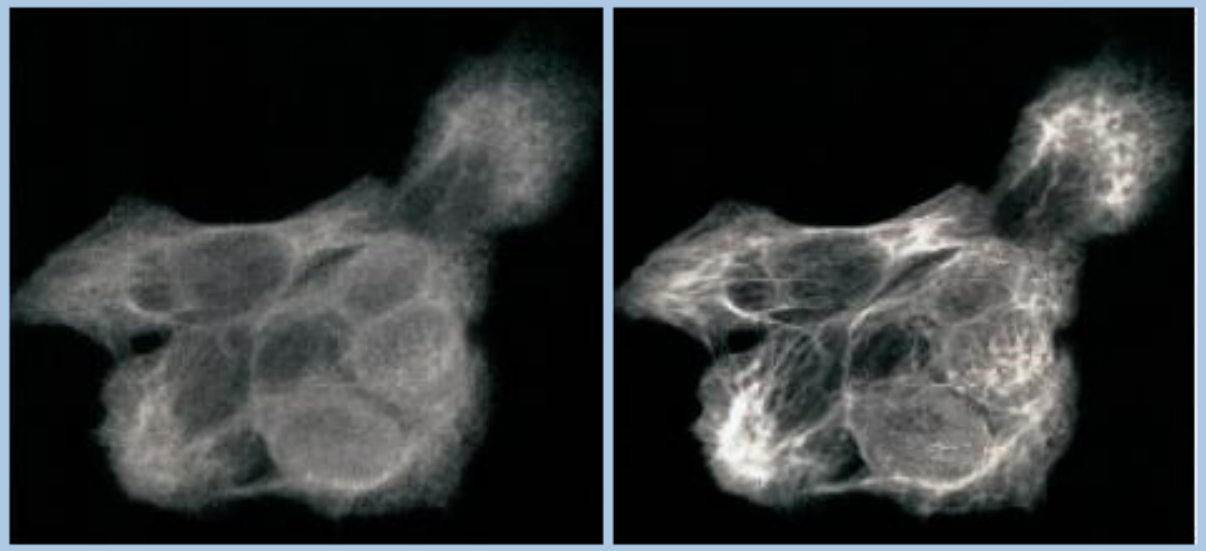
Using water immersion objectives for samples in liquid media captures more signal and preserves resolution better than refractive index mismatched oil immersion objectives.

oil immersion objective

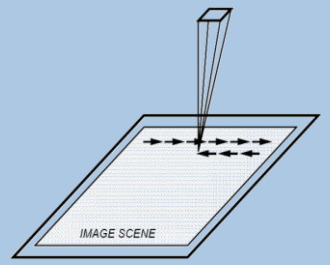
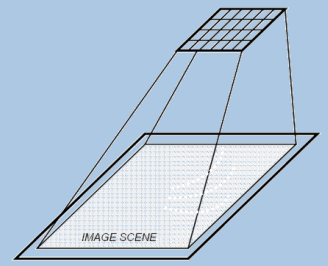
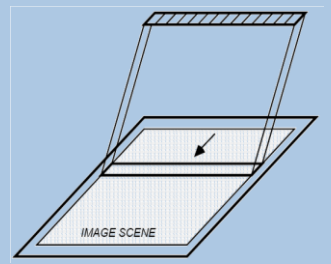
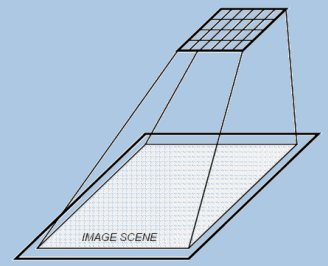
NA=1.4

water immersion objective

NA=1.2

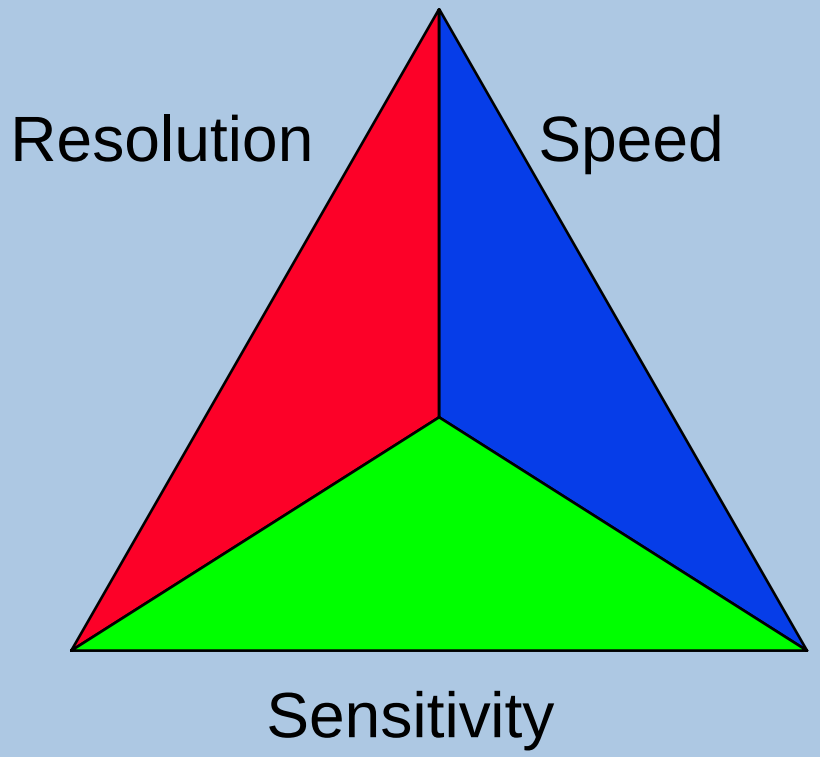


CONFOCAL MICROSCOPES

Point Scanning	Multi Point Scanner	Line Scanning	Area Scanning
			
Point Detector	Area Detector	Line Detector	Area Detector
Photomultiplier	CCD camera	Photomultiplier Array	CCD camera
Avalanche Photodiode	CMOS camera	CCD Line Array	CMOS camera
GaAsP			

RESOLUTION, SPEED, SENSITIVITY

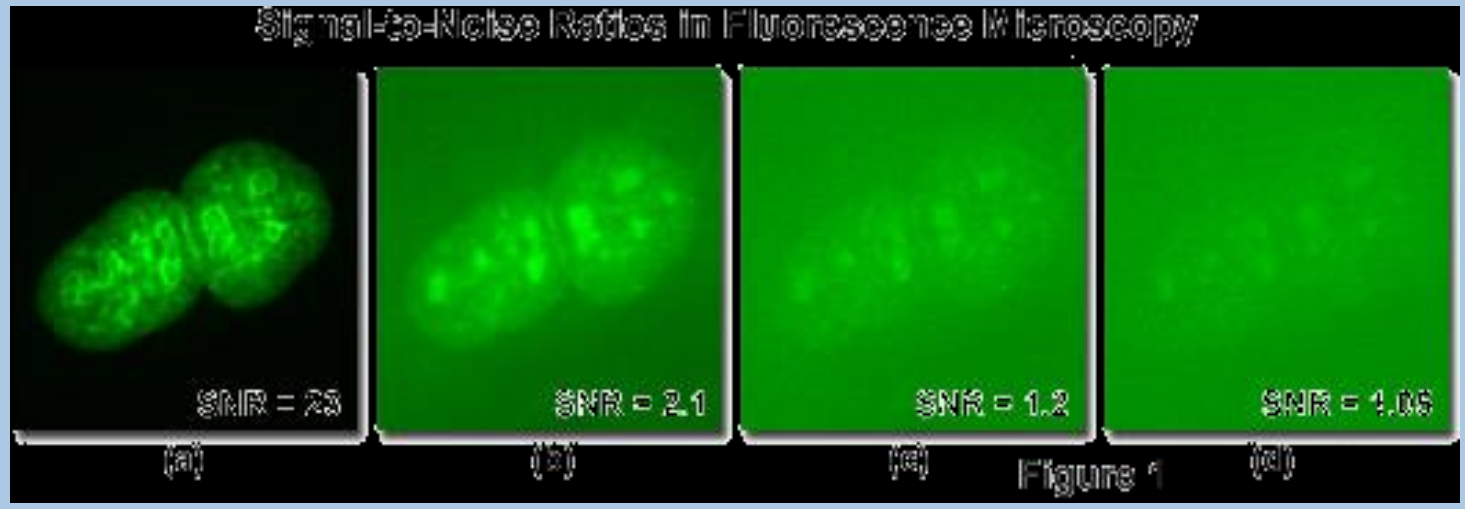
- Spatial Resolution
  - Depends on application → optical magnification and lens apperture
  - Smaller and more pixels = higher resolution
- Sensitivity
  - Larger pixels = higher sensitivity
  - Higher sensitivity = shorter exposure time
  - Shorter exposure time = higher frame rate
  - Larger pixels = lower resolution
- Speed
  - Less pixels = faster readout
  - Faster Readout = higher framerate
  - Less pixels = lower resolution
- Speed requires fast readout and high sensitivity
  - Light is the limit



SIGNAL TO NOISE RATIO

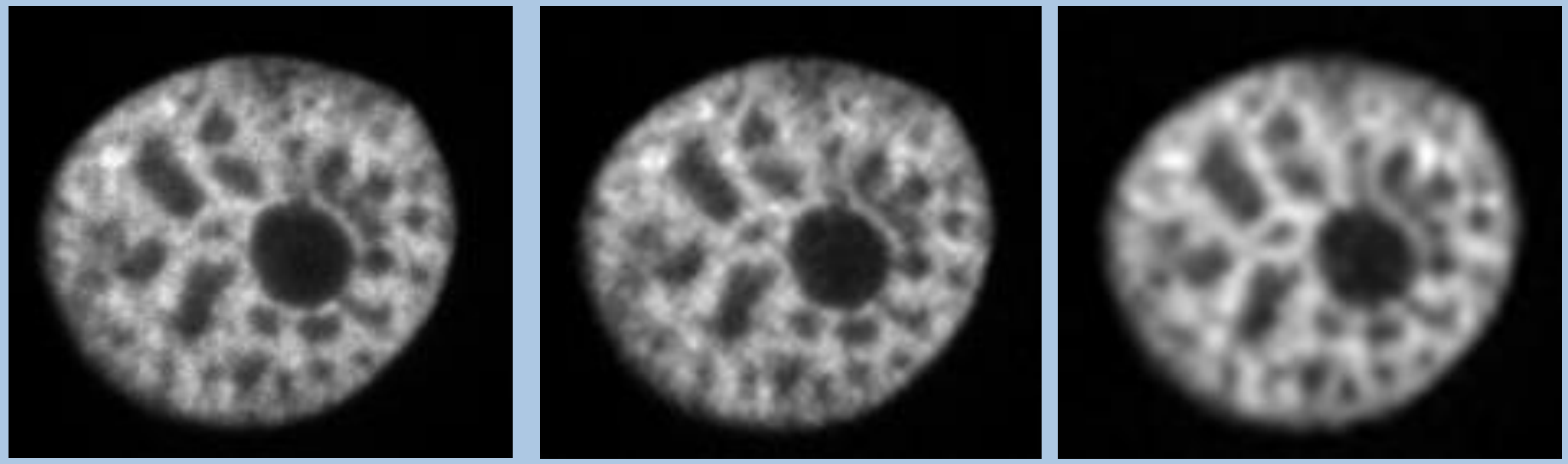
Camera Sensitivity -

Signal needs to be 2-3x brighter than background noise to be detected.



Increasing the exposure time (integration time) or increasing the illumination intensity produce more signal.

RESOLUTION VERSUS SPEED



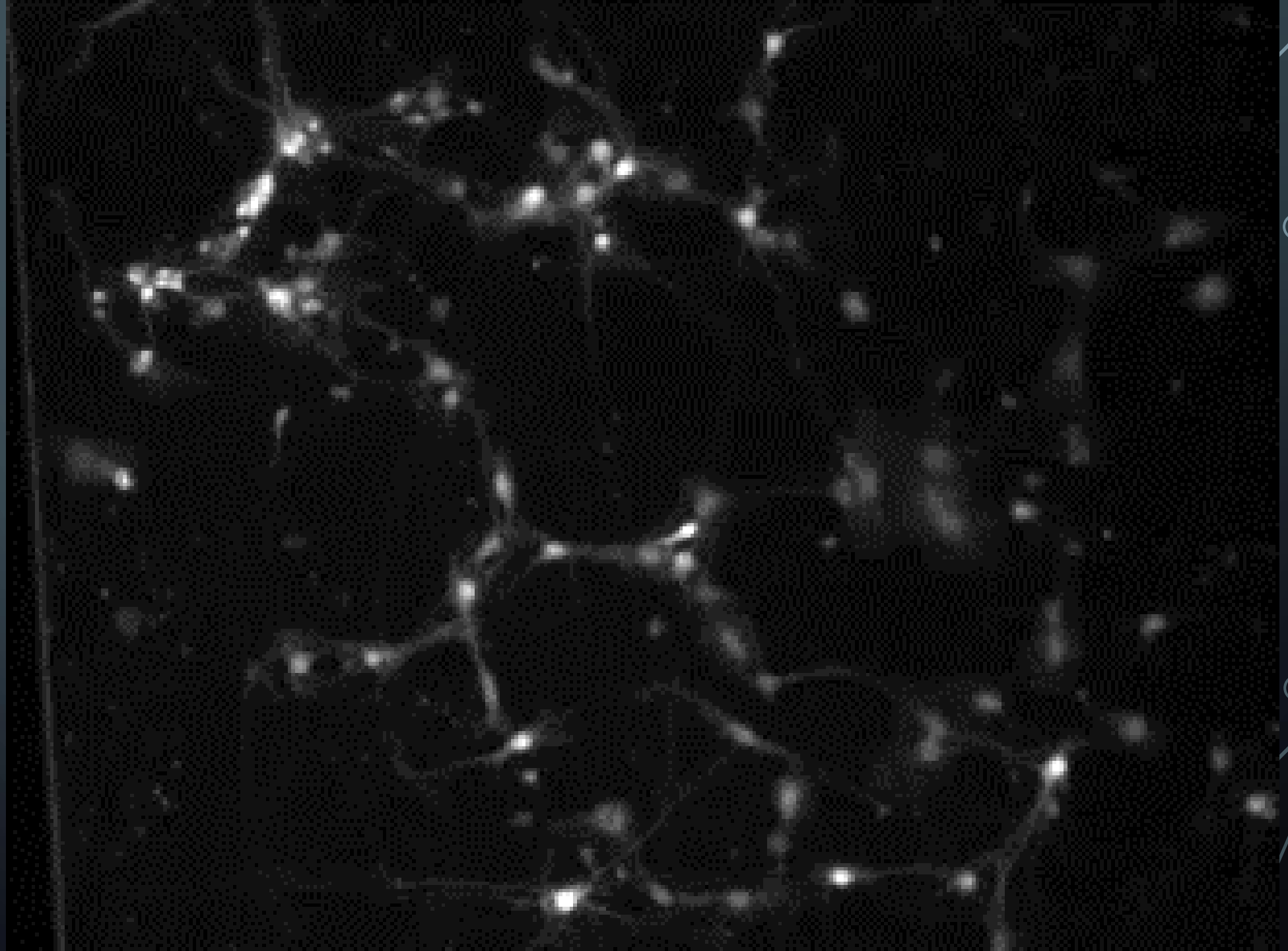
512ms 1x1 binning	117ms 2x2 binning	28ms 4x4 binning

Using binning shortens exposure times reducing cell phototoxicity and fluorophore photobleaching

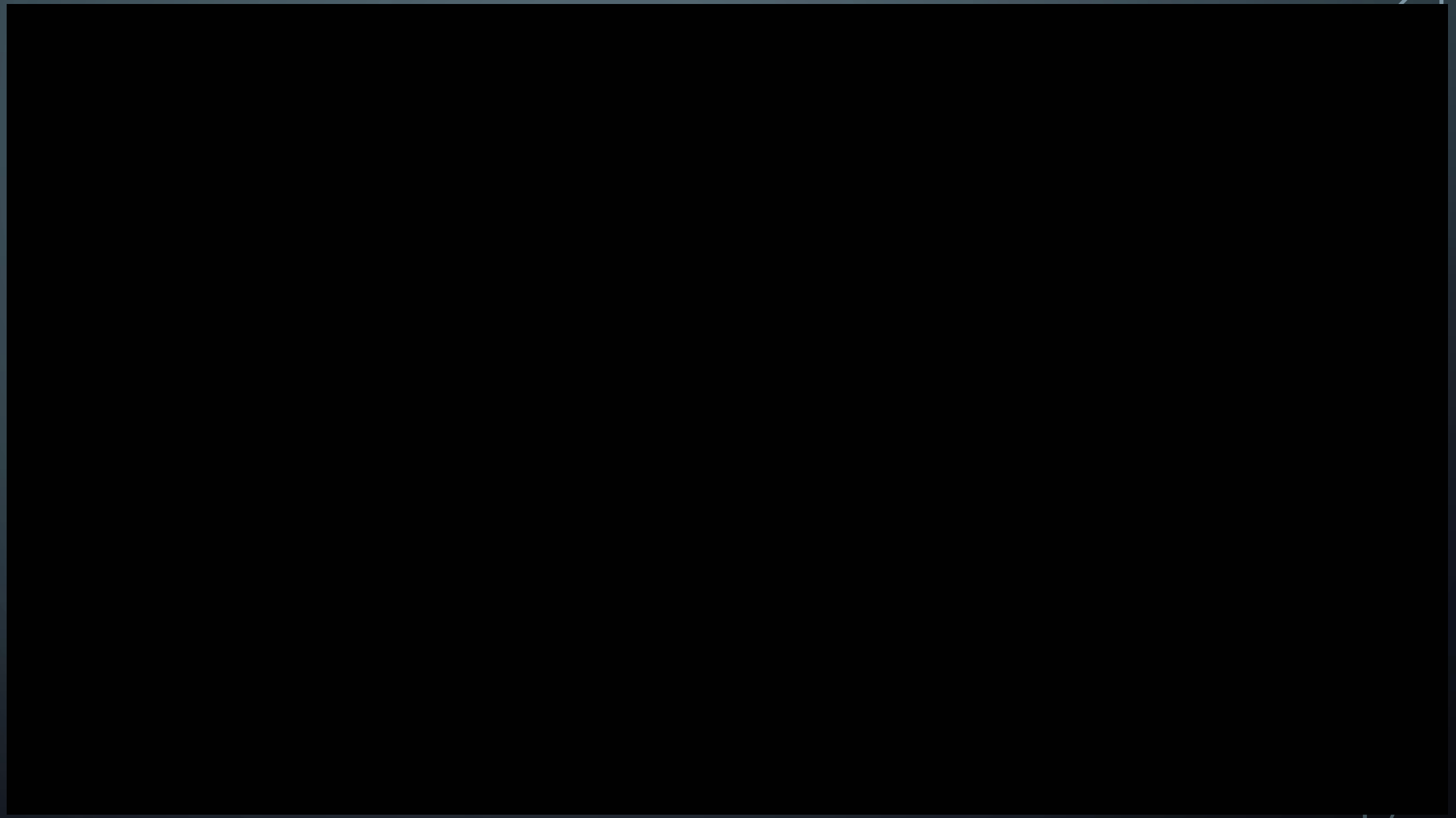
Ion imaging
observing changes in ion concentrations

A commonly performed method is ion imaging (calcium, chloride, magnesium) using either fluorescent dyes or proteins especially designed to change their emission behavior upon calcium binding.

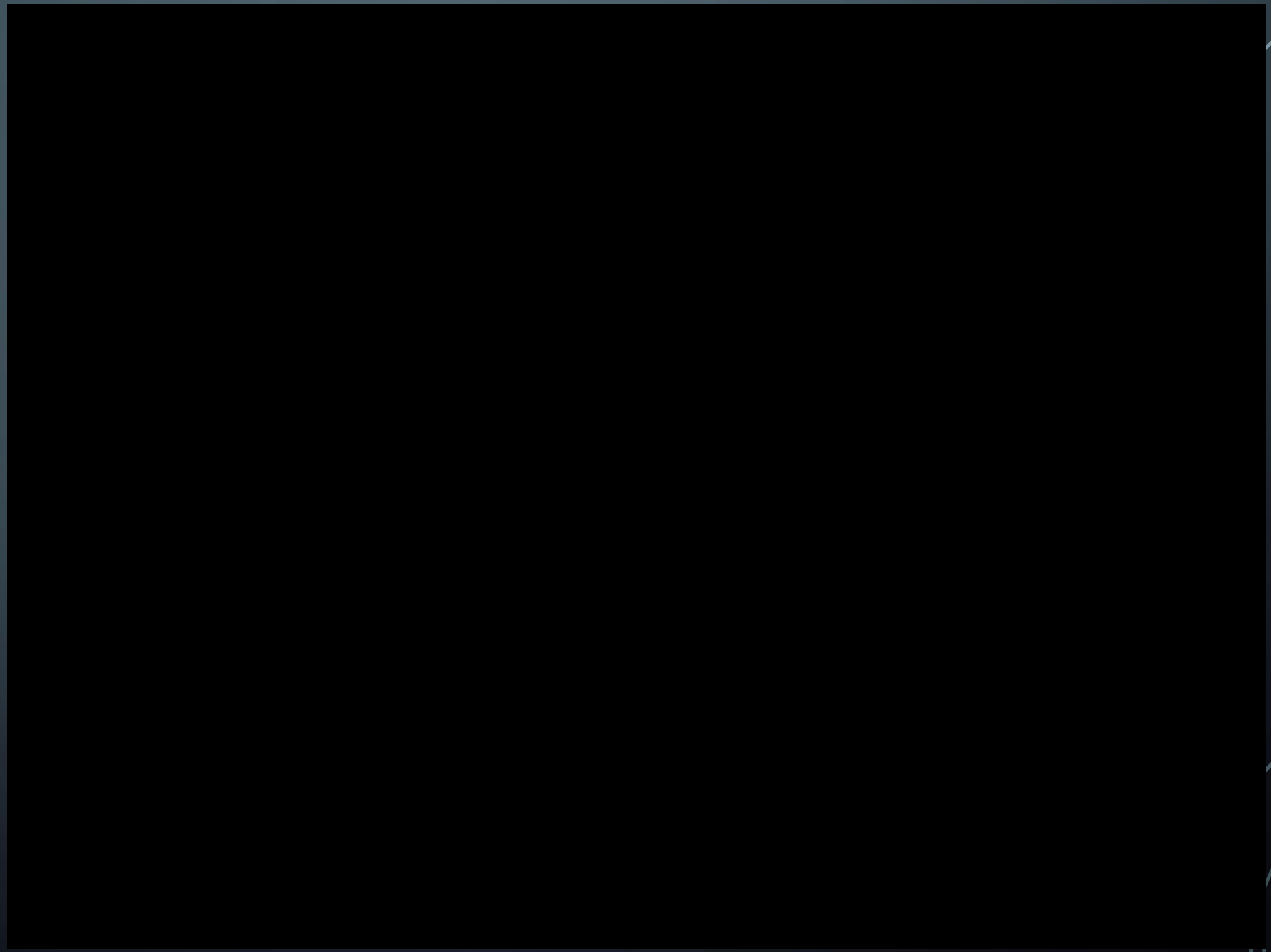
primary neurons
in vitro
calcium imaging



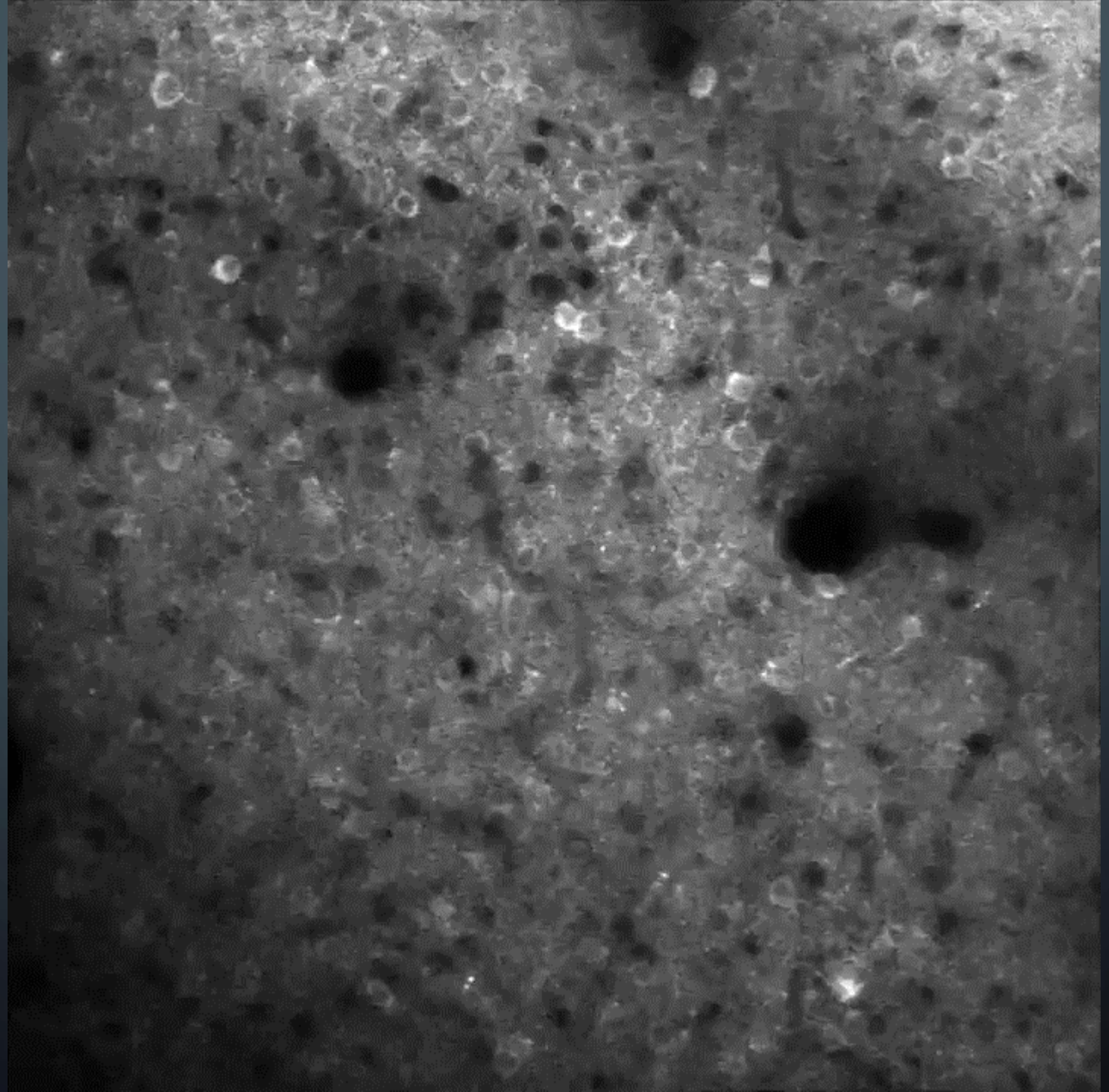
primary neurons in vitro calcium imaging



primary neurons
in vitro
calcium imaging



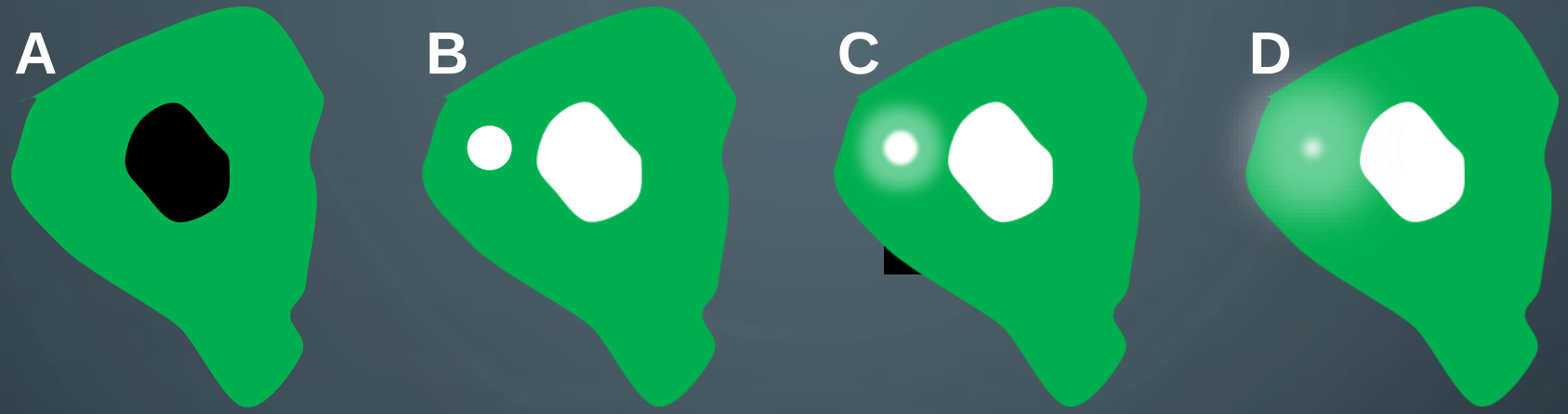
neurons
in vivo
calcium imaging

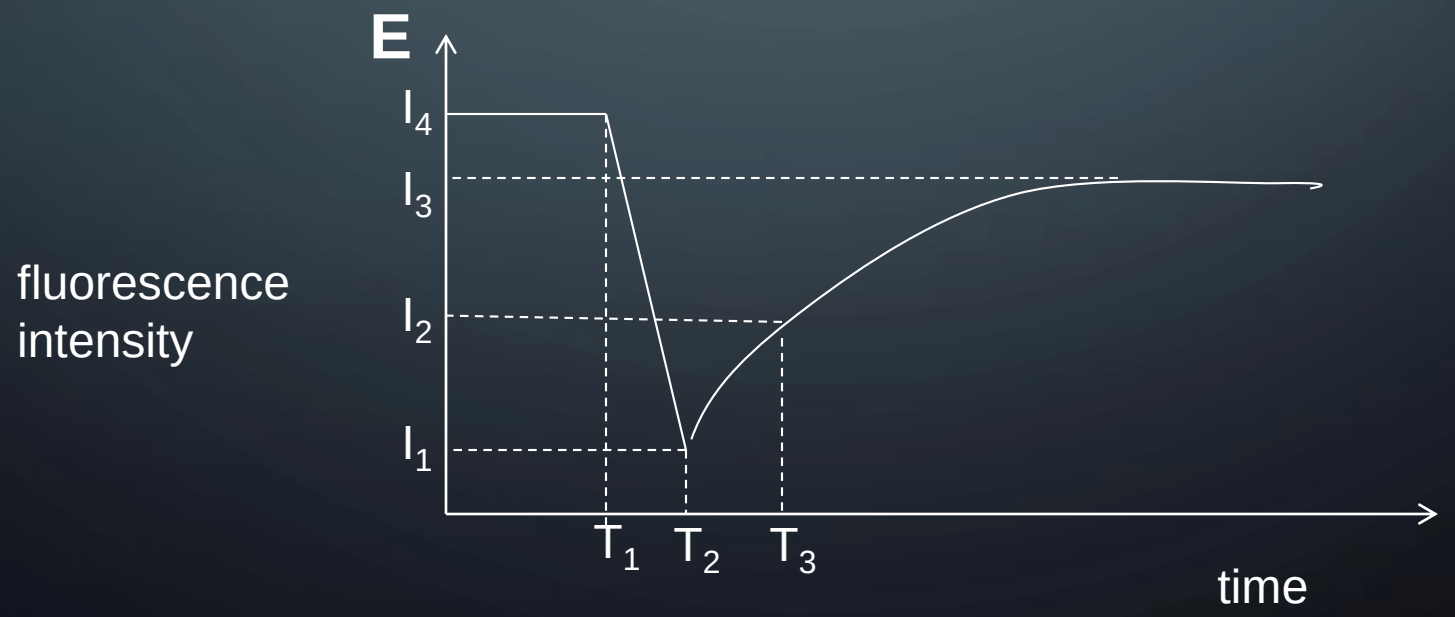


OTHER ADVANCED TECHNIQUES

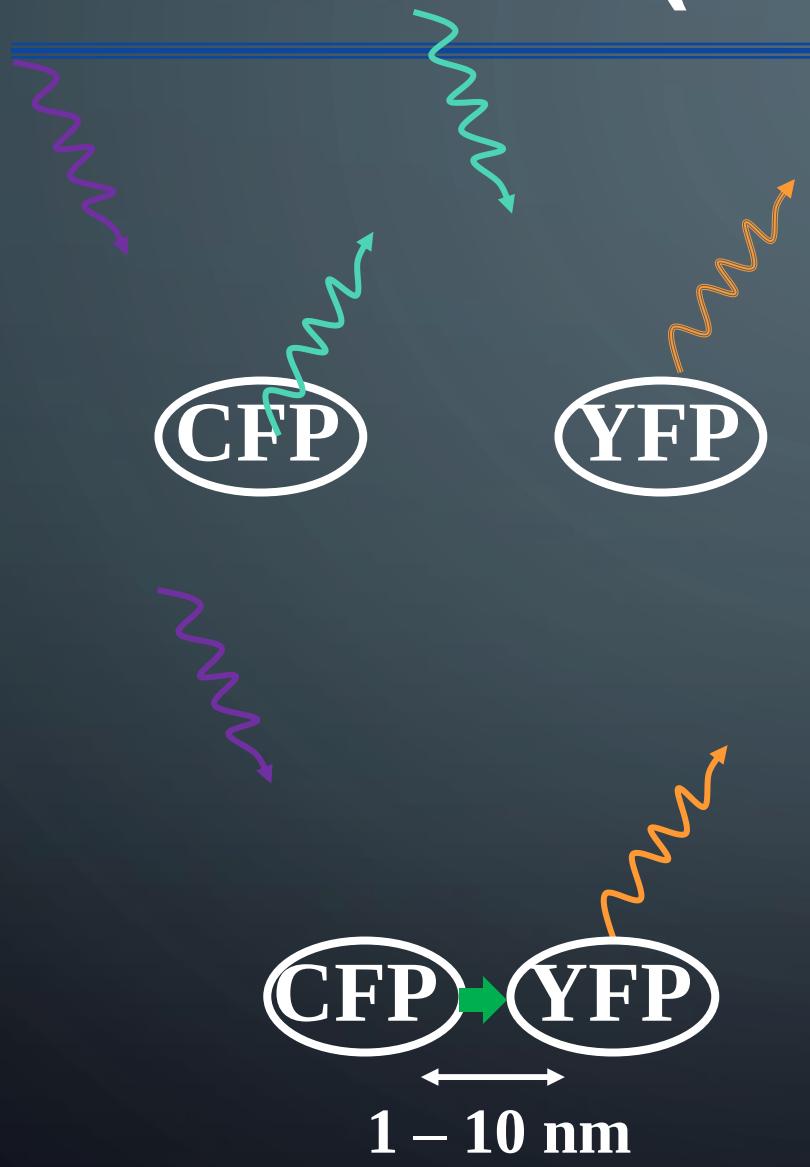
a. Fluorescence Recovery After Photobleaching
b. Förster Resonance Energy Transfer
c. Total Internal Reflection Fluorescence
d. Photo-Activation
e. Combining Technologies

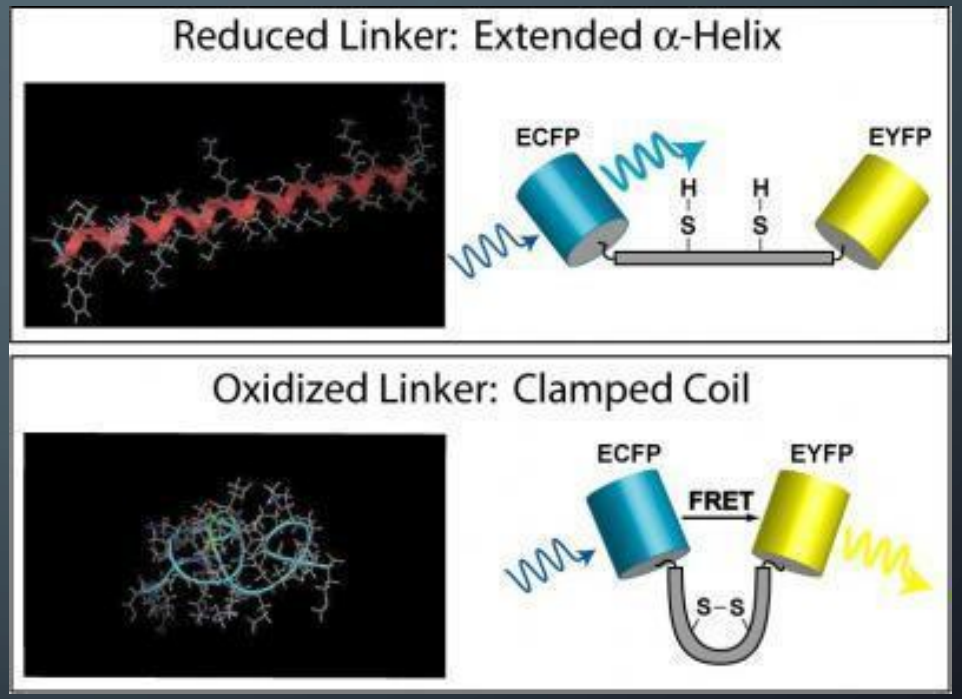
FLUORESCENCE RECOVERY AFTER PHOTOBLEACHING





FÖRSTER RESONANCE ENERGY TRANSFER (FRET)

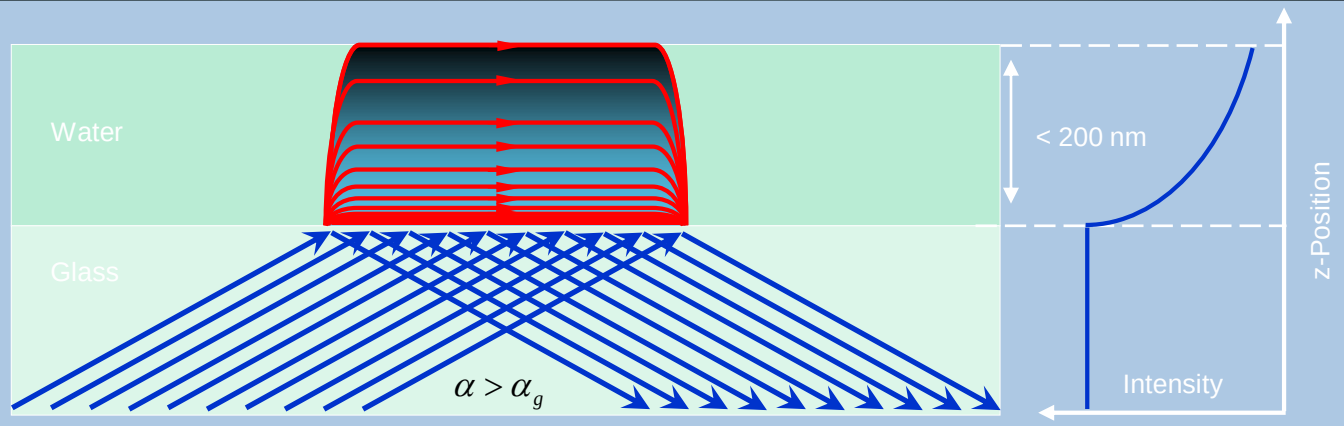




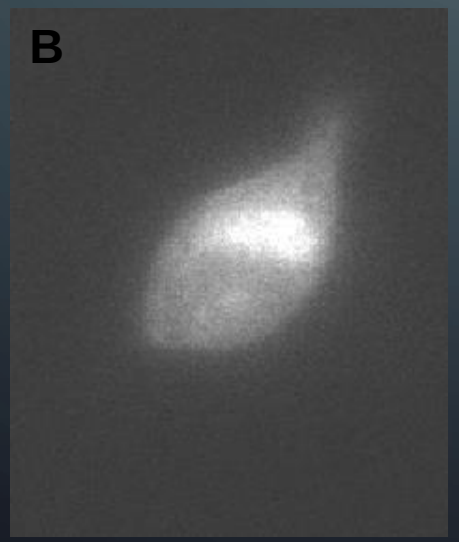
(Fig 1 in Exp Biol Med Feb 2008 vol 233, no 2, vii – viii)

TOTAL INTERNAL REFLECTION FLUORESCENCE (TIRF)

A



Only fluorophores very near the coverslip (100-200 nm) are excited.



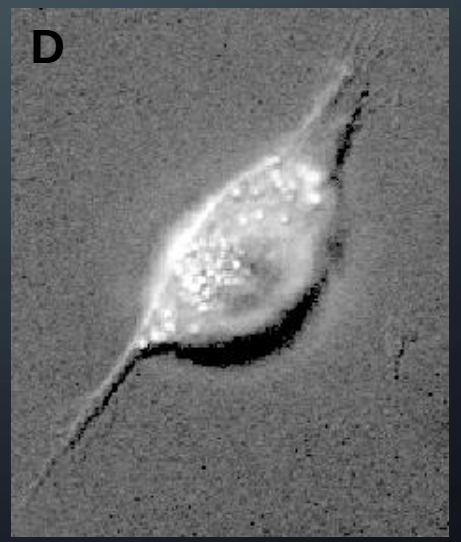
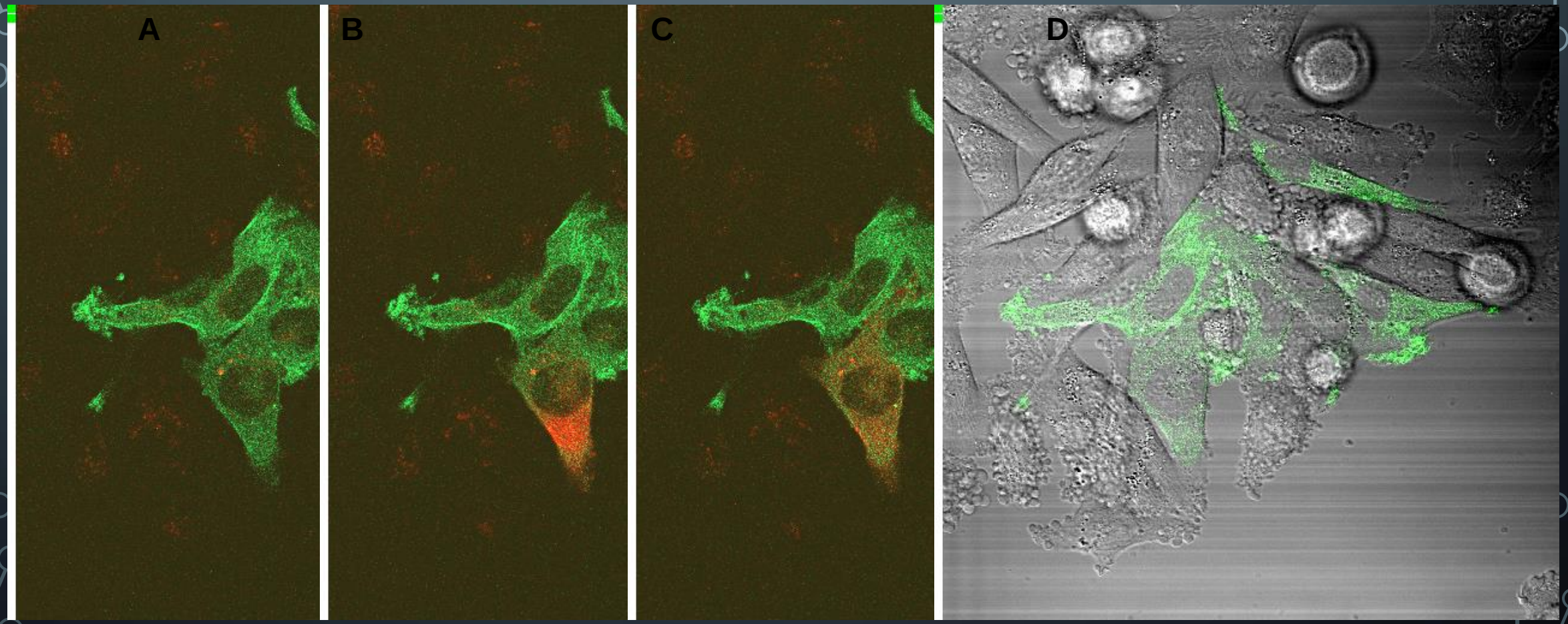


PHOTO-CONVERTIBLE FLUORESCENT ACTIN



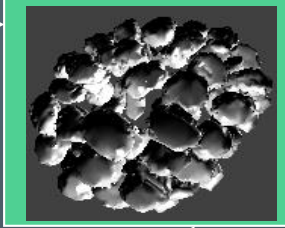
COMBINING TECHNIQUES

Living Animal Two Photon Microscopy

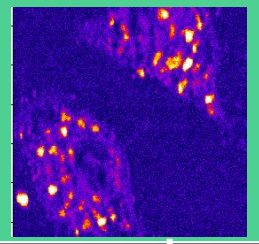
3D Microscope Reconstruction of Sectioned Tumors

H&E
Antibody
Laser capture

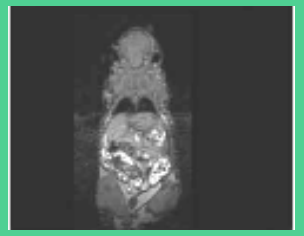
Confocal Imaging of Organo-typic Tumor Models



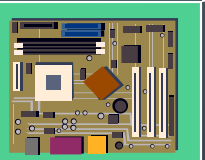
Non-Fluorescent Molecular Imaging



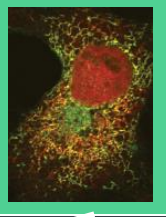
Whole Animal Imaging



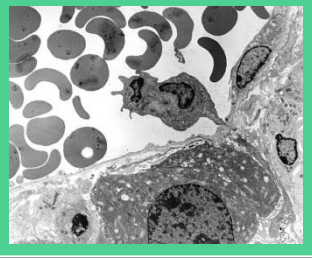
Computational Visualization, Quantification and Modeling



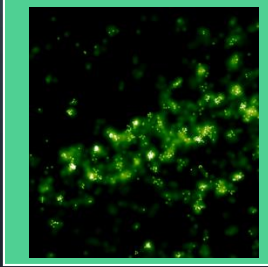
Molecular Dynamics in Individual Cells



3D EM of Tumor Cells



Super-Resolution Optical Microscopy



Summary

Biological application	Microscopy Approach	Abbreviation
Cell shape – cell migration – organelle kinetics	Transmission Microscopy : Bright Field, Dark Field, Phase Contrast, Differential Interference Contrast	BF/ PH/ DIC
Protein colocalization - dynamics, organisation and structure of cells , organelles, proteins	Epifluorescence / Confocal / Spinning disk	
3D imaging of live cells	Confocal/ Spinning disk	
3D imaging of tissues, model organisms, small animals – study structure and development	Multiphoton microscopy/ light sheet microscopy	
Quantification of ion concentrations (calcium, magnesium)	ion imaging	
Biosensing and protein-protein interactions	Forster resonance energy transfer	FRET
Biosensing and protein-protein interactions without light excitation	Bioluminescence resonance energy transfer	BRET
Protein diffusion and kinetics	Fluorescence recovery after photo-bleaching	FRAP
Protein diffusion with high spatio-temporal accuracy (µs-s and 300-400nm)	Fluorescence correlation spectroscopy	FCS
Protein diffusion with single molecule mapping and high spatio-temporal resolution	Single particle tracking	SPT
Protein diffusion with single molecule mapping and high spatio-temporal resolution in dense samples	SPT photoactivated localisation microscopy	sptPALM

Summary

Type	Common Application	Pro	Con
Fluorescent Proteins (FP)	Targeting probe to specific cellular compartments or to interact with particular proteins FRET	Direct in situ labeling Can be attached to a target genetically Very specific Low toxicity	Large Size Beta barrel shape may hinder molecules under study Photobleach Low signal intensity
Fluorescent Peptides	Similar applications as FP but utilized when size of FP may hinder study	Small size Low Toxicity	Targets Specific Interactions Often requires coupling to molecules
Organic Fluorophores	Can target cell structures Label mRNA sequences	Small Good for multiplexing Limited photostability Wide range of colors	Require coupling to another molecule Photobleach Hydrophobic Somewhat toxic Lower specificity
Indicator Probes	Indicate presence of ions and metals	Very specific	Often requires coupling to molecules
Quantum Dots	Long term imaging studies Real-time tracking over extended periods	Very photostable Good for multiplexing Intense signal Enable very sensitive detection	Coupling to another molecule necessary Interference

Platform	To improve efficiency	To improve sensitivity and S/N	To minimize light exposure
All	Hard-coated filters (see Box 1). Dye-specific filters. Avoid excess optical elements (lenses, DIC components). 100% light to detector port. High NA objectives. Spectral un-mixing. Avoid phase objectives.	**General:** Phenol-red-free medium. Restorative deconvolution. **Camera-based systems:** Avoid color cameras. Bin high-resolution cameras 2×2 (exposure times of 200-500 ms). Slow camera read times. Use EM-CCD for high speed (exposure times of <100 ms).	Keep excitation light levels low. Avoid blue dyes. Minimum resolution (60×, 1.4 NA): *x,y* ~0.1-0.2 µm *z* ~0.3-0.4 µm *t*=2.3× timescale of events. Minimum number of probes. Use oxygen-radical scavengers.
WFM	Remove DIC prism and analyzer when imaging fluorescence.	Perform post-acquisition restorative deconvolution (Fig. 3C,D).	Find cells with transmitted light. Use ND filters (<10% lamp power). Use UV- and IR-blocking filters. Use halogen lamps if possible.

CLSM	Use long-pass or wide band-pass filters and un-mix post acquisition. Open the pinhole ≥2 Airy units. Remove DIC prism.	High PMT voltage (≥800 V). Restorative deconvolution. Avoid spectral-array detectors.	Low laser power. AOTF laser blanking. No line or frame averaging. Fast scan speed (≥8 µs/pixel). Image regions of interest when possible. Recommended laser powers: 405 nm Avoid 488 nm <2% (30 mW) 514 nm <2% (30 mW) 543 nm <50% (1 mW) 633 nm <5% (5 mW)

RESOLUTION, SPEED, SENSITIVITY

- Spatial Resolution
  - Depends on application → optical magnification and lens apperture
  - Smaller and more pixels = higher resolution
- Sensitivity
  - Larger pixels = higher sensitivity
  - Higher sensitivity = shorter exposure time
  - Shorter exposure time = higher frame rate
  - Larger pixels = lower resolution
- Speed
  - Less pixels = faster readout
  - Faster Readout = higher framerate
  - Less pixels = lower resolution
- Speed requires fast readout and high sensitivity
  - Light is the limit