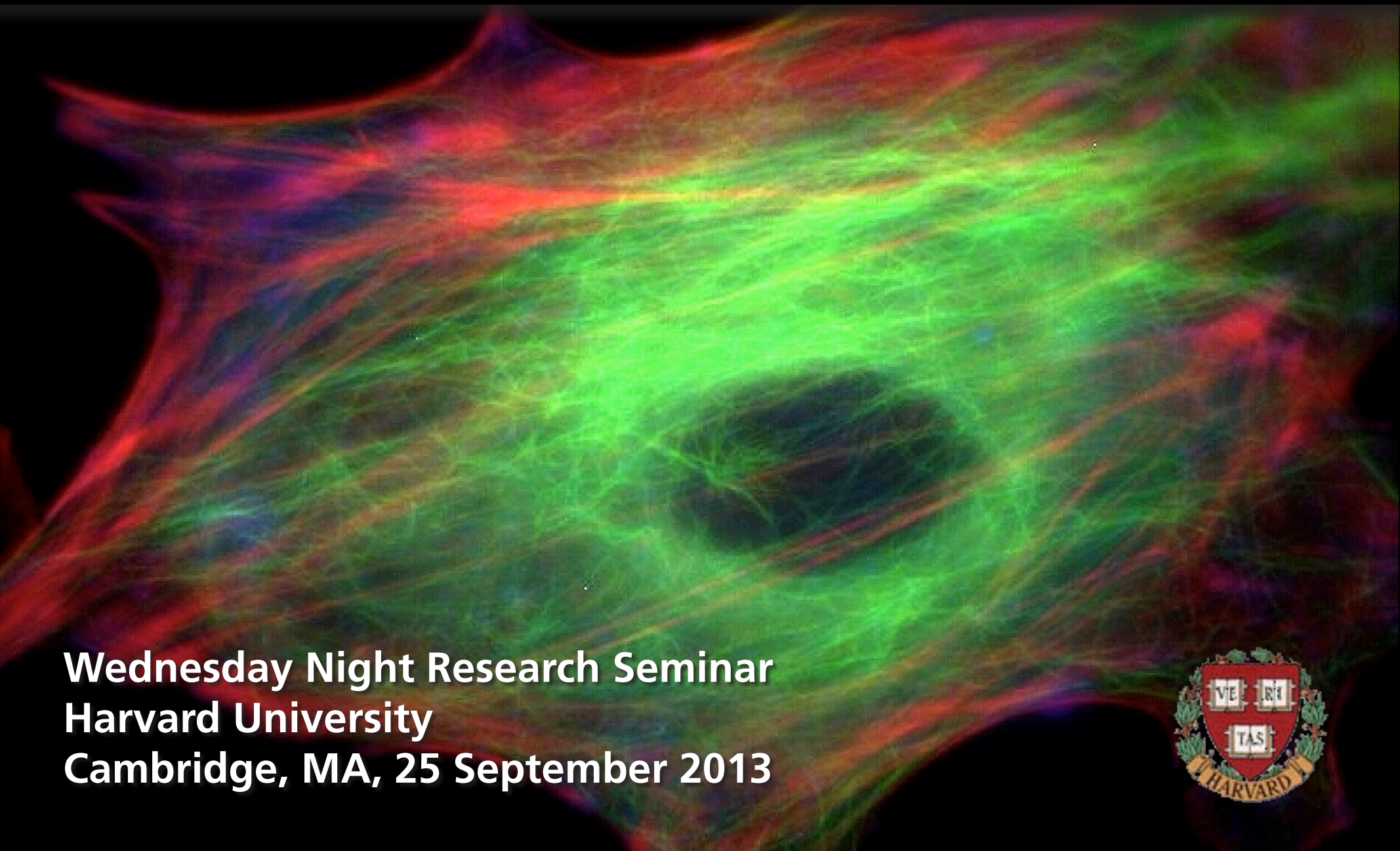


Femtosecond laser micromachining and subcellular surgery

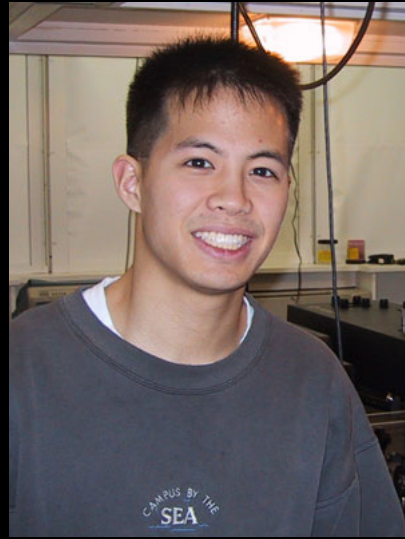


Wednesday Night Research Seminar
Harvard University
Cambridge, MA, 25 September 2013





Iva Maxwell



Sam Chung



Valeria Nuzzo



Alexander Heisterkamp



eric_mazur

and also....

**Nabiha Saklayen
Sébastien Courvoisier**

**Dr. Eli Glezer
Prof. Chris Schaffer
Prof. Nozomi Nishimura
Debayoti Datta
Dr. Jonathan Ashcom
Jeremy Hwang
Dr. Nan Shen
Roanna Ruiz
Anja Schmalz
Dr. Prakriti Tayalia**

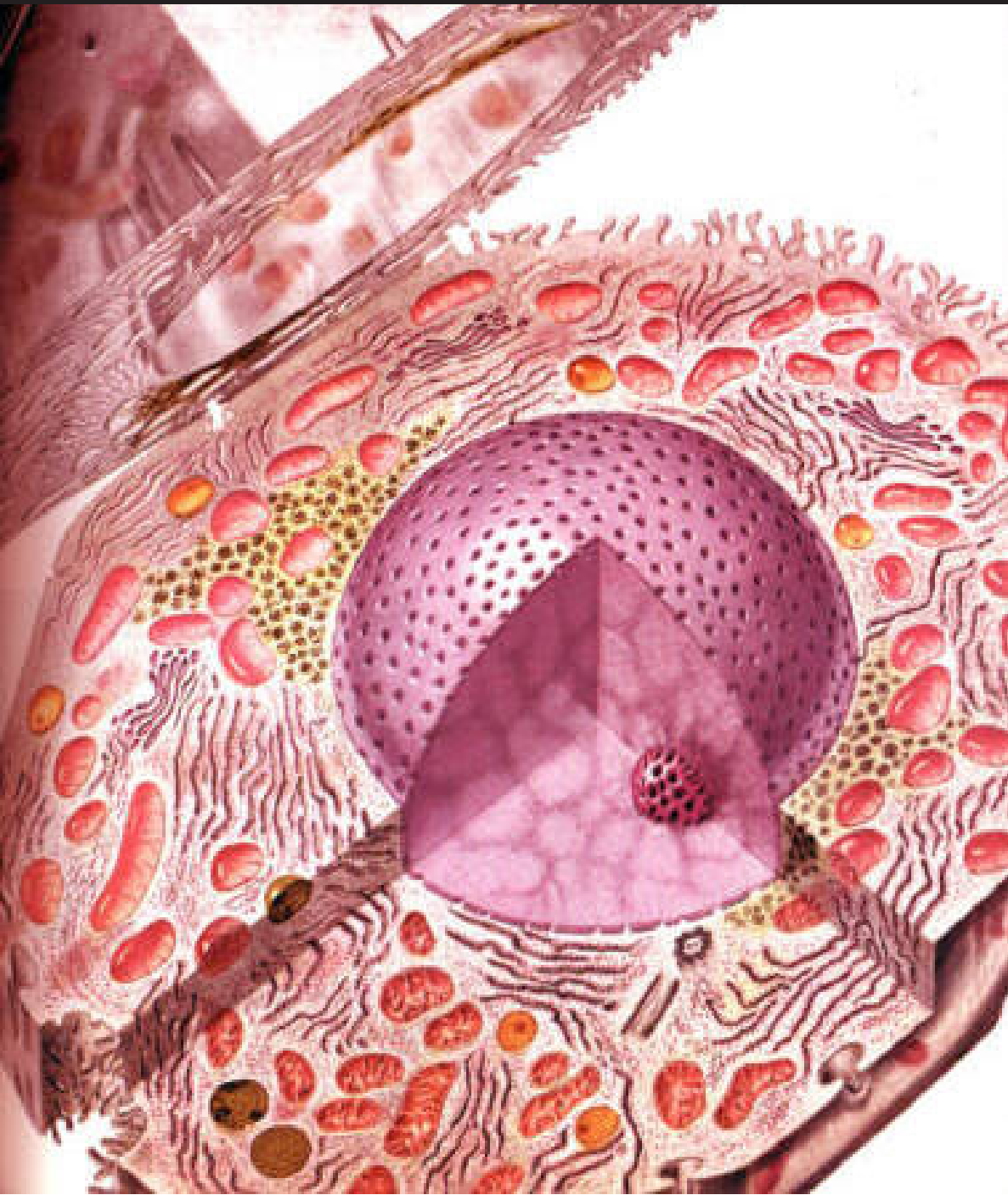
**Prof. Donald Ingber (Harvard Medical School)
Prof. Phil LeDuc (Carnegie Mellon University)
Prof. Sanjay Kumar (UC Berkeley)
Prof. Aravi Samuel (Harvard University)
Prof. Jean Underwood (UMass Worcester)
Prof. Daniel Needleman (Harvard University)**

Introduction

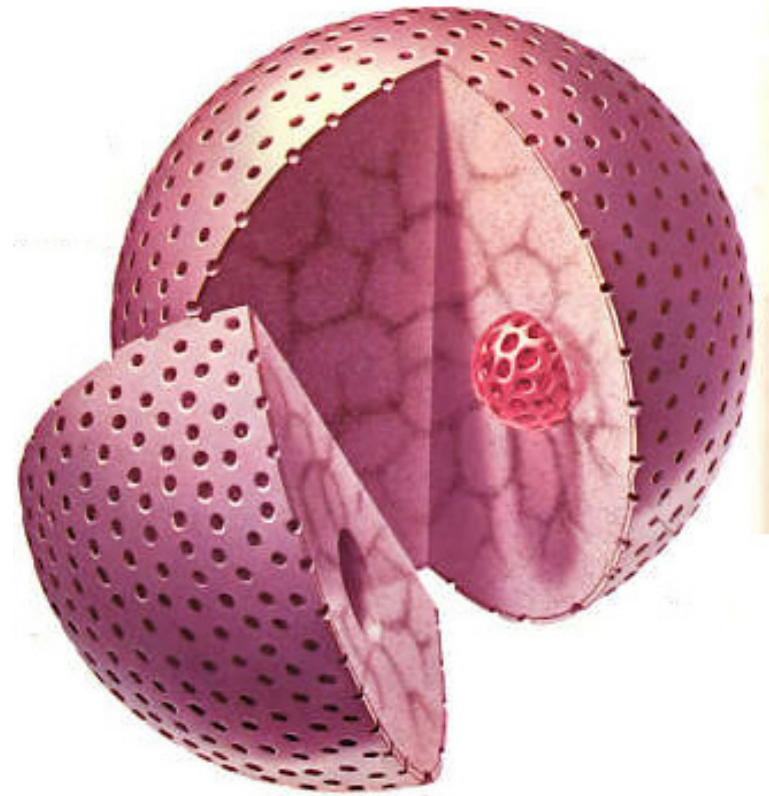
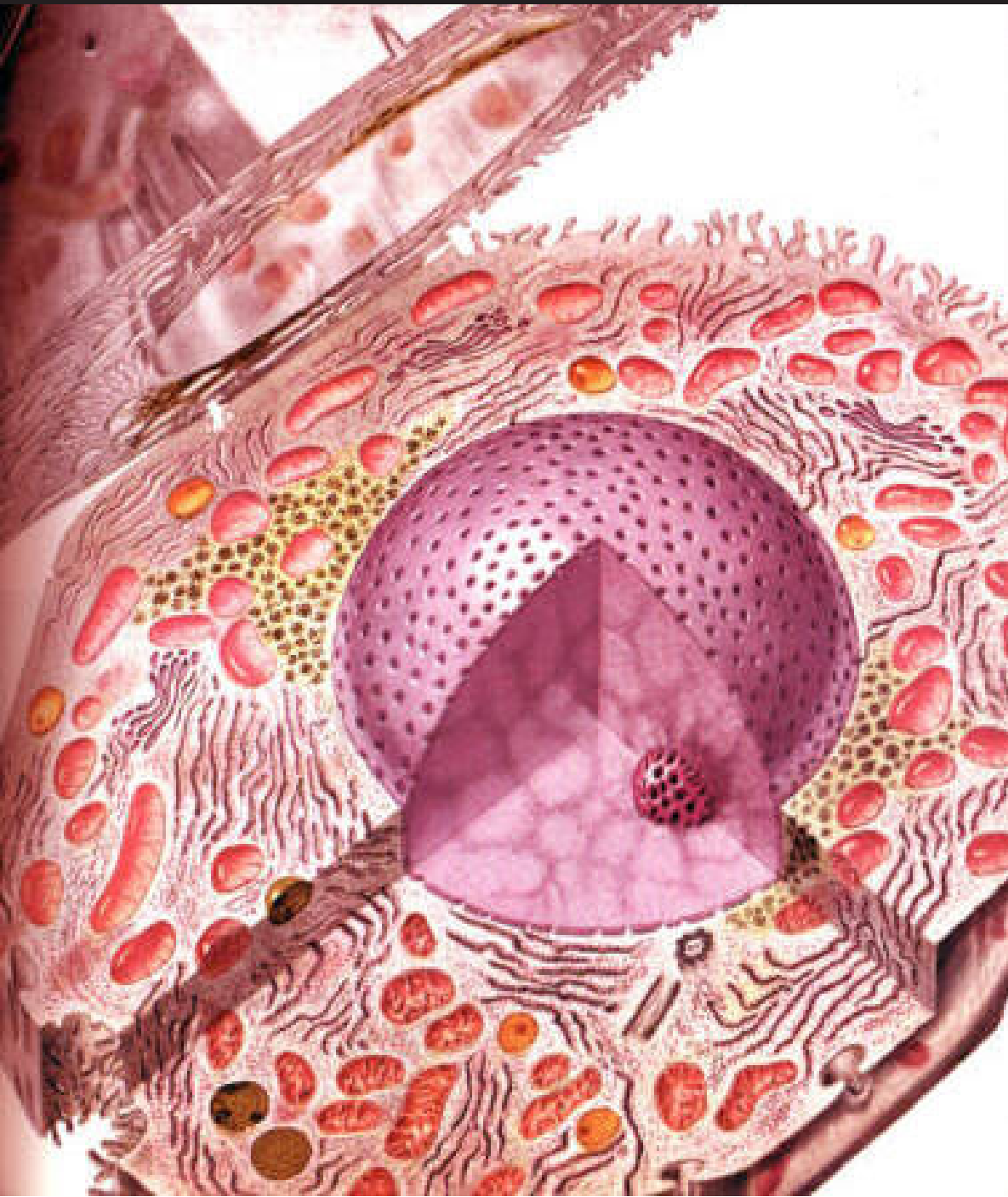
living systems require interdisciplinary tools

A microscopic view of several red blood cells (erythrocytes) against a black background. The cells are biconcave discs, appearing as bright red, textured spheres with a darker center. They are scattered across the frame, with some overlapping. The lighting highlights the granular texture of the cell surfaces.

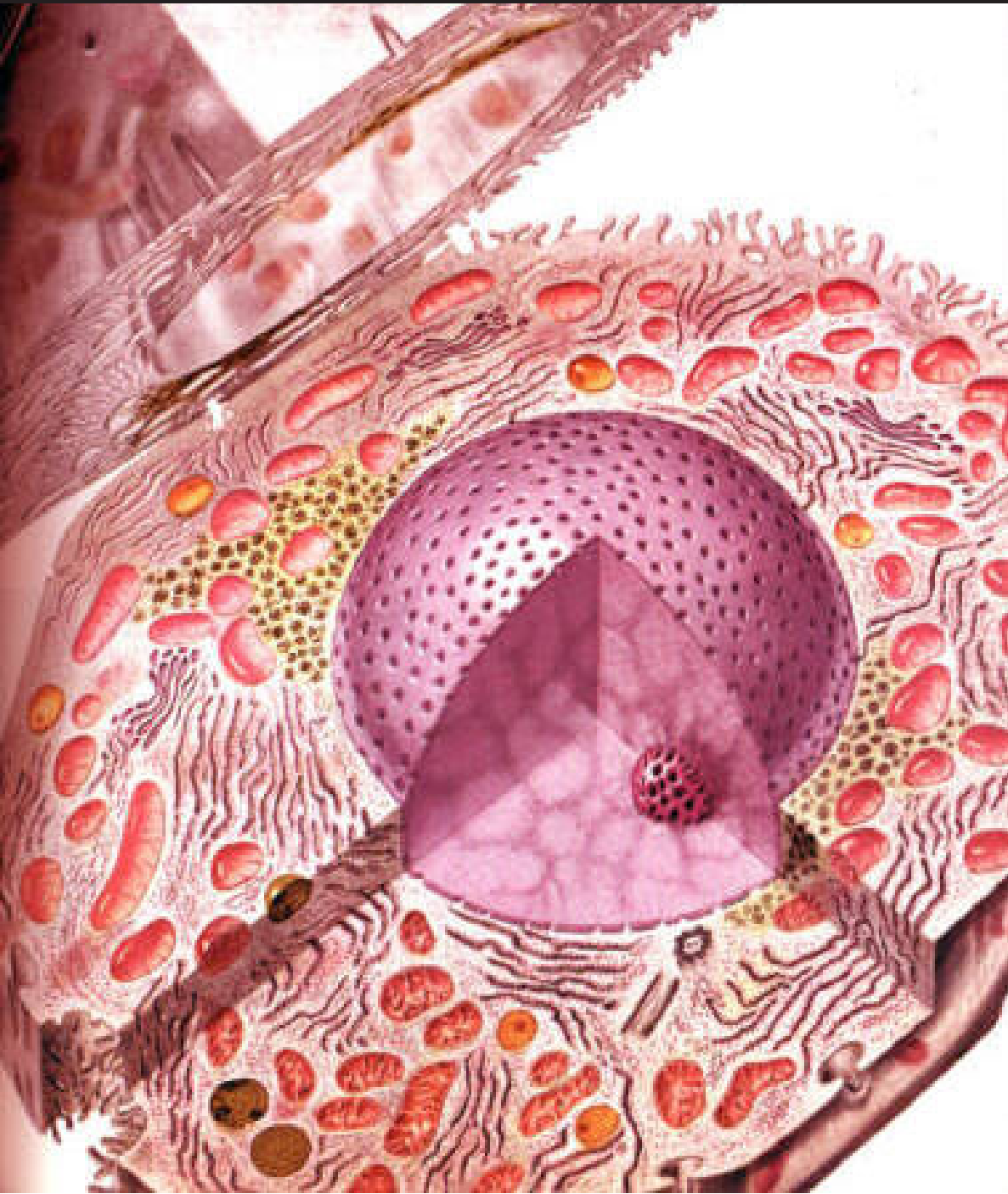
Introduction



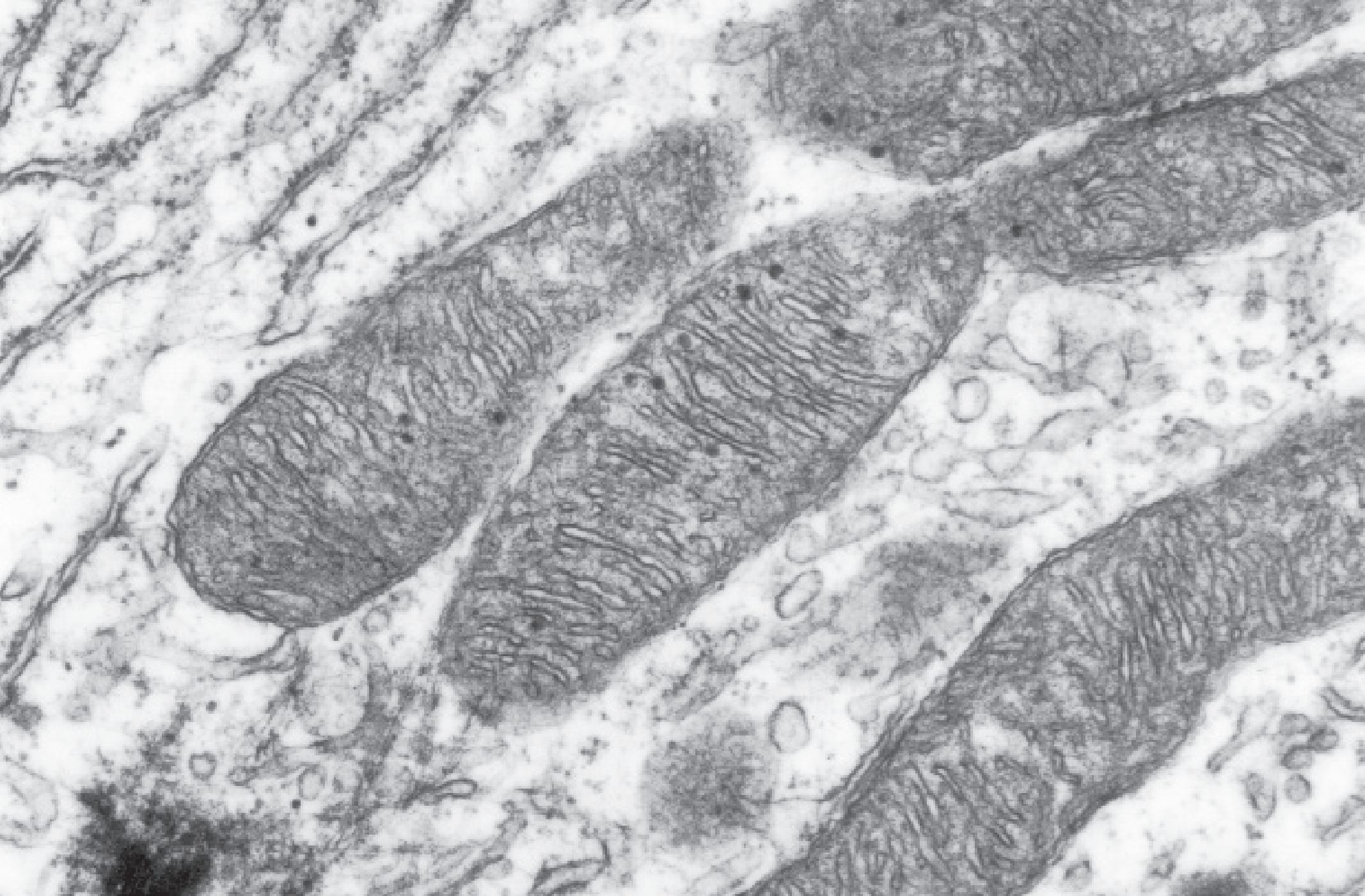
Introduction



Introduction



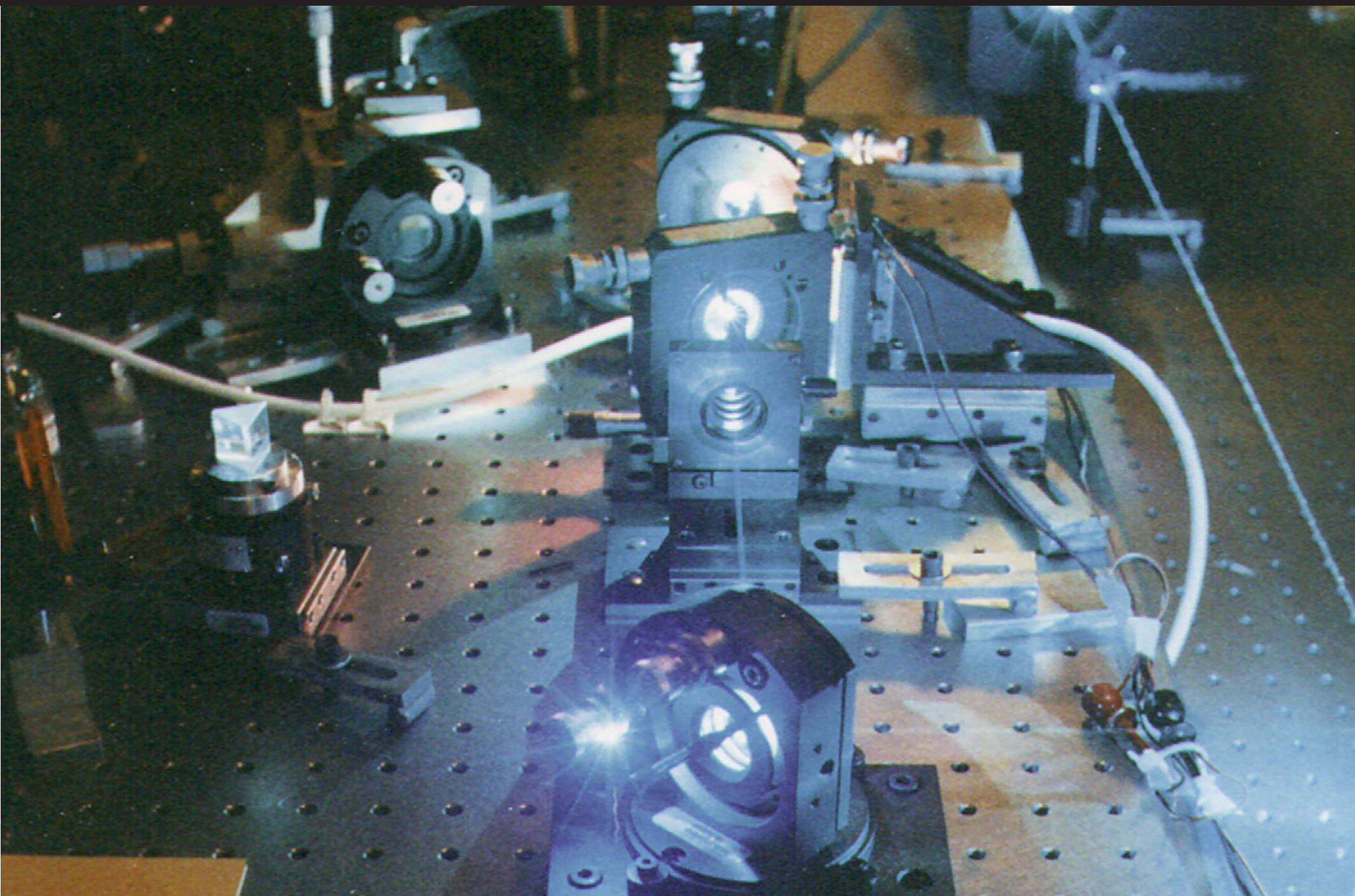
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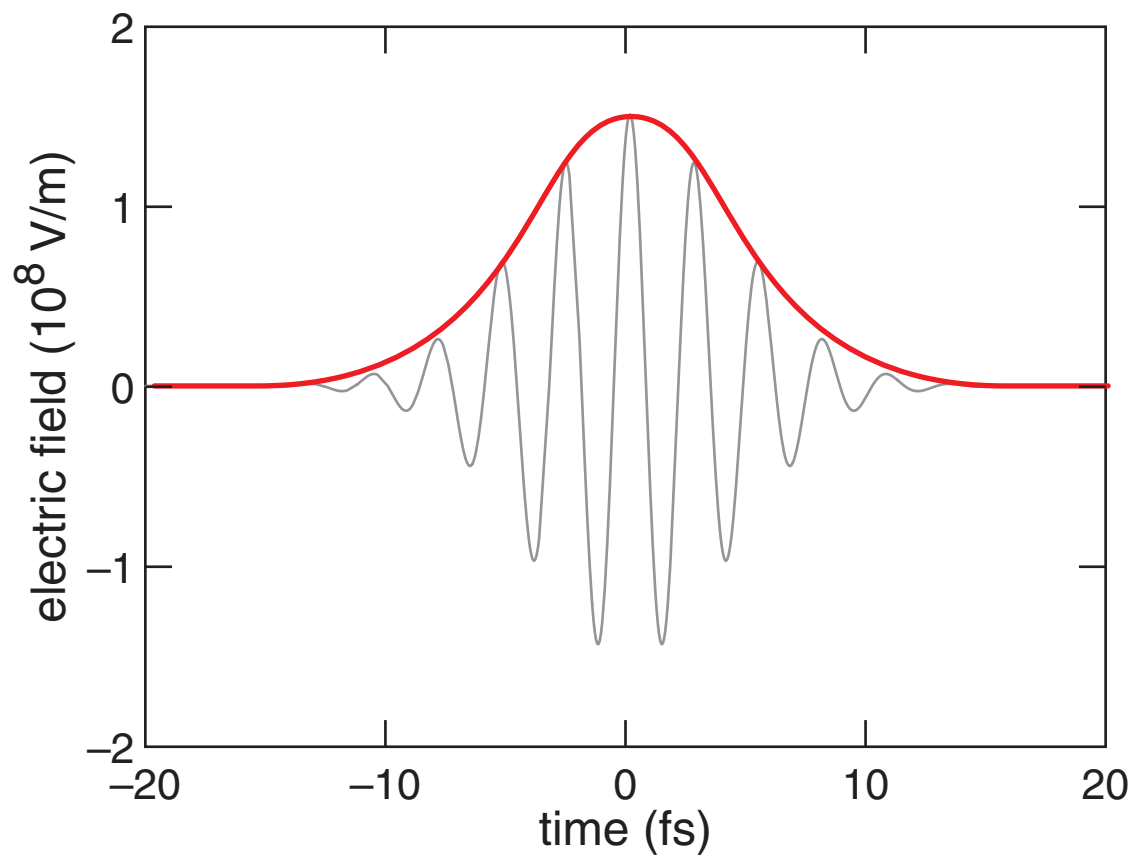
Introduction

why use femtosecond pulses?

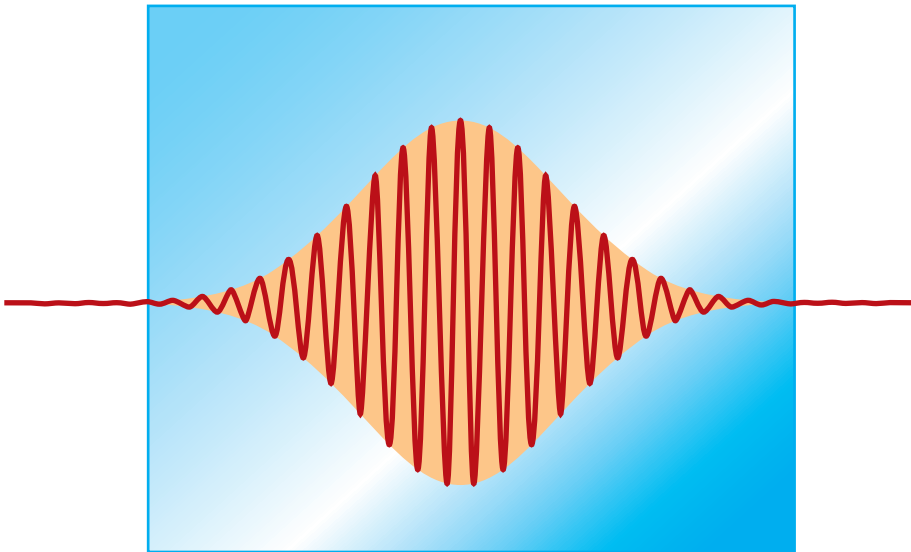
Introduction



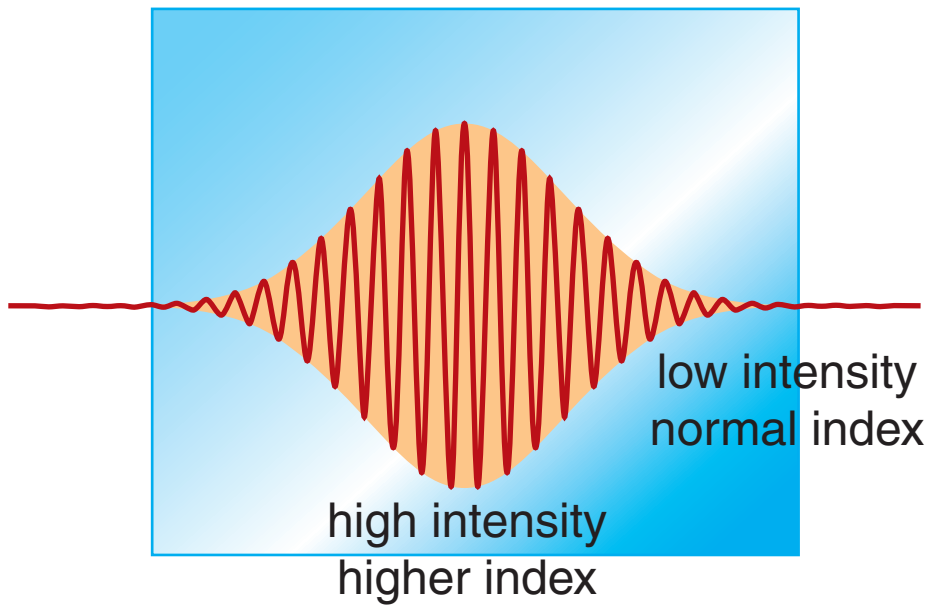
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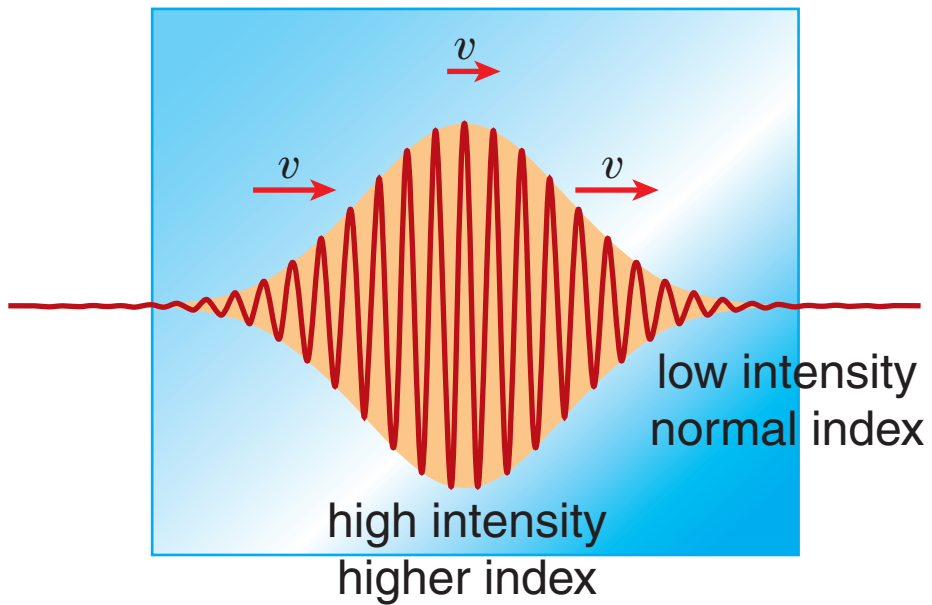
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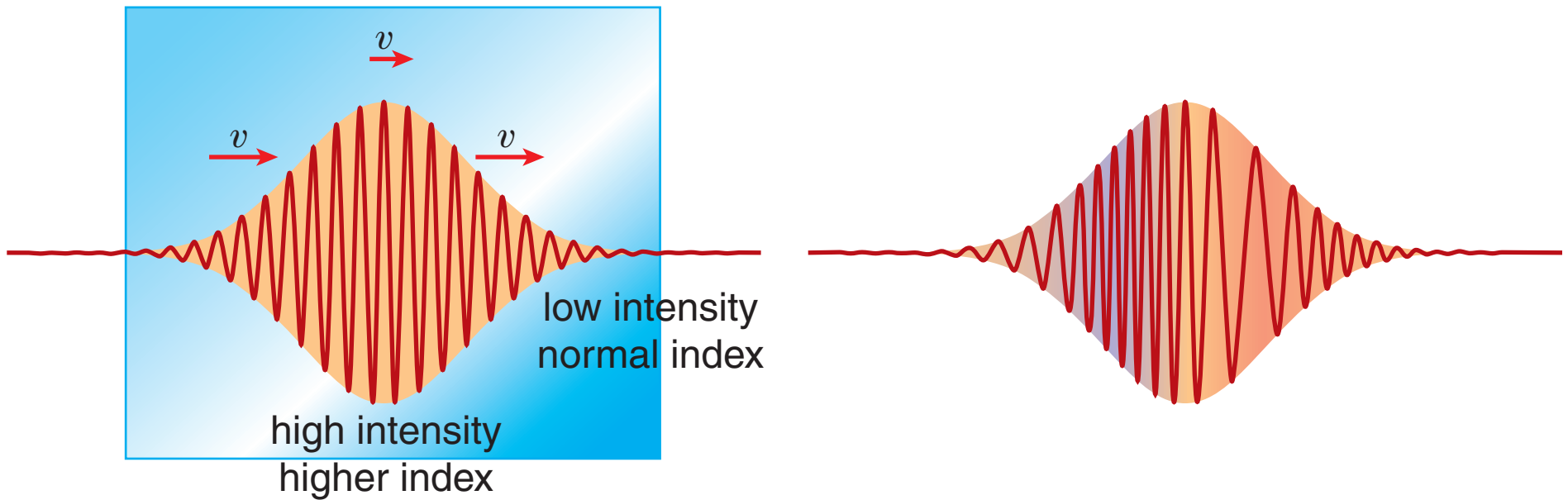
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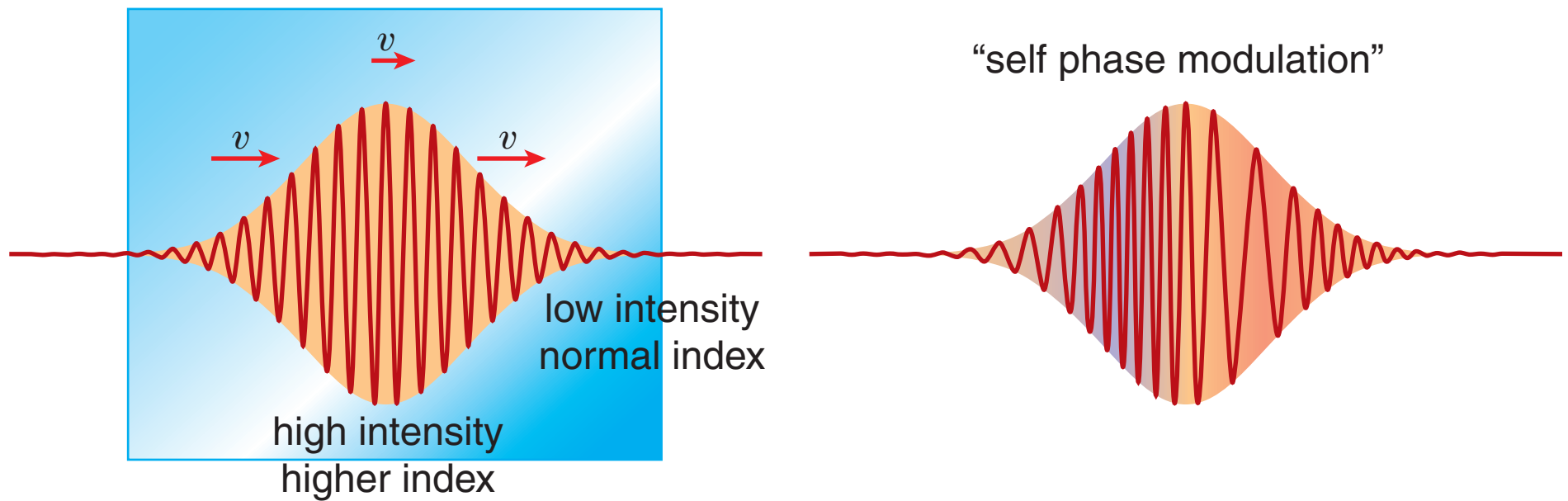
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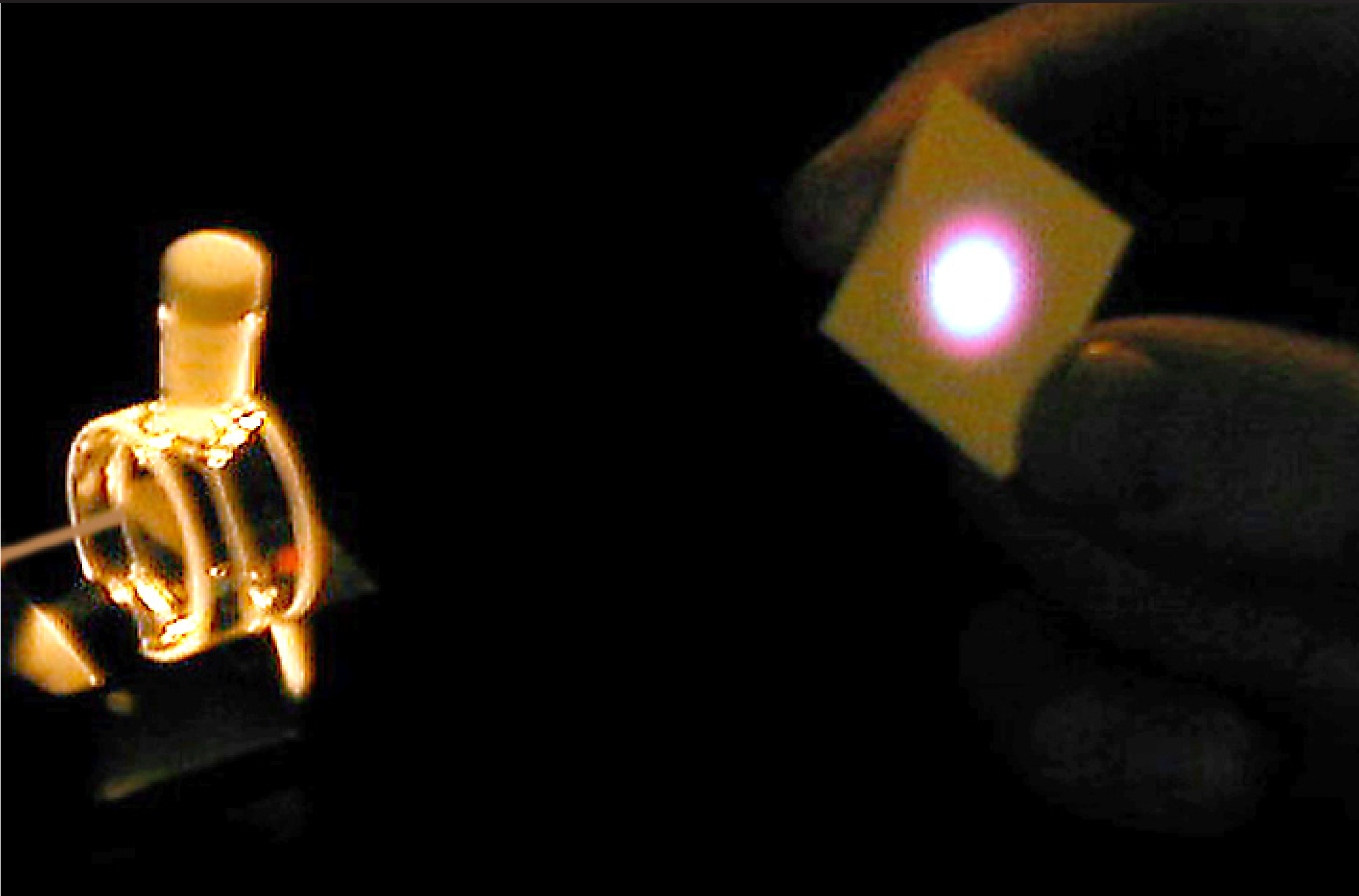
Introduction



Introduction

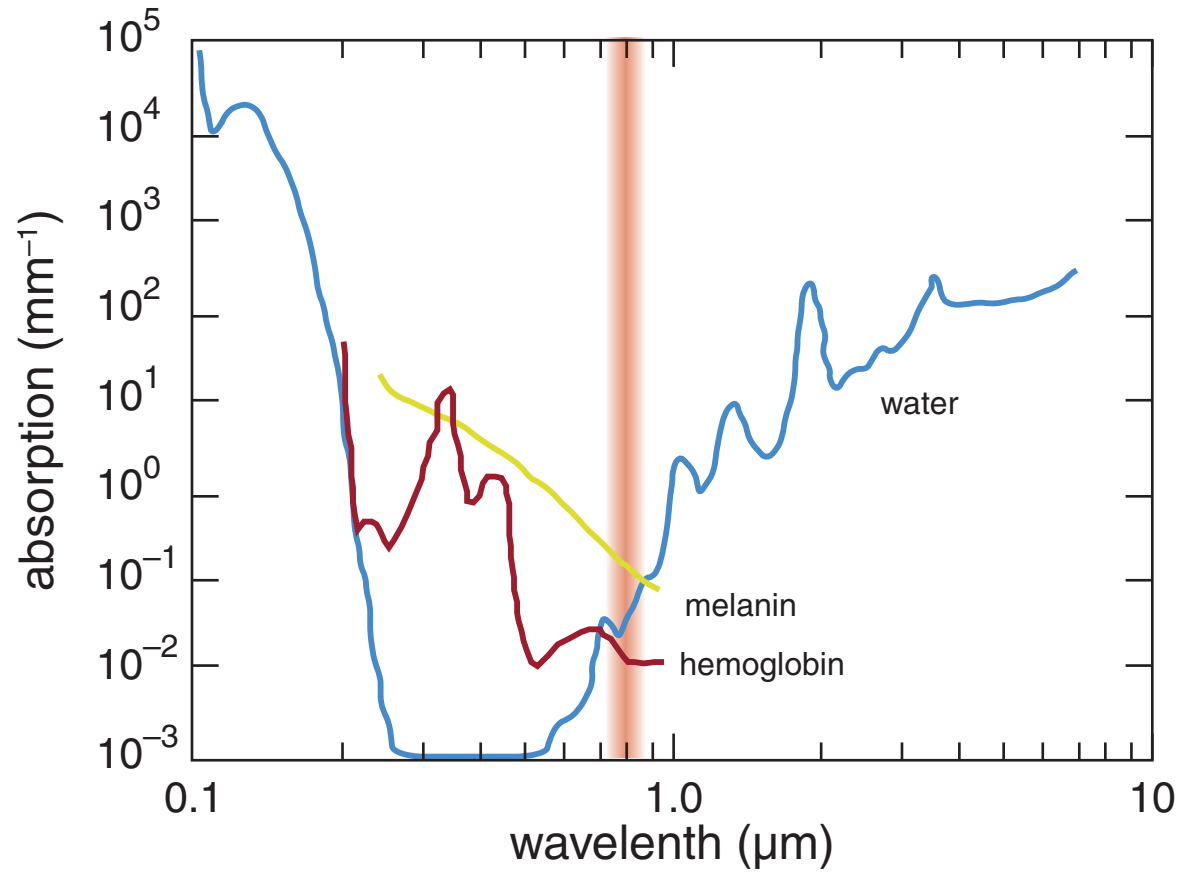


Introduction

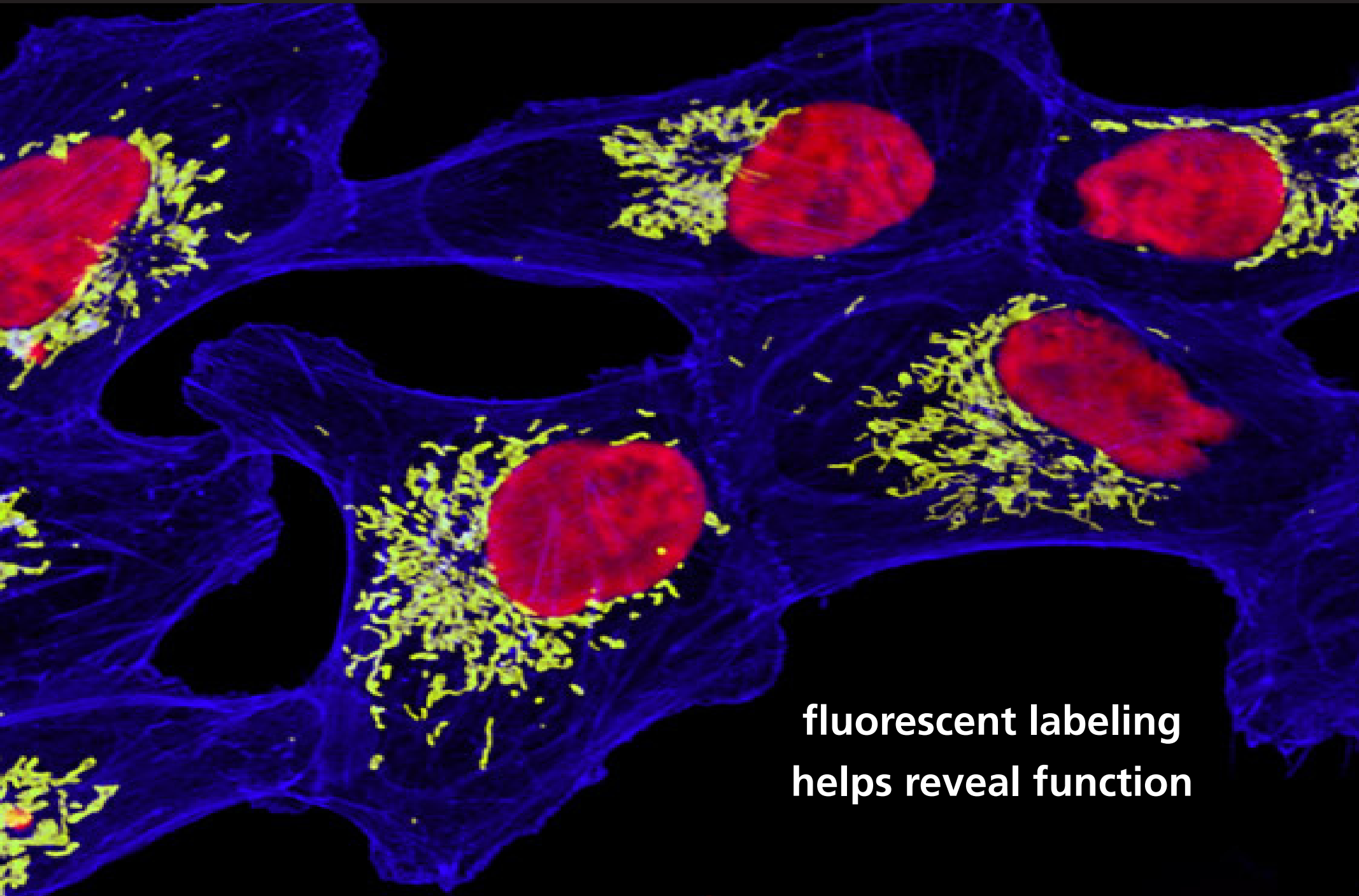


Introduction

tissue is nearly transparent at 800 nm

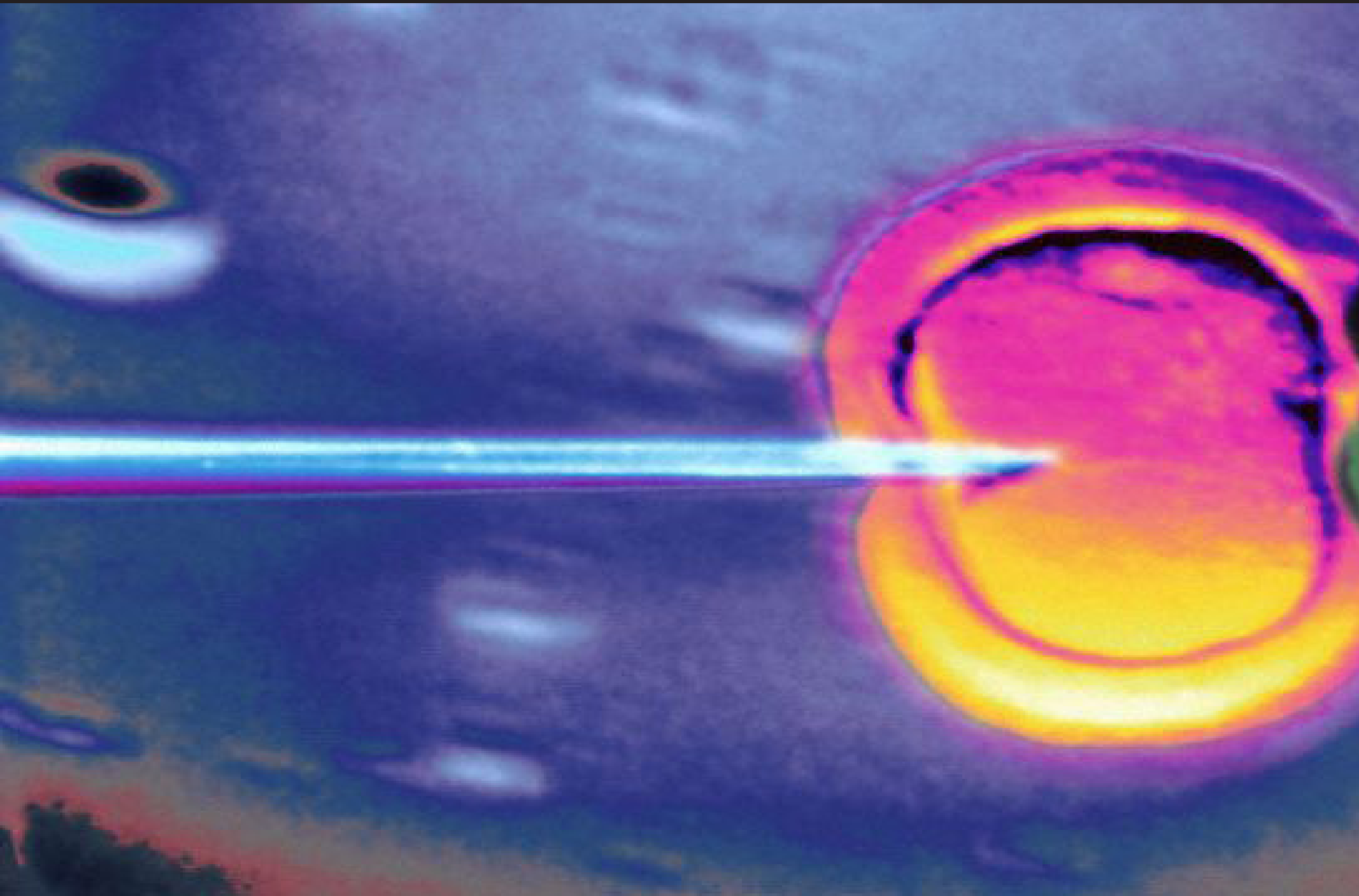


Introduction



**fluorescent labeling
helps reveal function**

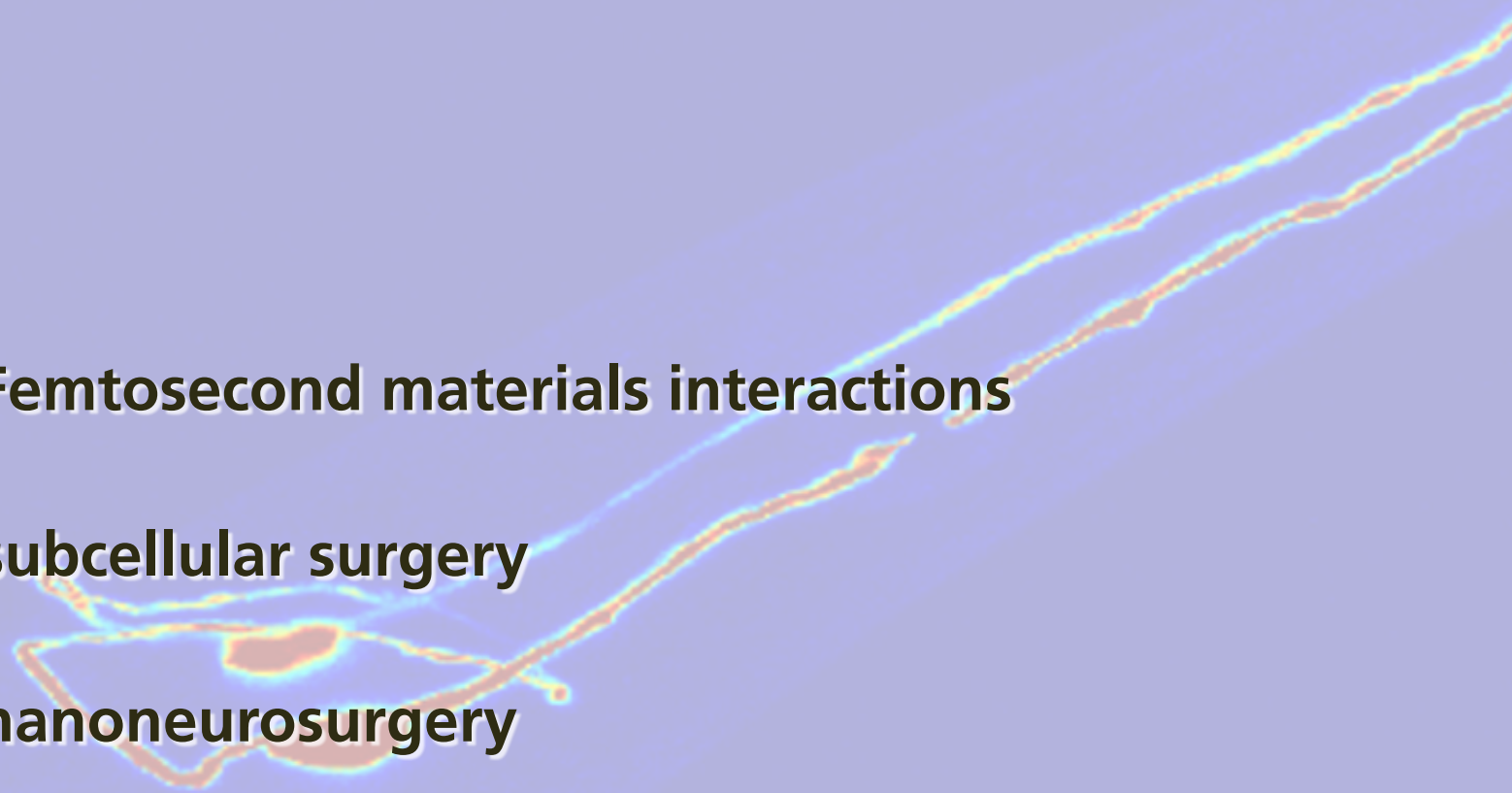
Introduction



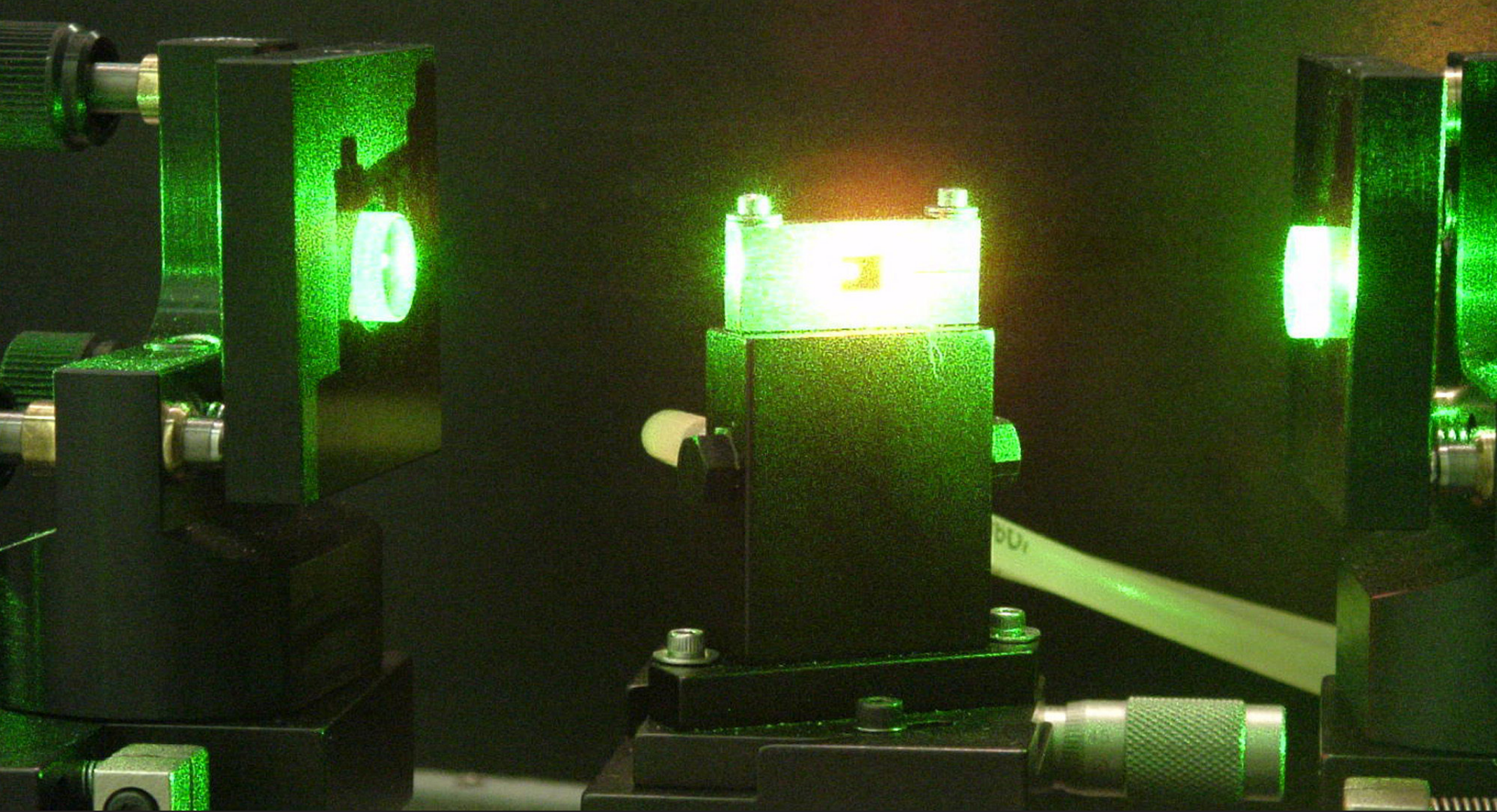
Introduction

- **standard biochemical tools: species selective**
- **fs 'nanosurgery': site-specific**

Outline

- Femtosecond materials interactions
 - subcellular surgery
 - nanoneurosurgery
- 

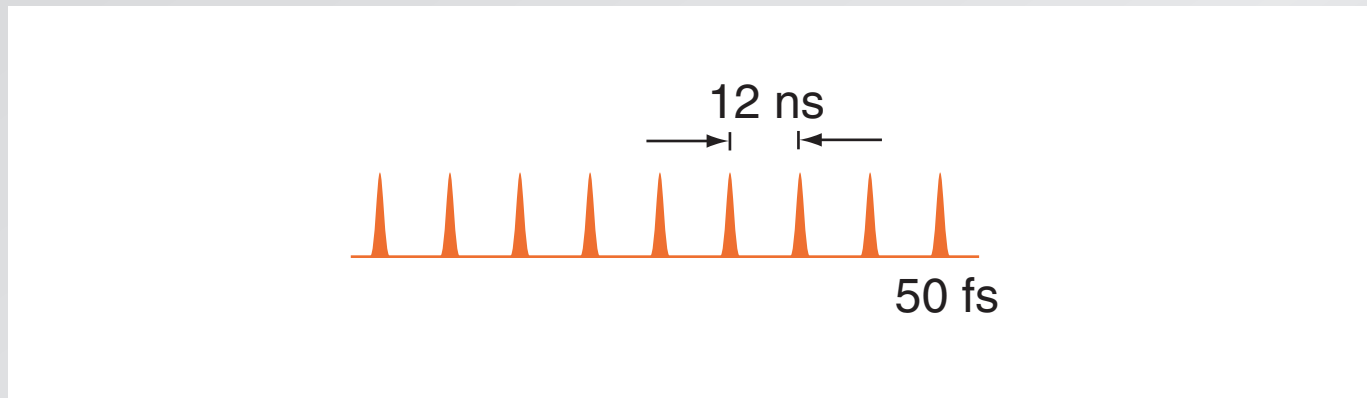
Femtosecond materials interactions



Ti: sapphire laser

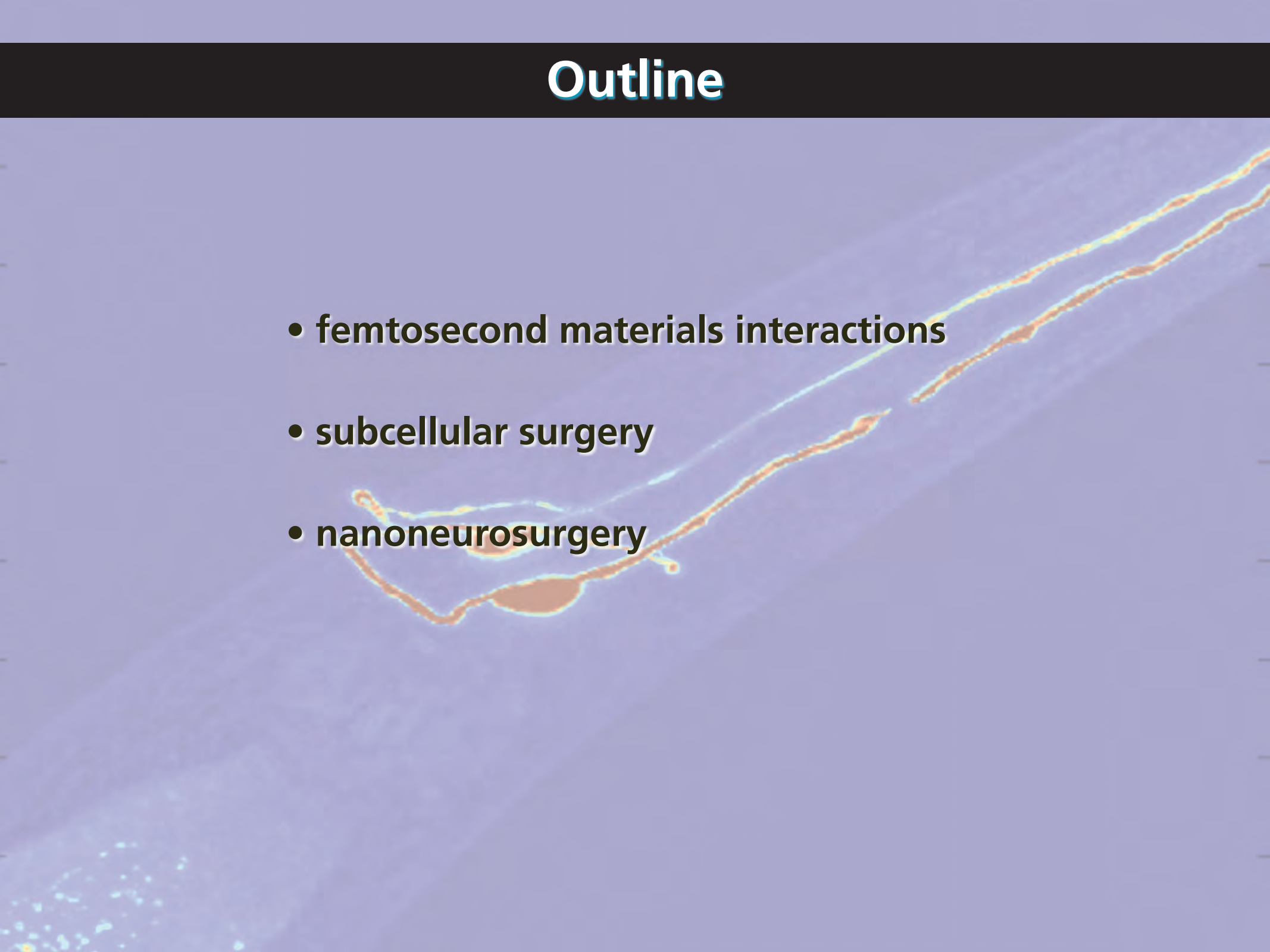
Femtosecond materials interactions

Ti:sapphire lasers



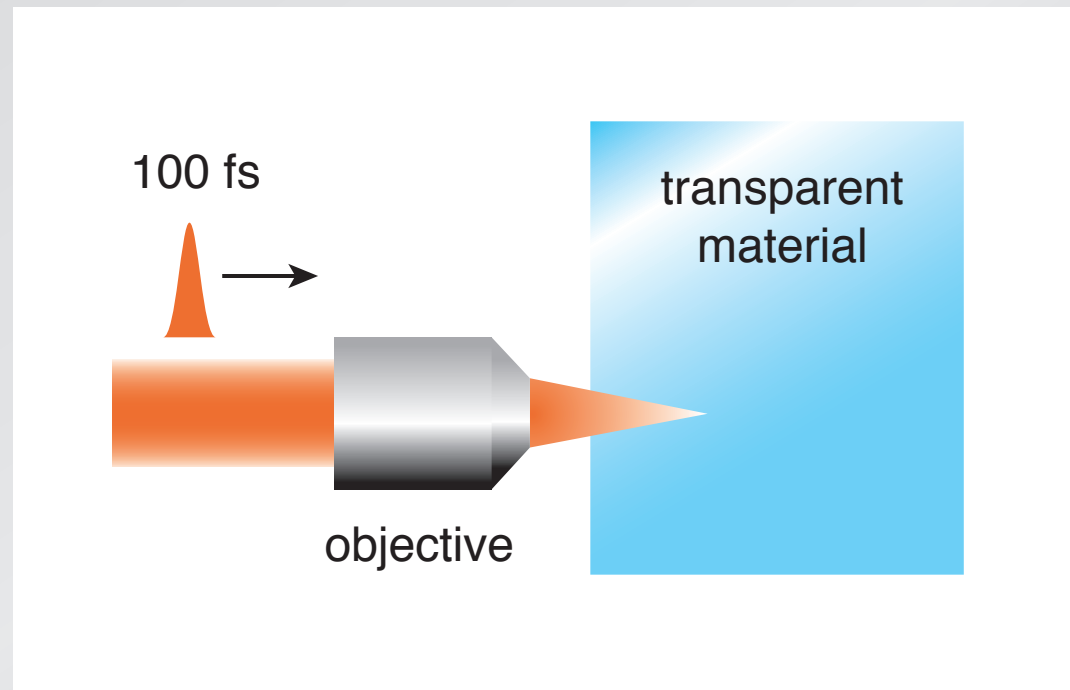
pulse duration:	50 fs	repetition rate:	80 MHz
average power:	1 W	peak power:	10^{10} W
energy per pulse:	1 mJ	wavelength:	800 nm

Outline

- femtosecond materials interactions
 - subcellular surgery
 - nanoneurosurgery
- 

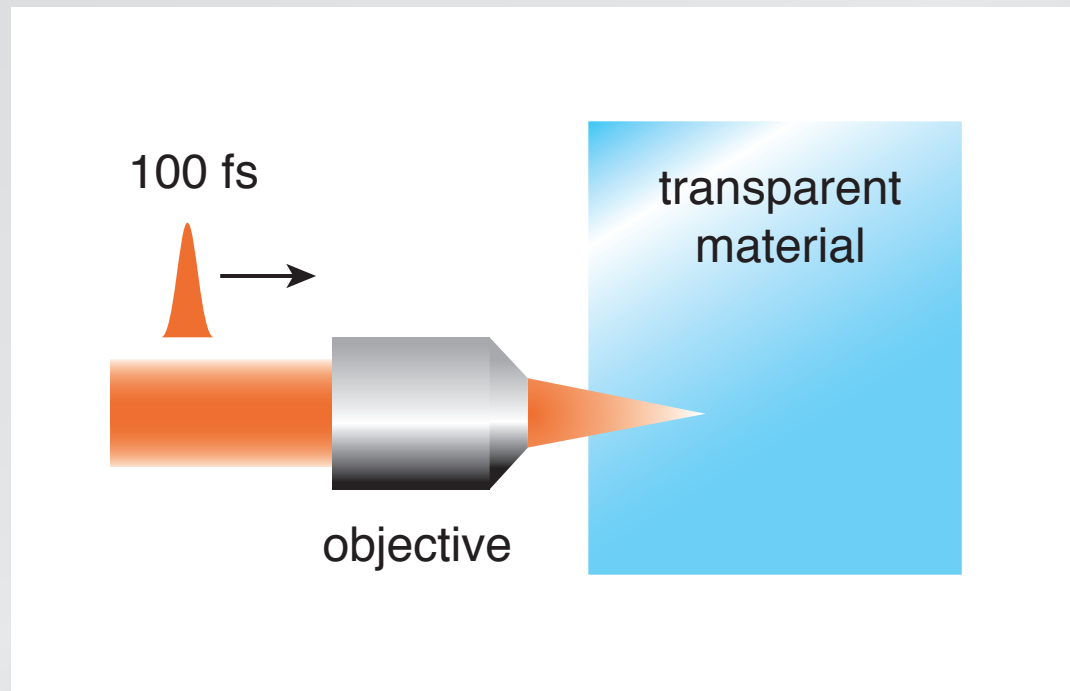
Femtosecond materials interactions

focus laser beam inside material



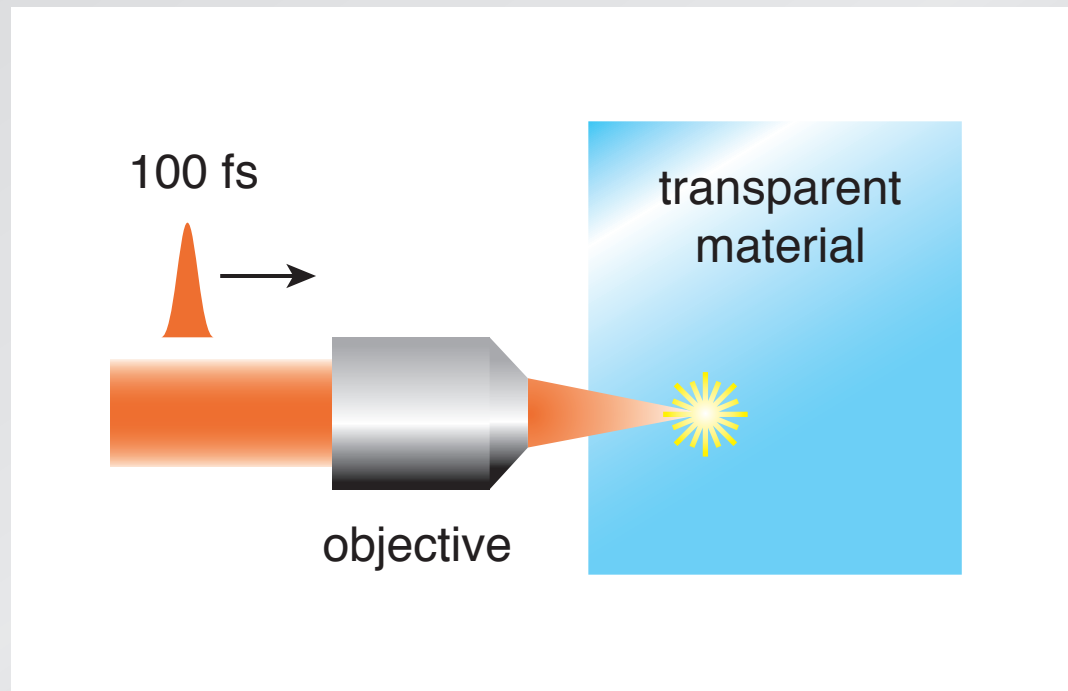
Femtosecond materials interactions

high intensity at focus...



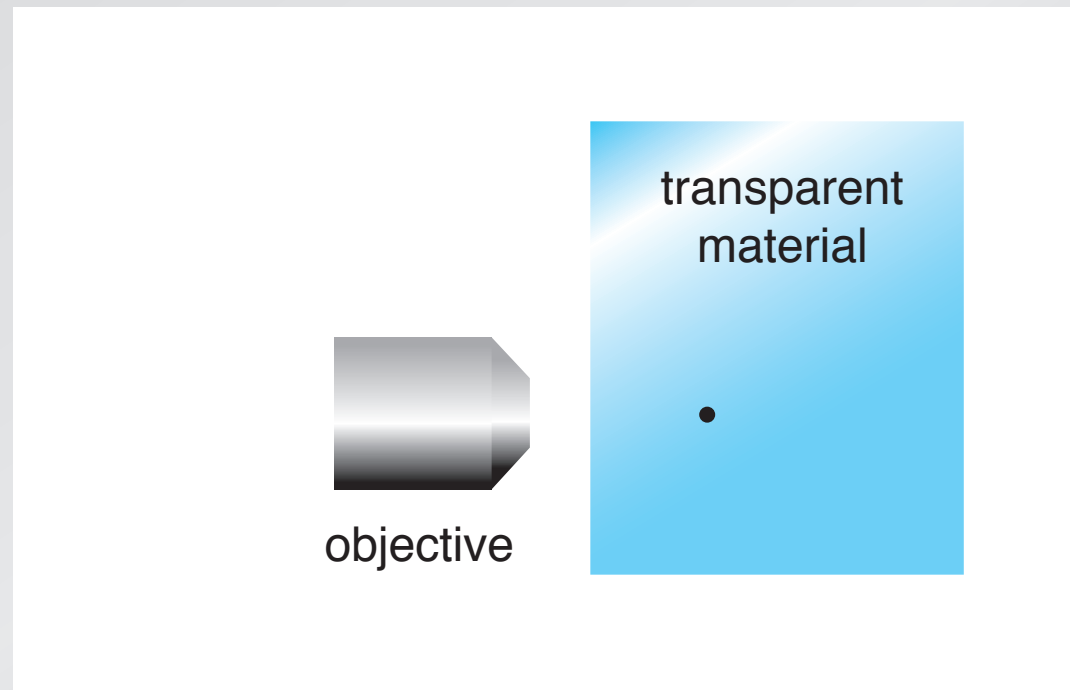
Femtosecond materials interactions

...causes nonlinear ionization...

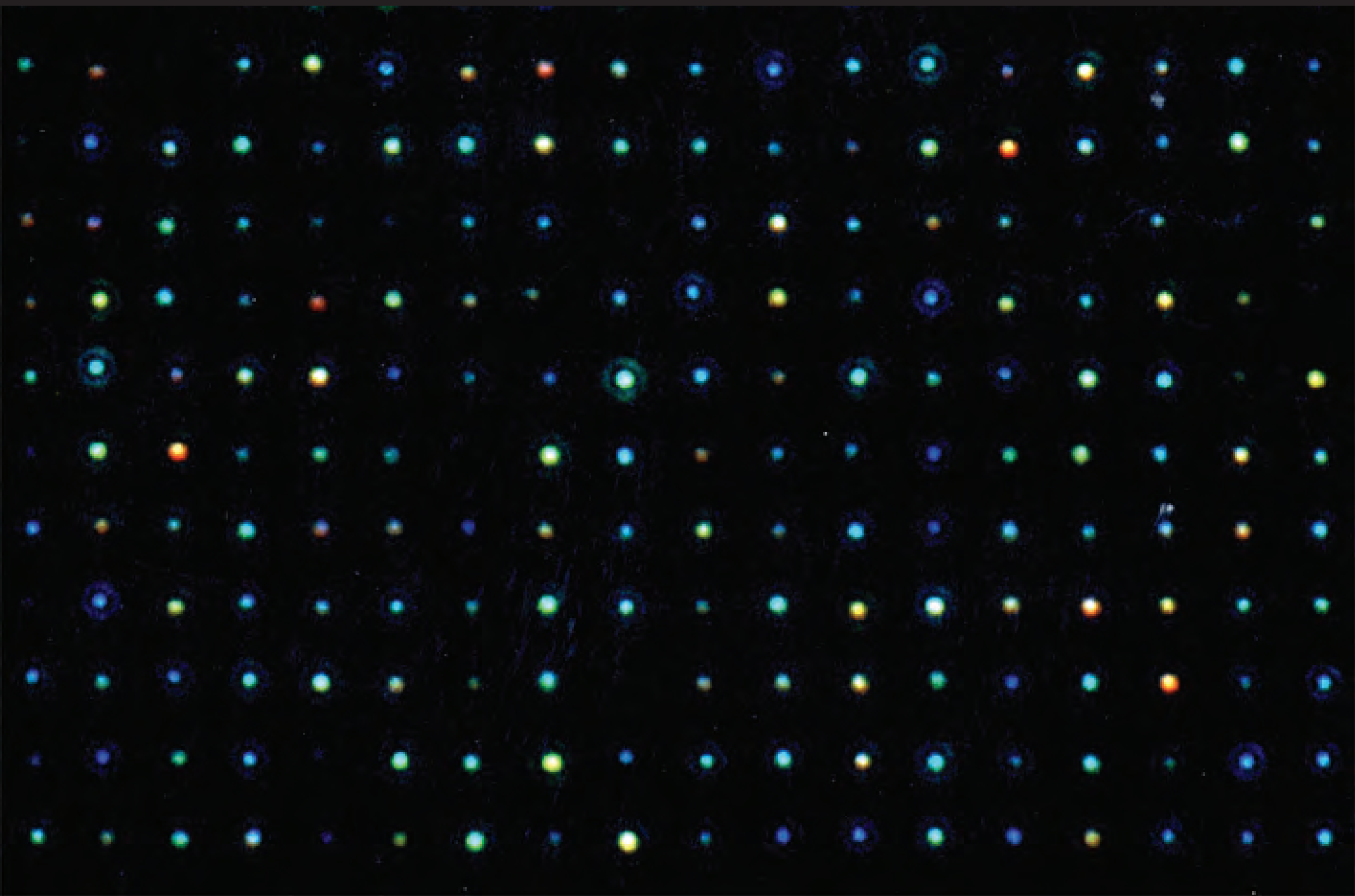


Femtosecond materials interactions

and 'microexplosion' causes microscopic damage...



Femtosecond materials interactions

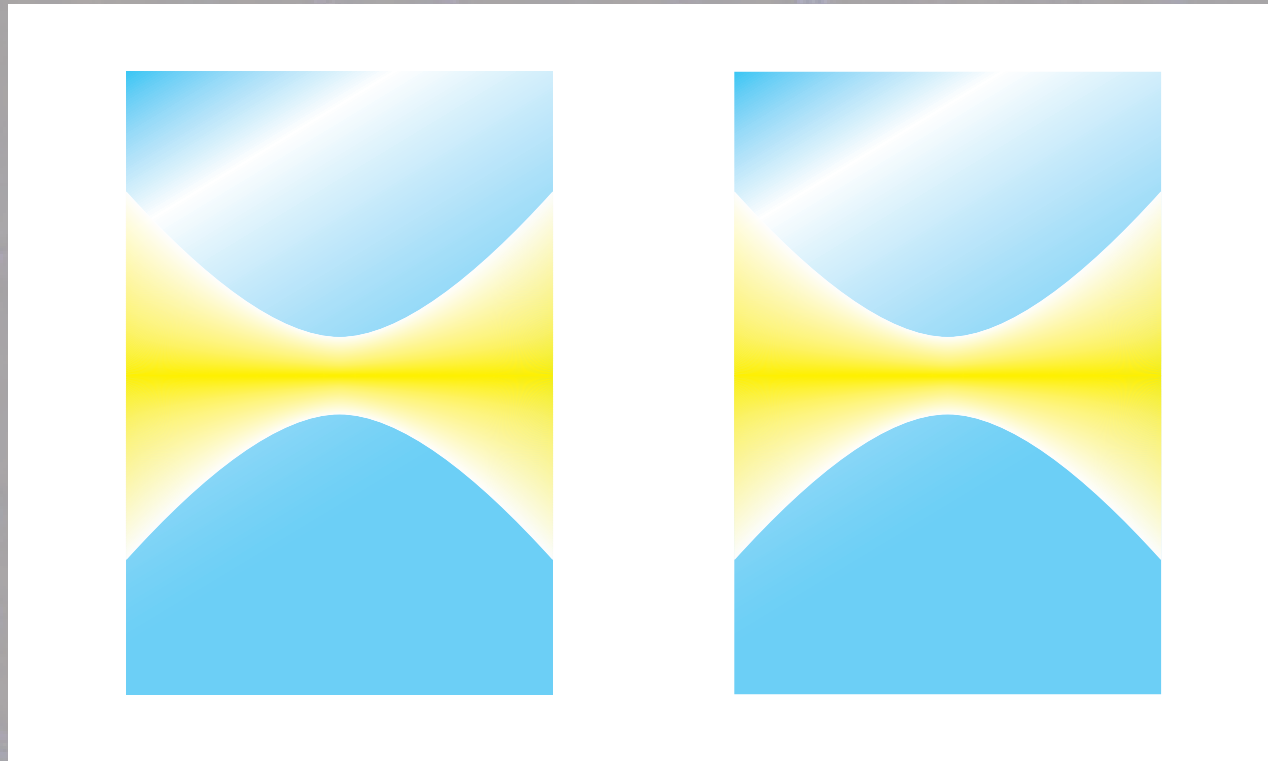


Femtosecond materials interactions

photon energy $<$ bandgap \longrightarrow nonlinear interaction

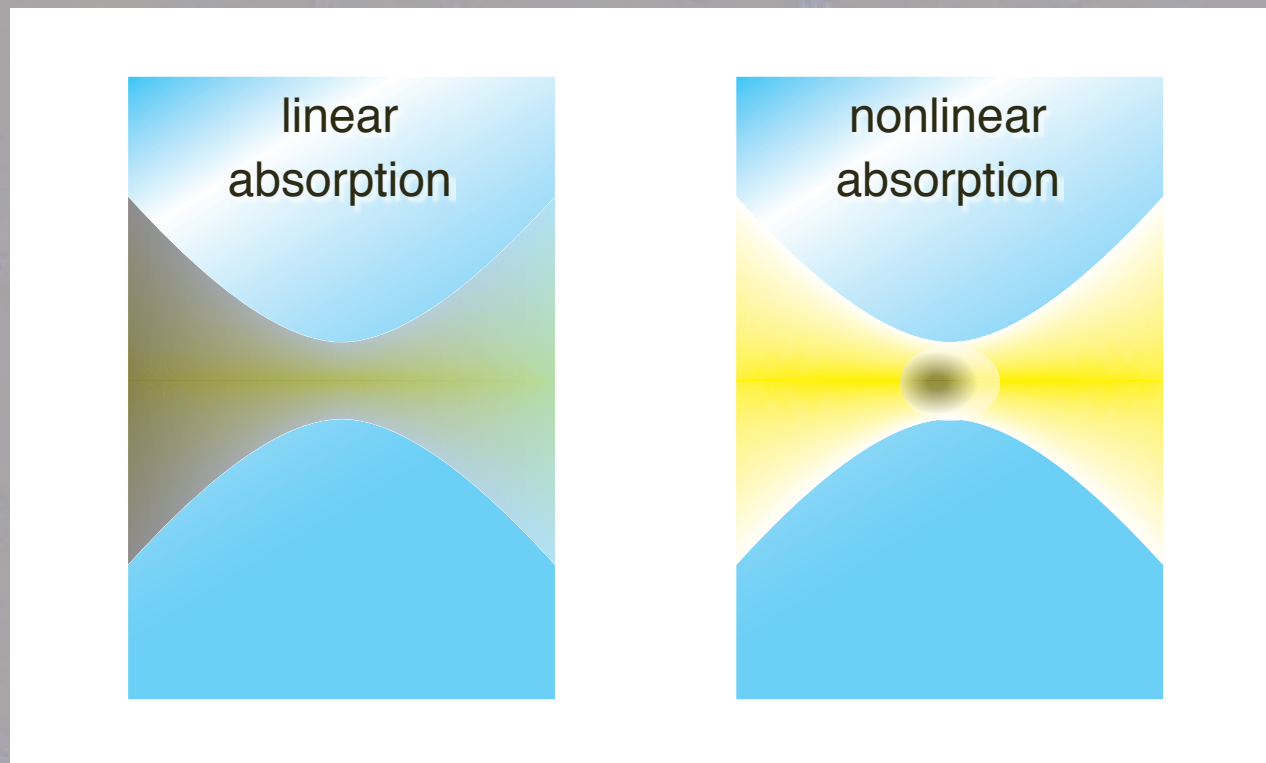
Femtosecond materials interactions

nonlinear interaction provides bulk confinement

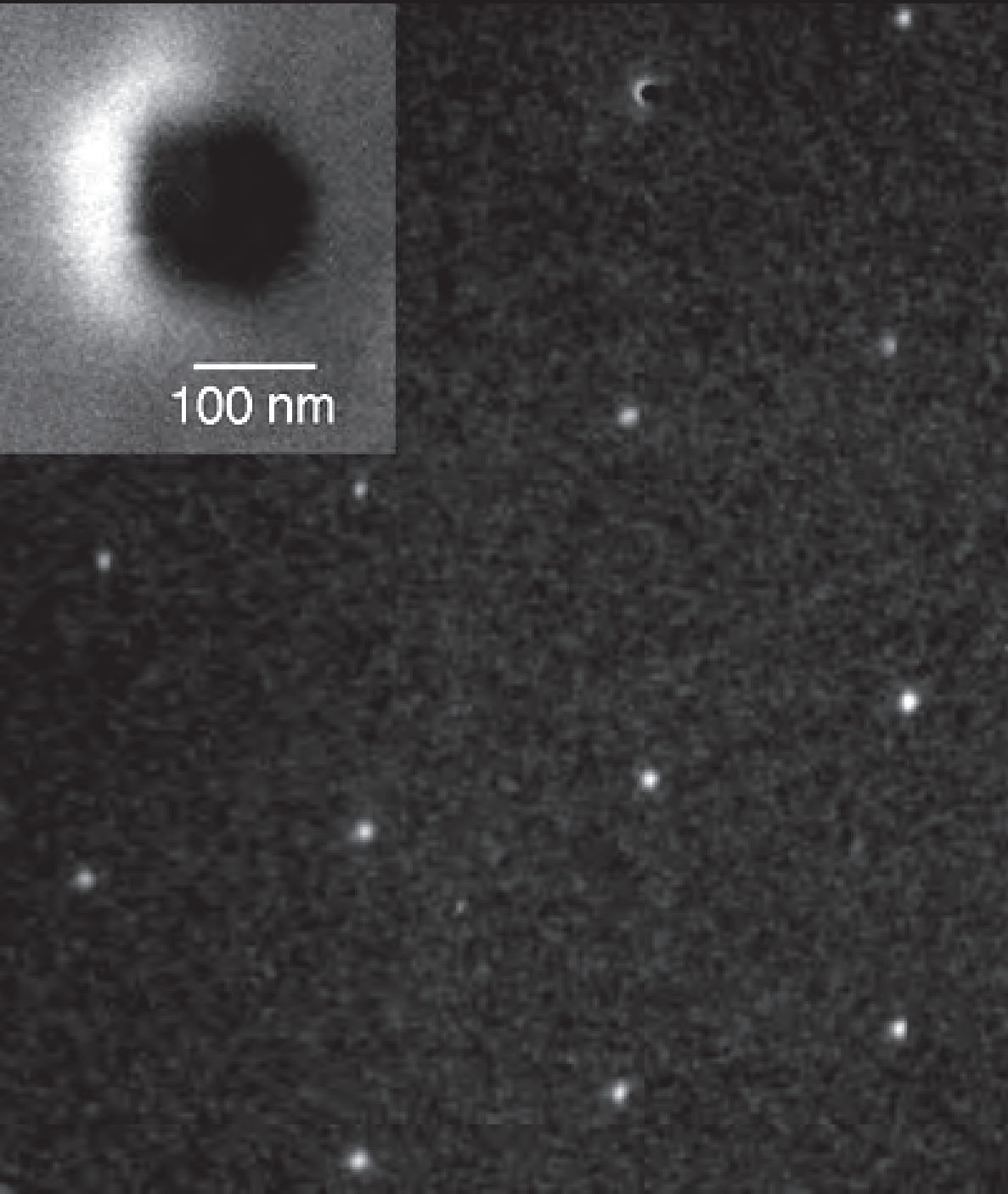


Femtosecond materials interactions

nonlinear interaction provides bulk confinement



Femtosecond materials interactions

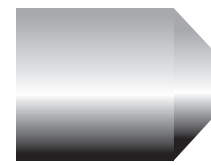


SEM & AFM:

- 100-nm cavities
- little colateral damage

Femtosecond materials interactions

Dark-field scattering



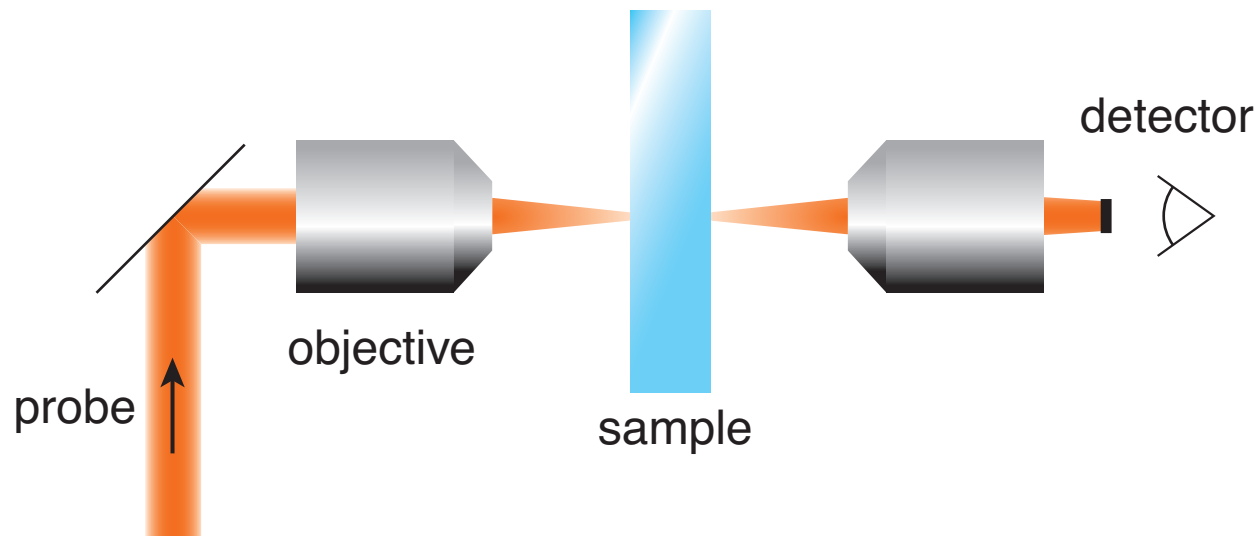
objective



sample

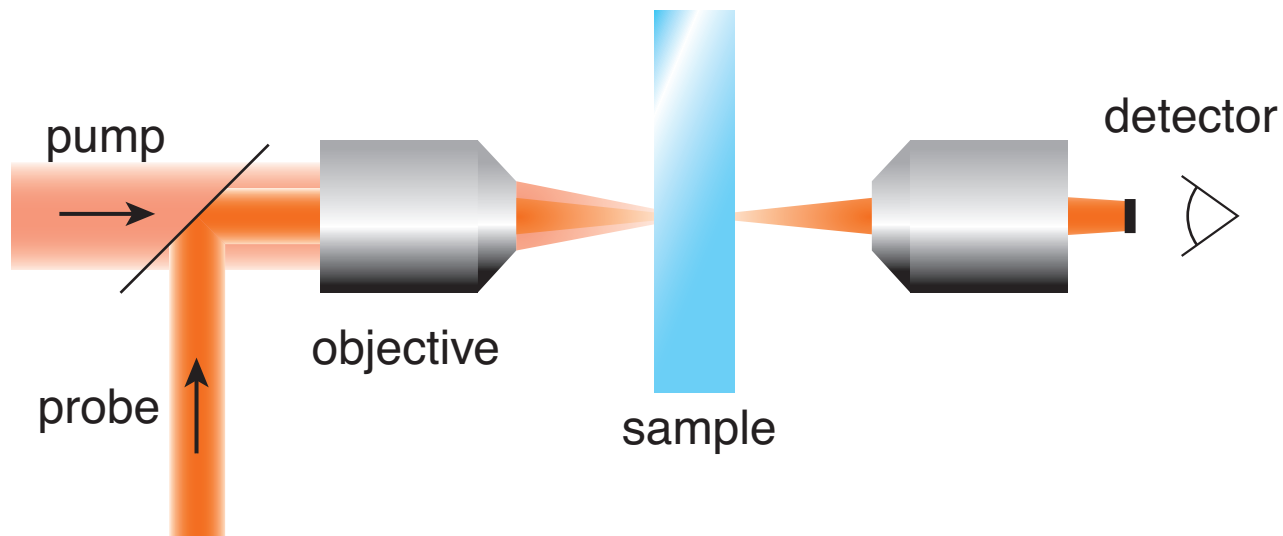
Femtosecond materials interactions

block probe beam...



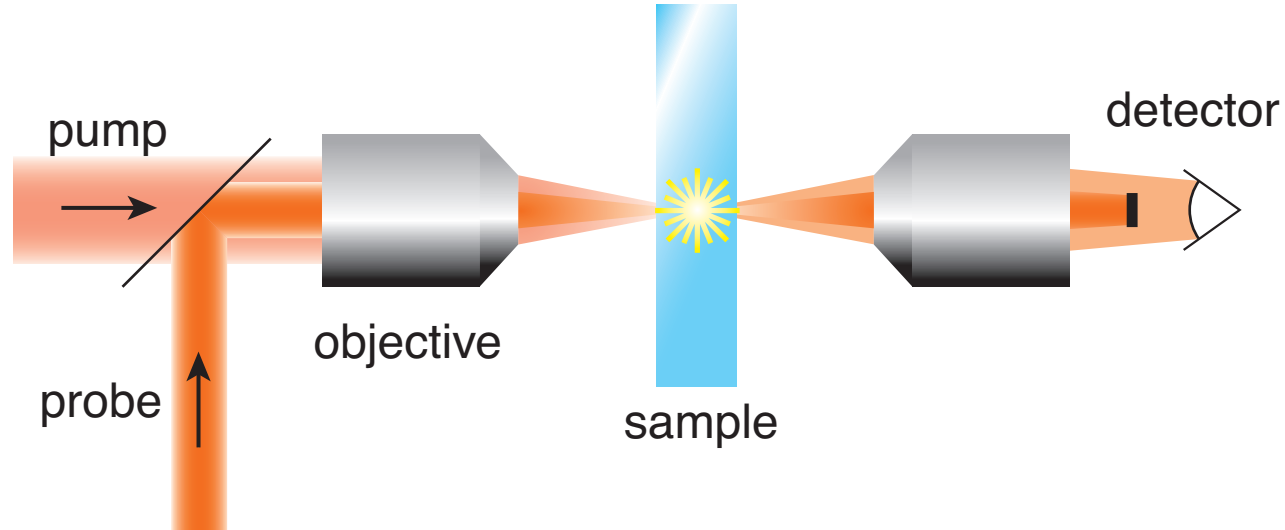
Femtosecond materials interactions

... bring in pump beam...



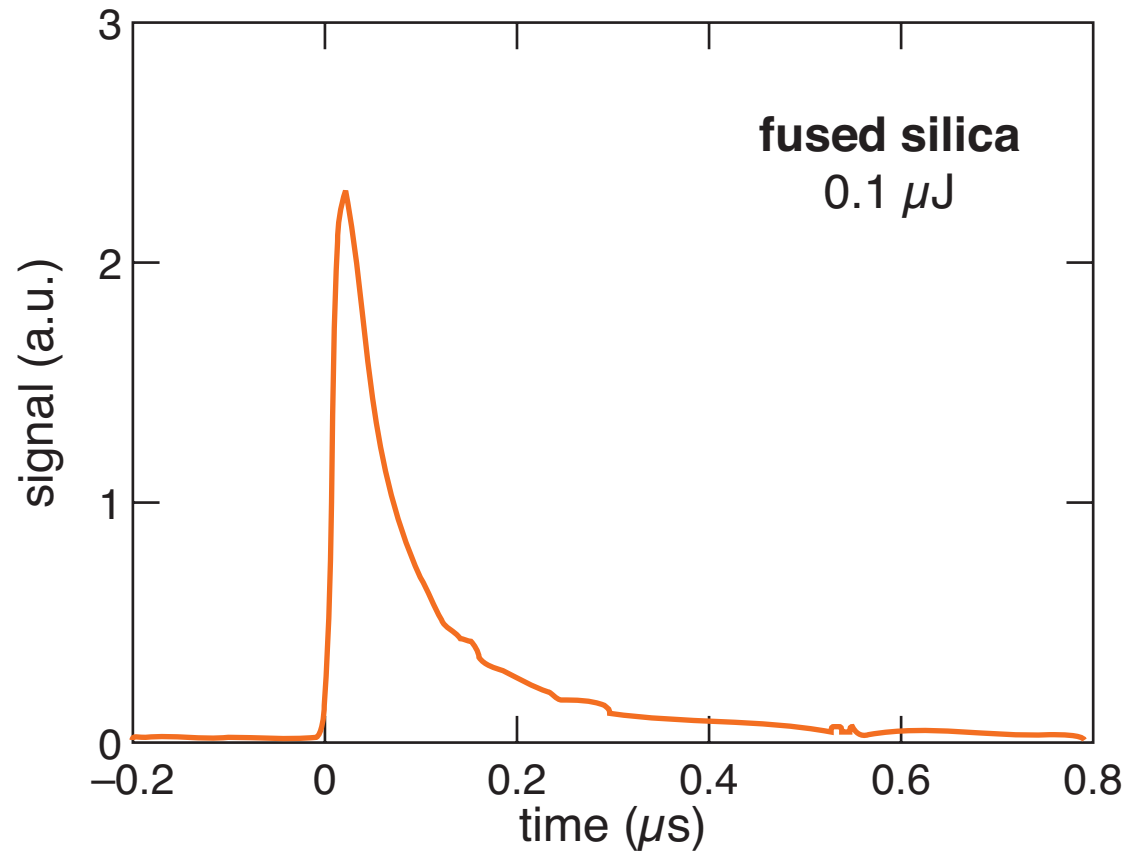
Femtosecond materials interactions

... damage scatters probe beam



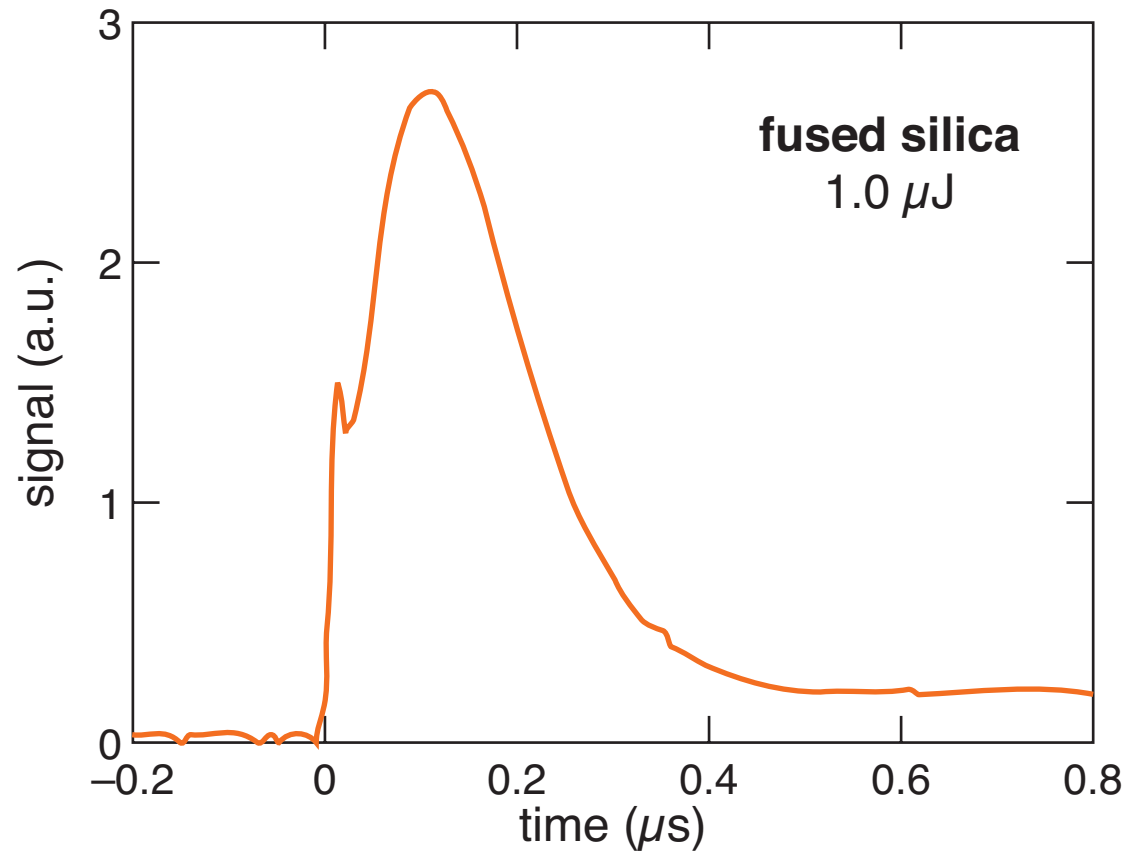
Femtosecond materials interactions

scattered signal



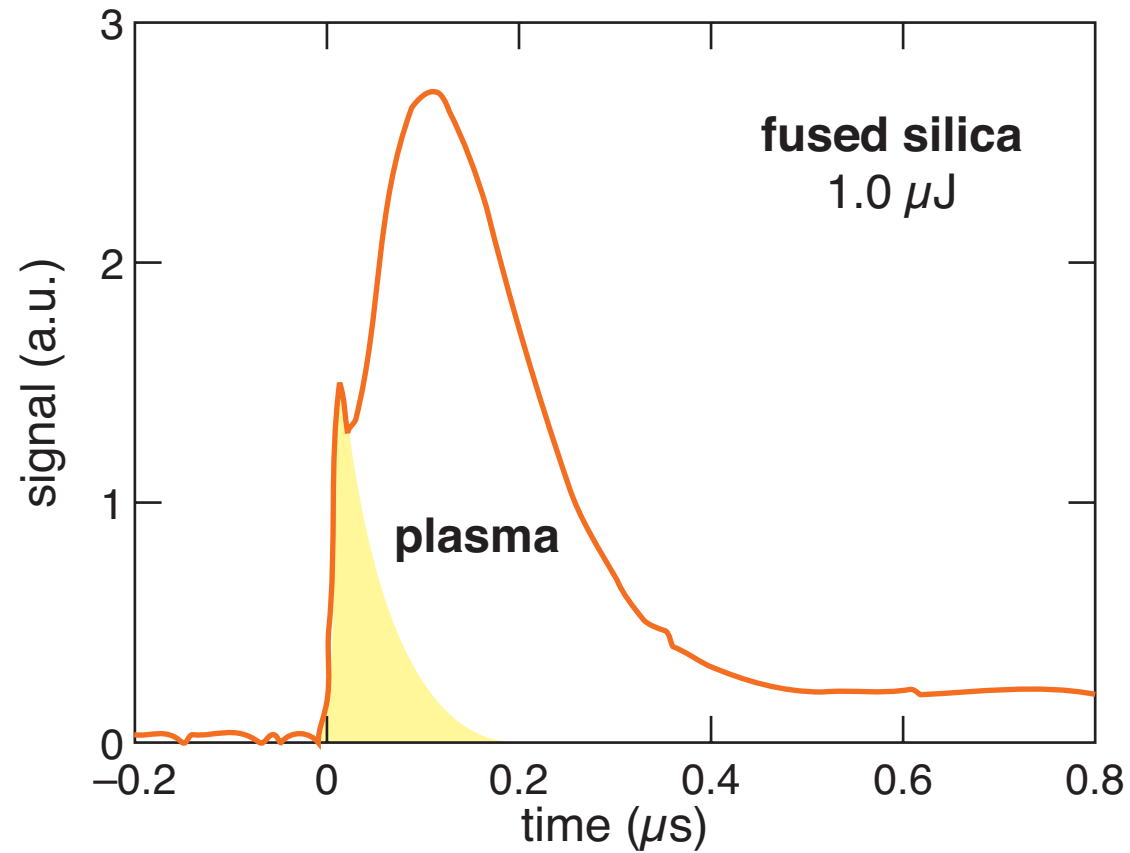
Femtosecond materials interactions

scattered signal



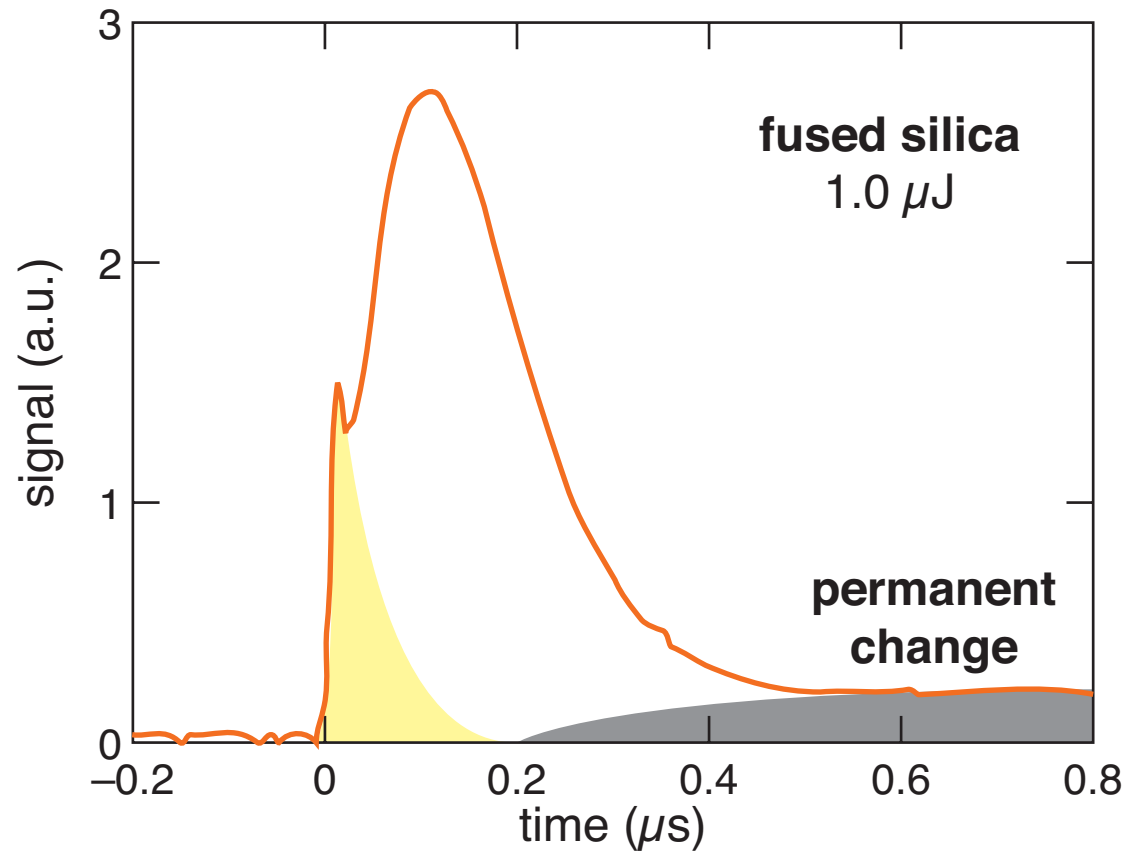
Femtosecond materials interactions

scattered signal



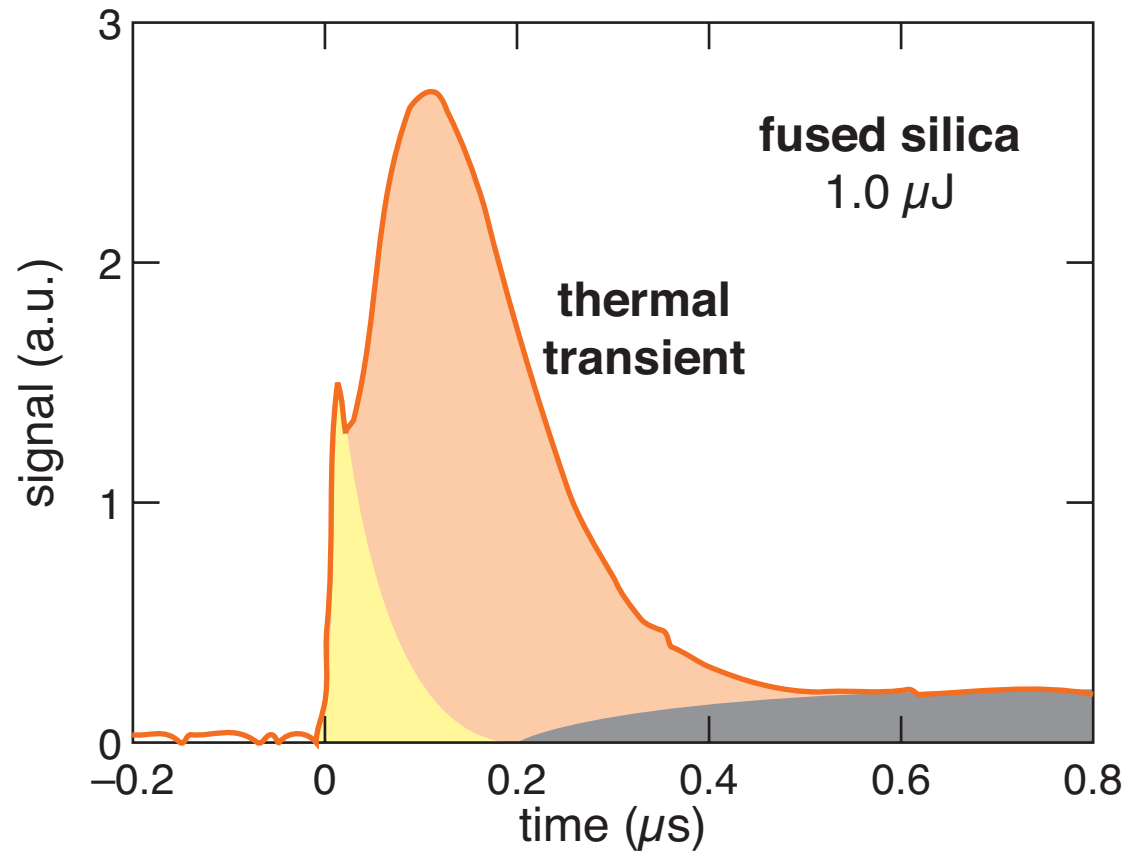
Femtosecond materials interactions

scattered signal



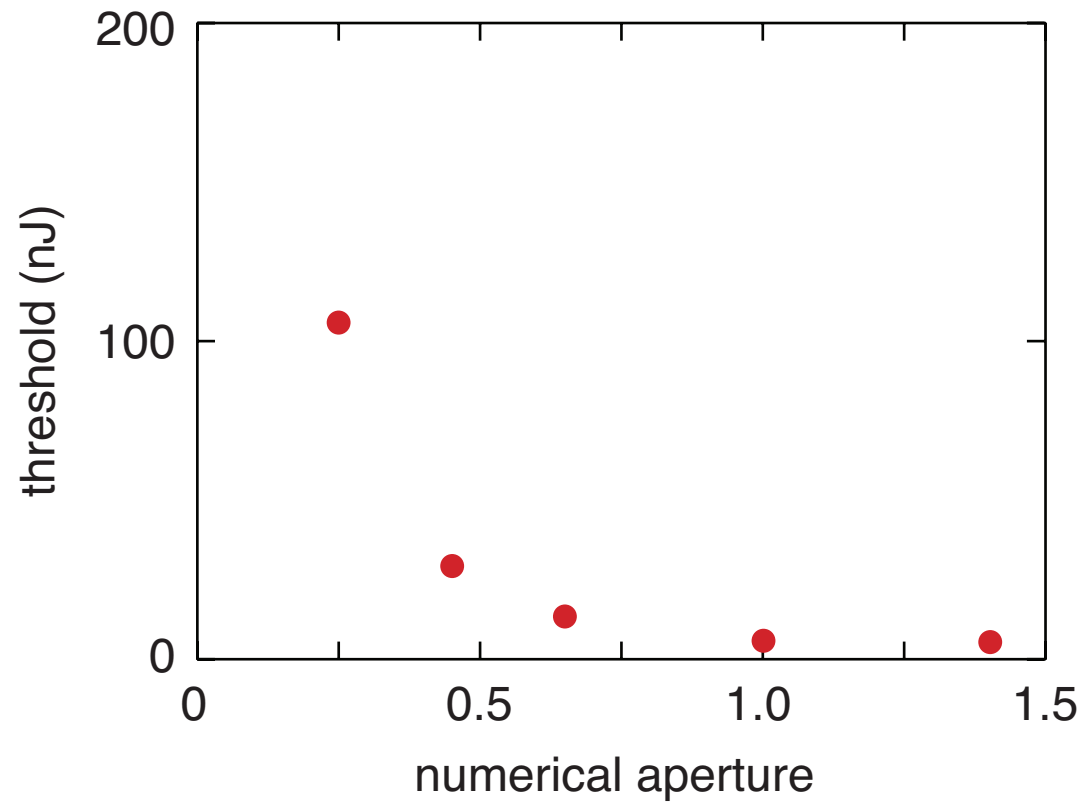
Femtosecond materials interactions

scattered signal



Femtosecond materials interactions

vary numerical aperture



Femtosecond materials interactions

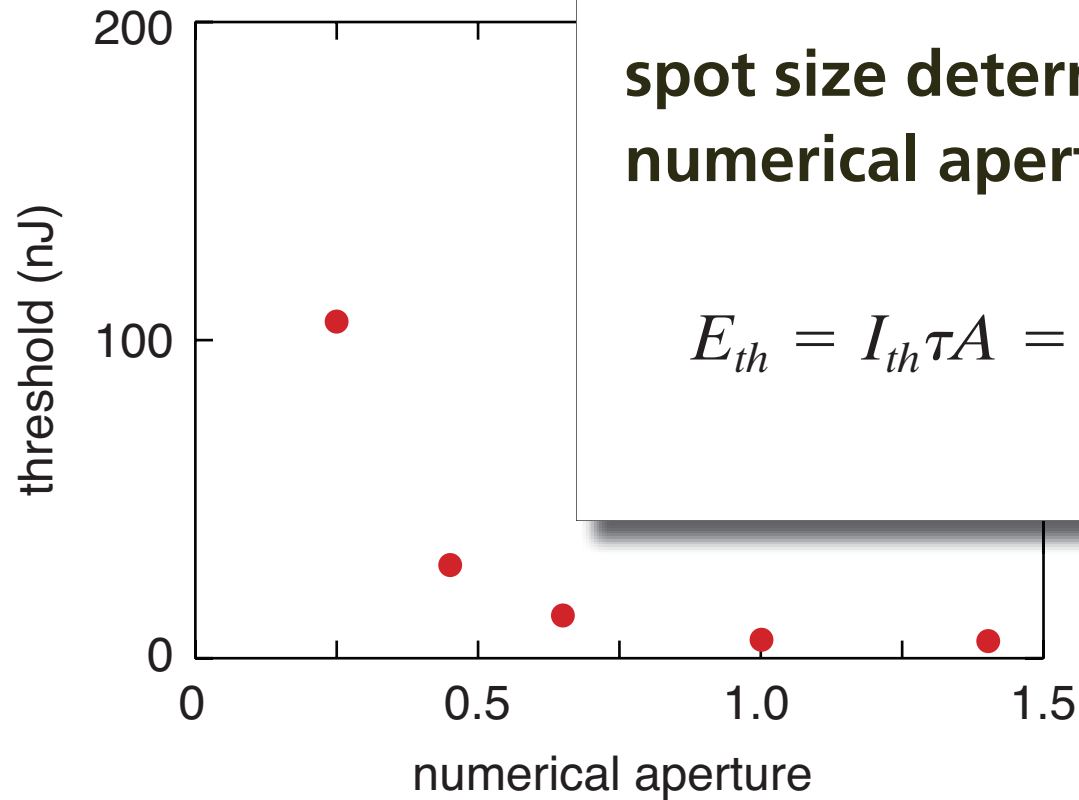
vary numerical

intensity threshold:

$$E_{th} = I_{th} \tau A$$

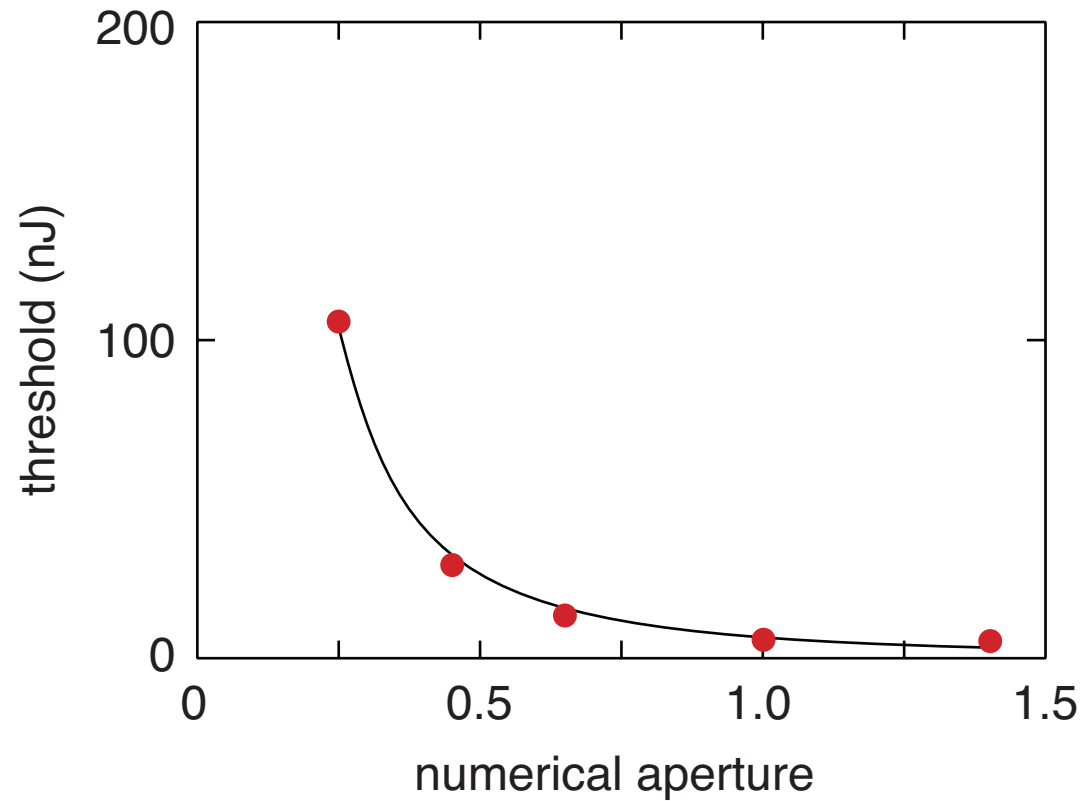
spot size determined by
numerical aperture:

$$E_{th} = I_{th} \tau A = \frac{I_{th} \tau \lambda^2}{\pi (\text{NA})^2}$$



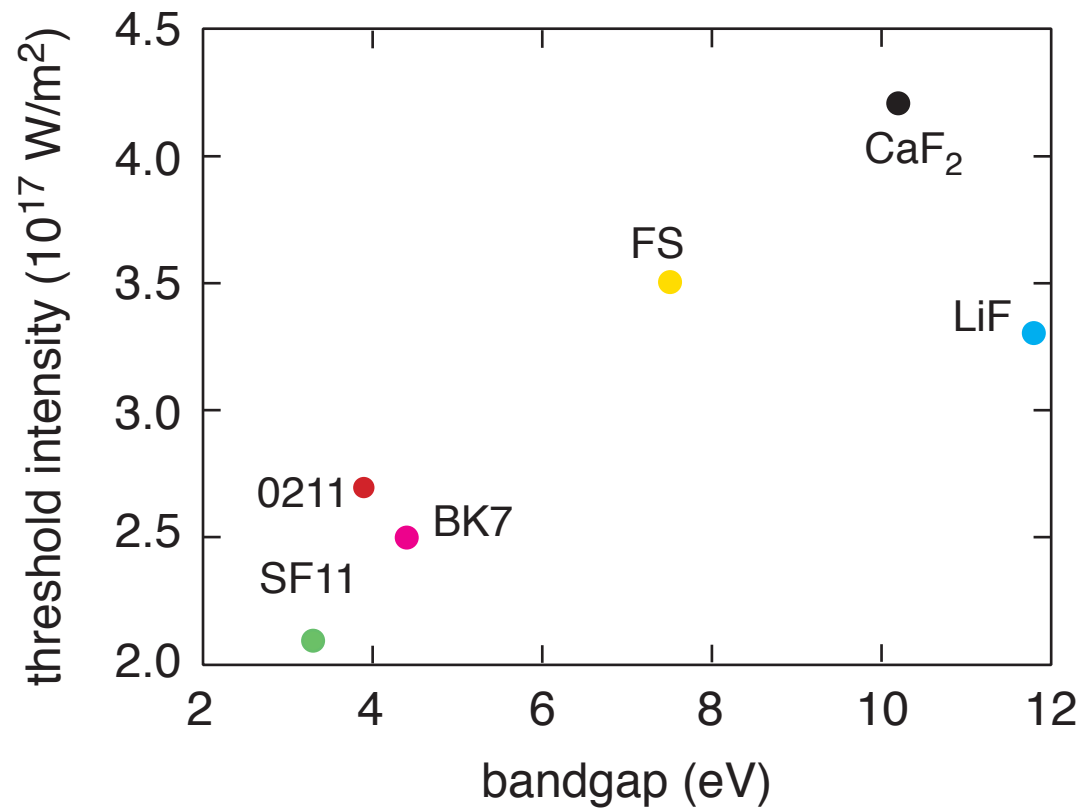
Femtosecond materials interactions

fit gives threshold intensity: $I_{th} = 2.5 \times 10^{17} \text{ W/m}^2$



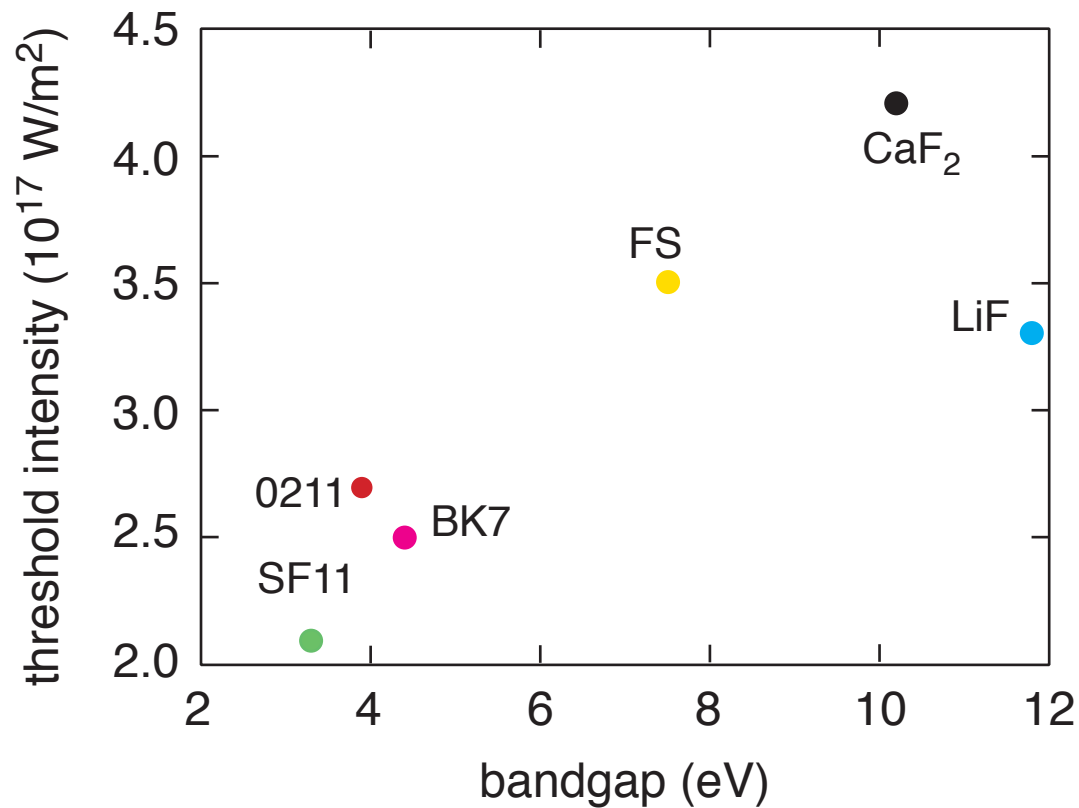
Femtosecond materials interactions

vary material...



Femtosecond materials interactions

...threshold varies with band gap (but not much!)



Femtosecond materials interactions

- **nonlinear interaction**
- **disrupt matter inside bulk**
- **ablation at very low energy**

Outline

- femtosecond materials interactions
 - **subcellular surgery**
 - nanoneurosurgery
- 
- A microscopic image of a biological structure, possibly a cell or tissue, with several regions highlighted in orange and yellow. The structure is elongated and has a complex, branching appearance. The background is a light blue color with some faint, scattered blue and yellow spots.

Subcellular surgery

Q: can we ablate material on the subcellular scale?

Subcellular surgery

Requirements:

- submicrometer precision (in bulk)
- no damage to neighboring structures
- independent of structure/organelle type

Subcellular surgery

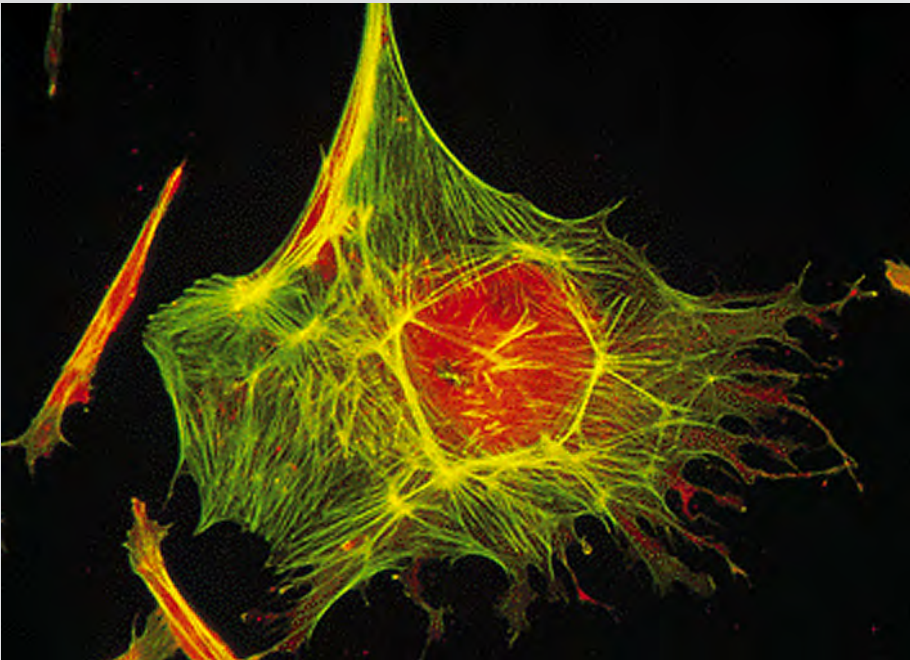
Cytoskeleton

- gives a cell its shape
- provides a scaffold for organelles
- responsible cell motion and attachment
- facilitates intracellular transport and signaling
- required for cell division

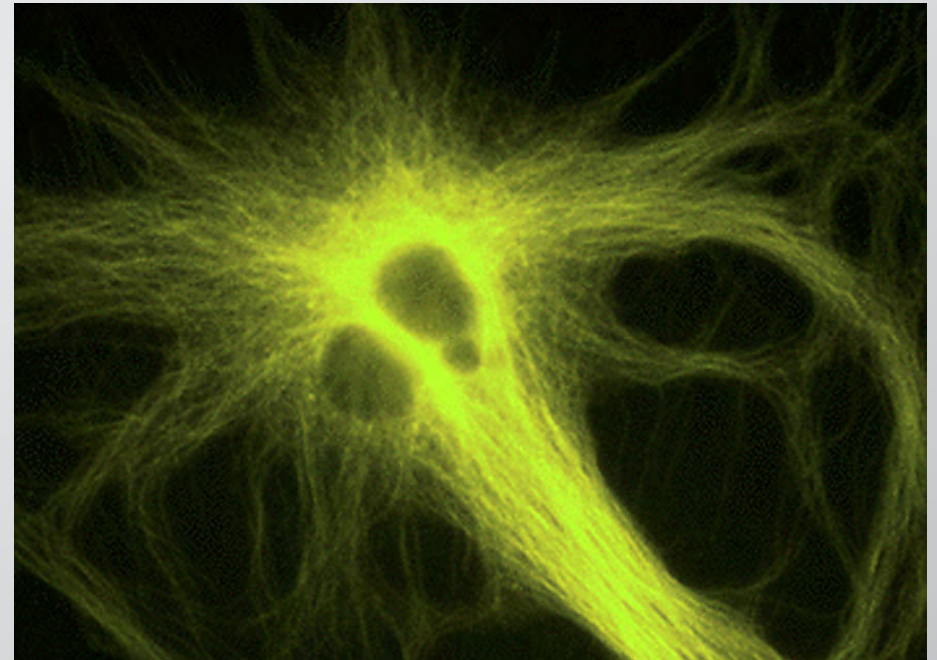
Subcellular surgery

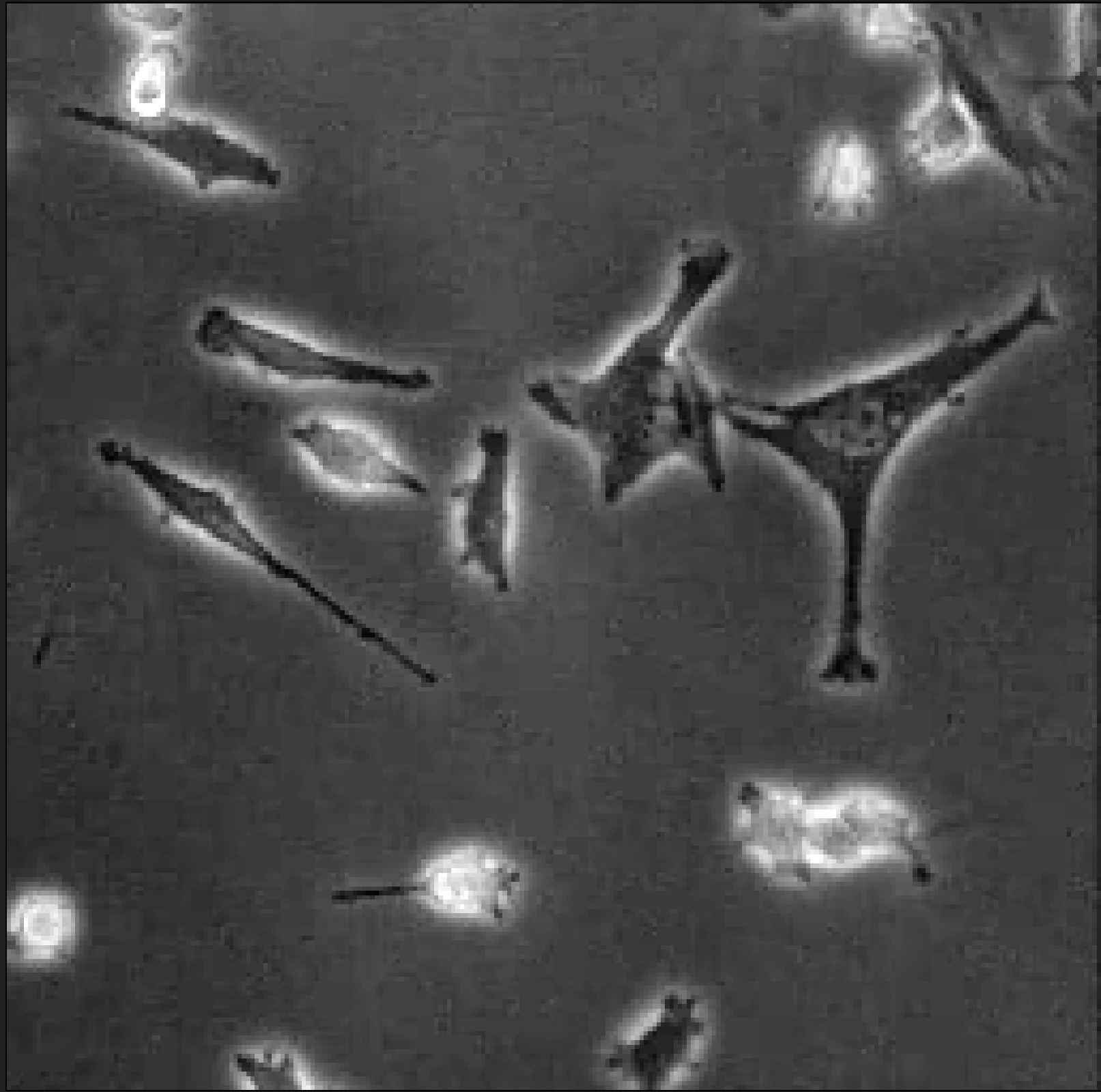
two components

actin fibers

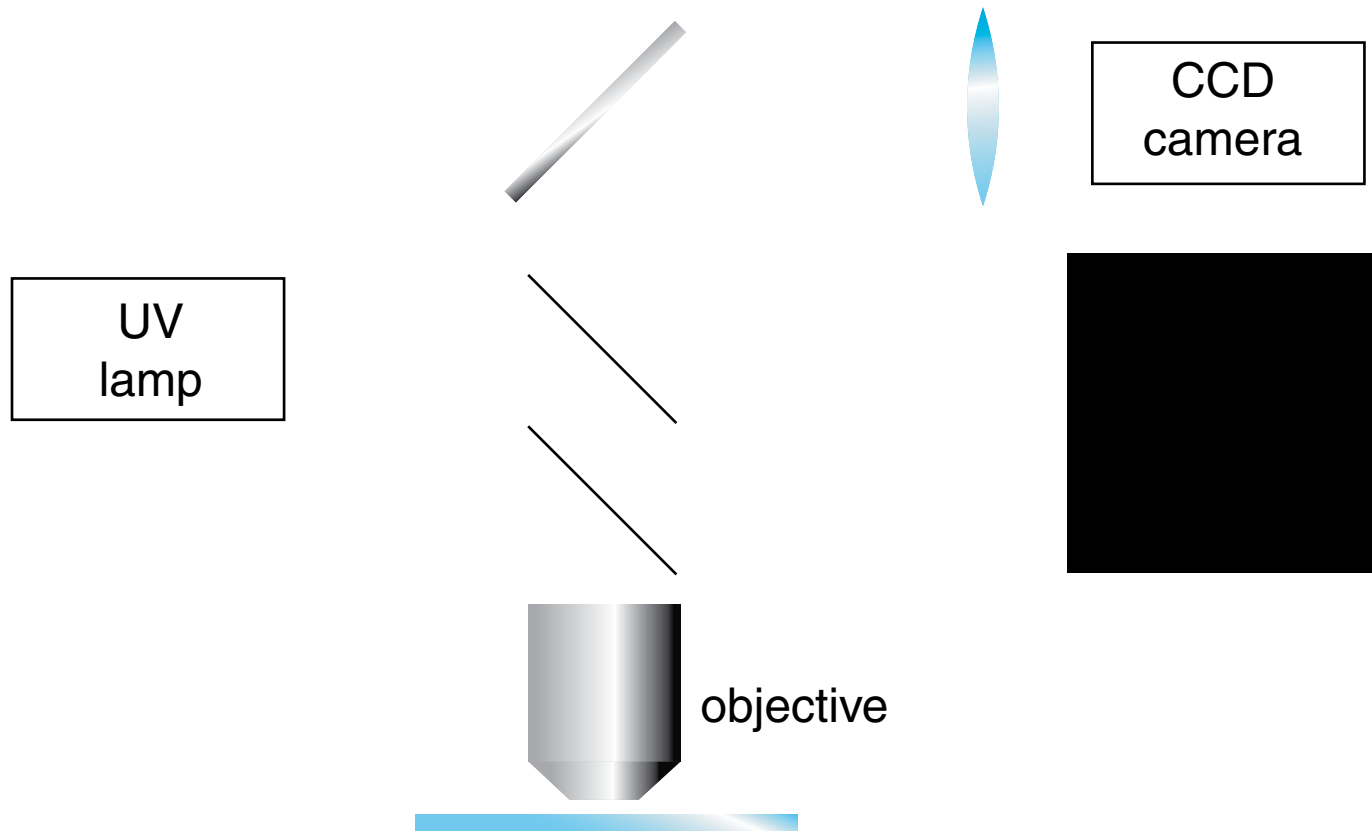


microtubules



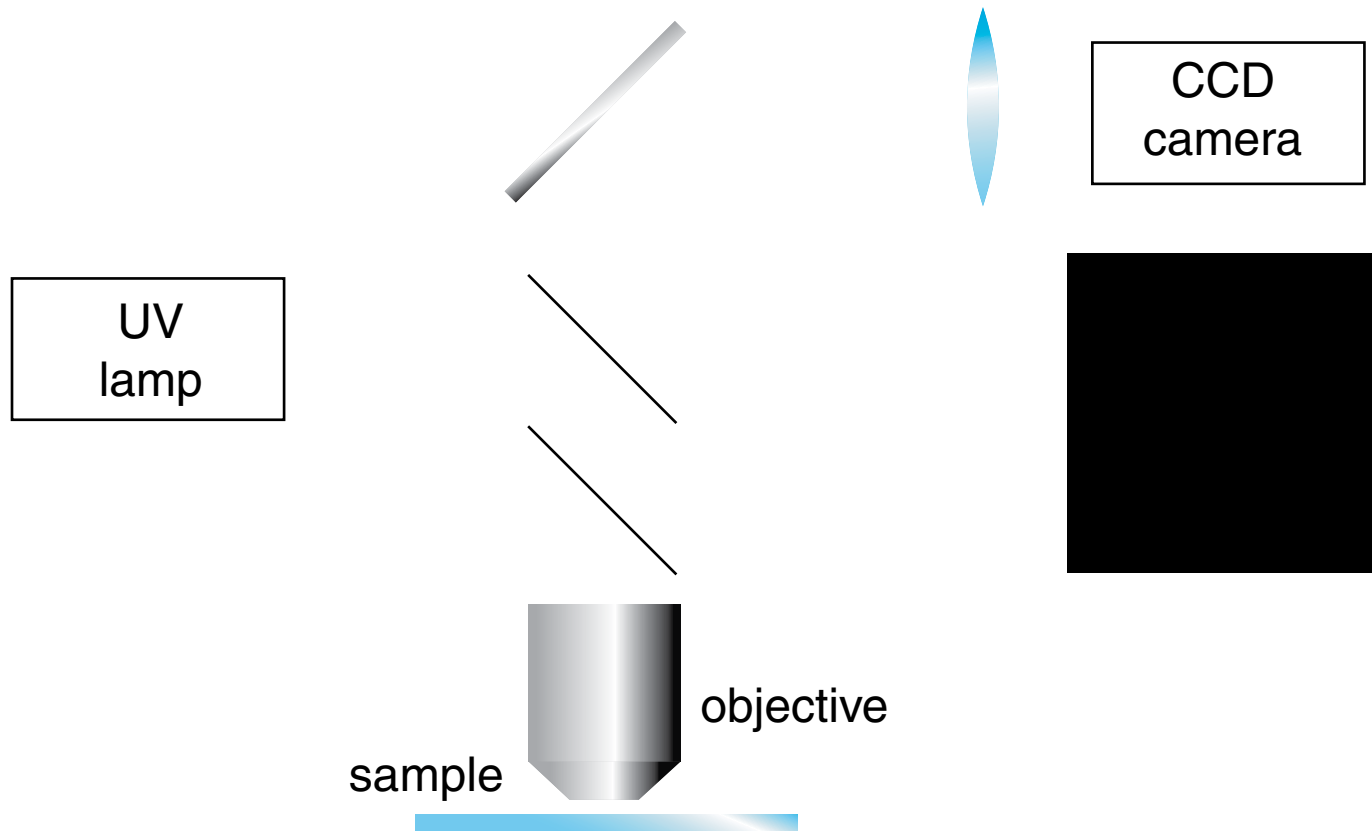


Subcellular surgery



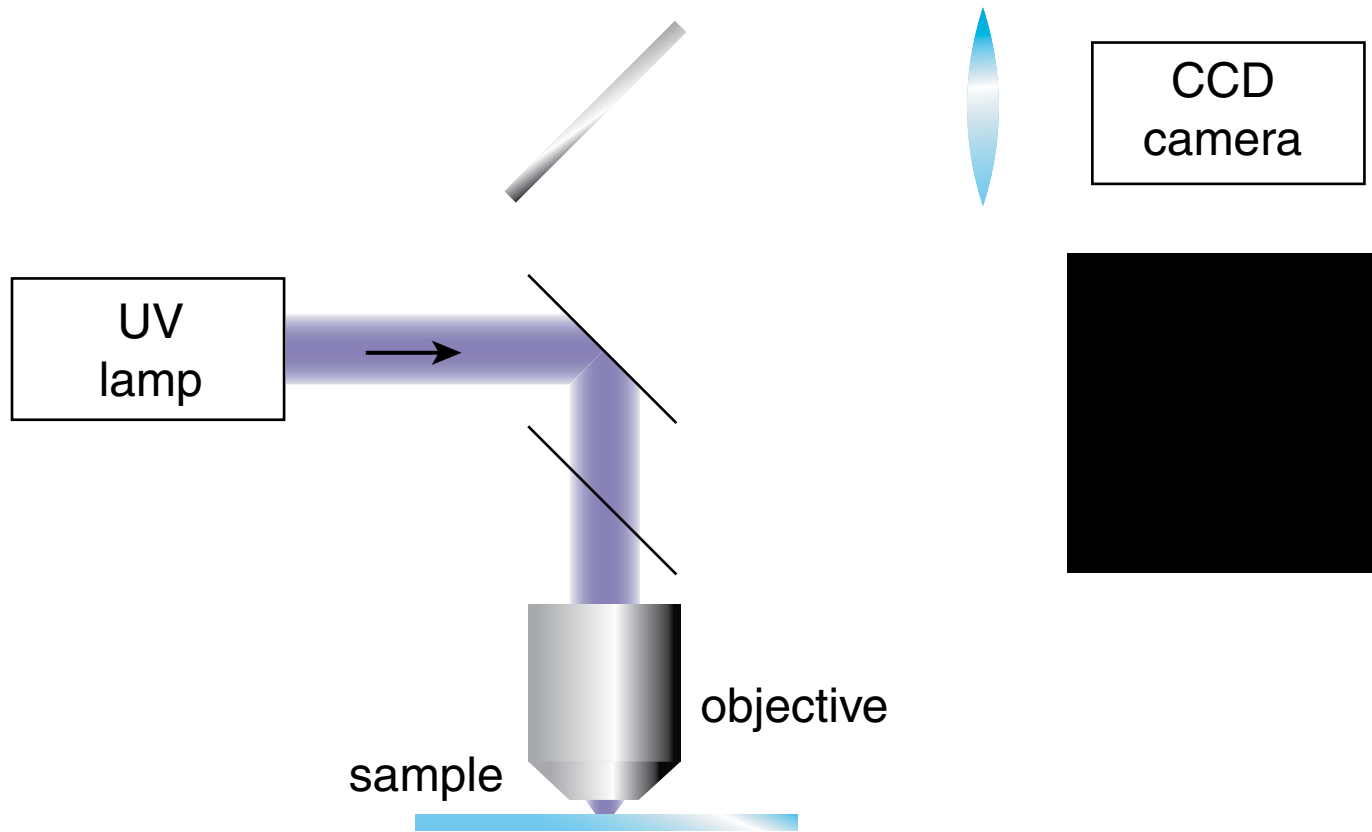
epi-fluorescence microscope

Subcellular surgery



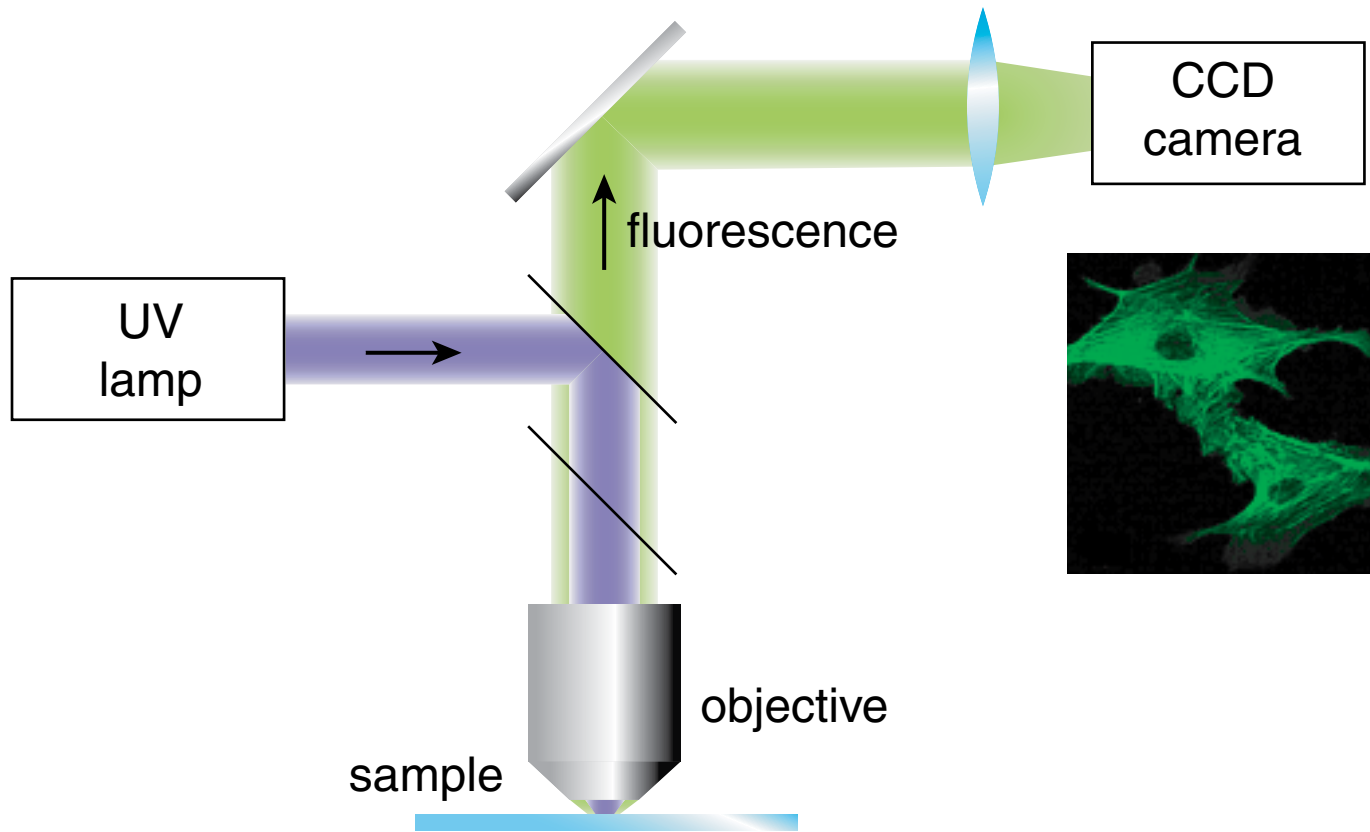
fluorescently label sample

Subcellular surgery



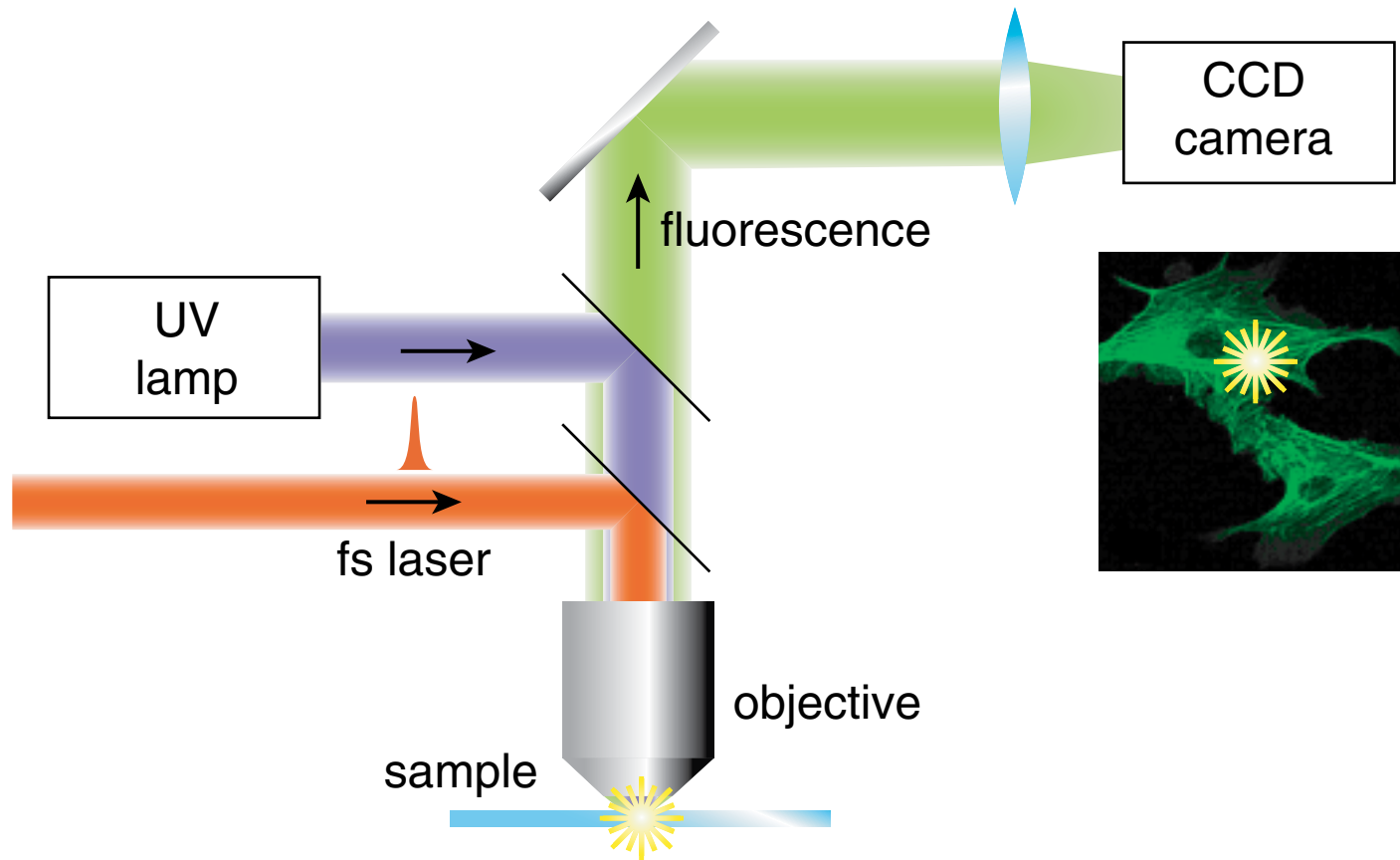
UV illumination...

Subcellular surgery



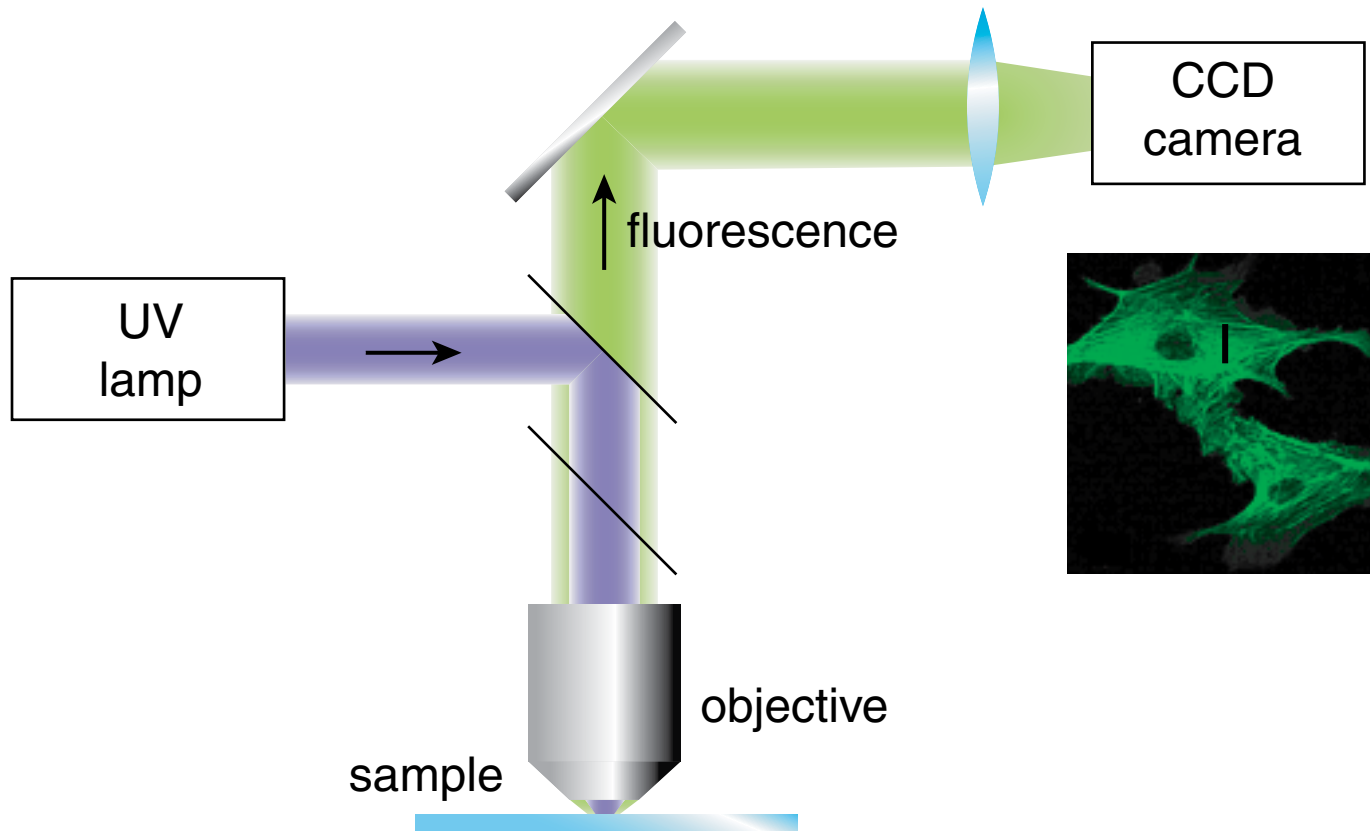
...causes fluorescence

Subcellular surgery



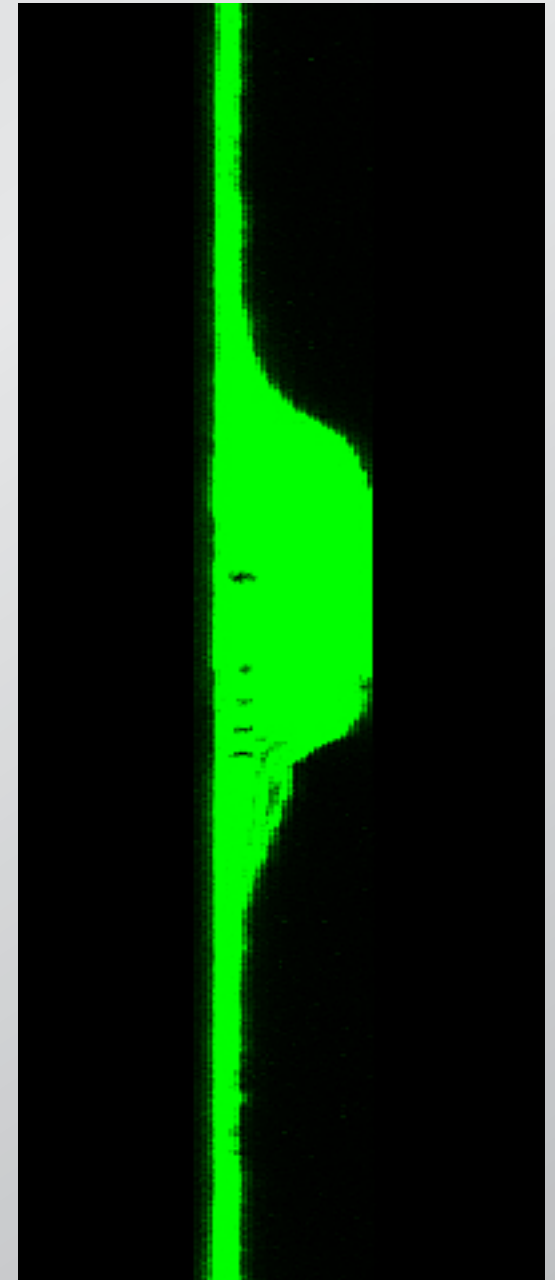
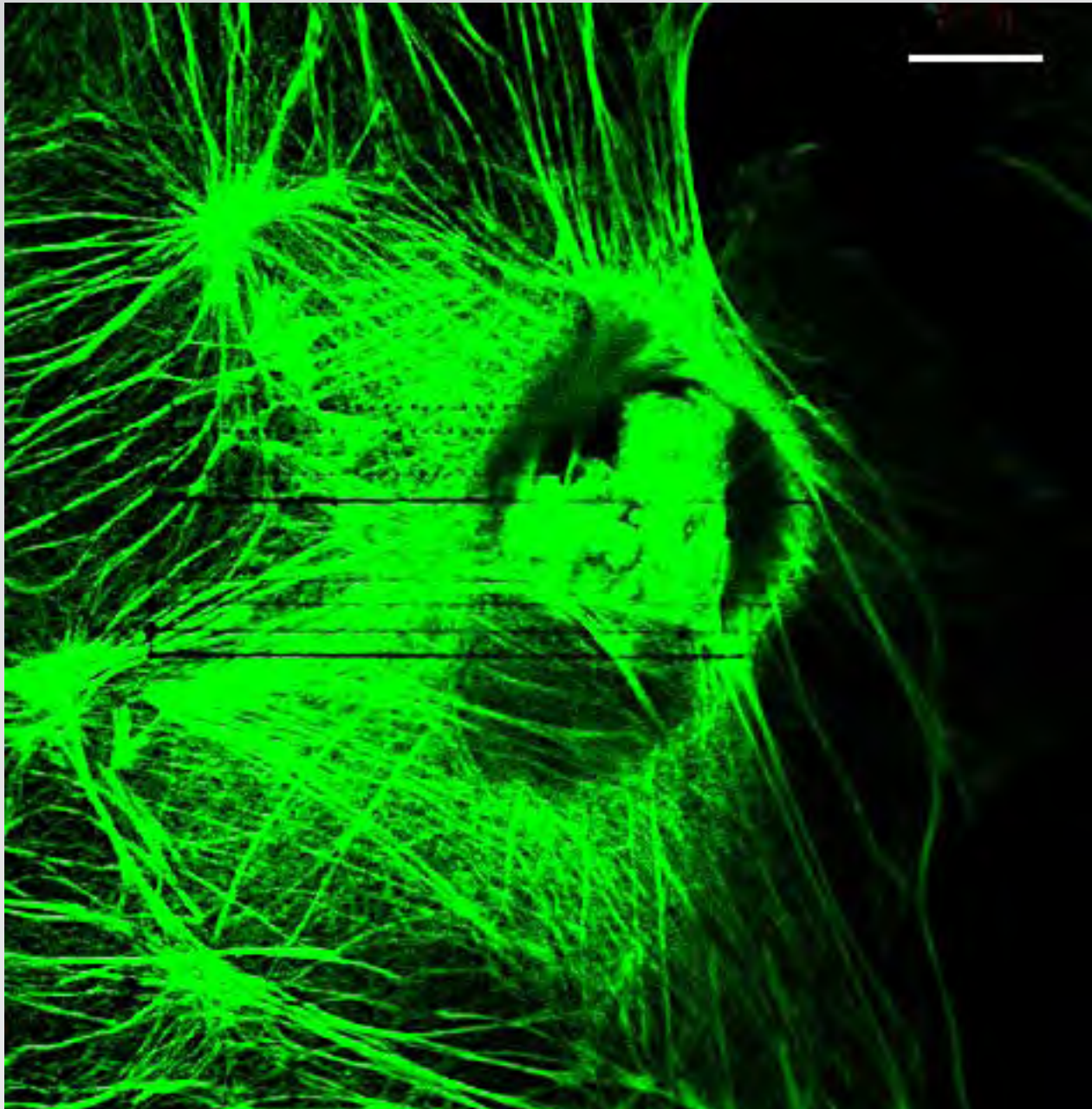
irradiate with fs laser beam

Subcellular surgery

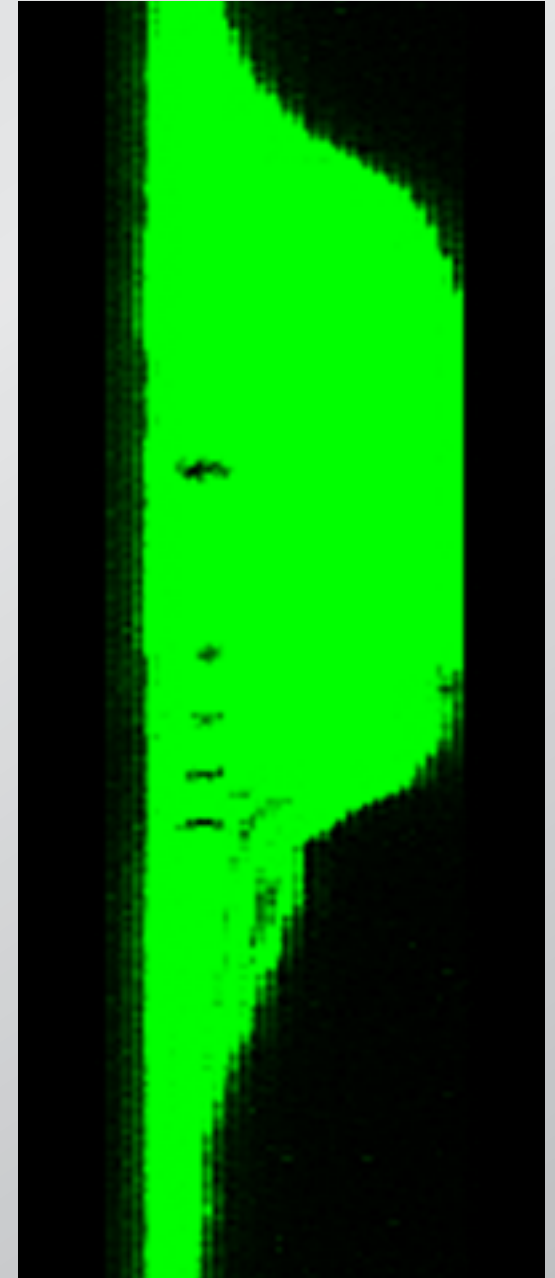
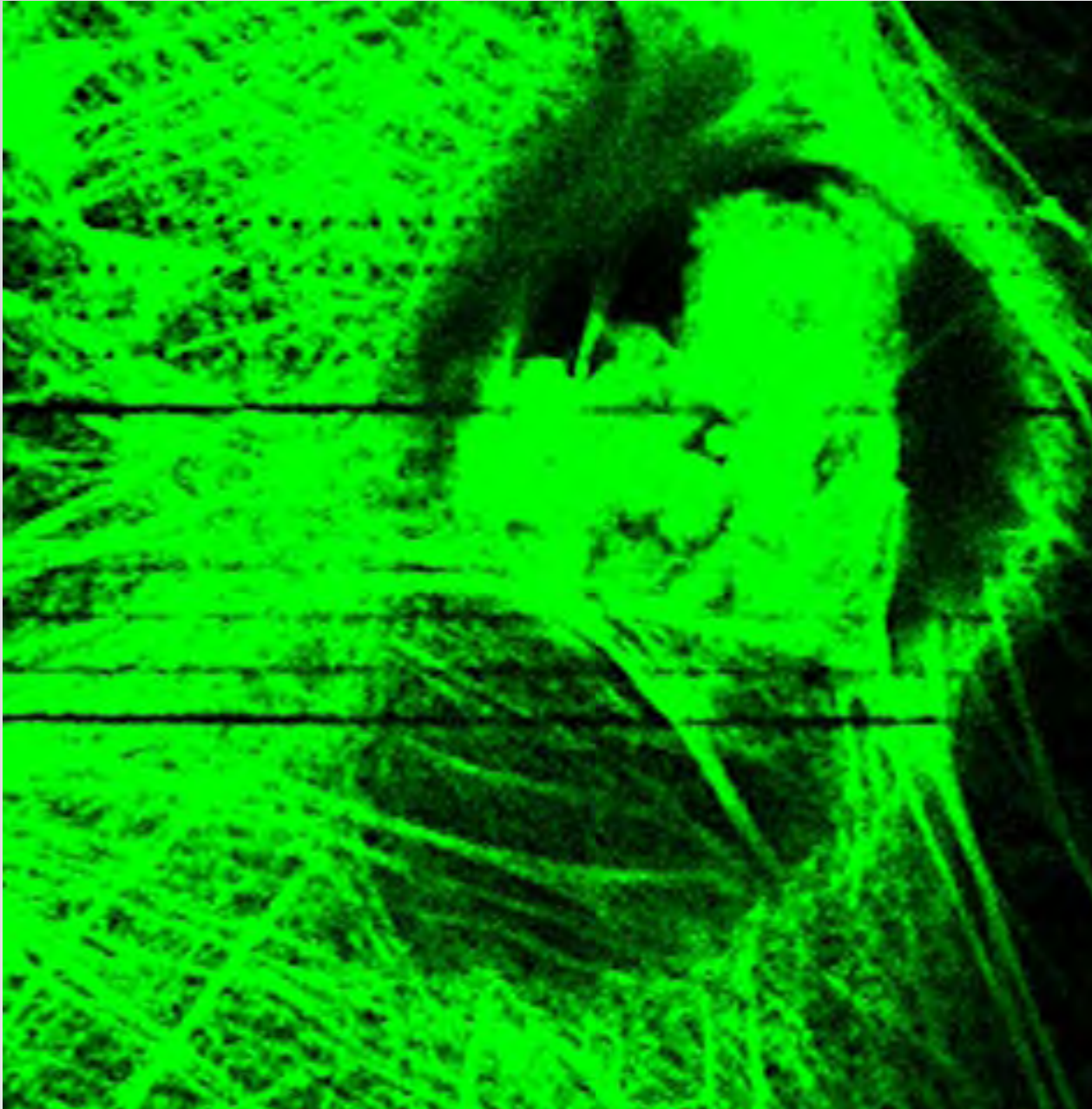


examine resulting ablation

Subcellular surgery

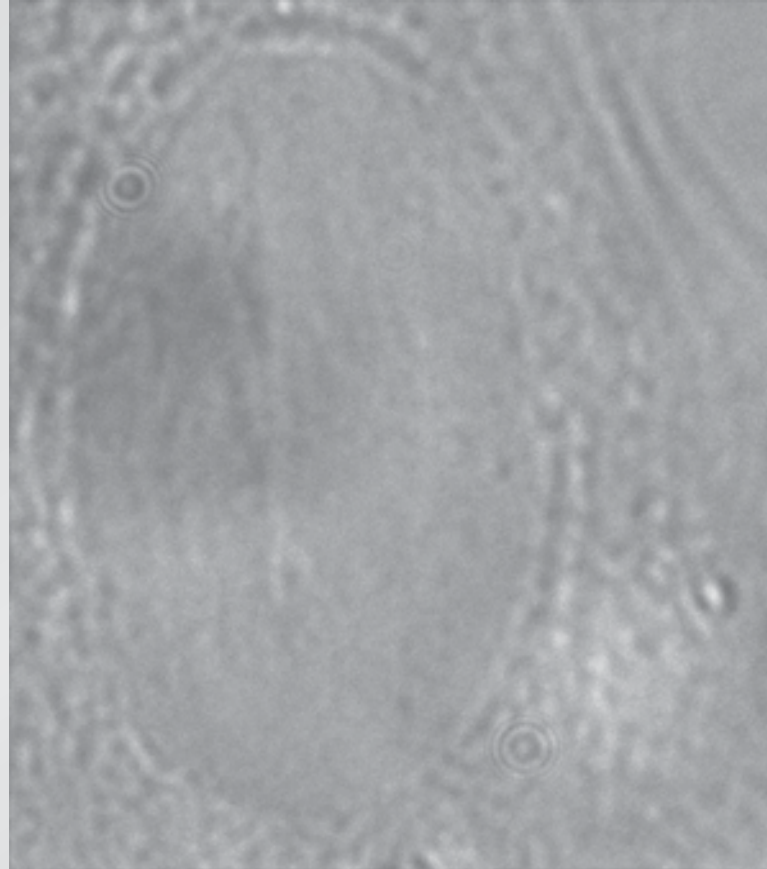


Subcellular surgery



Subcellular surgery

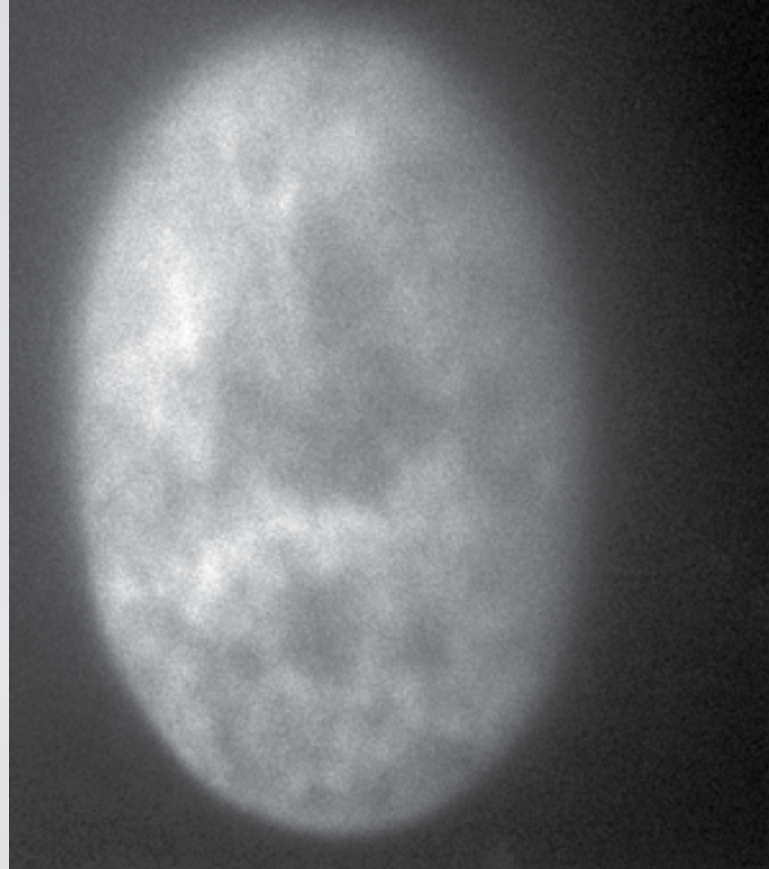
nucleus of fixed endothelial cell



white light microscopy

Subcellular surgery

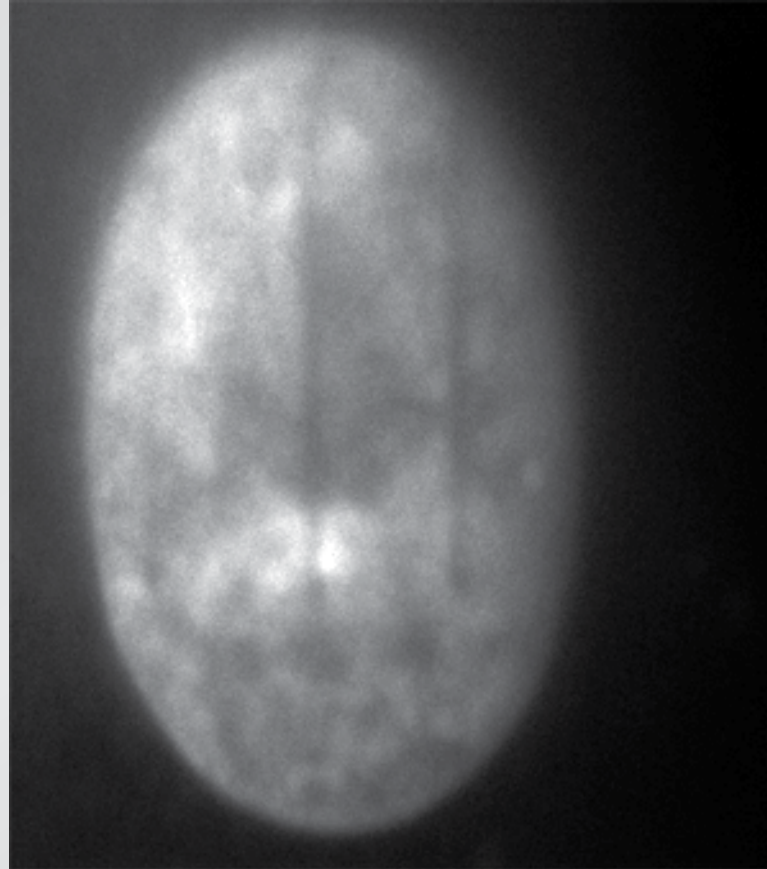
nucleus of fixed endothelial cell



fluorescence microscopy

Subcellular surgery

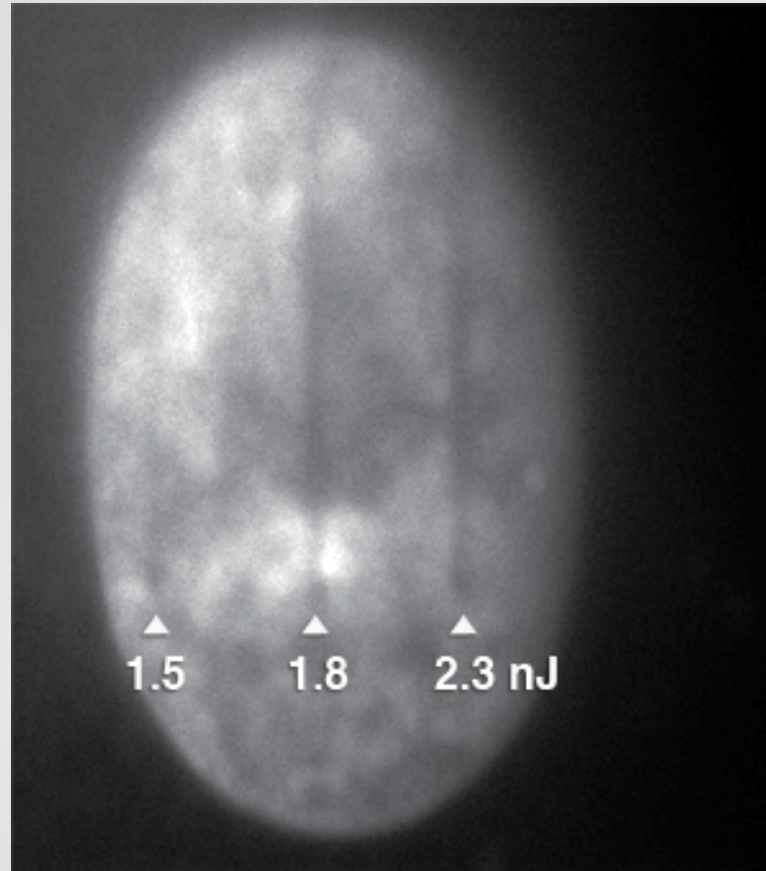
irradiate with fs laser



fluorescence microscopy

Subcellular surgery

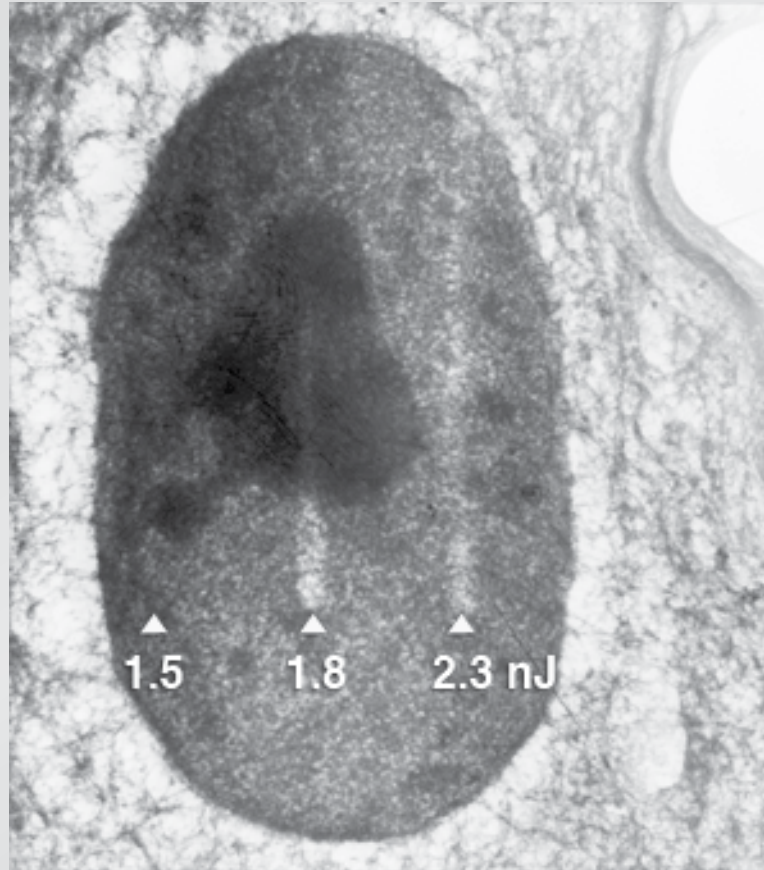
irradiate with fs laser



fluorescence microscopy

Subcellular surgery

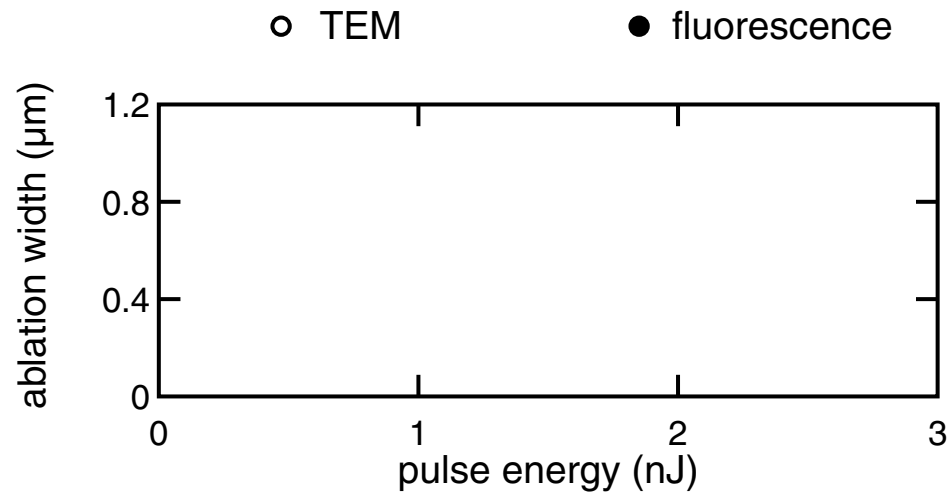
bleaching or ablation?



TEM image

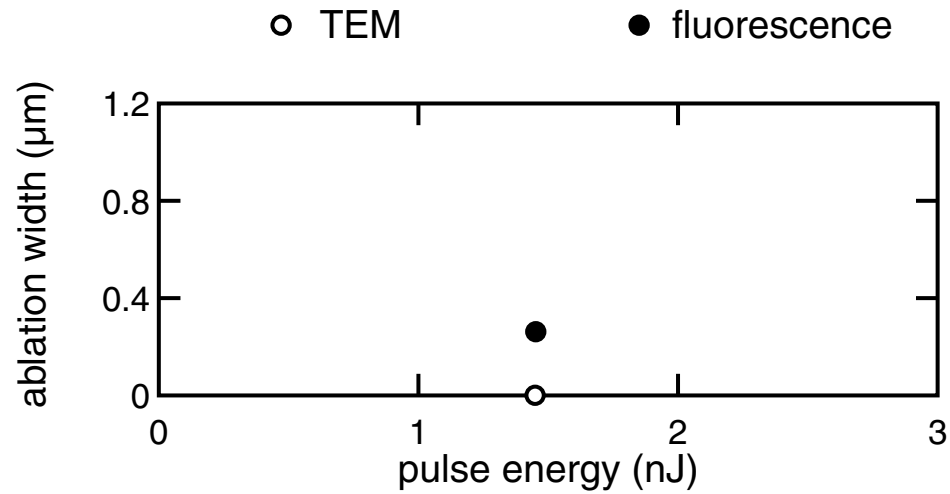
Subcellular surgery

three regions of interaction



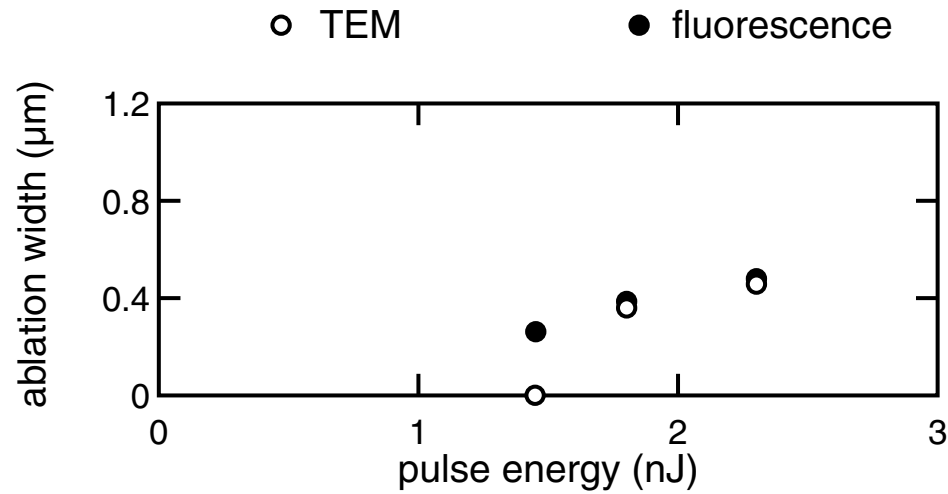
Subcellular surgery

three regions of interaction



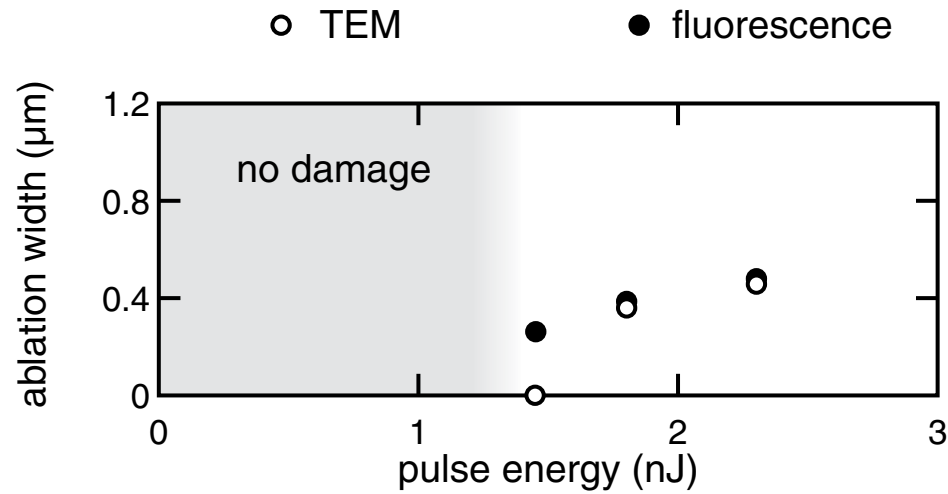
Subcellular surgery

three regions of interaction



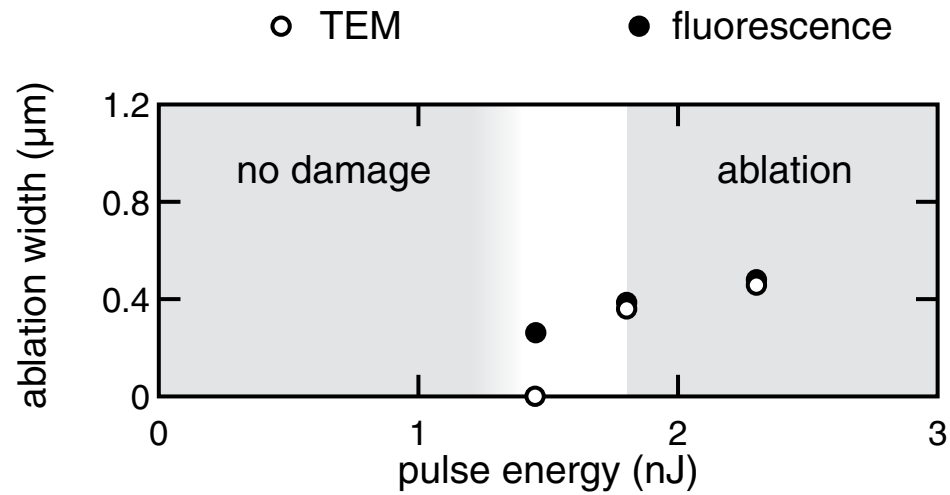
Subcellular surgery

three regions of interaction



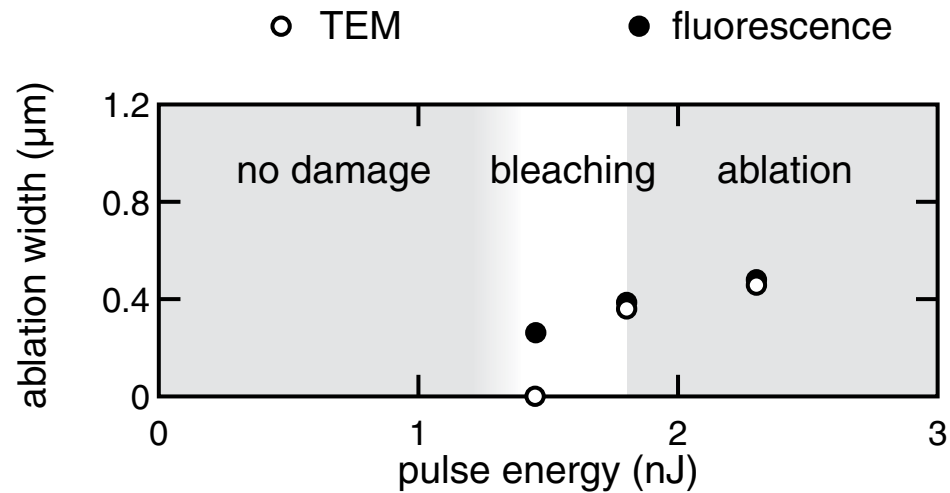
Subcellular surgery

three regions of interaction



Subcellular surgery

three regions of interaction



Subcellular surgery

Definitive proof of ablation

- ablation width as small as 100 nm
- ablation threshold varies slightly
- ablation threshold 20% above bleaching threshold

Subcellular surgery

Definitive proof of ablation

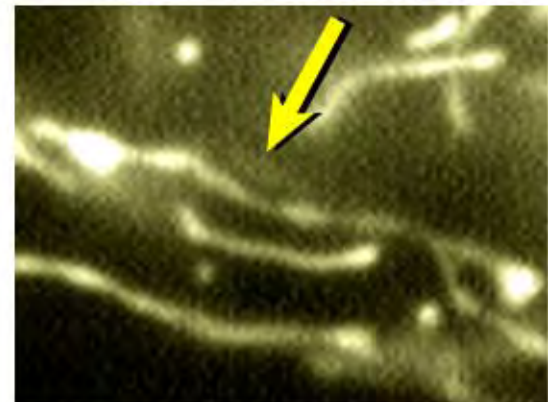
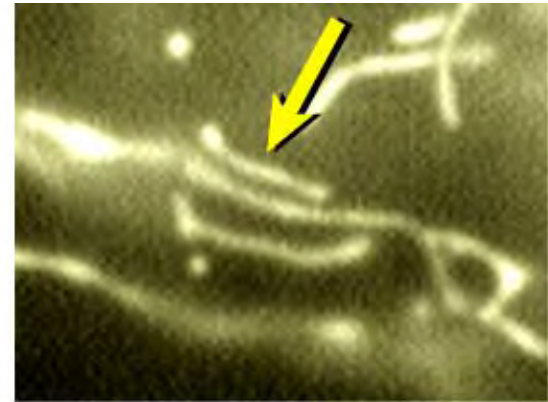
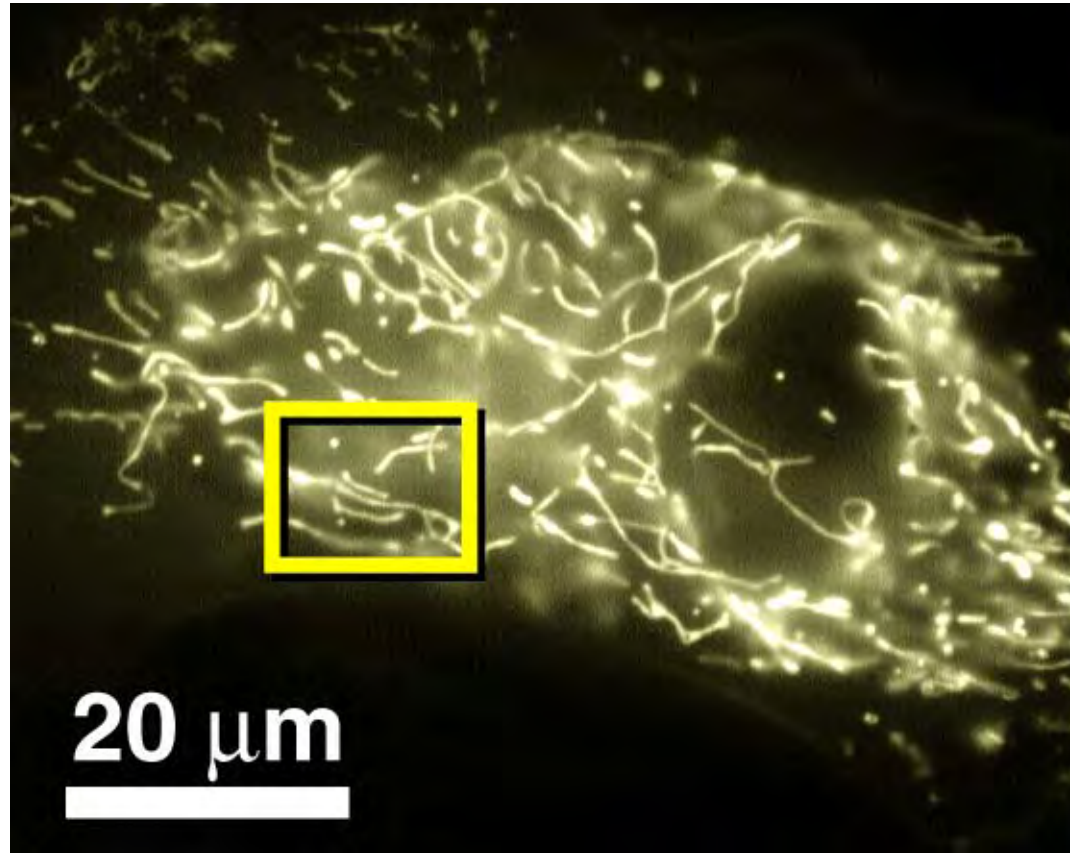
- ablation width as small as 100 nm
- ablation threshold varies slightly
- ablation threshold 20% above bleaching threshold

Subcellular surgery

Q: subcellular surgery on live cells?

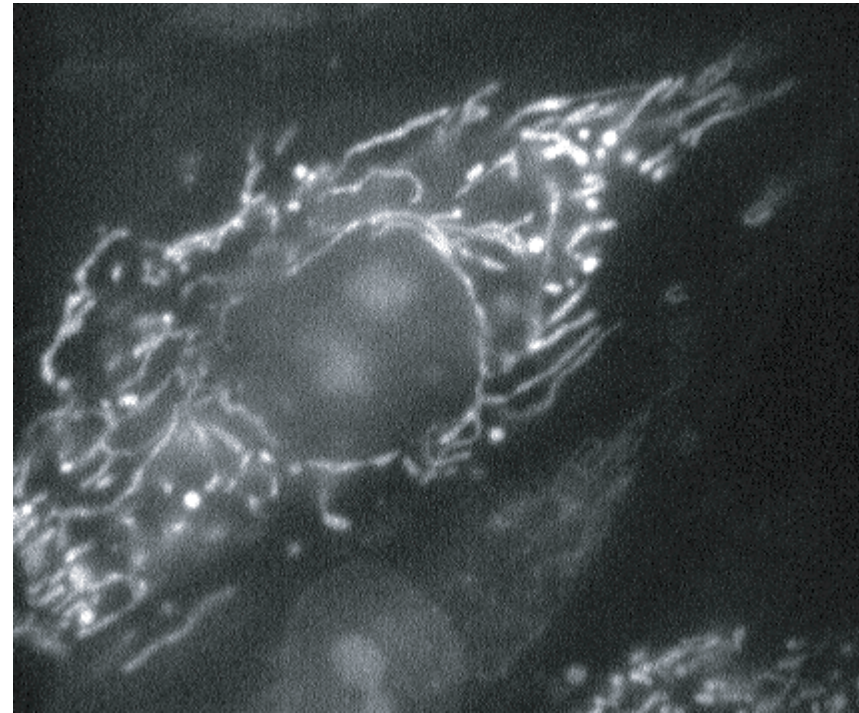
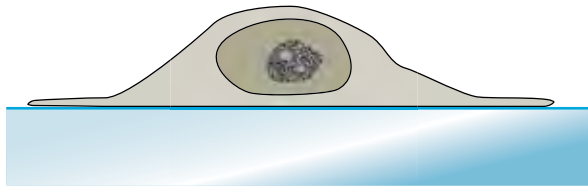
A fluorescence microscopy image showing a cell with a highly branched and interconnected cytoskeleton. The cytoskeleton is stained with a bright yellow-green fluorescent dye, making it stand out against the darker background of the cell and surrounding environment. The structure appears as a dense network of thin, thread-like filaments.

Subcellular surgery



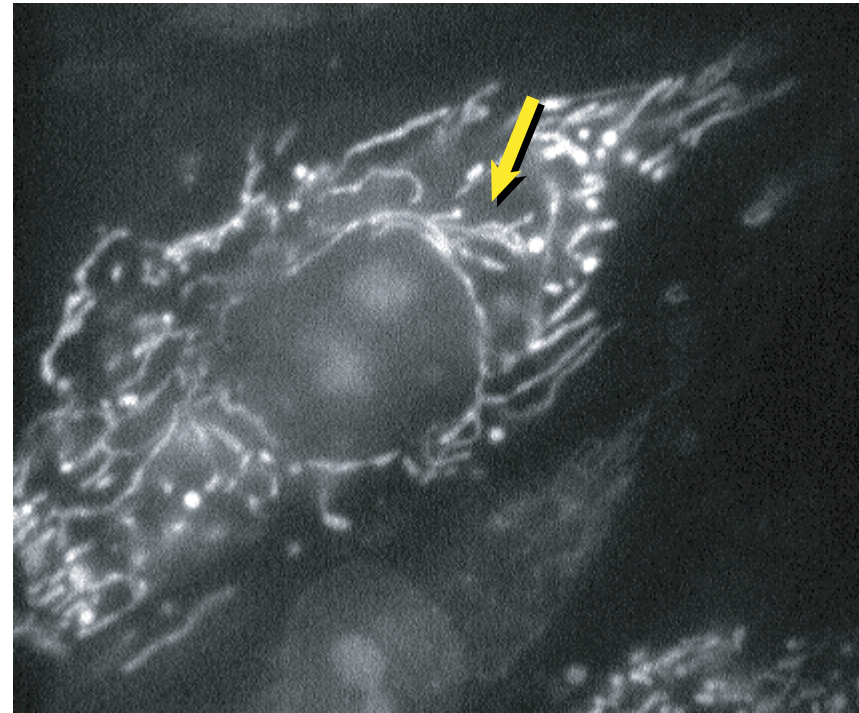
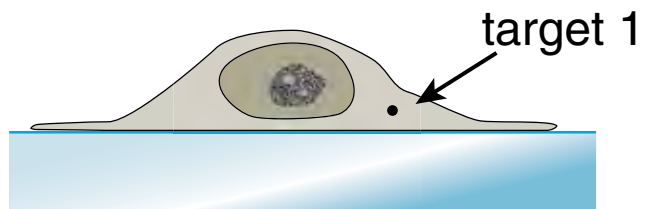
Subcellular surgery

ethyidium bromide test



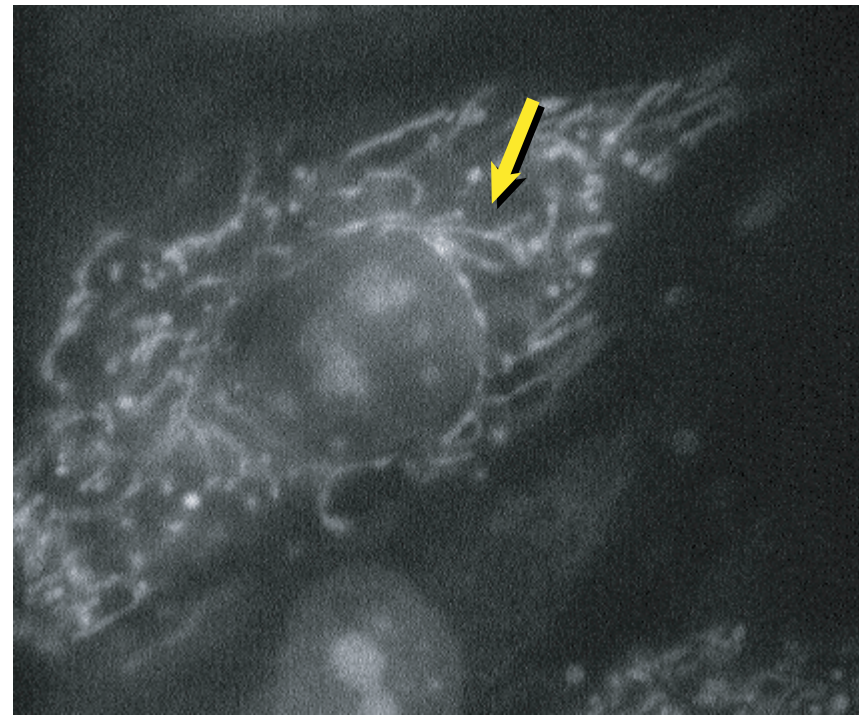
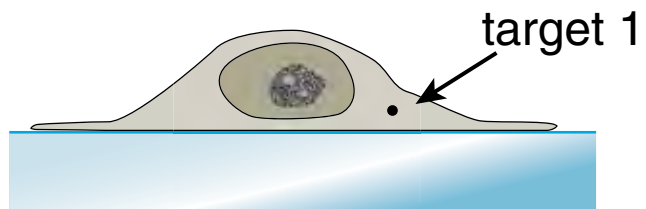
Subcellular surgery

ethyidium bromide test



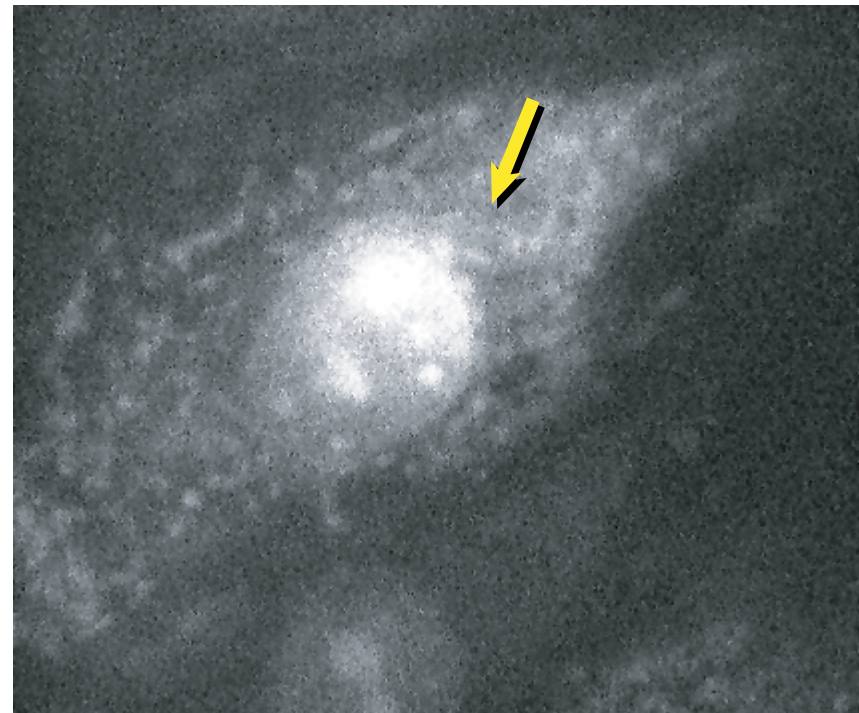
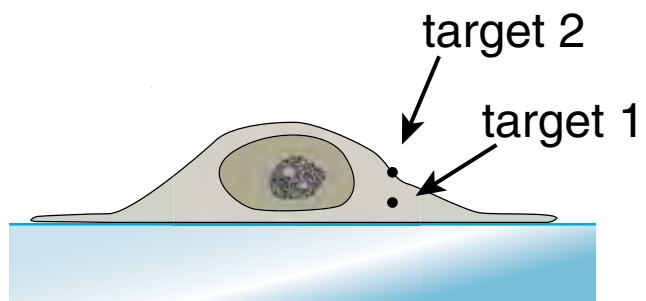
Subcellular surgery

ethyidium bromide test



Subcellular surgery

ethyidium bromide test

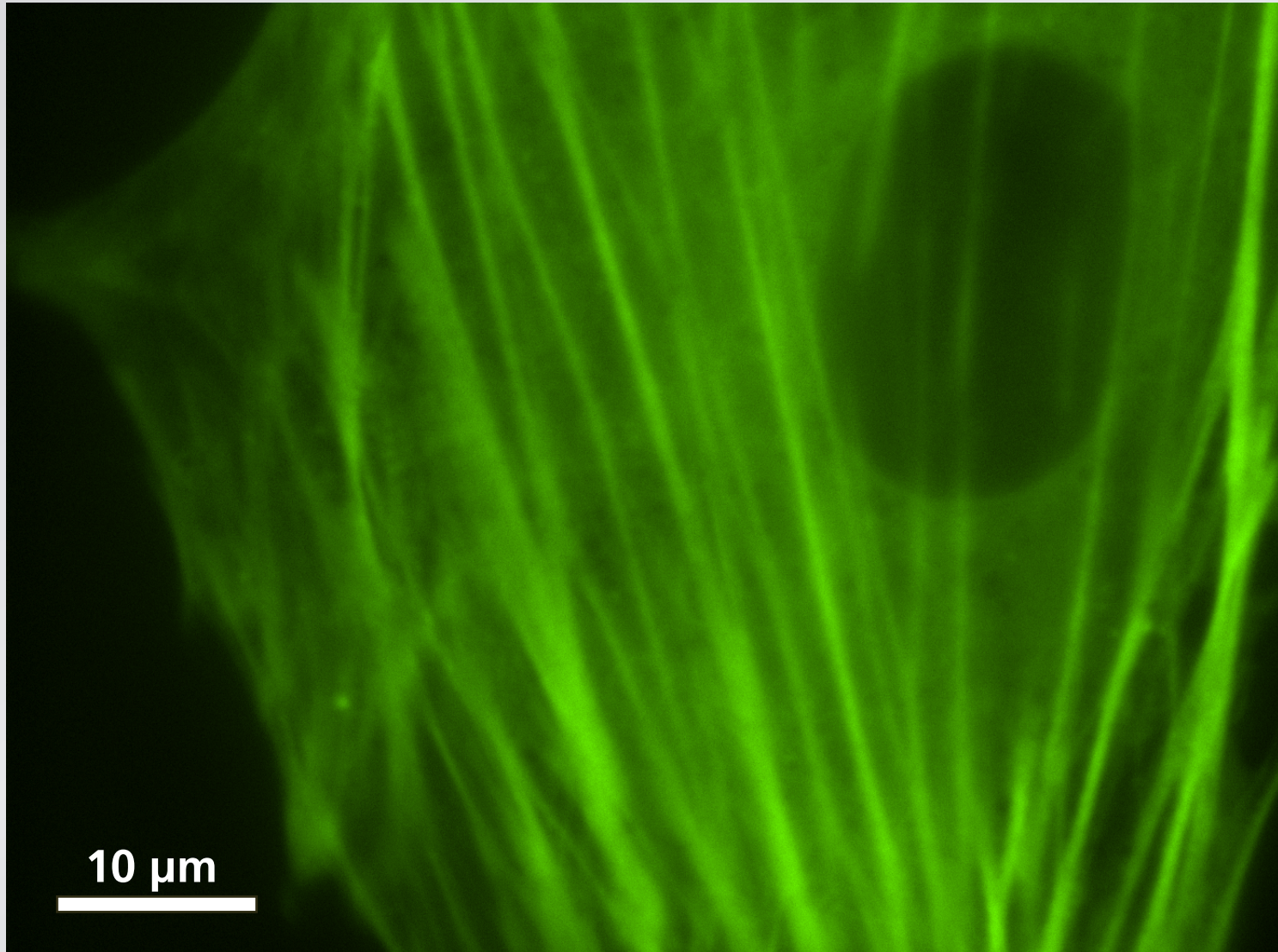


Subcellular surgery

Q: can we probe the dynamics of the cytoskeleton?

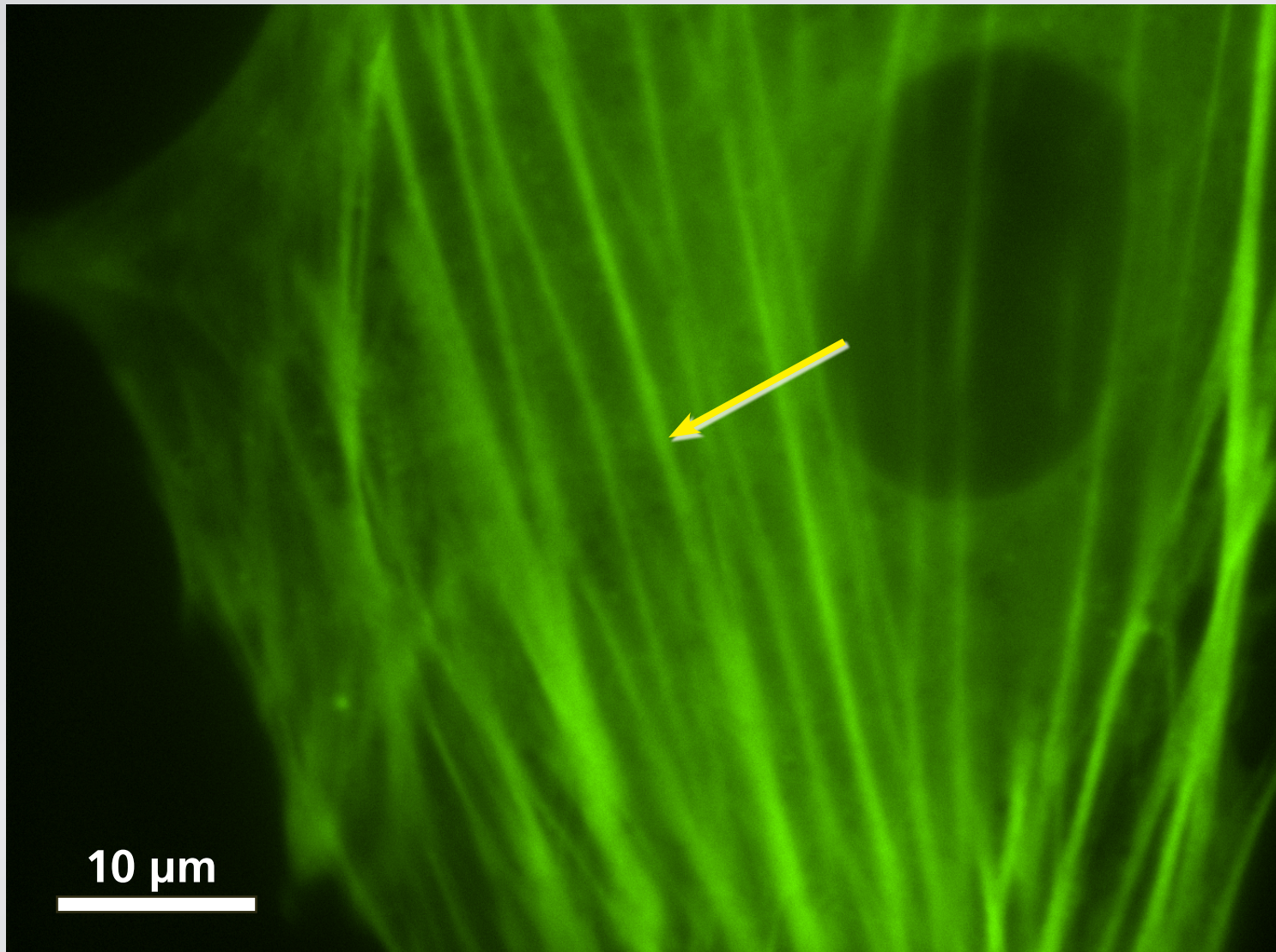
Subcellular surgery

YFP-labeled actin fiber network of a live cell



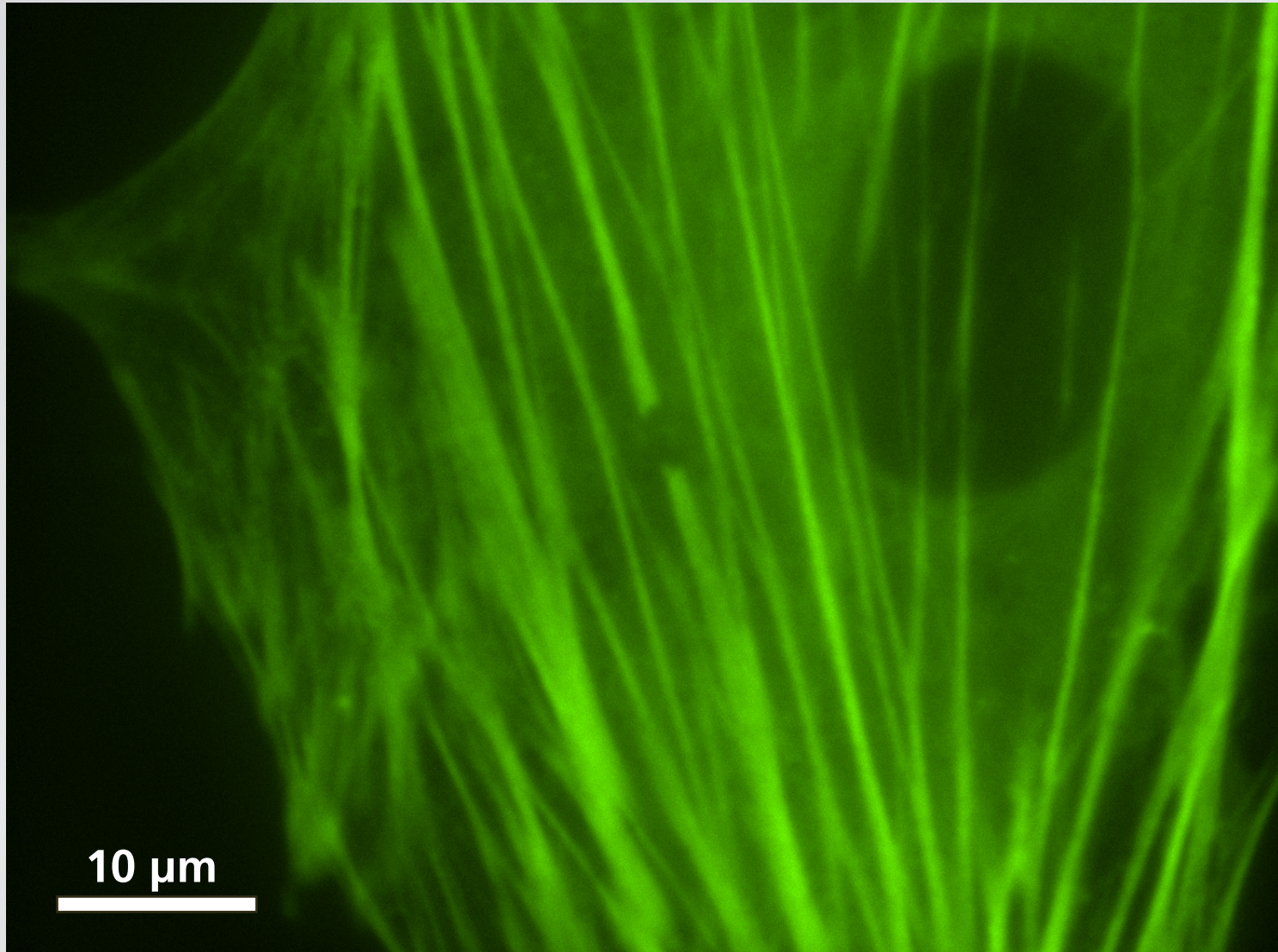
Subcellular surgery

cut a single fiber bundle



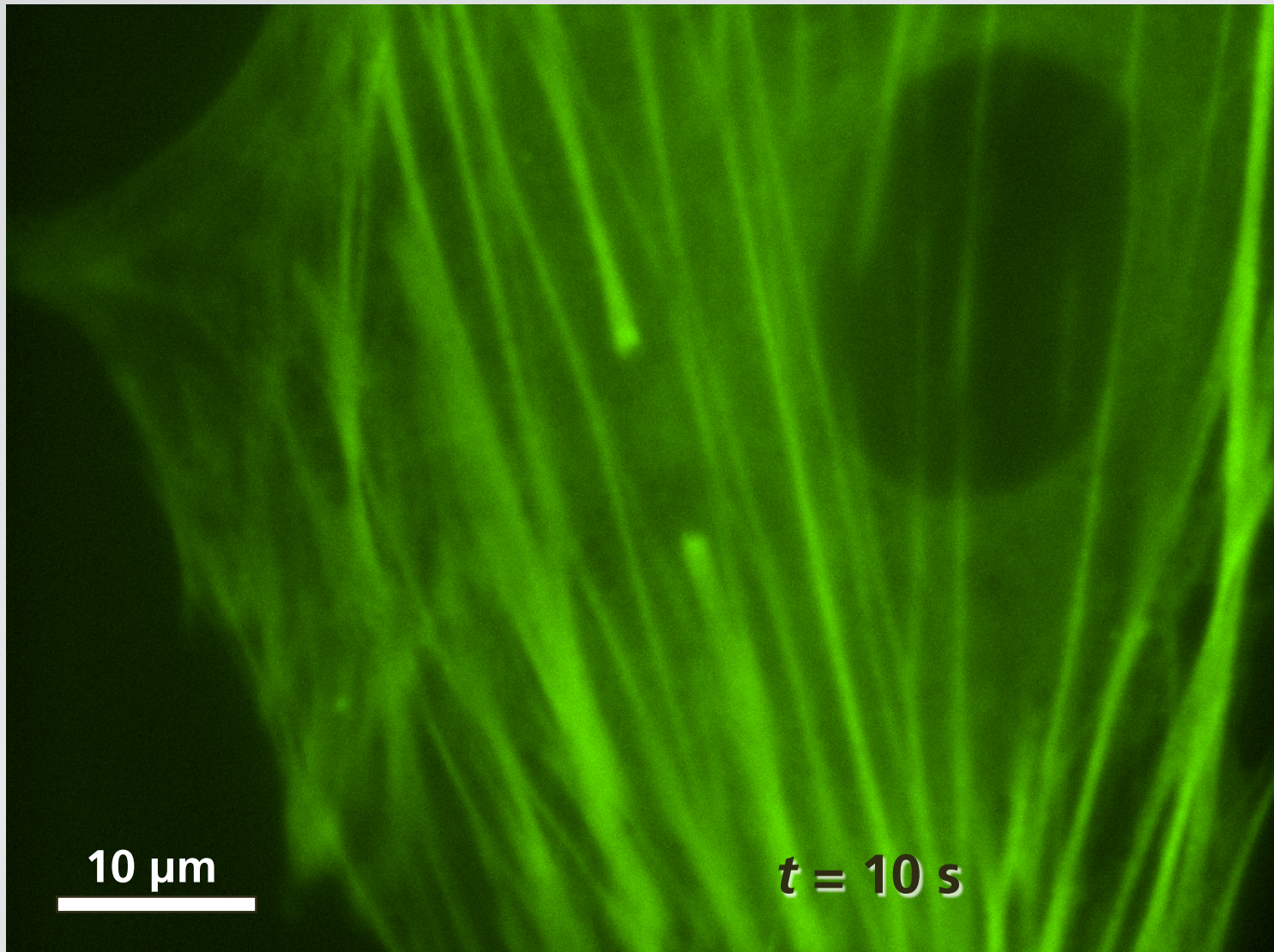
Subcellular surgery

cut a single fiber bundle



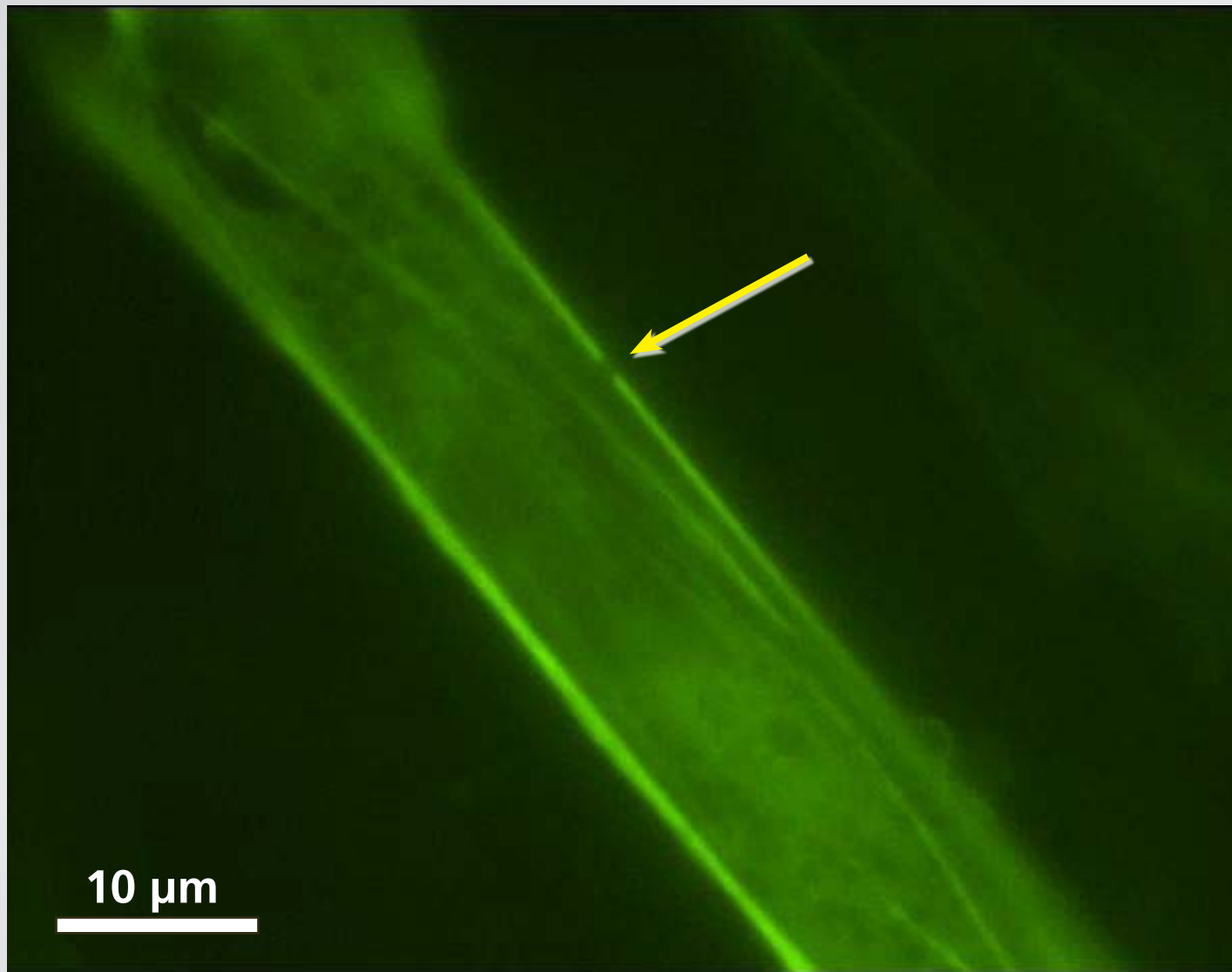
Subcellular surgery

gap widens with time



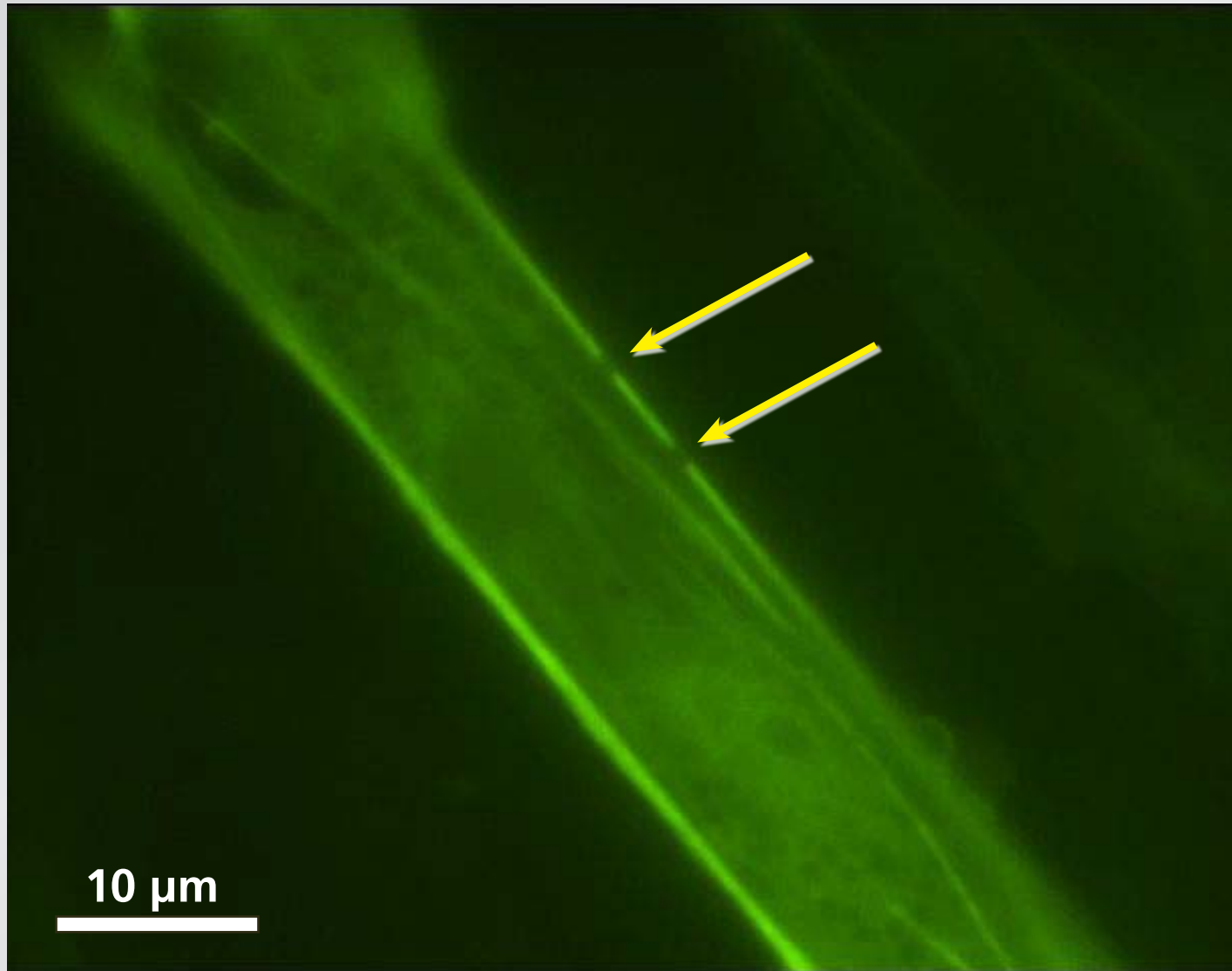
Subcellular surgery

retraction or depolymerization?



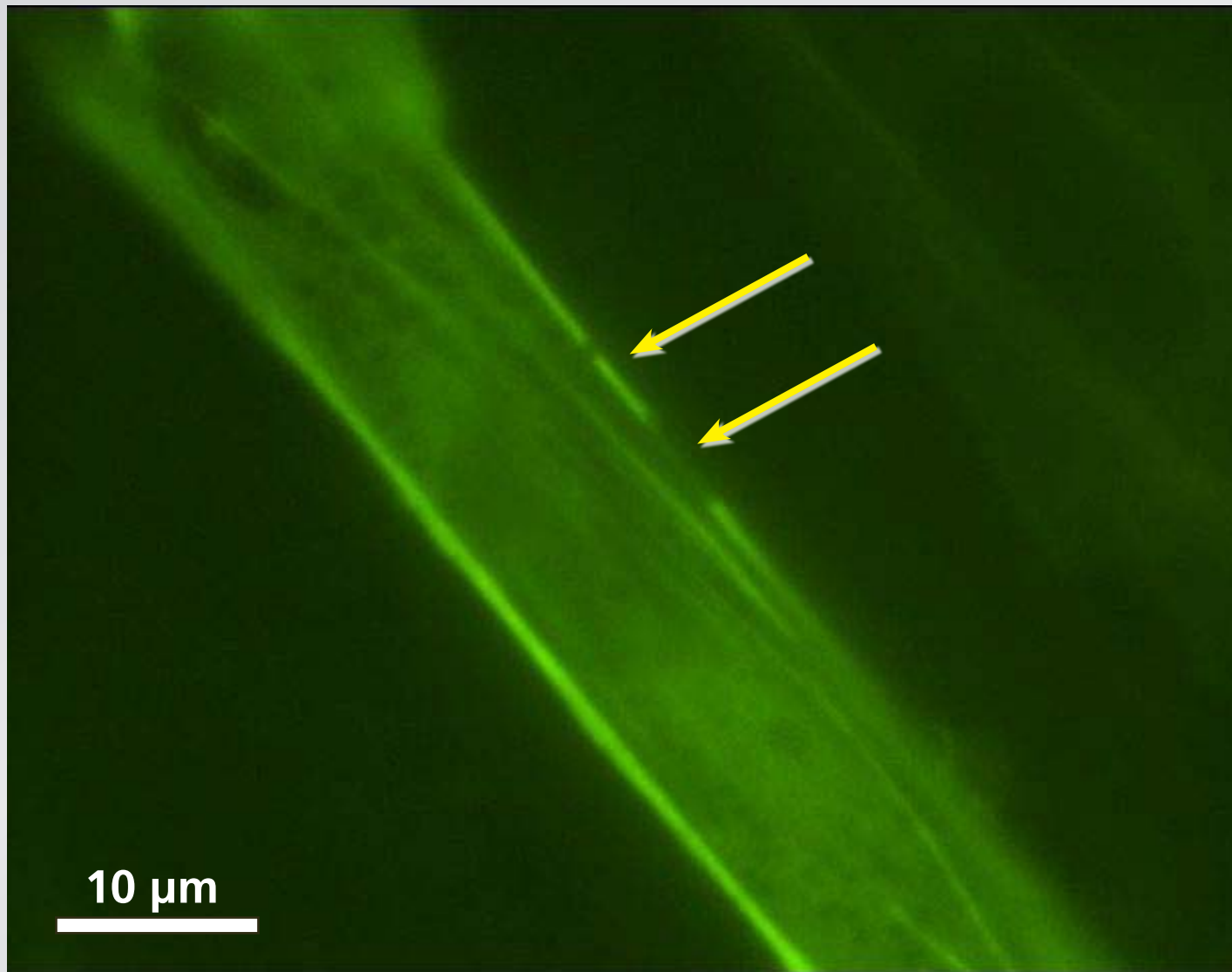
Subcellular surgery

retraction or depolymerization?



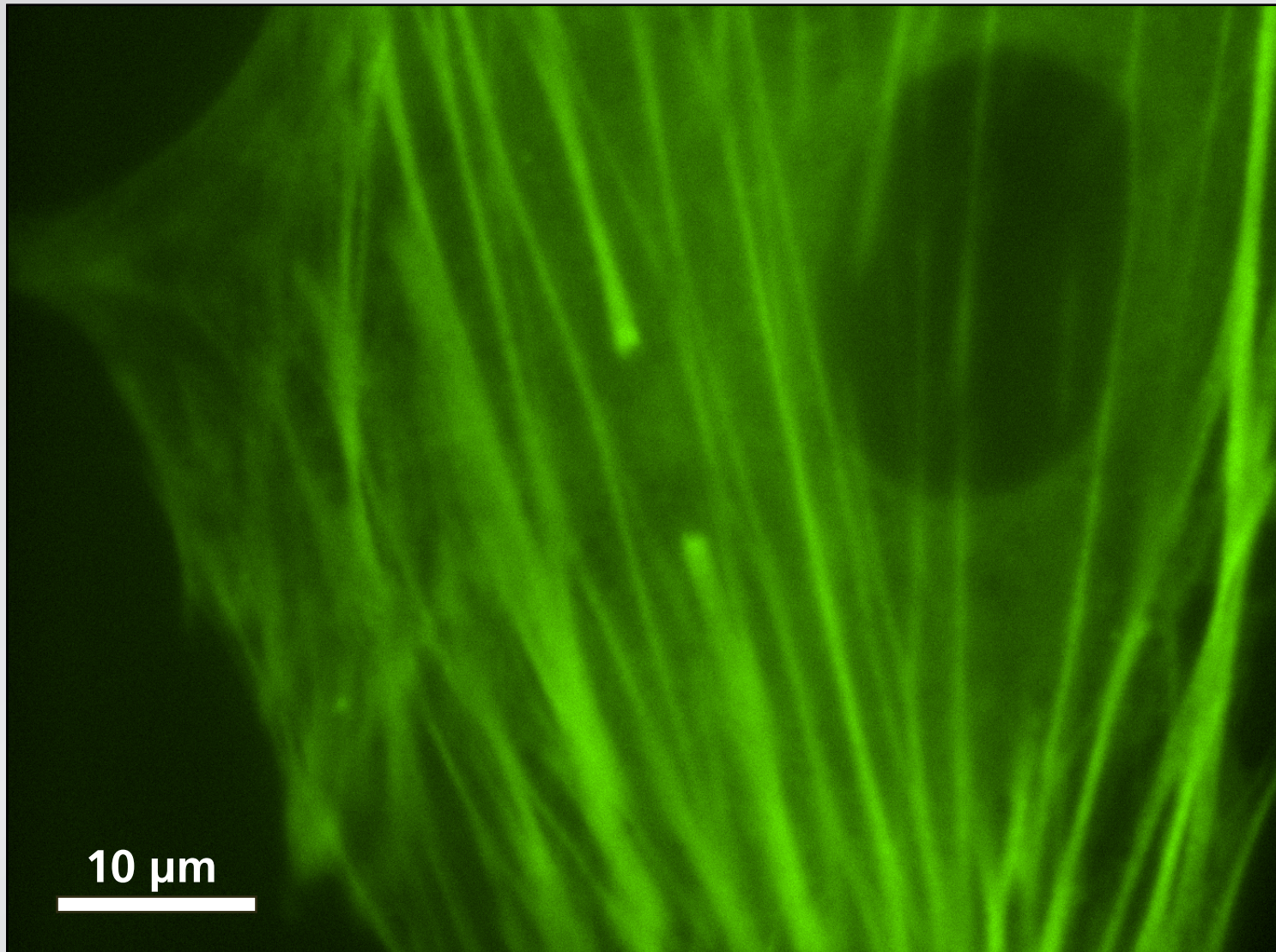
Subcellular surgery

retraction!

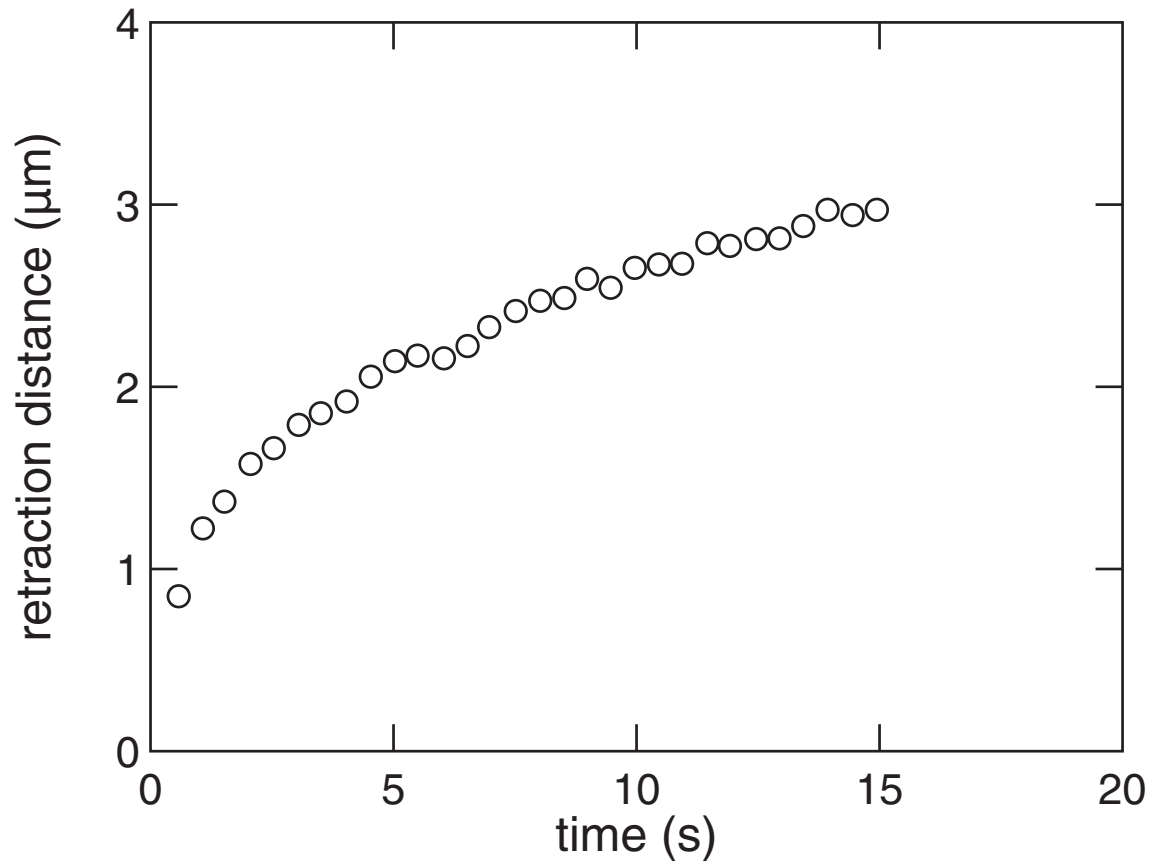


Subcellular surgery

dynamics provides information on *in vivo* mechanics

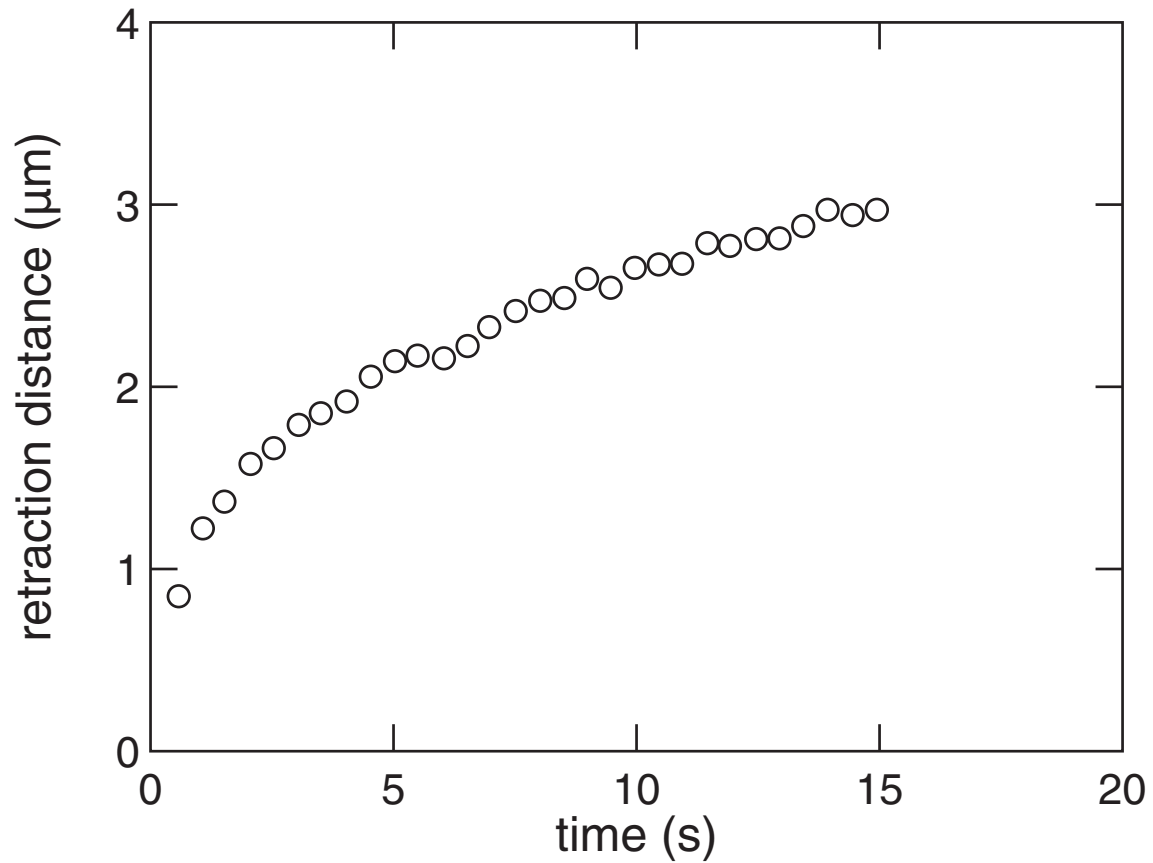


Subcellular surgery



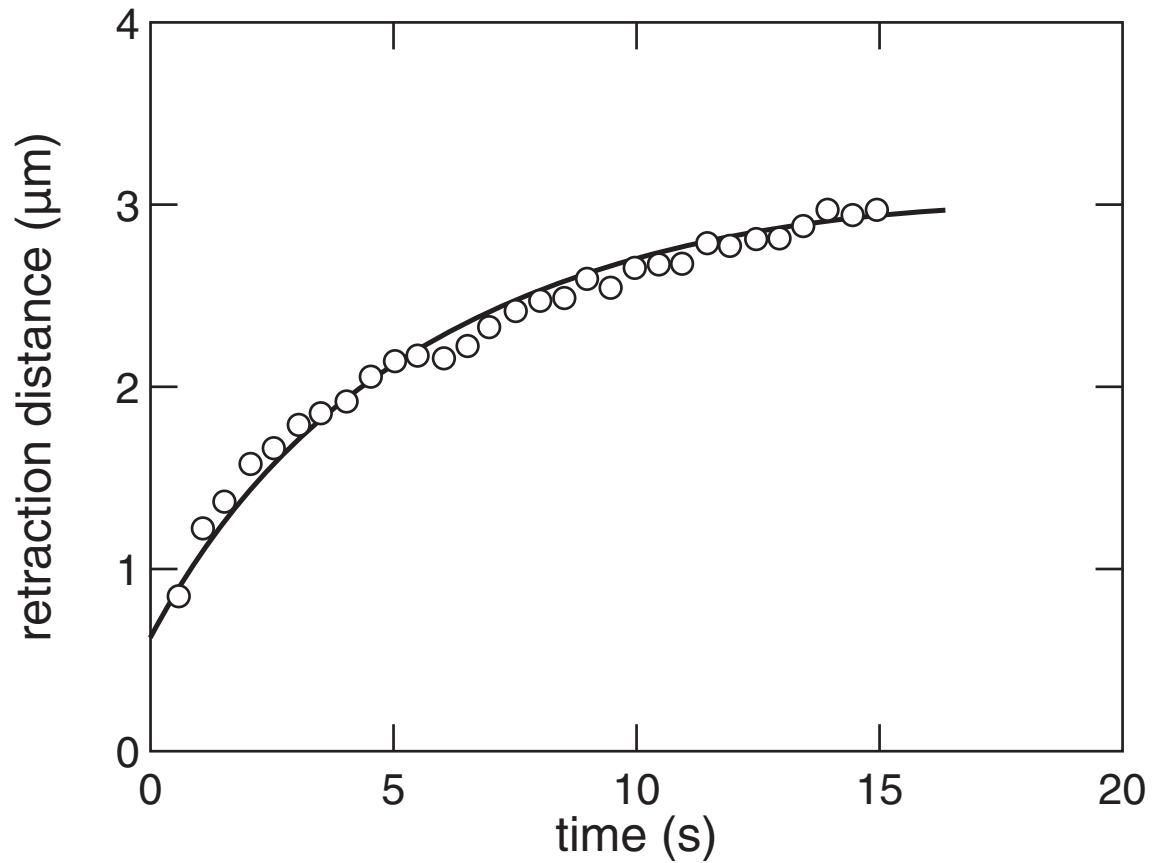
Subcellular surgery

overdamped spring: $\Delta L = L_{\infty}(1 - e^{-t/\tau}) + L_0$



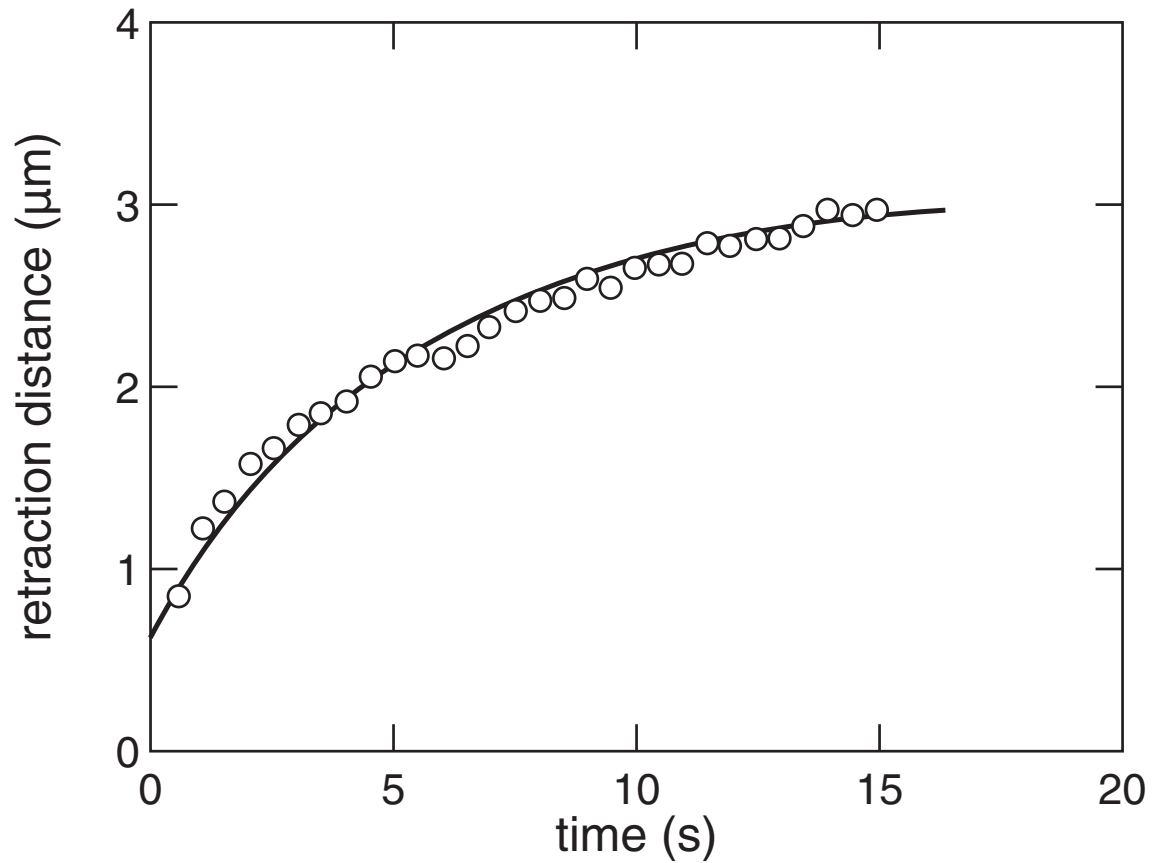
Subcellular surgery

overdamped spring: $\Delta L = L_{\infty}(1 - e^{-t/\tau}) + L_0$



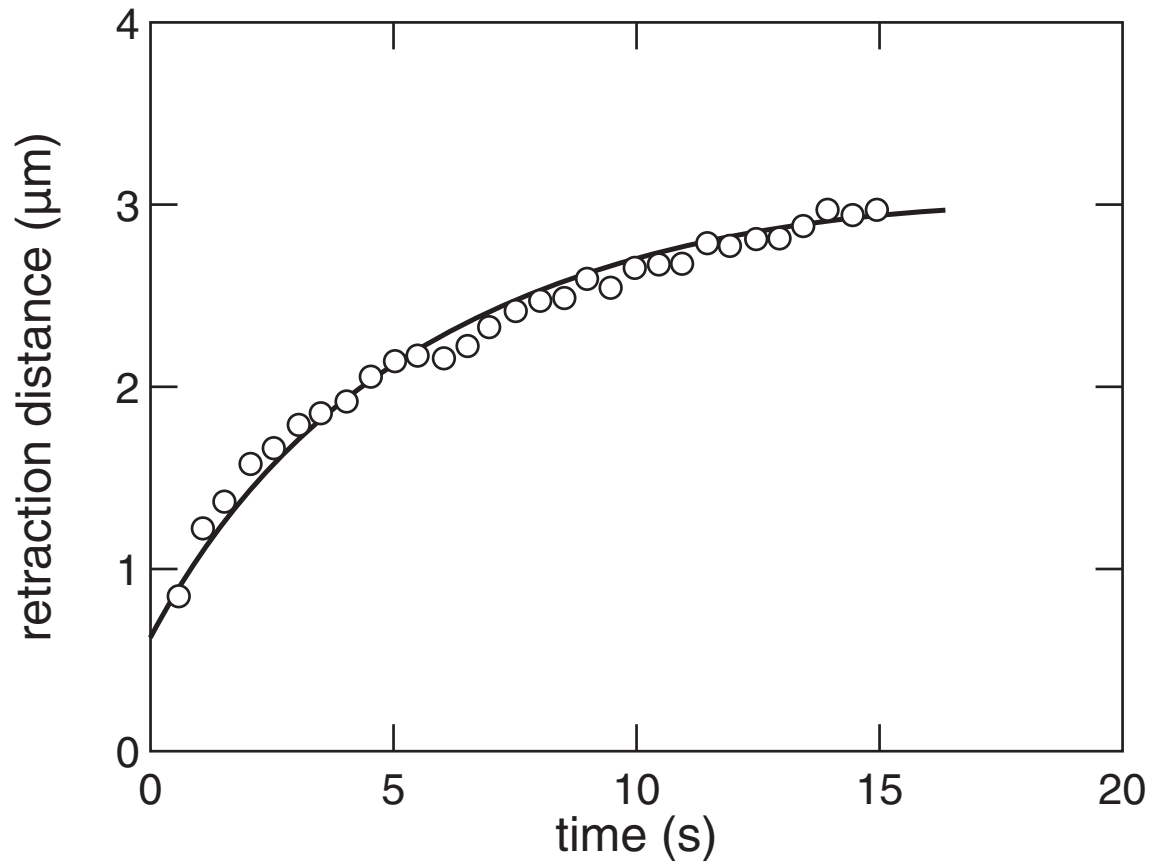
Subcellular surgery

L_0 and τ independent of fiber width!



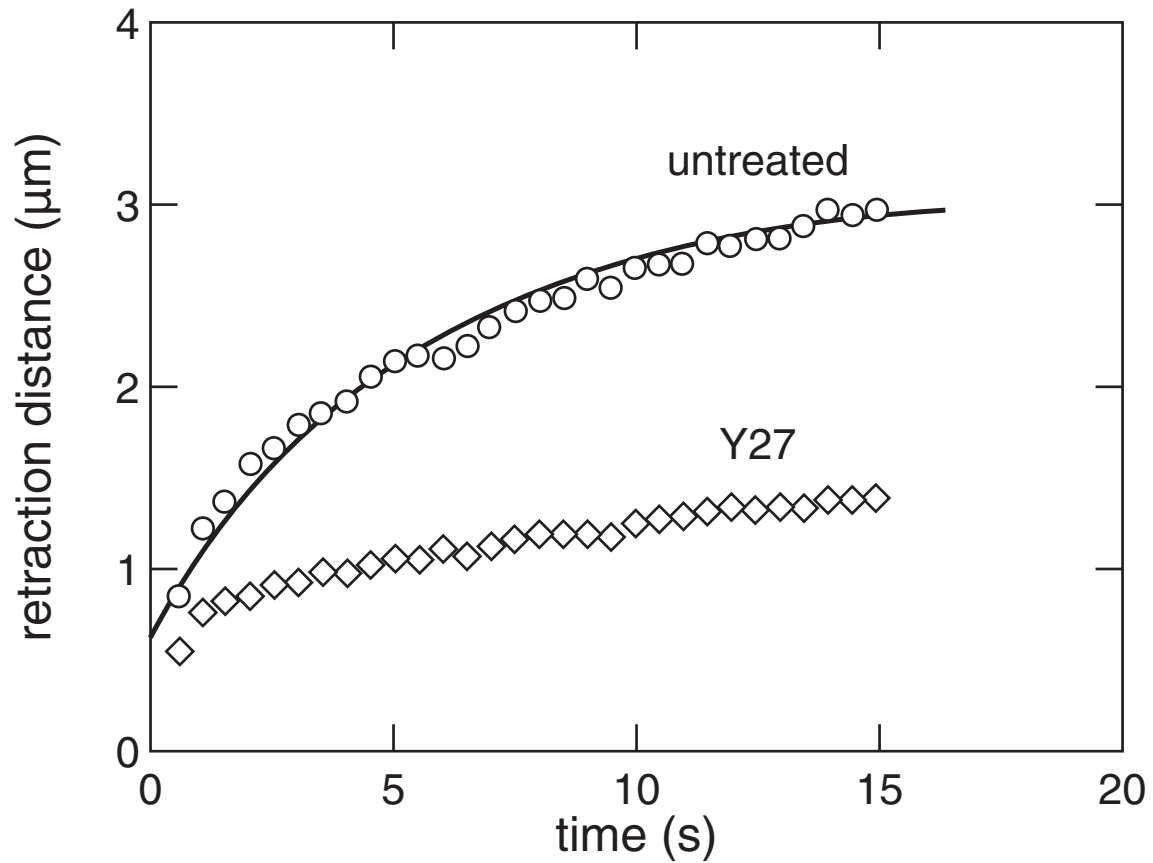
Subcellular surgery

tension in actin filaments is generated by myosin motors



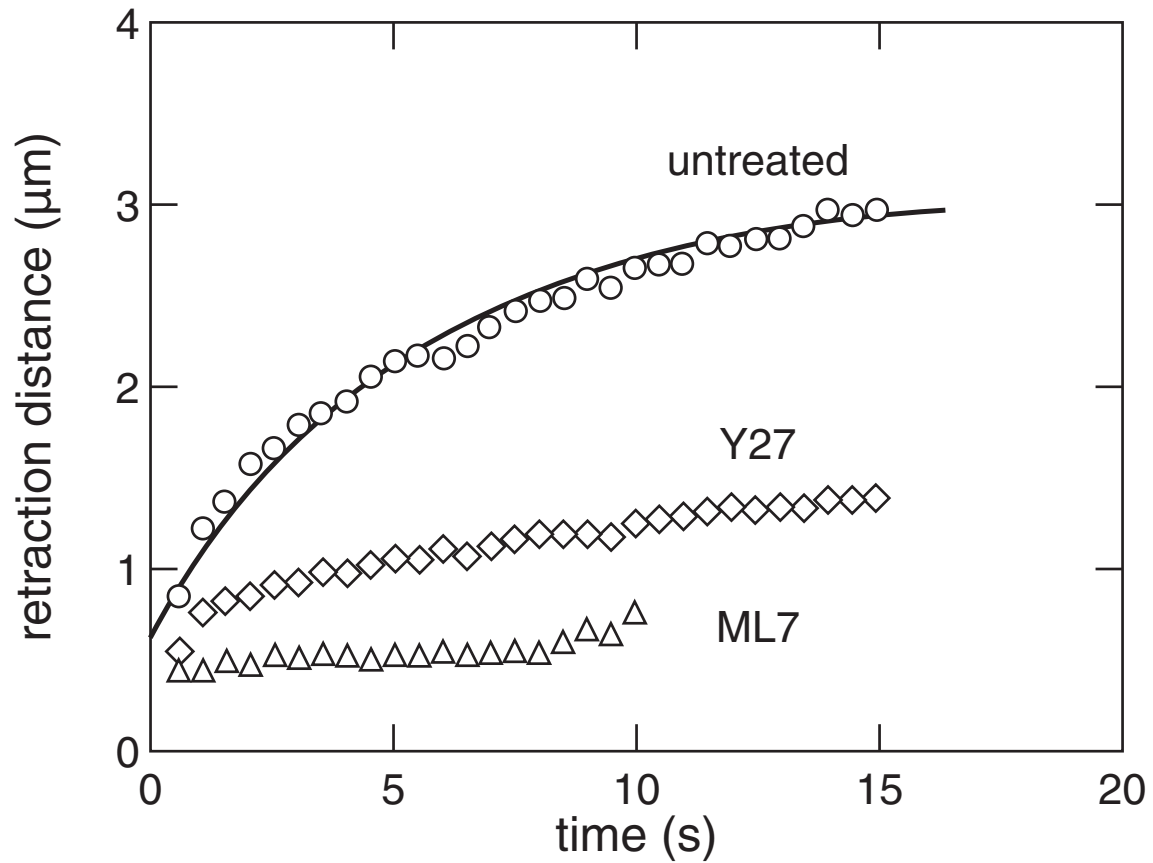
Subcellular surgery

Y27: inhibits some myosin activity



Subcellular surgery

ML7: direct inhibitor of myosin activity



Outline

- femtosecond materials interactions
 - subcellular surgery
 - **nanoneurosurgery**
- 

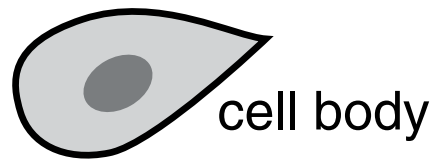
Nanoneurosurgery

Q: can we probe the neurological origins of behavior?



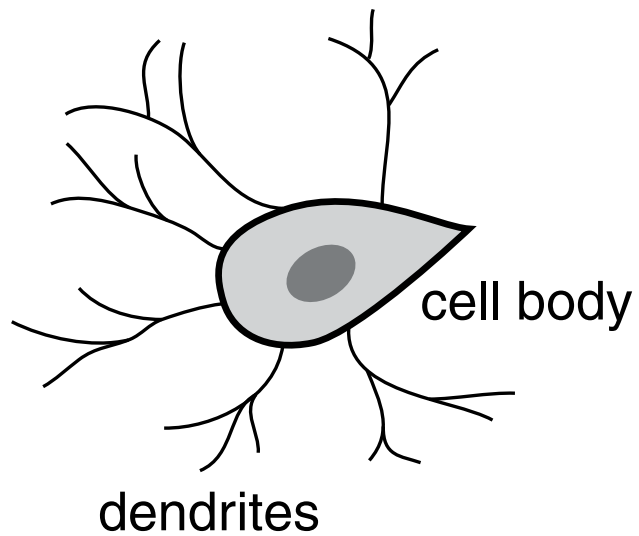
Nanoneurosurgery

neuron basics



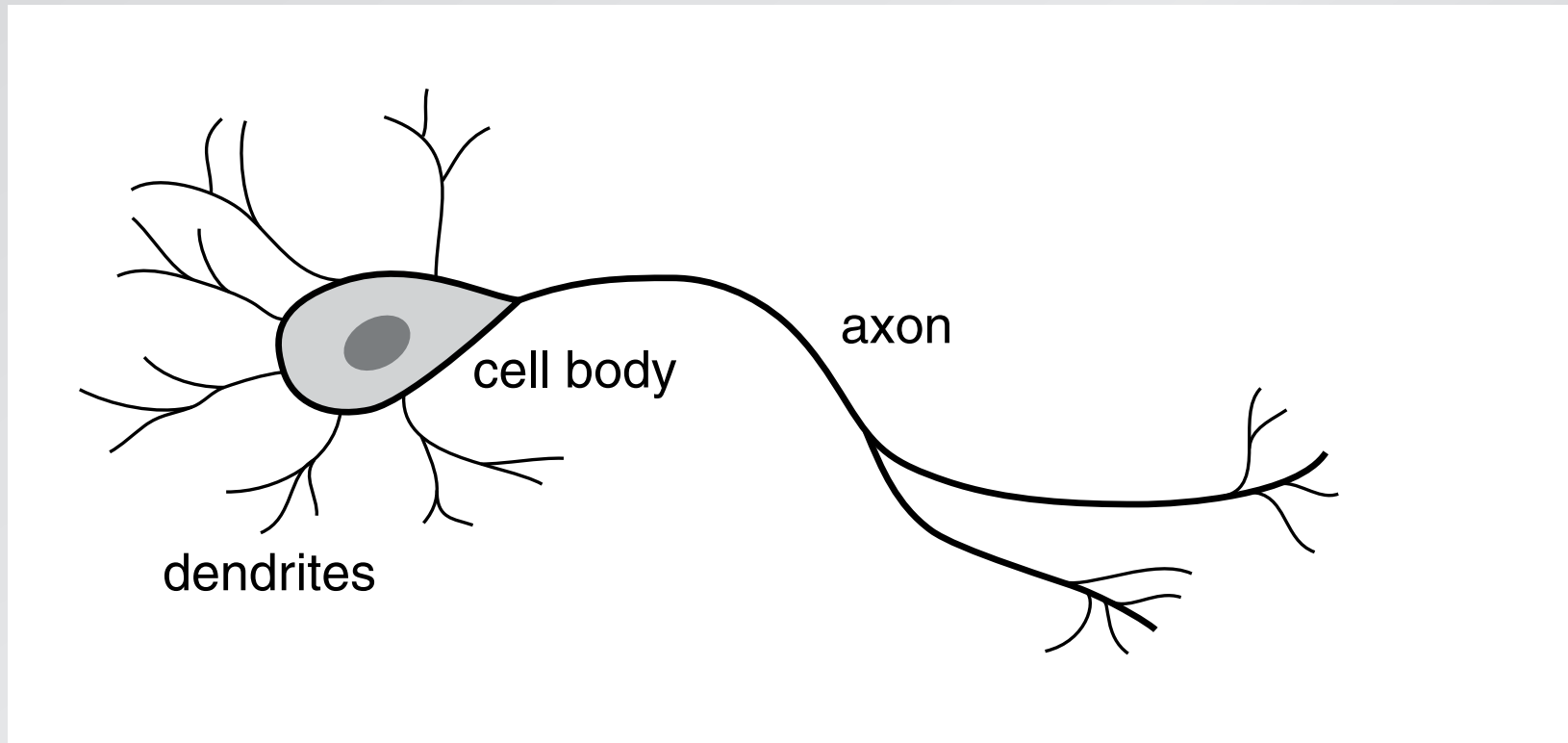
Nanoneurosurgery

neuron basics



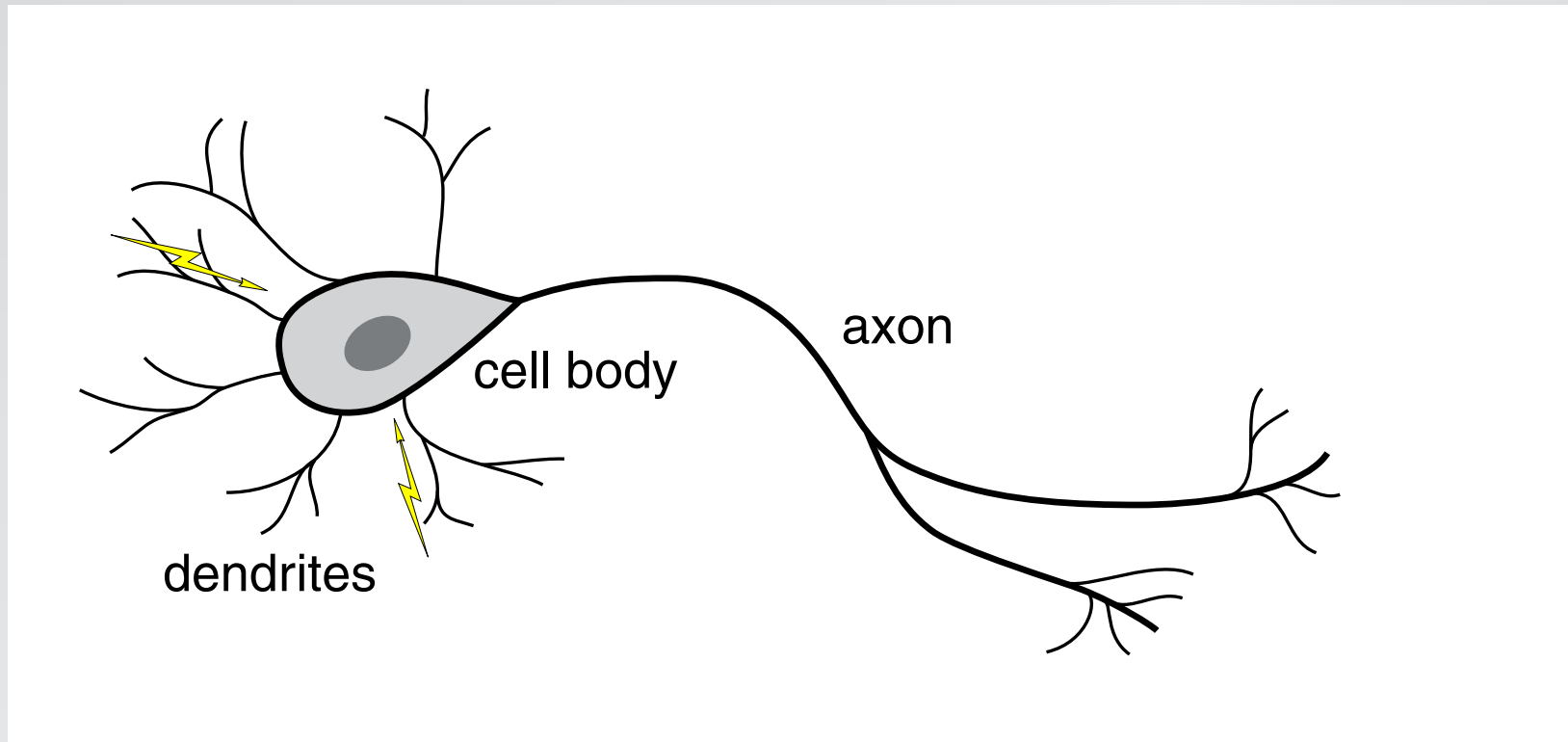
Nanoneurosurgery

neuron basics



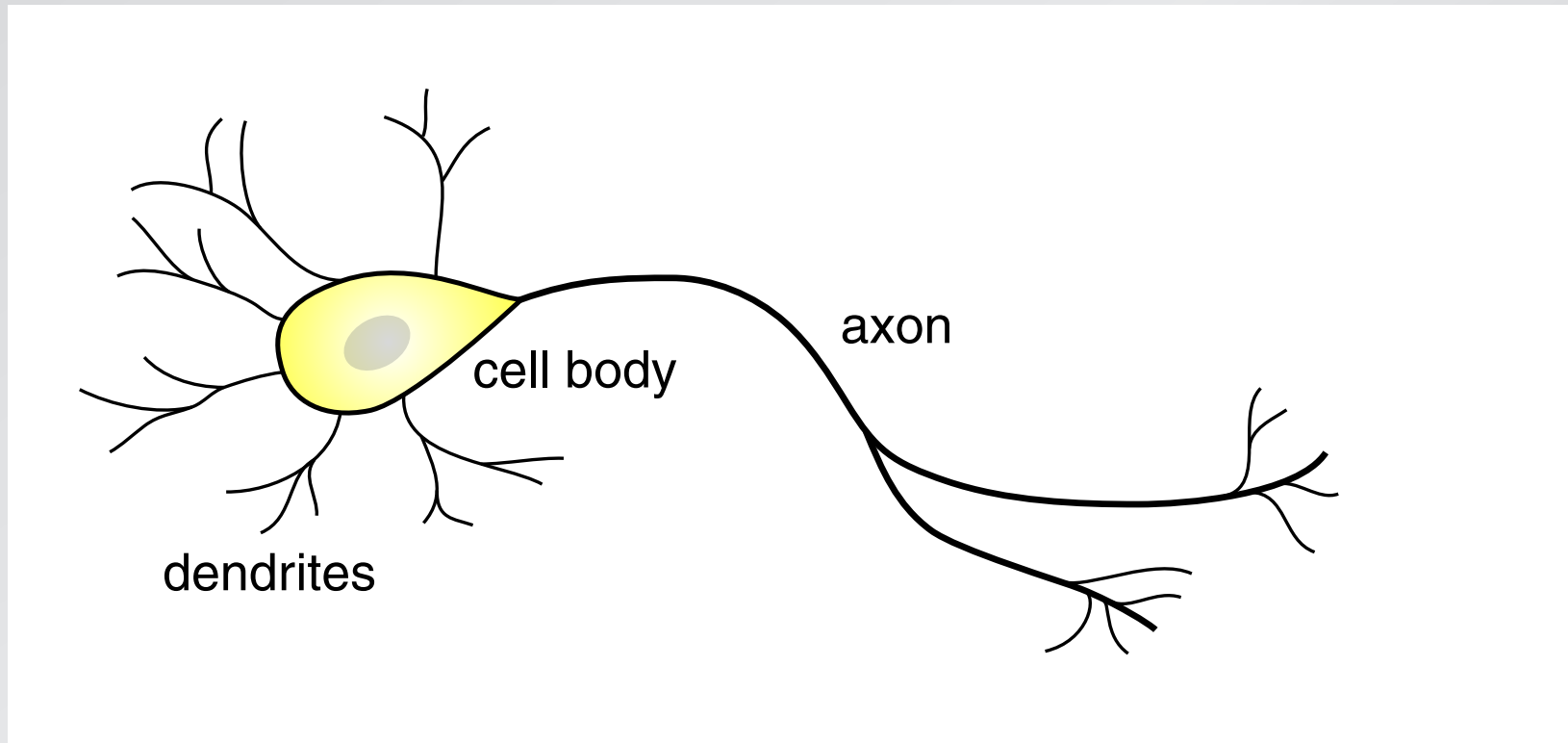
Nanoneurosurgery

neuron basics



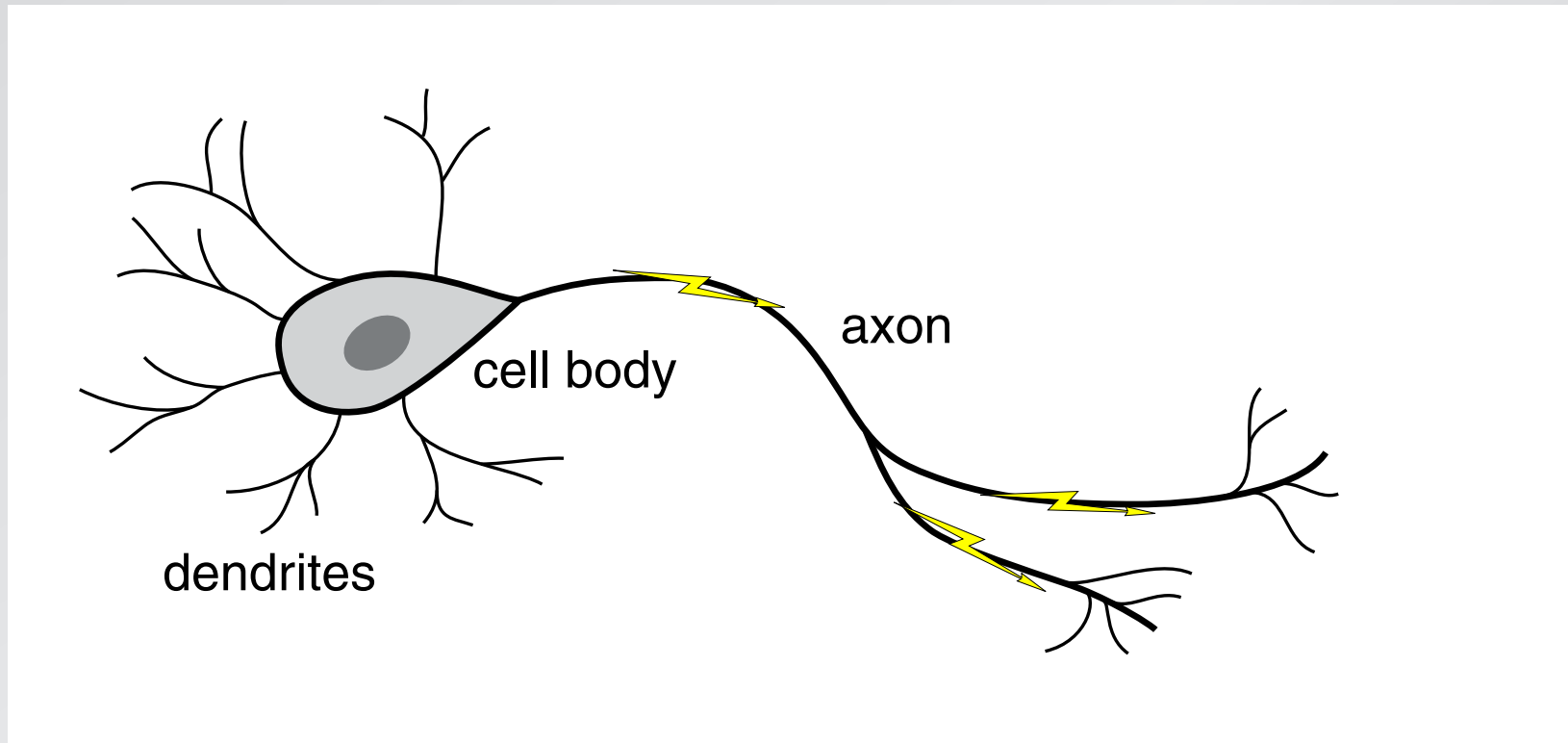
Nanoneurosurgery

neuron basics



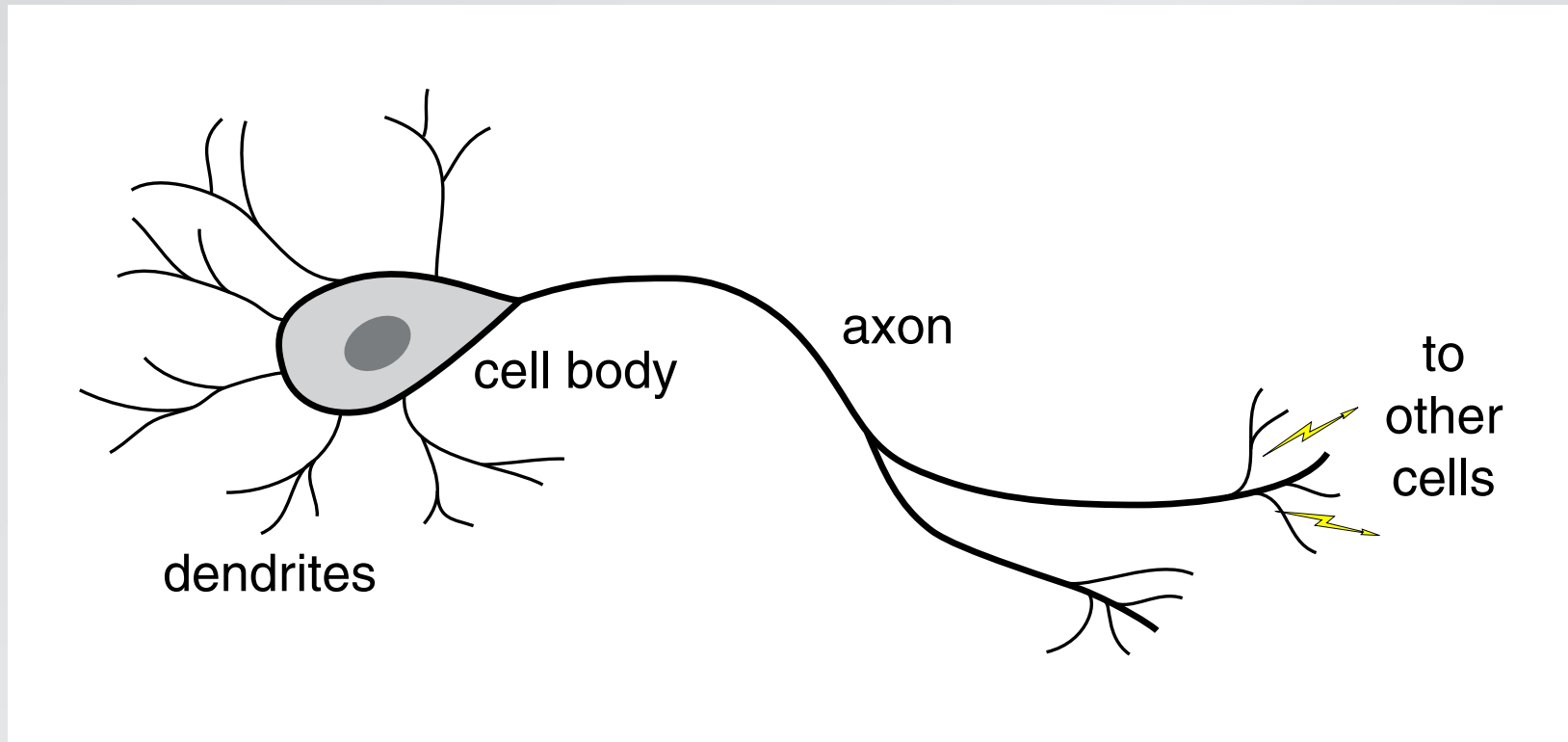
Nanoneurosurgery

neuron basics



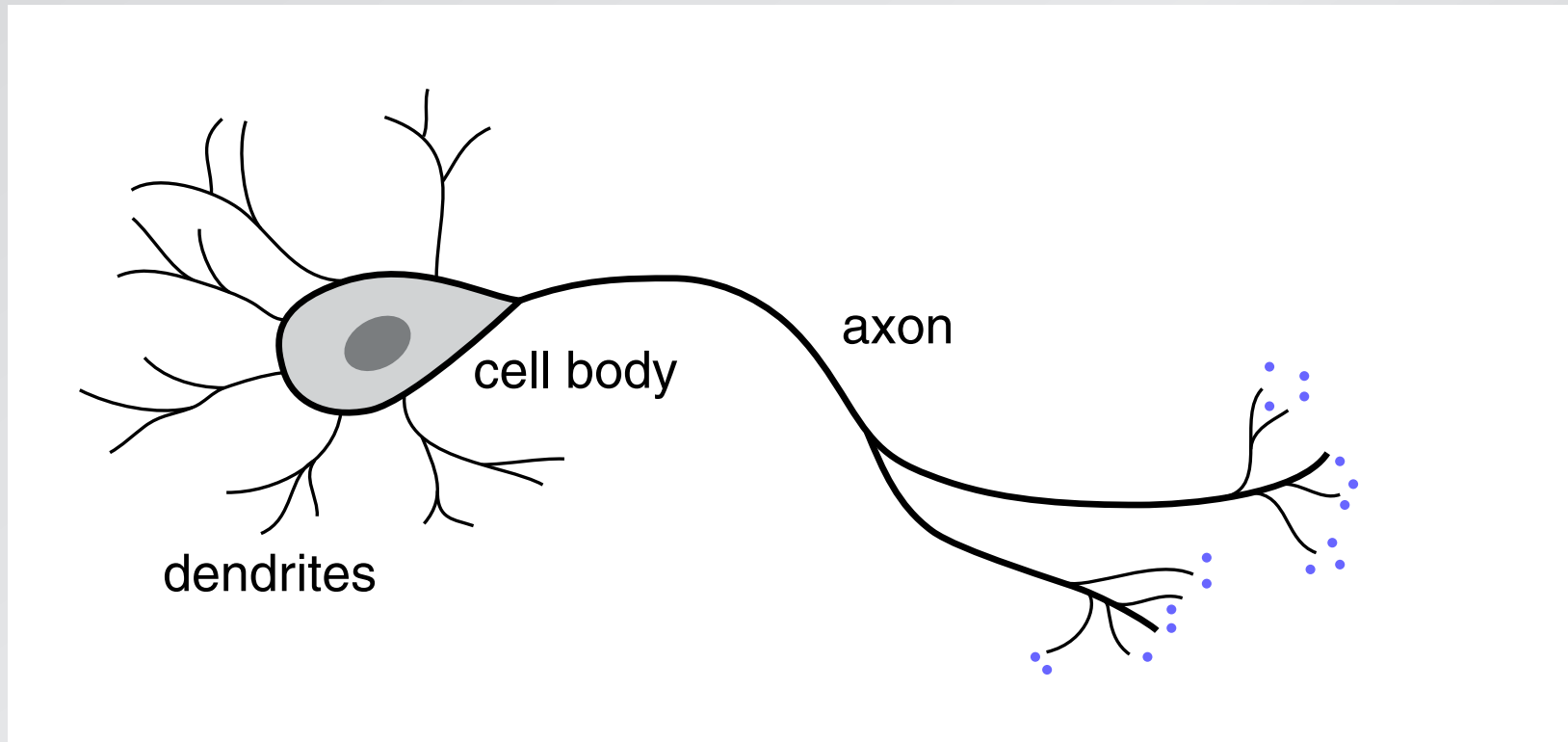
Nanoneurosurgery

neuron basics



Nanoneurosurgery

neuron basics



Nanoneurosurgery

Caenorhabditis elegans



Juergen Berger & Ralph Sommer
Max-Planck Institute for Developmental Biology

Nanoneurosurgery

Caenorhabditis elegans

- simple model organism
- similarities to higher organisms
- genome fully sequenced
- easy to handle

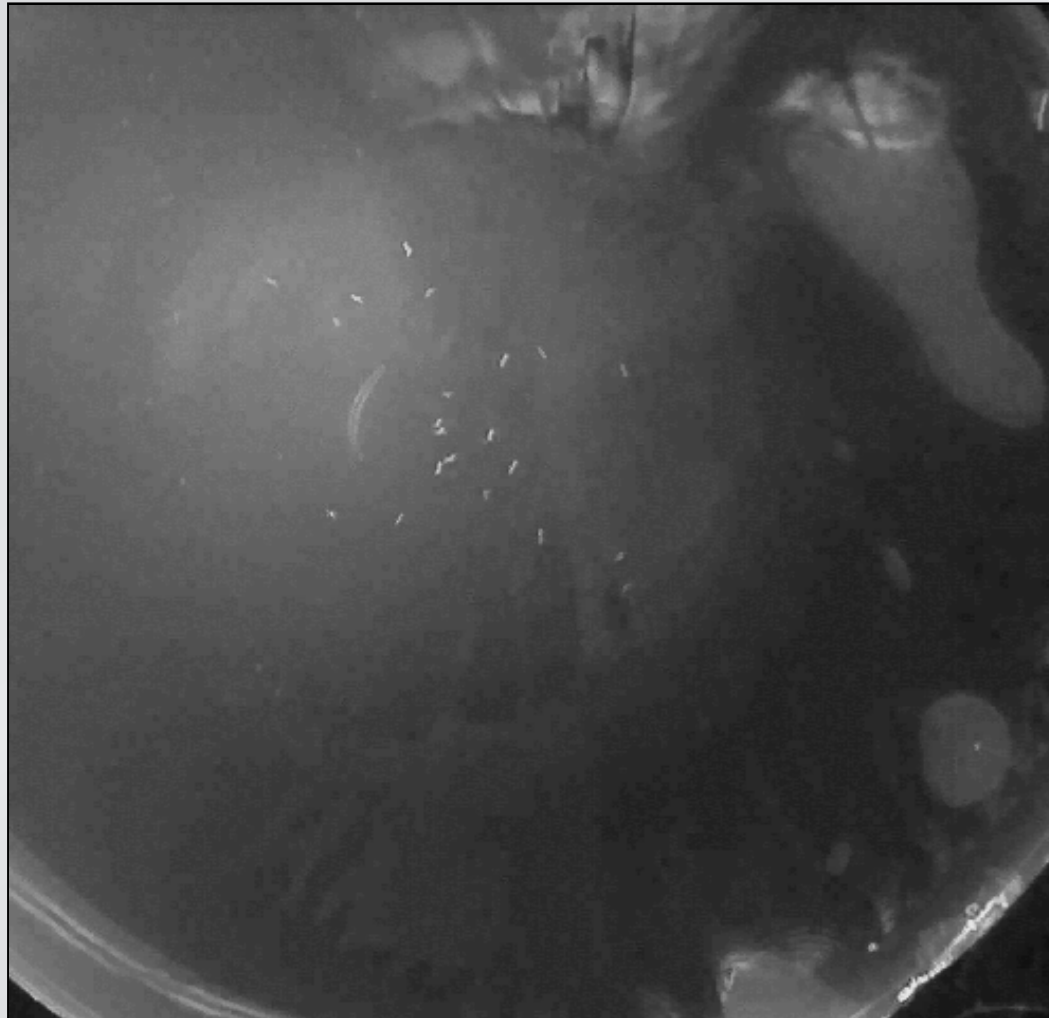
Nanoneurosurgery

Caenorhabditis elegans

- 80 μm x 1 mm
- about 1000 cells
- 302 neurons
- invariant wiring diagram
- neuronal system completely encodes behavior

Nanoneurosurgery

Caenorhabditis elegans



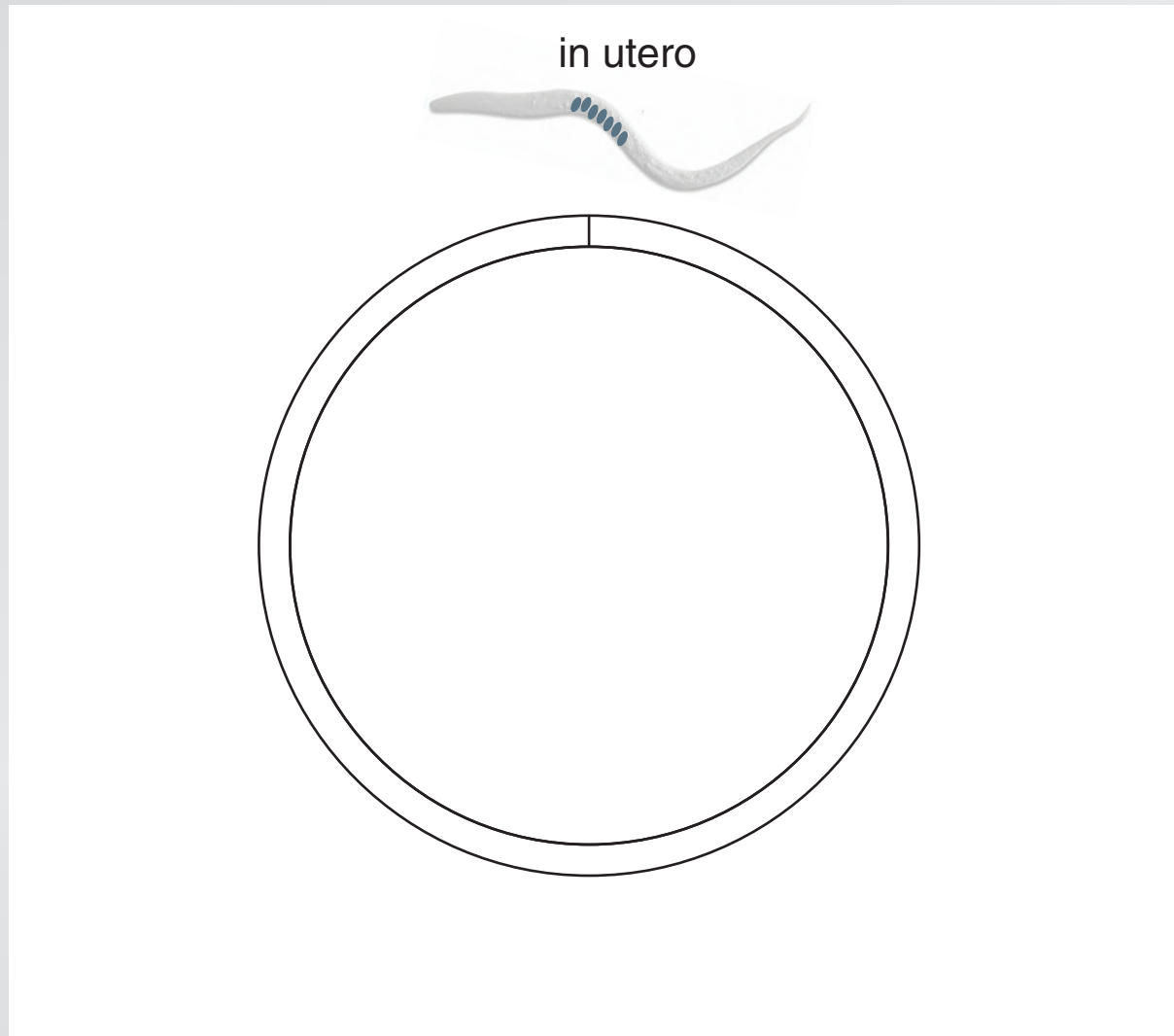
Nanoneurosurgery

Caenorhabditis elegans



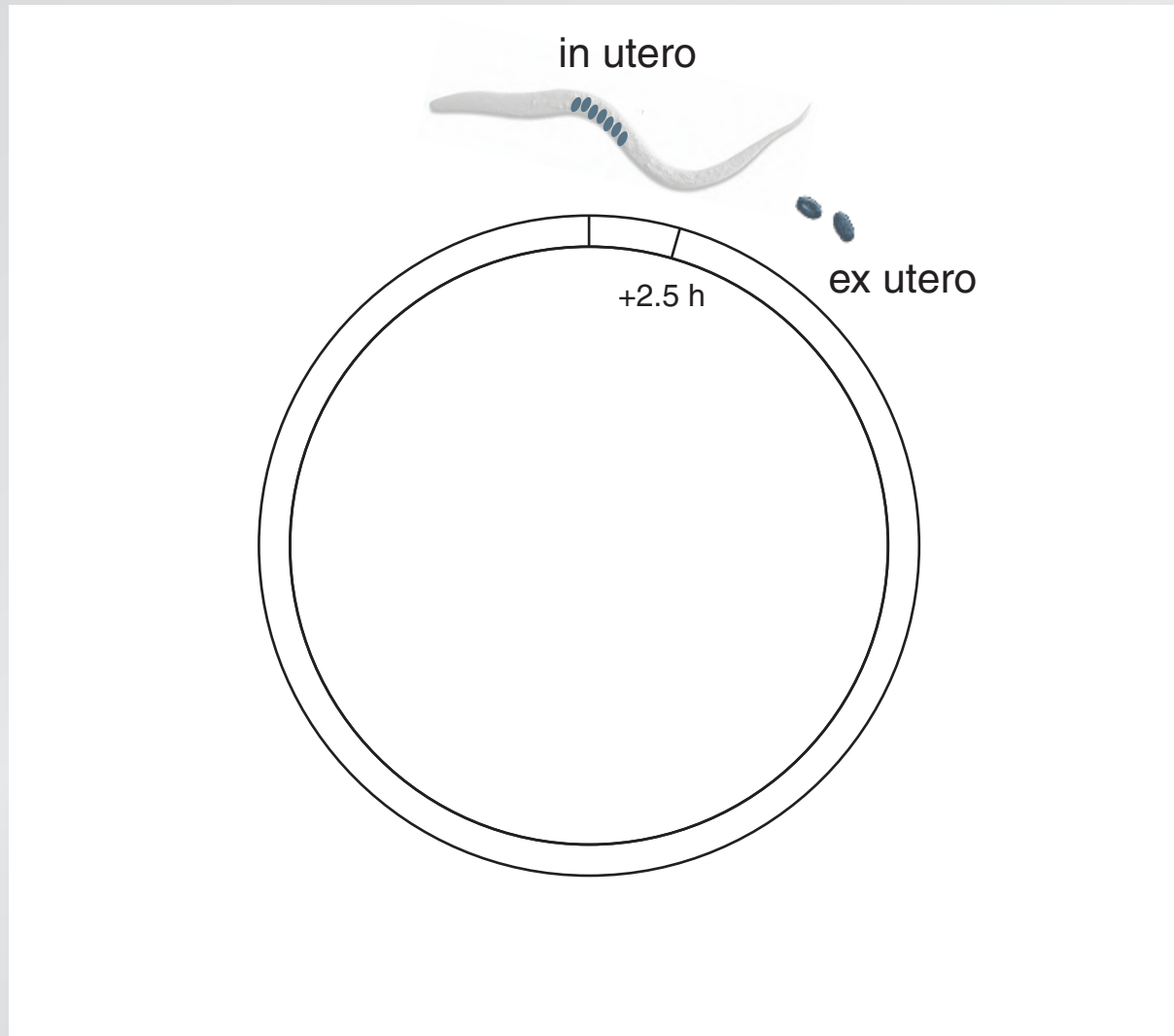
Nanoneurosurgery

C. elegans life cycle



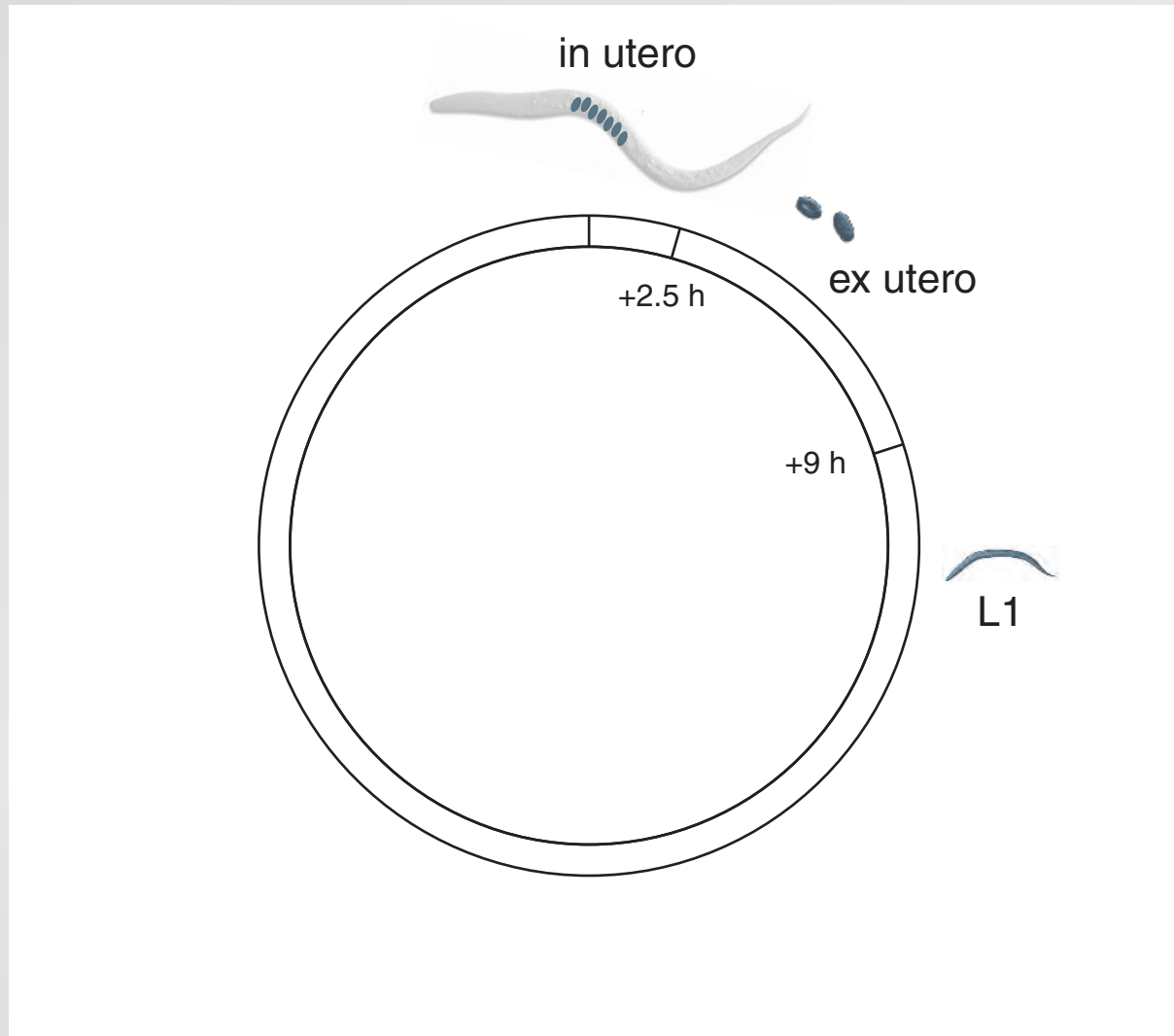
Nanoneurosurgery

C. elegans life cycle



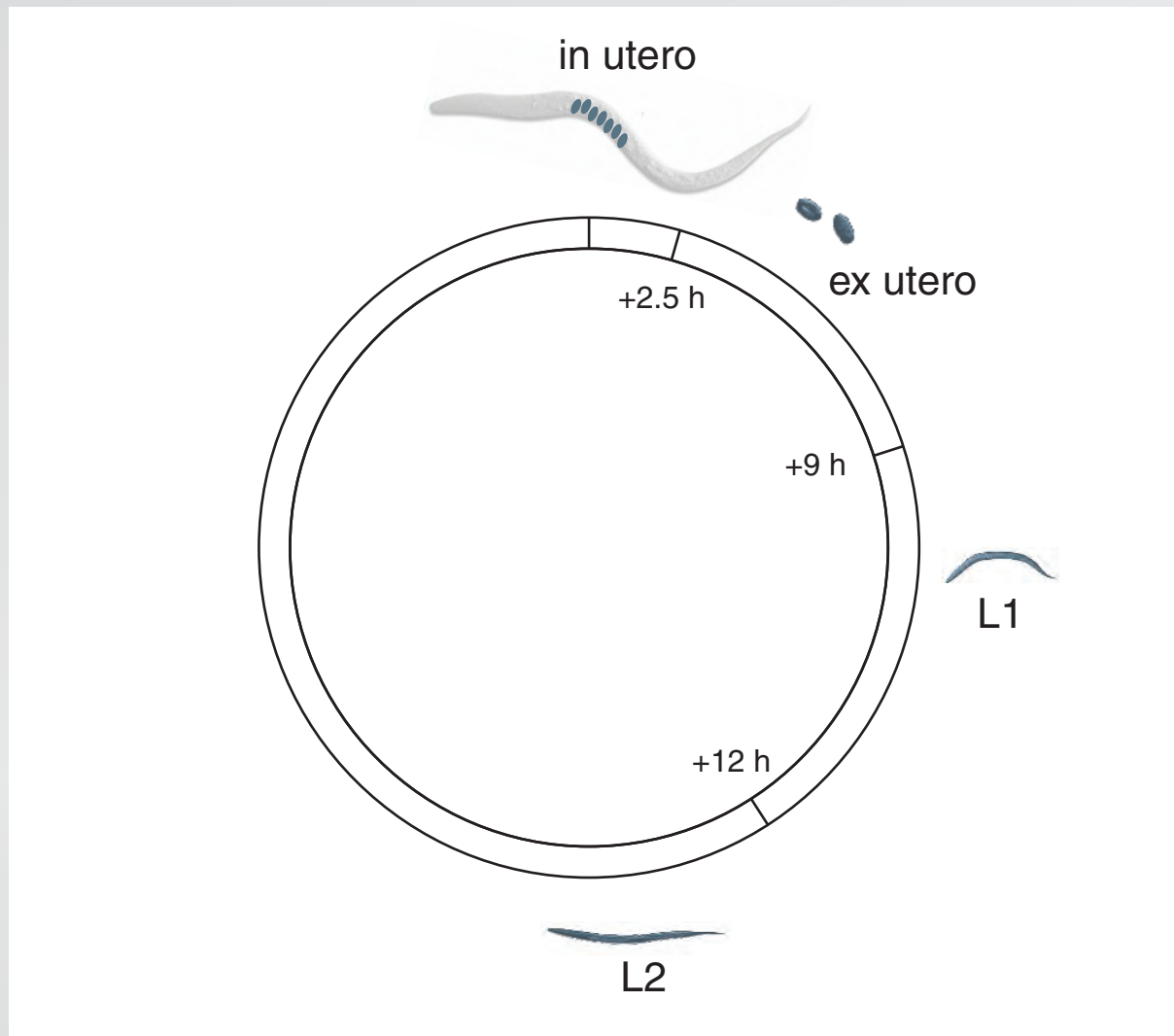
Nanoneurosurgery

C. elegans life cycle



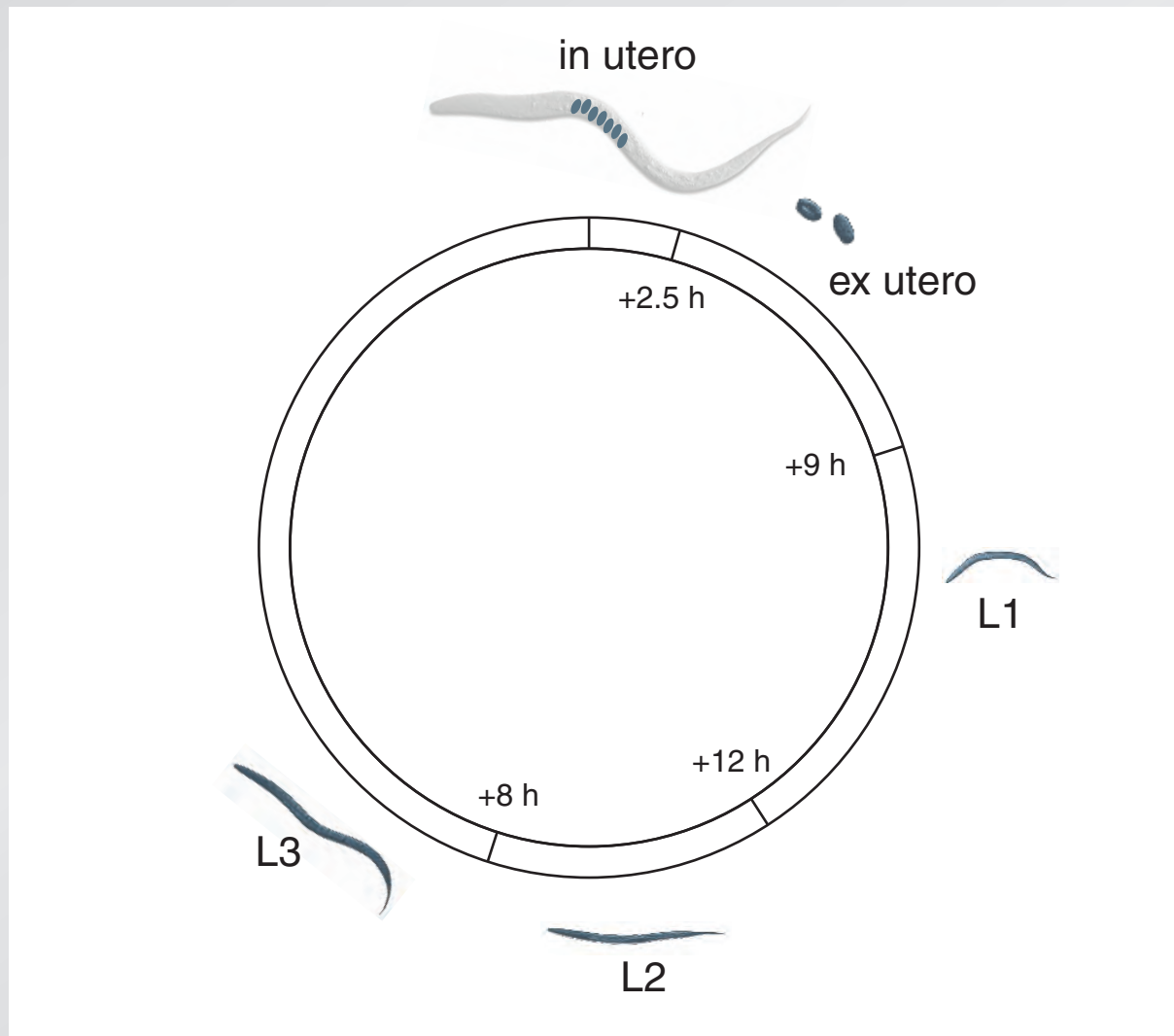
Nanoneurosurgery

C. elegans life cycle



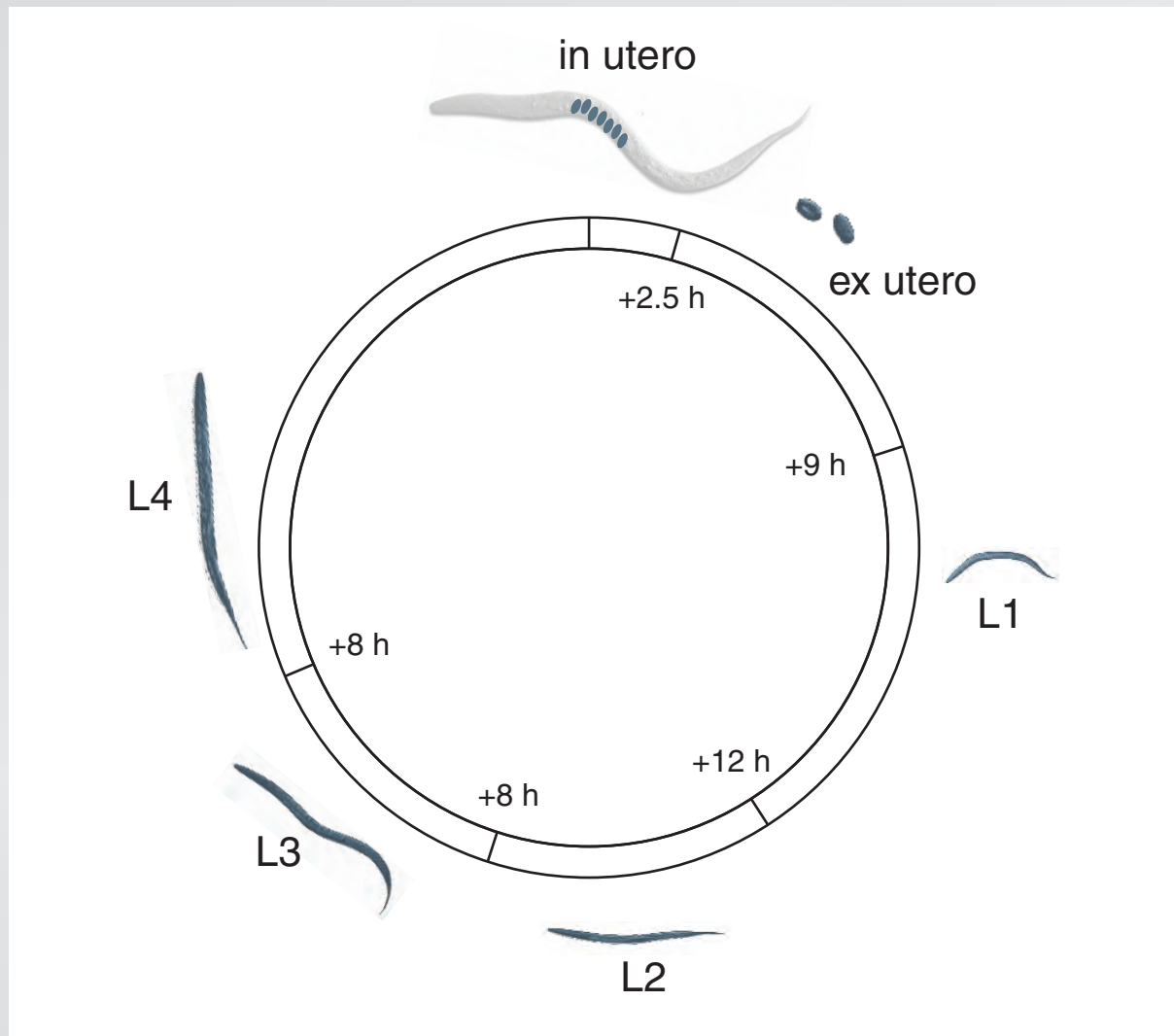
Nanoneurosurgery

C. elegans life cycle



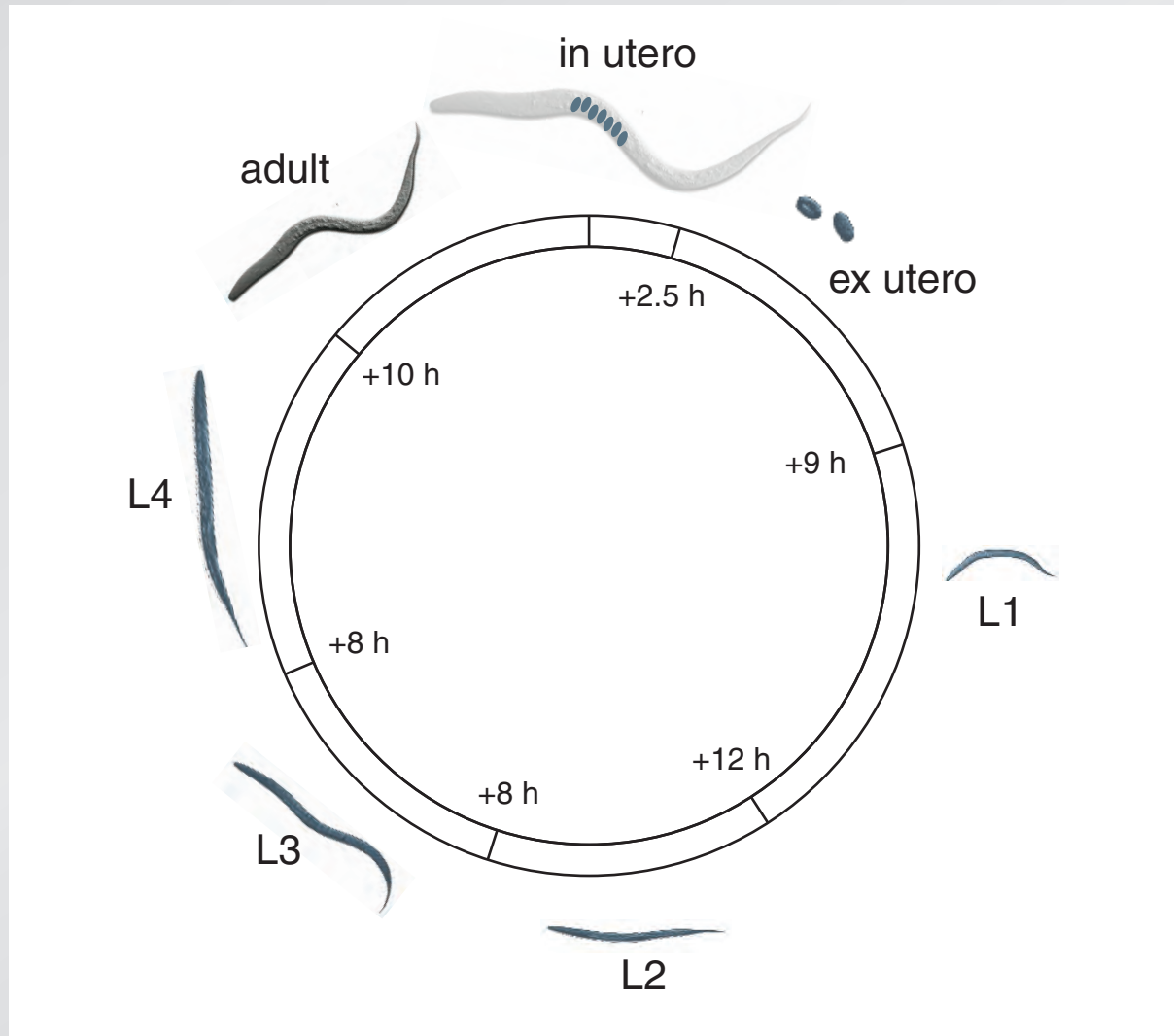
Nanoneurosurgery

C. elegans life cycle



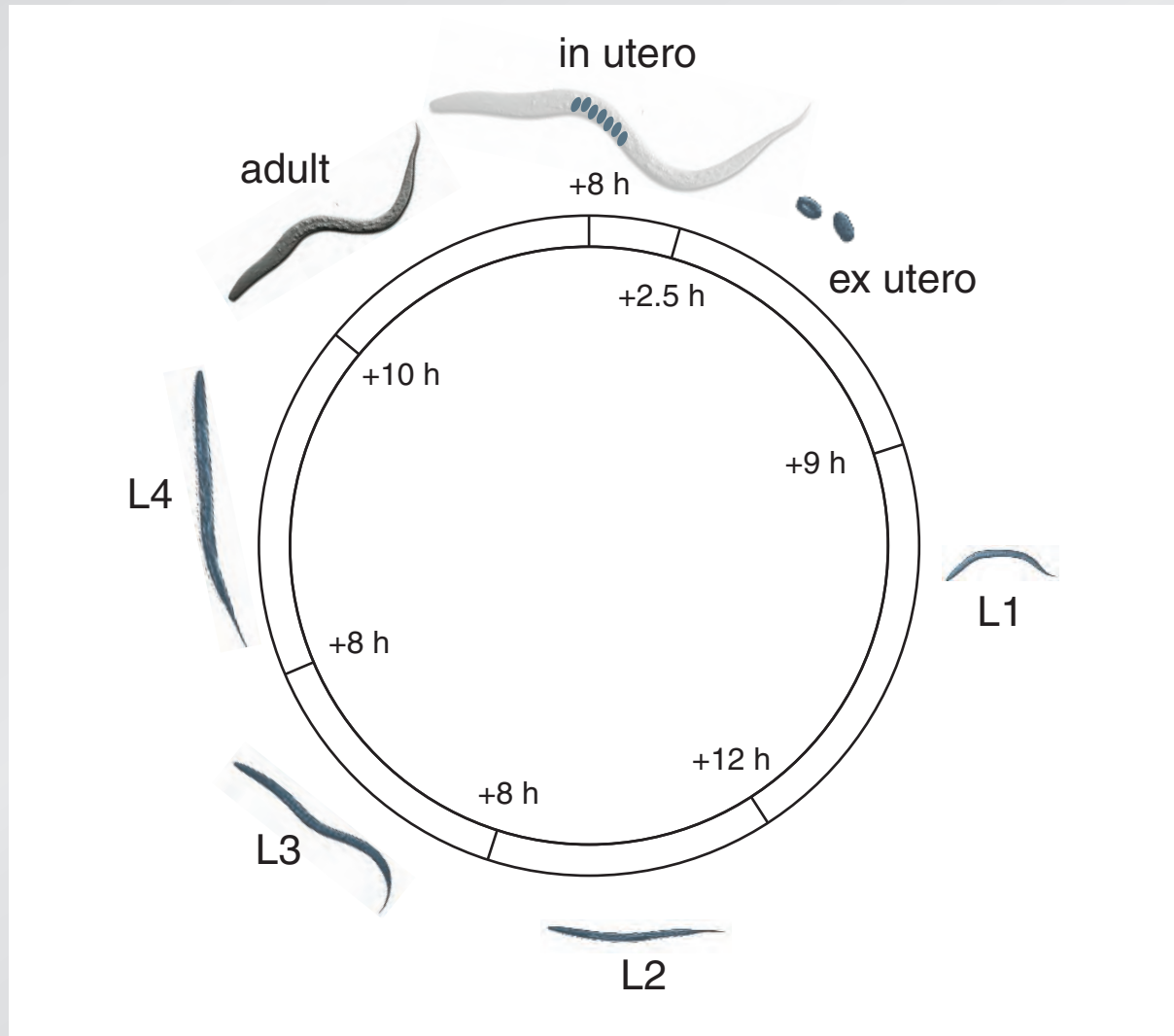
Nanoneurosurgery

C. elegans life cycle



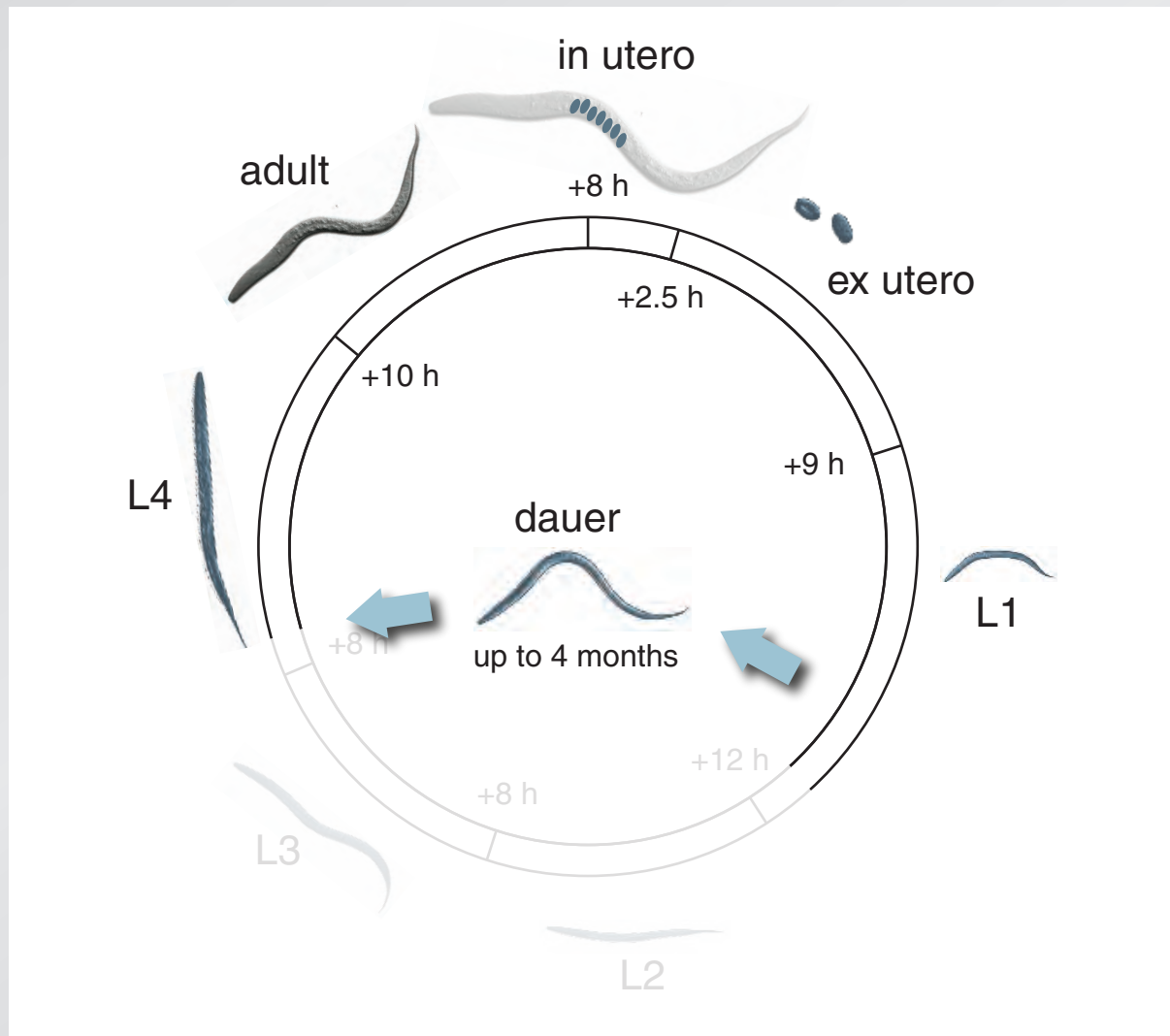
Nanoneurosurgery

C. elegans life cycle



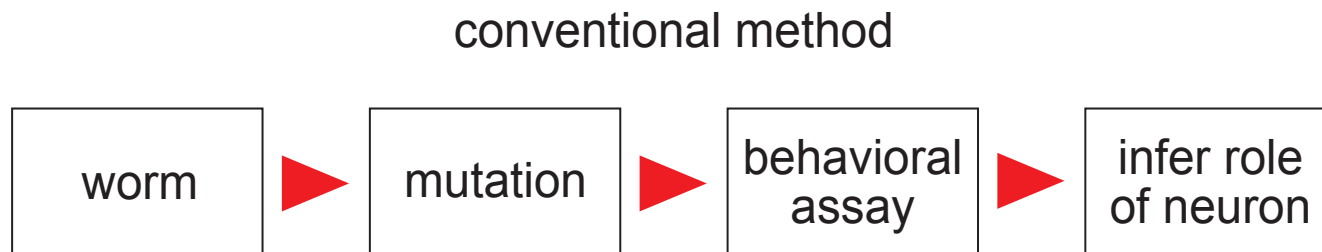
Nanoneurosurgery

C. elegans life cycle



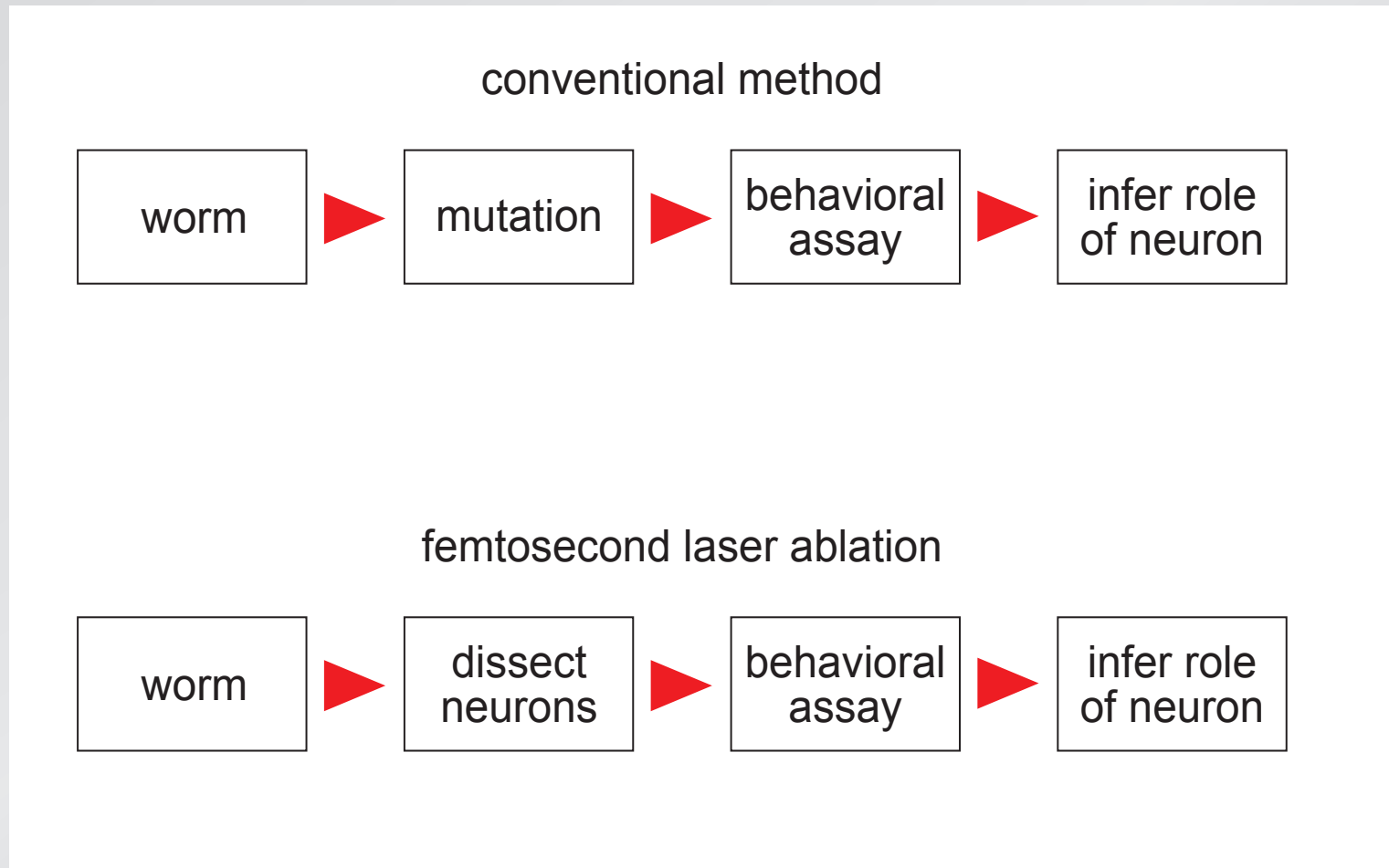
Nanoneurosurgery

Mapping behavior to neurons



Nanoneurosurgery

Mapping behavior to neurons



Nanoneurosurgery

ASH neurons

- responsible for chemical sensing
- ciliary projections extend through skin
- one on each side

Nanoneurosurgery

ASH neurons



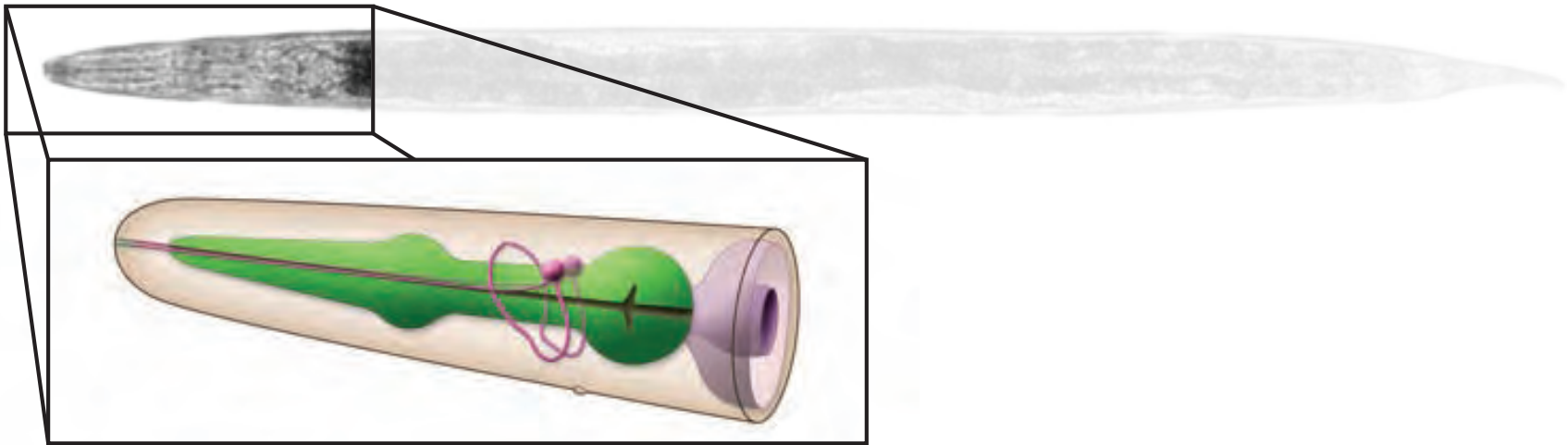
Nanoneurosurgery

ASH neurons



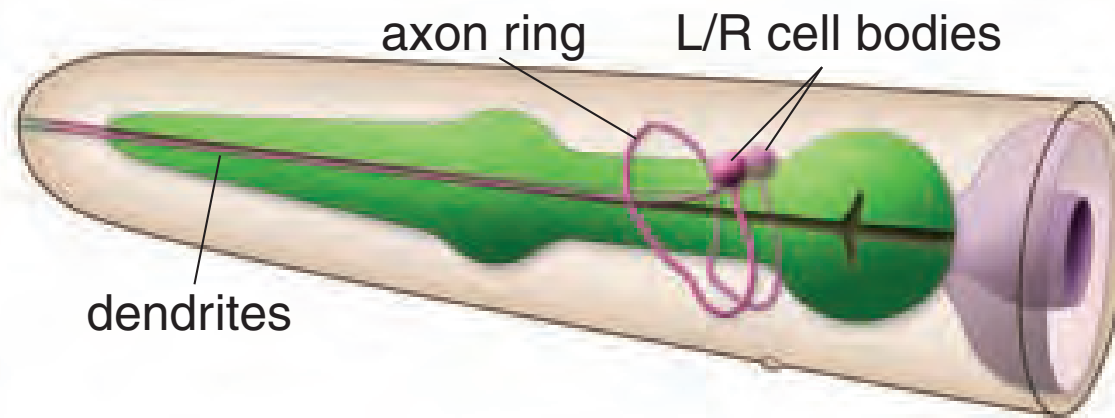
Nanoneurosurgery

ASH neurons



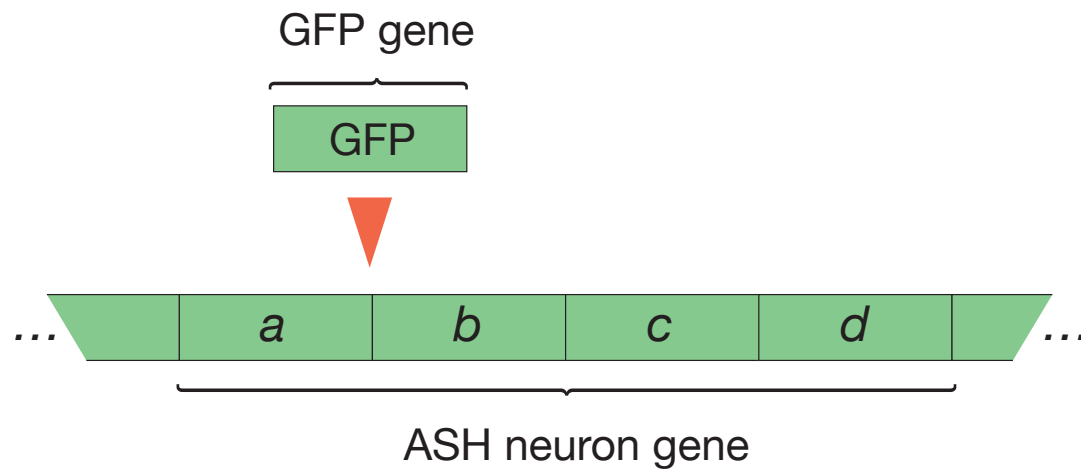
Nanoneurosurgery

ASH neurons



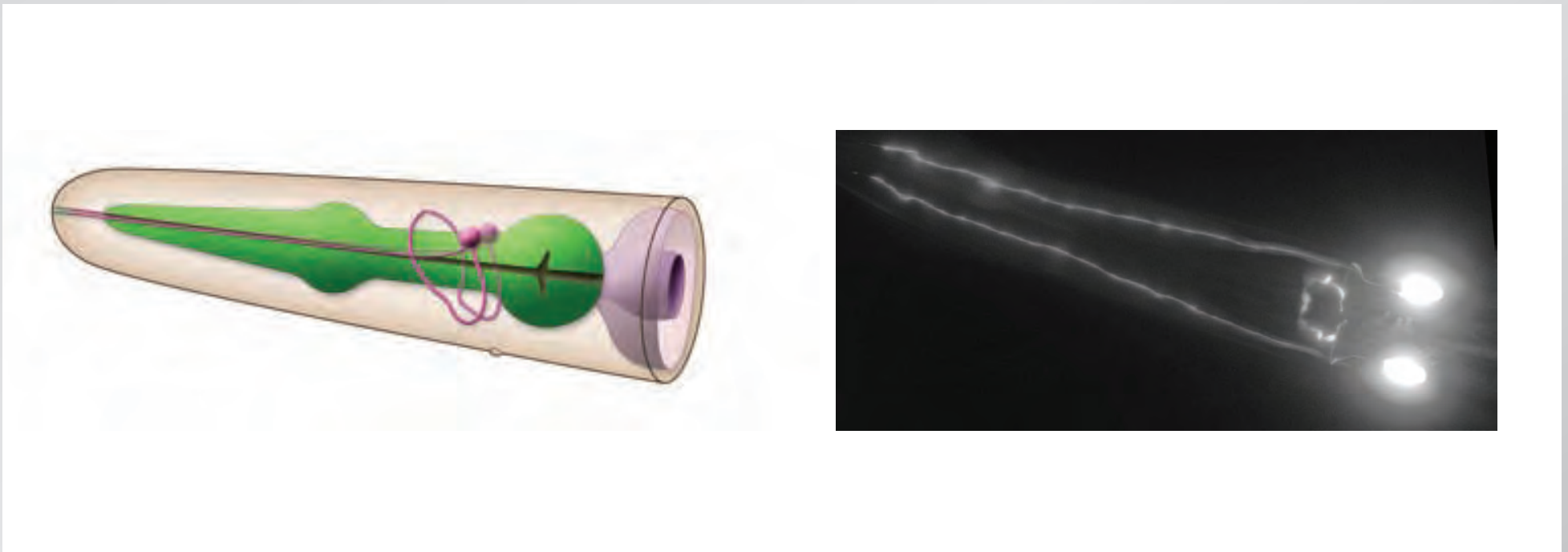
Nanoneurosurgery

make ASH neurons express GFP



Nanoneurosurgery

make ASH neurons express GFP



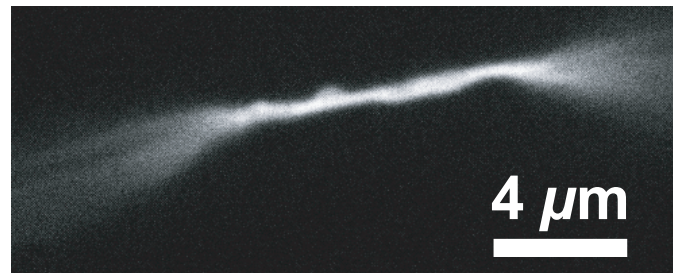
Nanoneurosurgery

GFP: absorbs UV, emits green



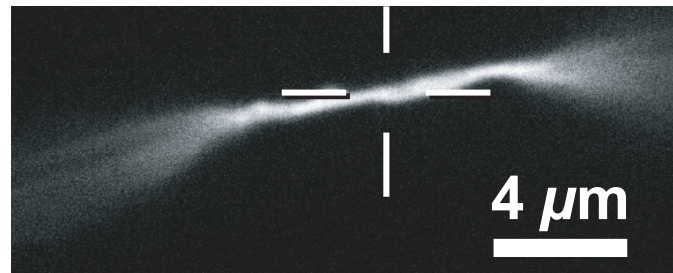
Nanoneurosurgery

retraction of cut dendrite (6 nJ)



Nanoneurosurgery

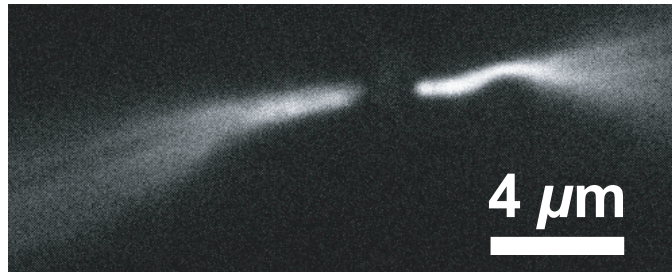
retraction of cut dendrite (6 nJ)



Nanoneurosurgery

retraction of cut dendrite (6 nJ)

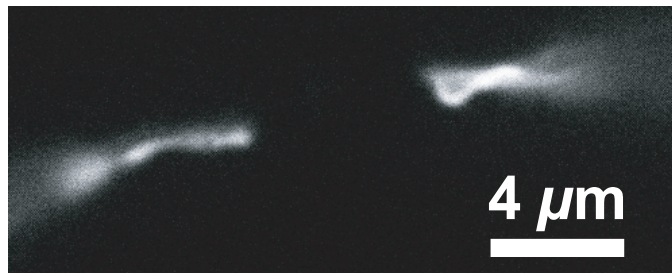
$t = 30 \text{ s}$



Nanoneurosurgery

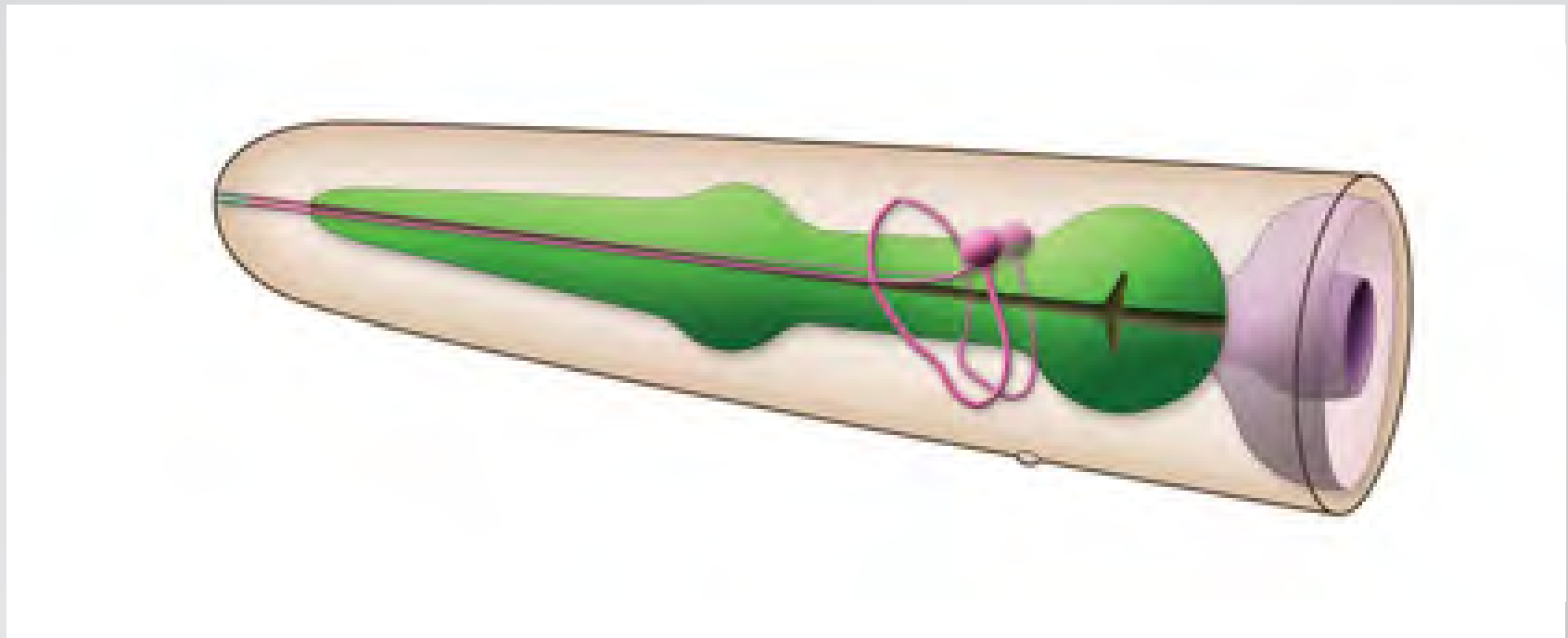
retraction of cut dendrite (6 nJ)

$t = 3 \text{ min}$



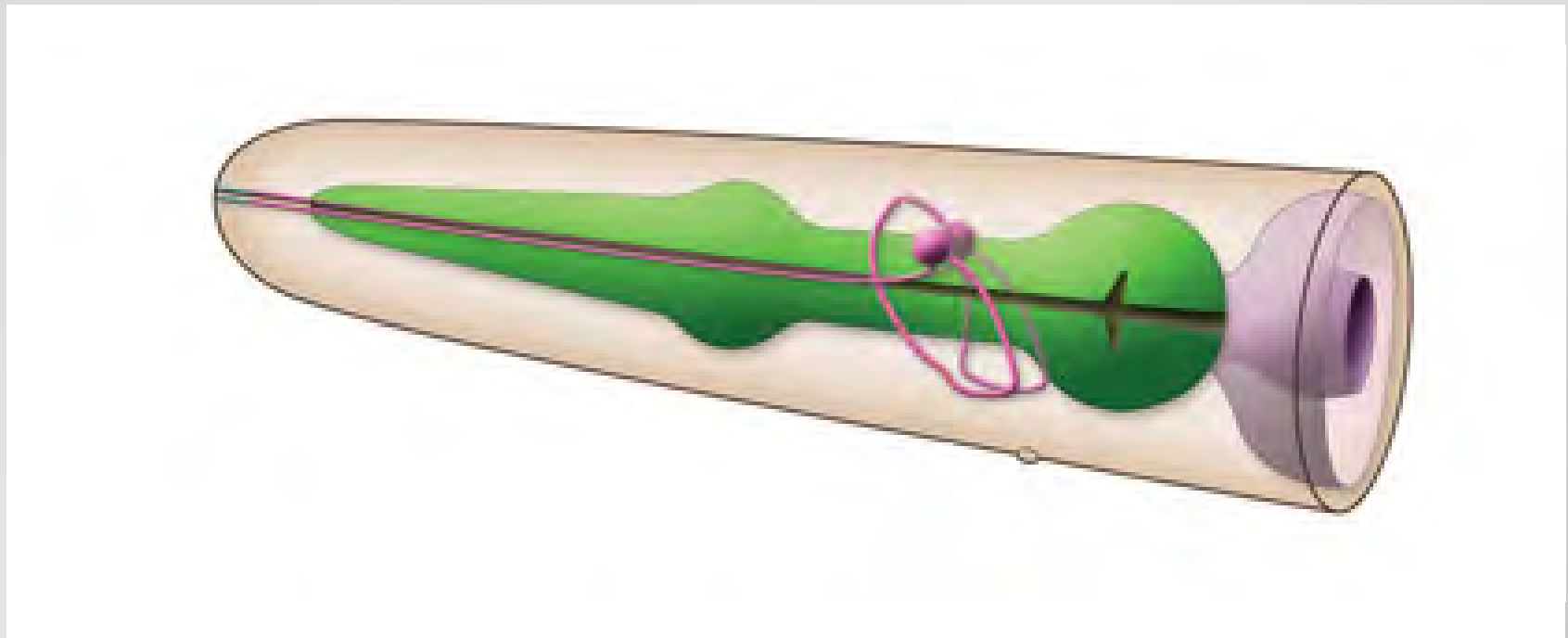
Nanoneurosurgery

ASH neurons



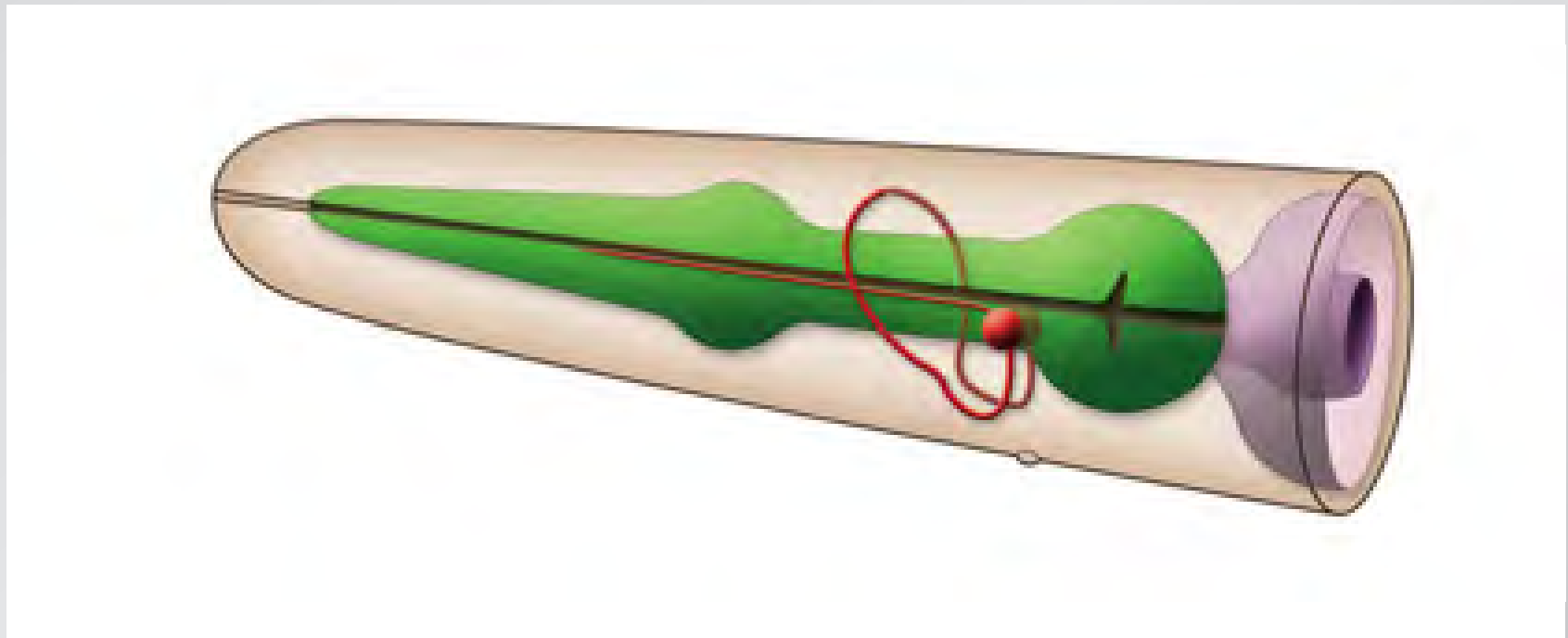
Nanoneurosurgery

ASK neurons



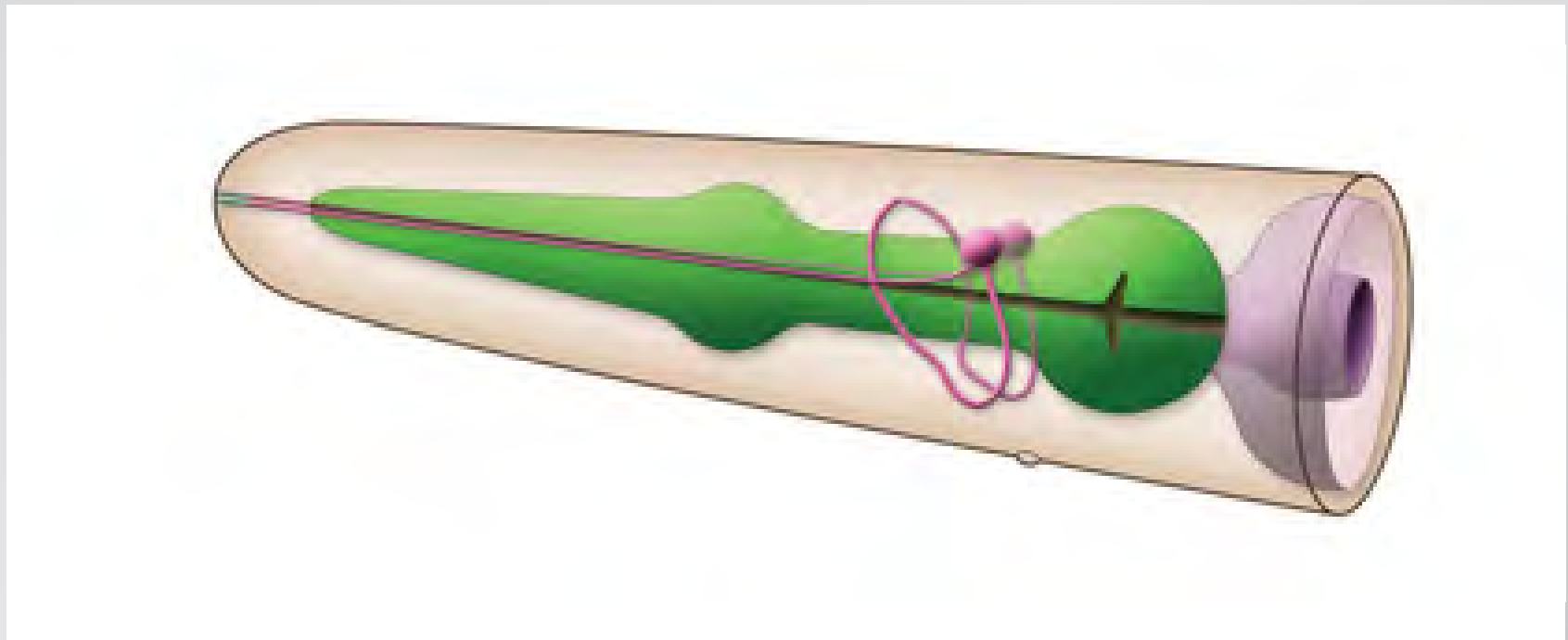
Nanoneurosurgery

AUA neurons



Nanoneurosurgery

ASI neurons

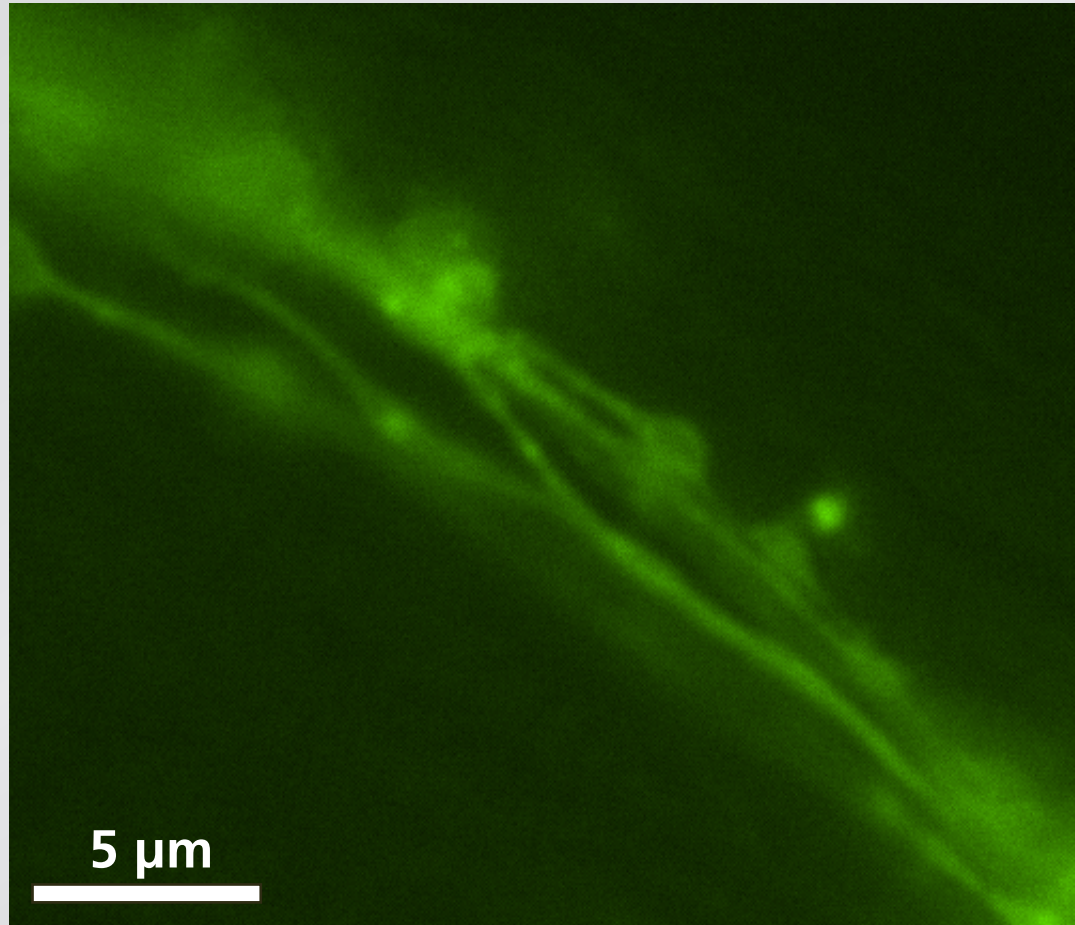


Nanoneurosurgery

need exquisite precision!

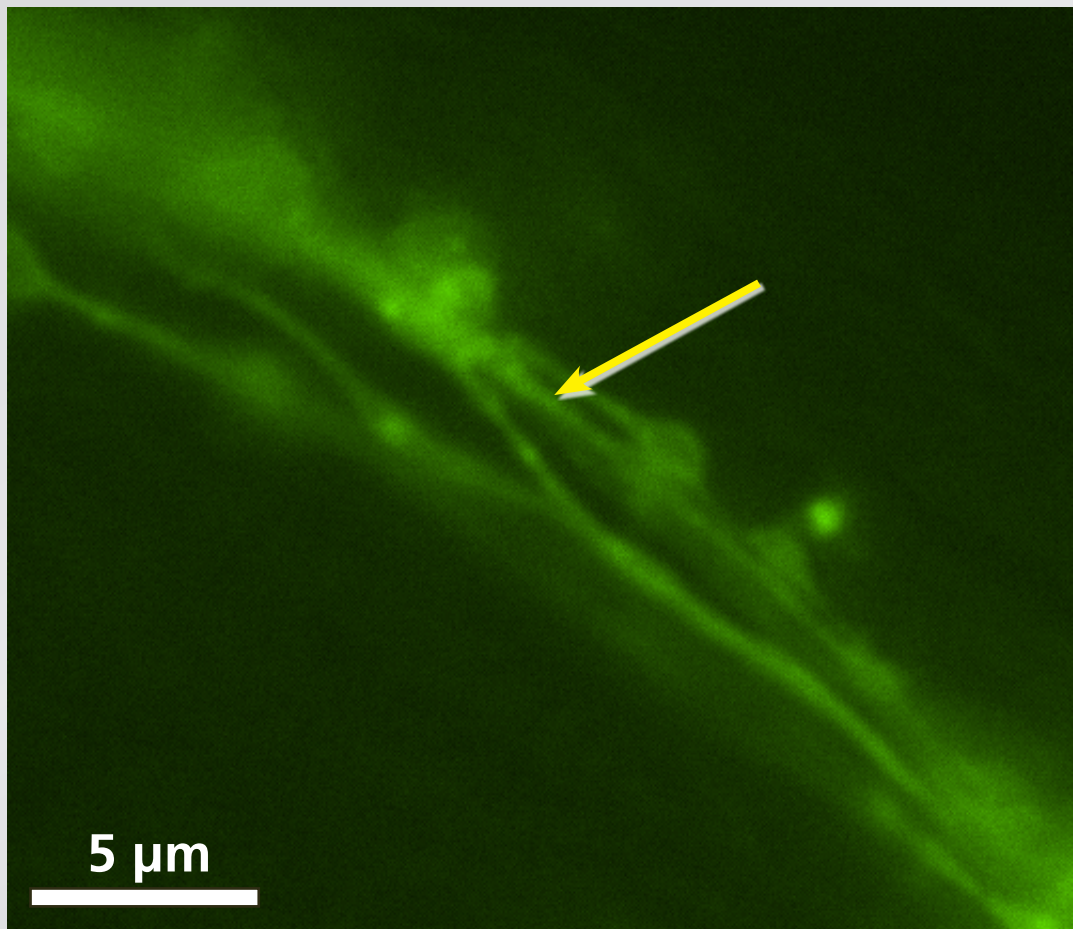
Nanoneurosurgery

DiO-stained bundle of dendrites



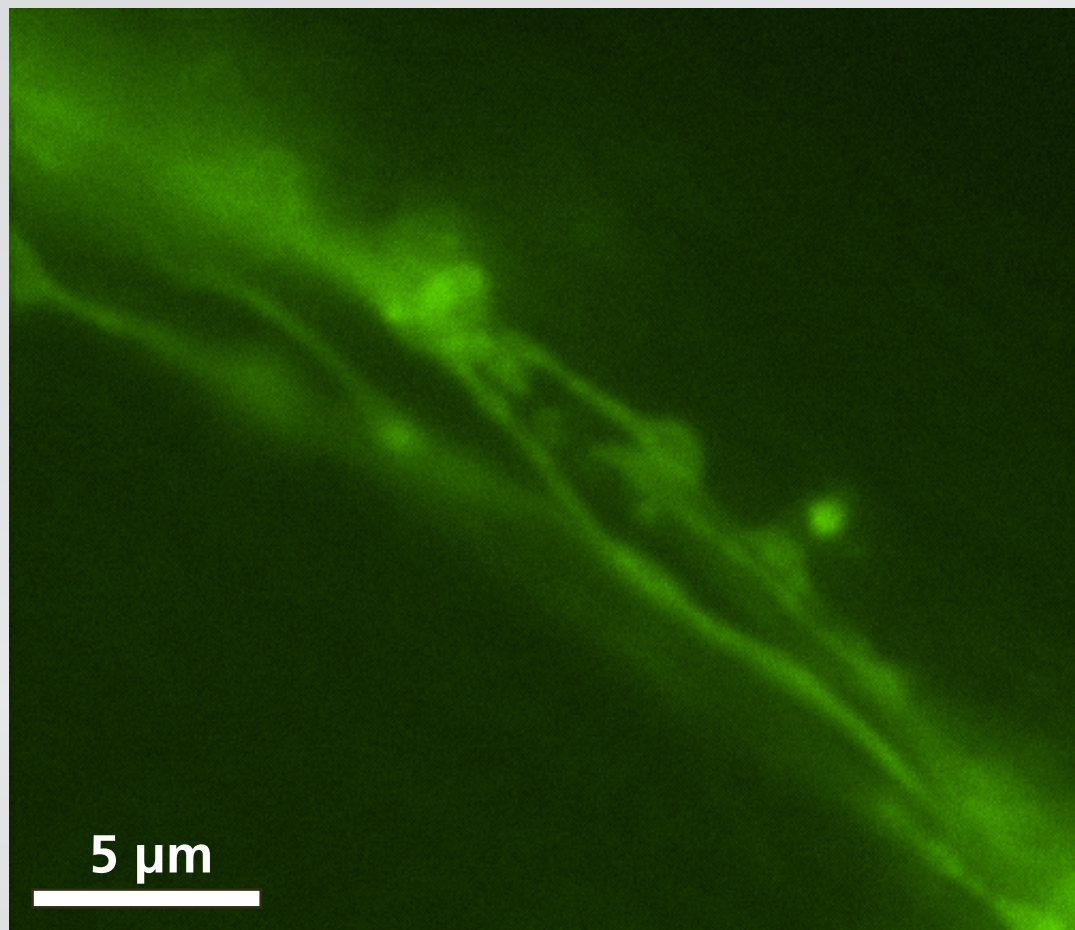
Nanoneurosurgery

cut single dendrite in bundle (3 nJ)



Nanoneurosurgery

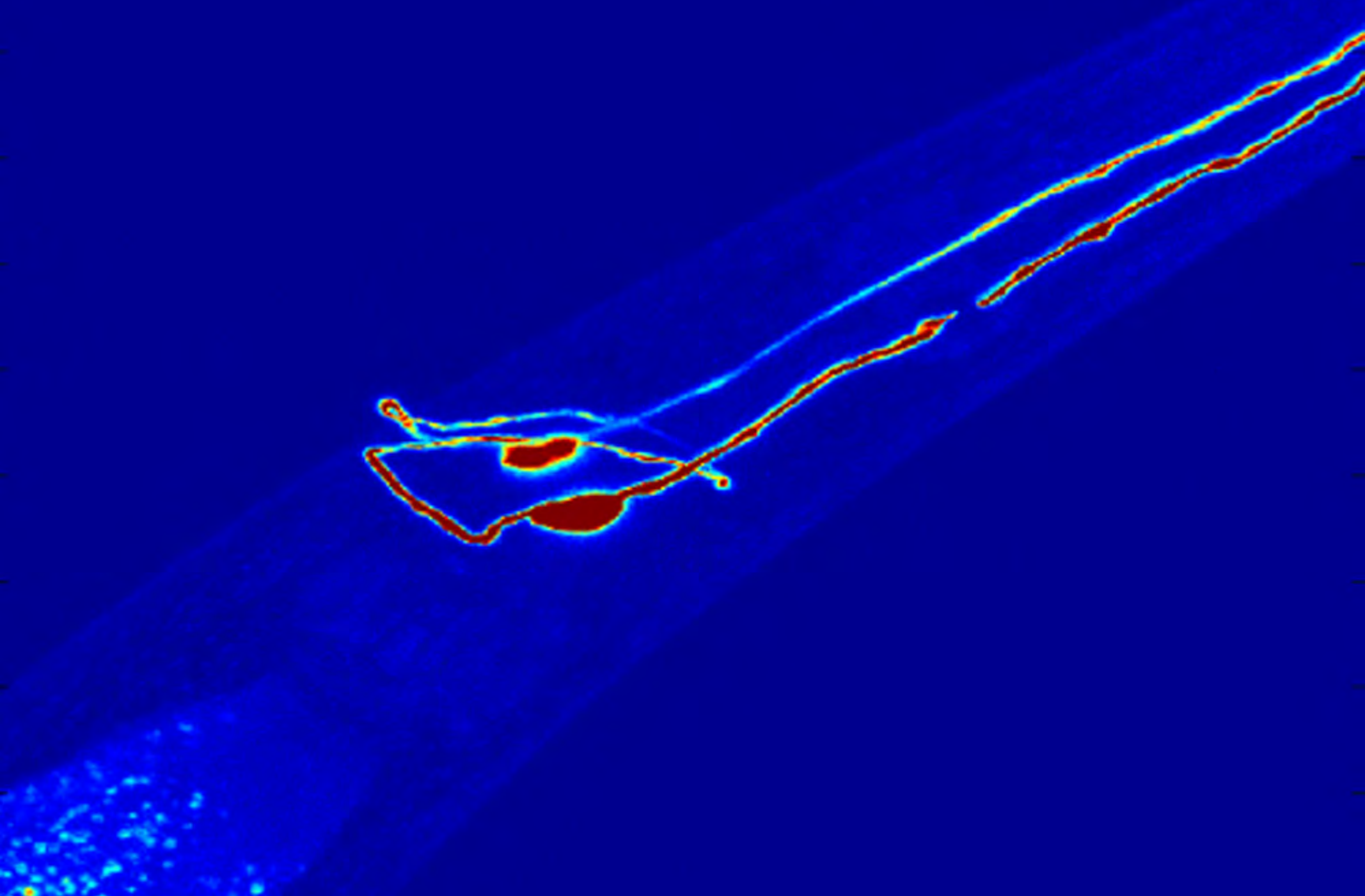
no damage to neighboring dendrites



Nanoneurosurgery

revive worm, reimage 1 day later

Nanoneurosurgery



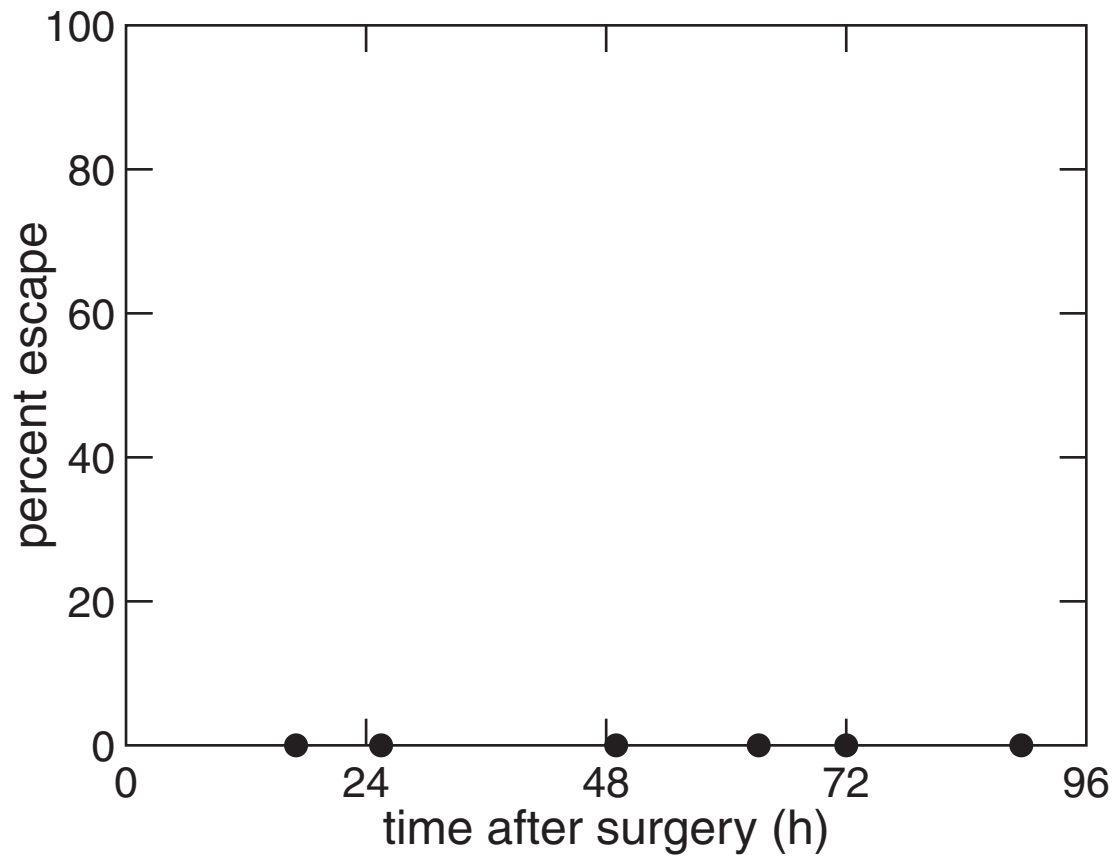
Nanoneurosurgery

osmolarity assay



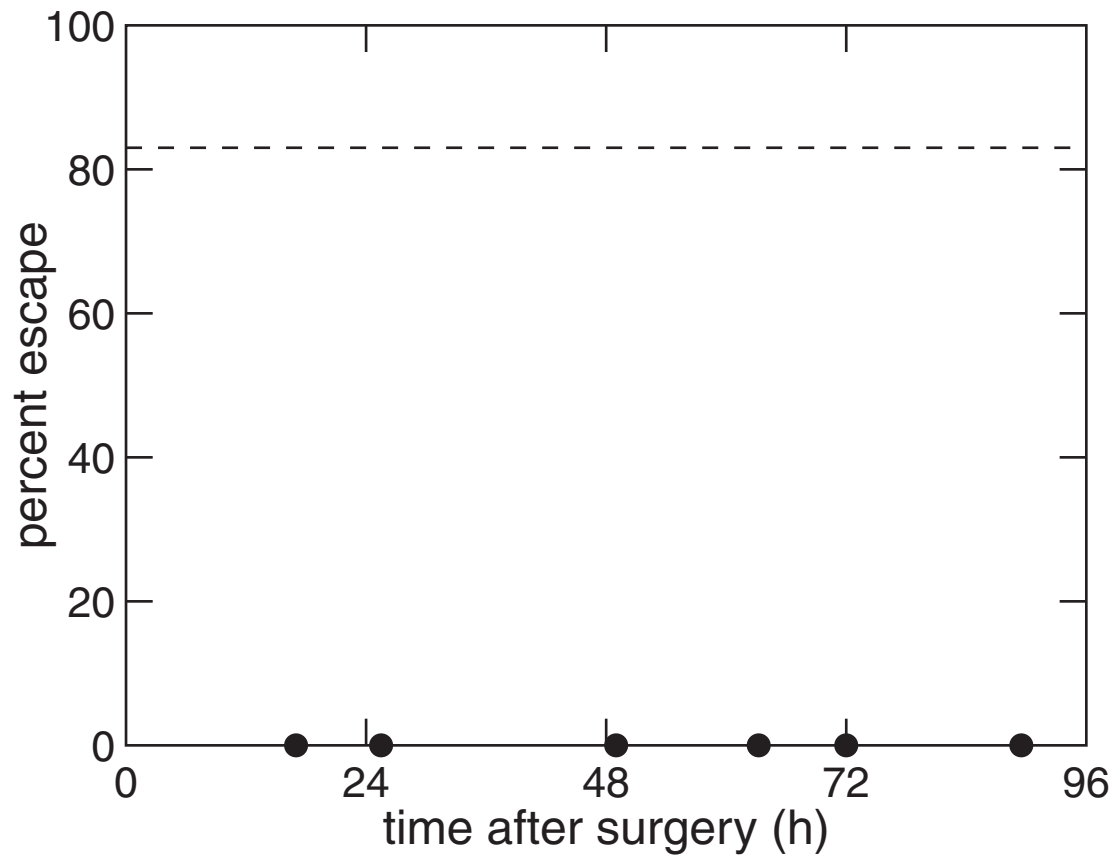
Nanoneurosurgery

escape rate after 'mock' surgery



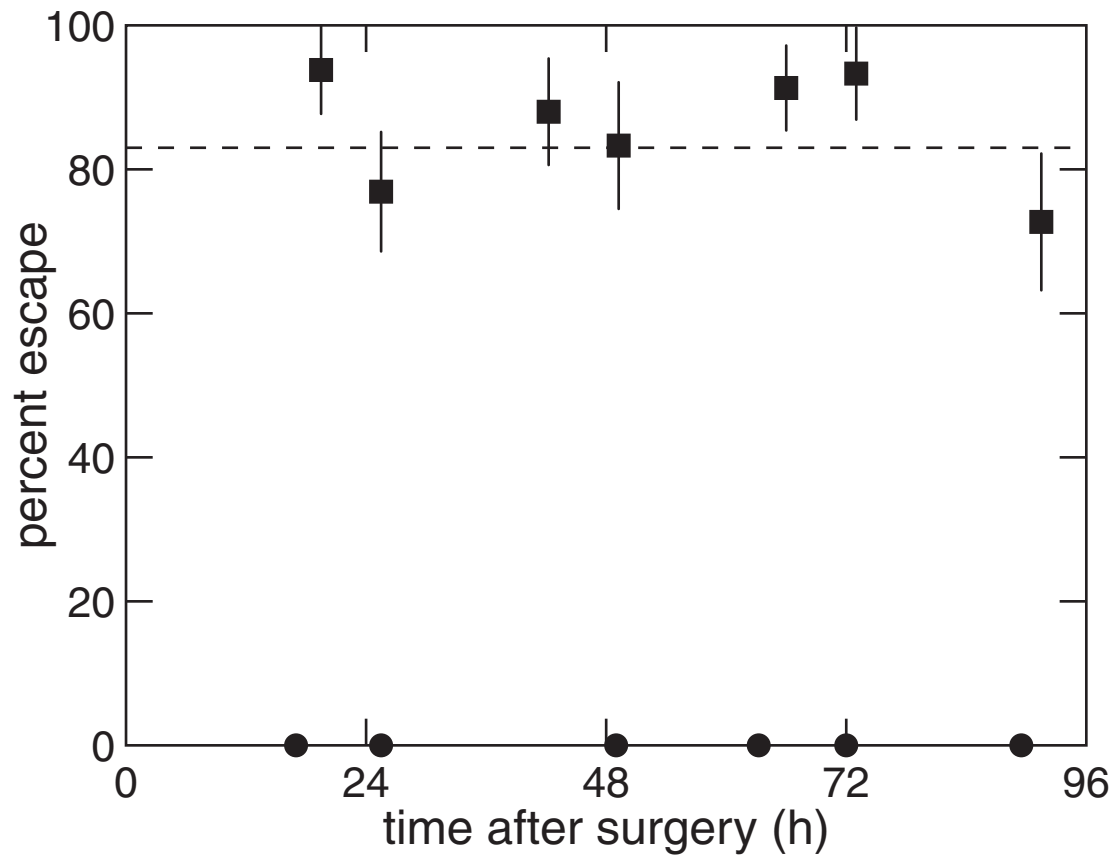
Nanoneurosurgery

escape rate of ASH-lacking mutant



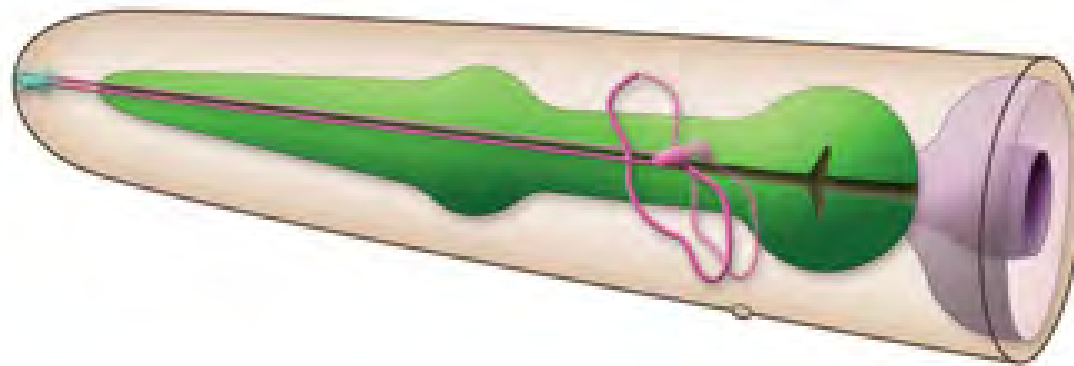
Nanoneurosurgery

escape rate after ASH-ablation surgery

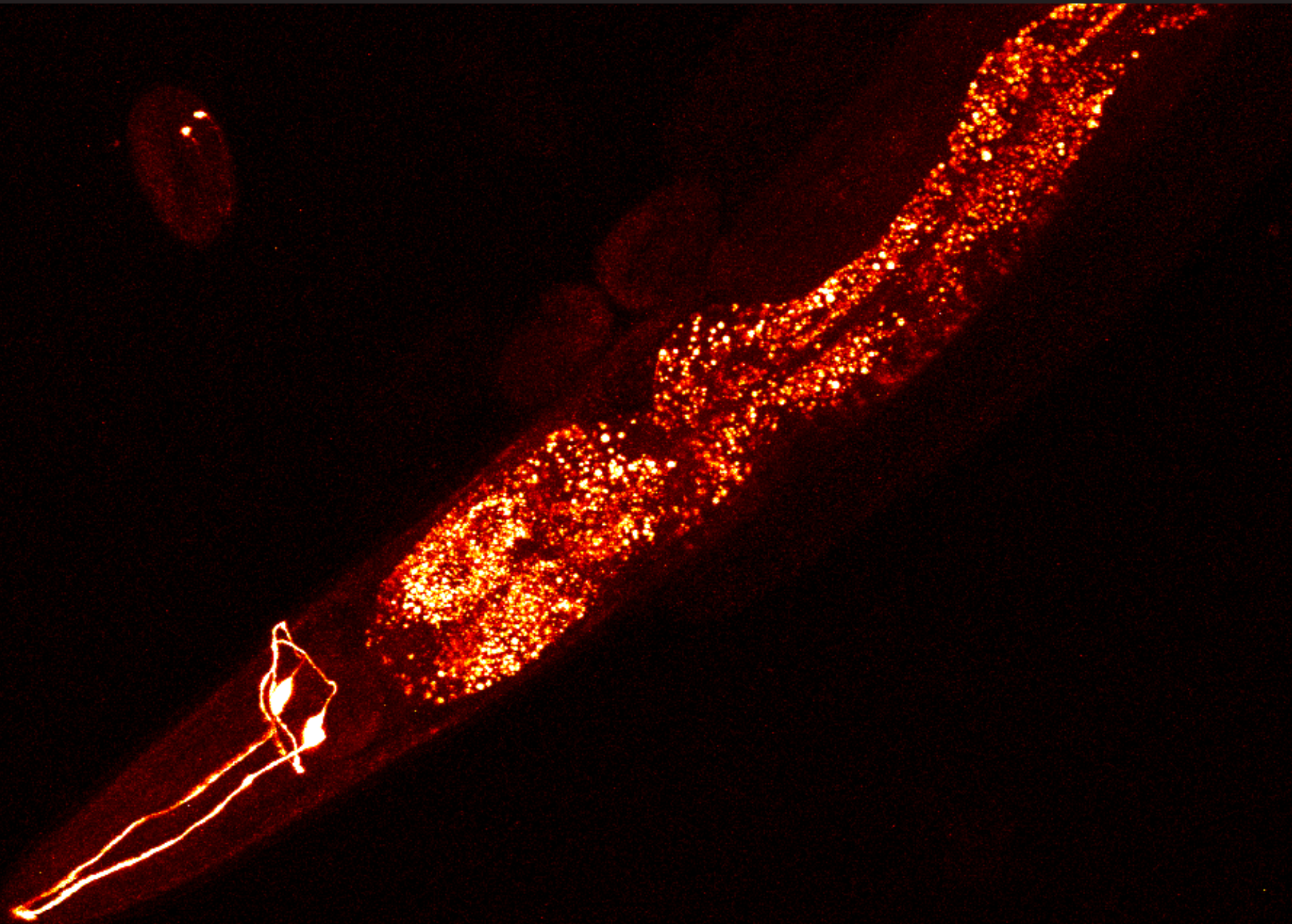


Nanoneurosurgery

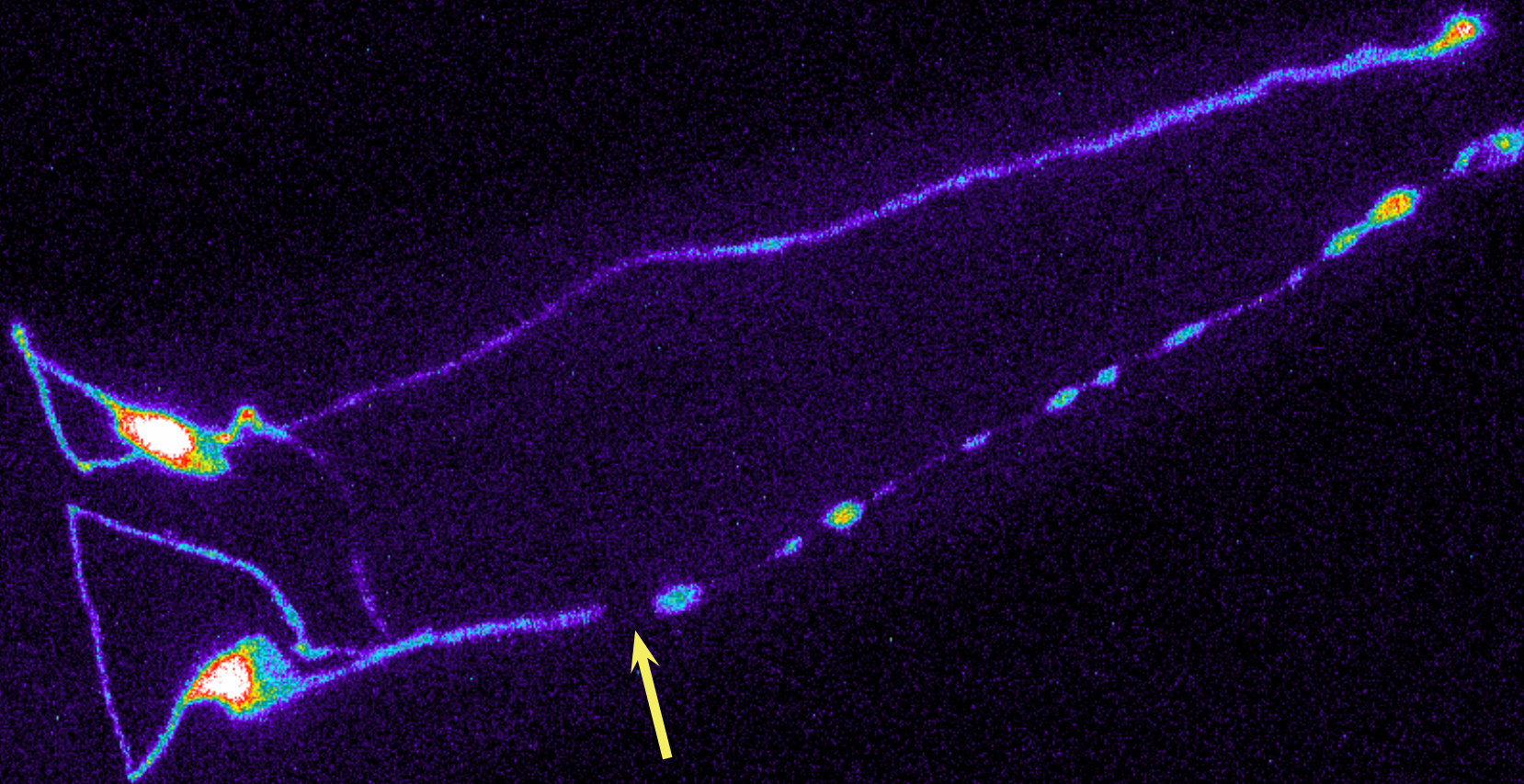
AFD neurons (temperature sensors)



Nanoneurosurgery



Nanoneurosurgery



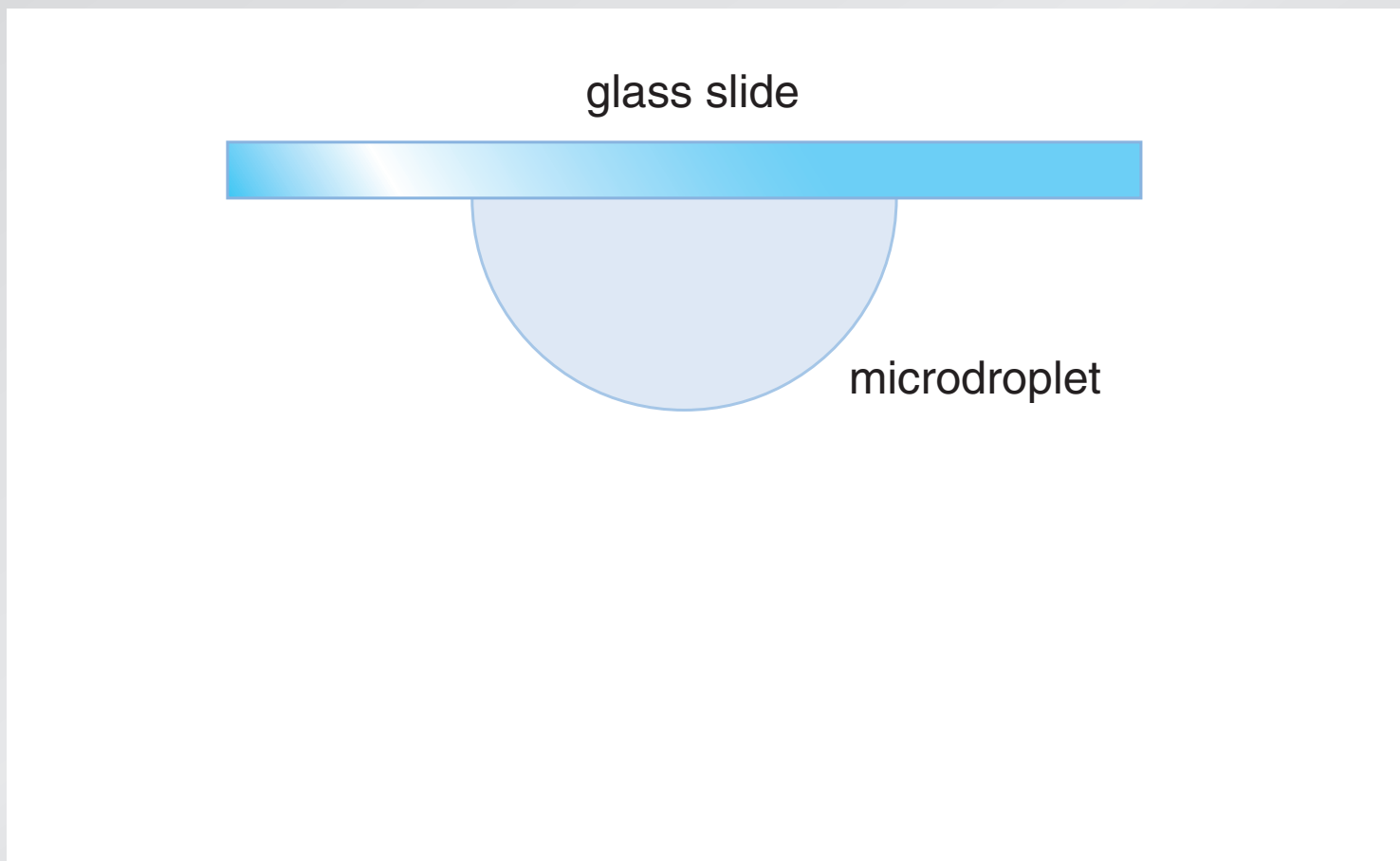
Nanoneurosurgery

Q: where does the ASH sense temperature?



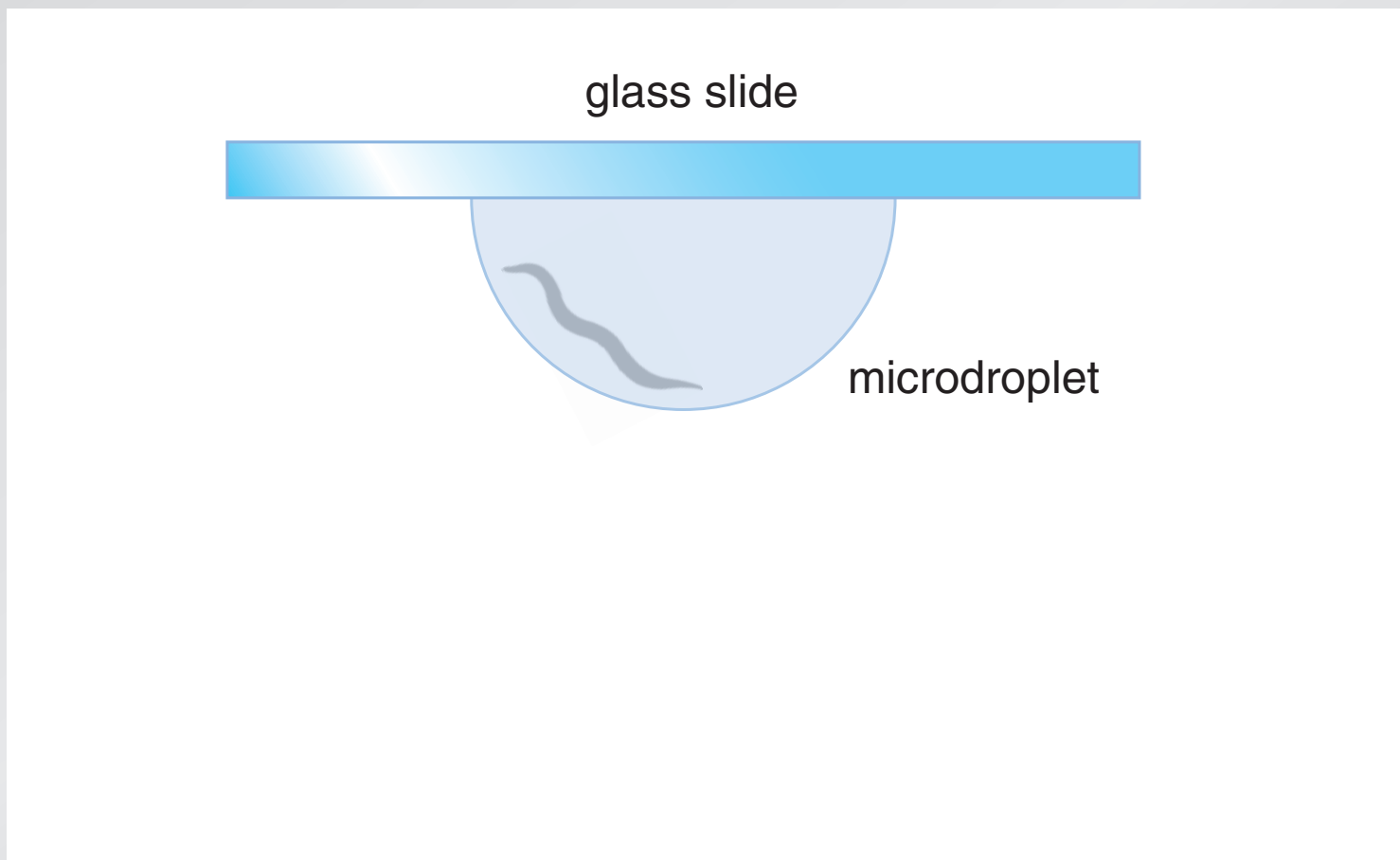
Nanoneurosurgery

microdroplet assay



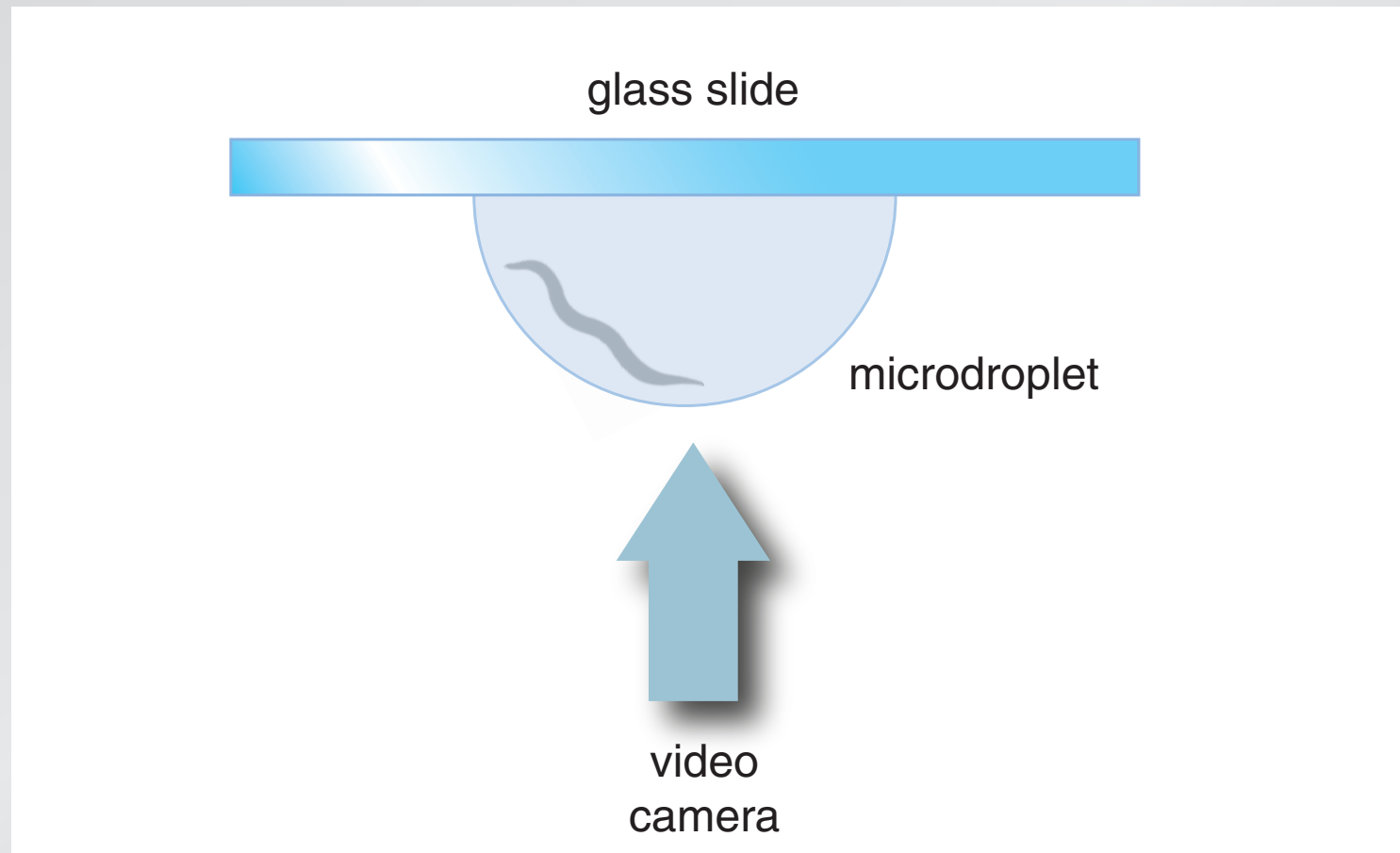
Nanoneurosurgery

microdroplet assay



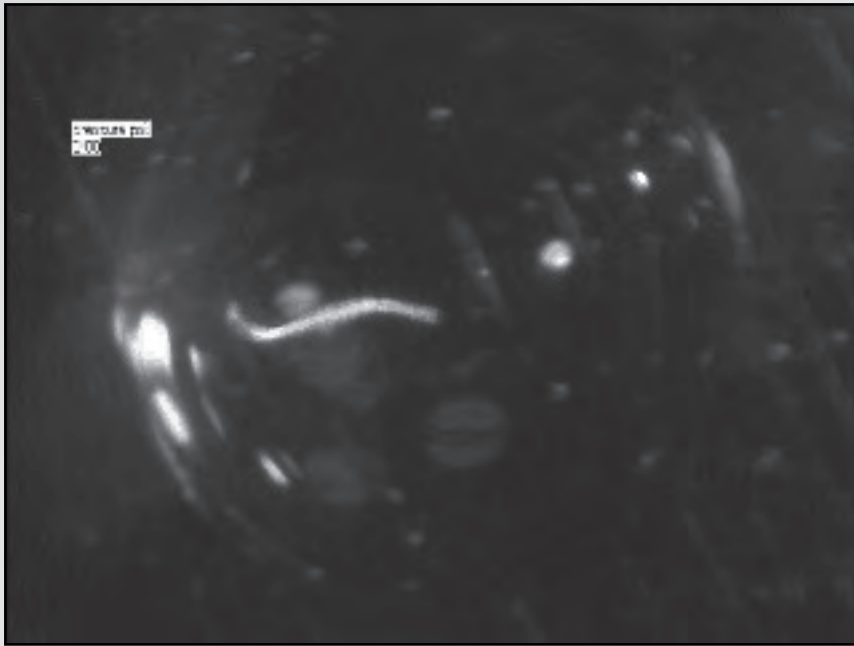
Nanoneurosurgery

microdroplet assay

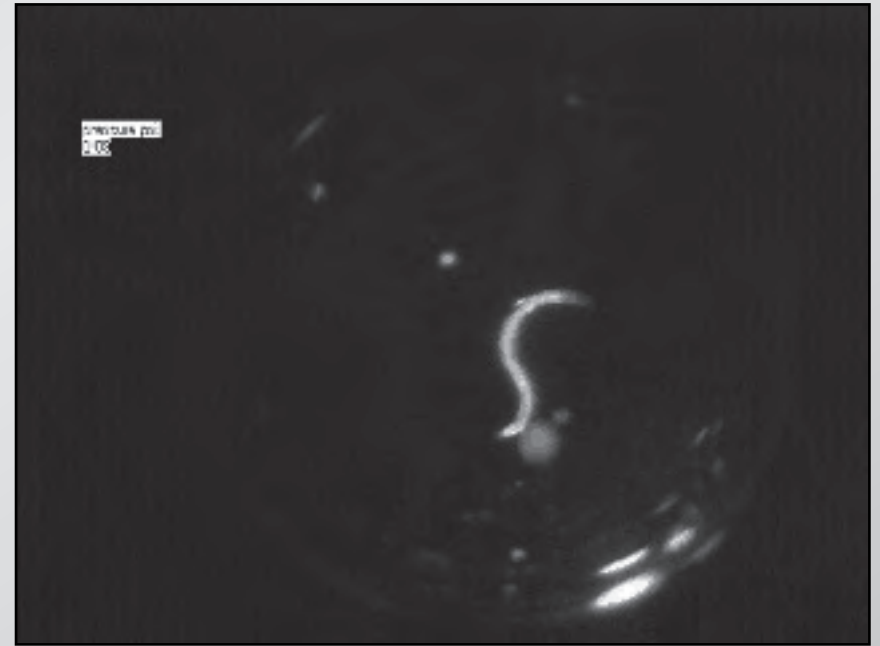


Nanoneurosurgery

surgery results in quantifiable behavior changes



before



after

Nanoneurosurgery

temperature sensing occurs at tip of dendrite

Cell transfection

brief communications

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Austin, Texas 78712-1086, USA

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Supplementary information accompanies this communication on Nature's website.
Competing financial interests: declared none.

Cell biology

Targeted transfection by femtosecond laser

The challenge for successful delivery of foreign DNA into cells *in vitro*, a key technique in cell and molecular biology with important biomedical implications, is to improve transfection efficiency while leaving the cell's architecture intact. Here we show that a variety of mammalian cells can be directly transfected with DNA without perturbing their structure by first creating a tiny, localized perforation in the membrane using ultrashort (femtosecond), high-intensity, near-infrared laser pulses. Not only does this superior optical technique give high transfection efficiency and cell survival, but it also allows simultaneous evaluation of the integration and expression of the introduced gene.

Previous techniques that have been developed for transfection of cells with DNA¹ include carrier-mediated transfer² and transfer by plasma-membrane permeabilization³, as well as direct transfer⁴, but the efficiency of all as direct delivery by these methods is low. However, none allows simultaneous evaluation of the integration and expression of the introduced gene.

mediated by intense near-infrared femtosecond laser pulses. Cells were suspended inside a sterile miniaturized cell chamber in 0.5 ml culture medium containing 0.2 µg plasmid DNA vector pEGFP-N1 (4.7 kilobases) encoding enhanced green fluorescent protein (EGFP)⁷. Transmission images of cells were obtained at low power (< 5 µW), and the near-infrared laser beam was then focused (under the same microscope) on the edge of the membrane of a target cell, which was exposed to an enhanced mean laser power of 50–100 mW for 16 ms so that transfection could occur. More than 200 cells of each type were targeted in each of replicate experiments; it took 10–15 s to prepare for the transfection and expression efficiency of the EGFP gene *in situ* by time-lapse two-photon fluorescence imaging⁸ at a mean laser power of < 1 mW over a period of 72 h, as well as by two-photon fluorescence-lifetime imaging (TPFLIM)⁹. Figure 1 shows that diffraction-limited focusing of intense femtosecond near-infrared laser pulses selectively facilitates transfection of the target cells, but not of the adjacent cells. Expression of EGFP in the transfected cells is also demonstrated by TPFLIM, and the measured fluorescence lifetime of about 2.4 ns is consistent with that reported for CHO cells expressing EGFP¹⁰.

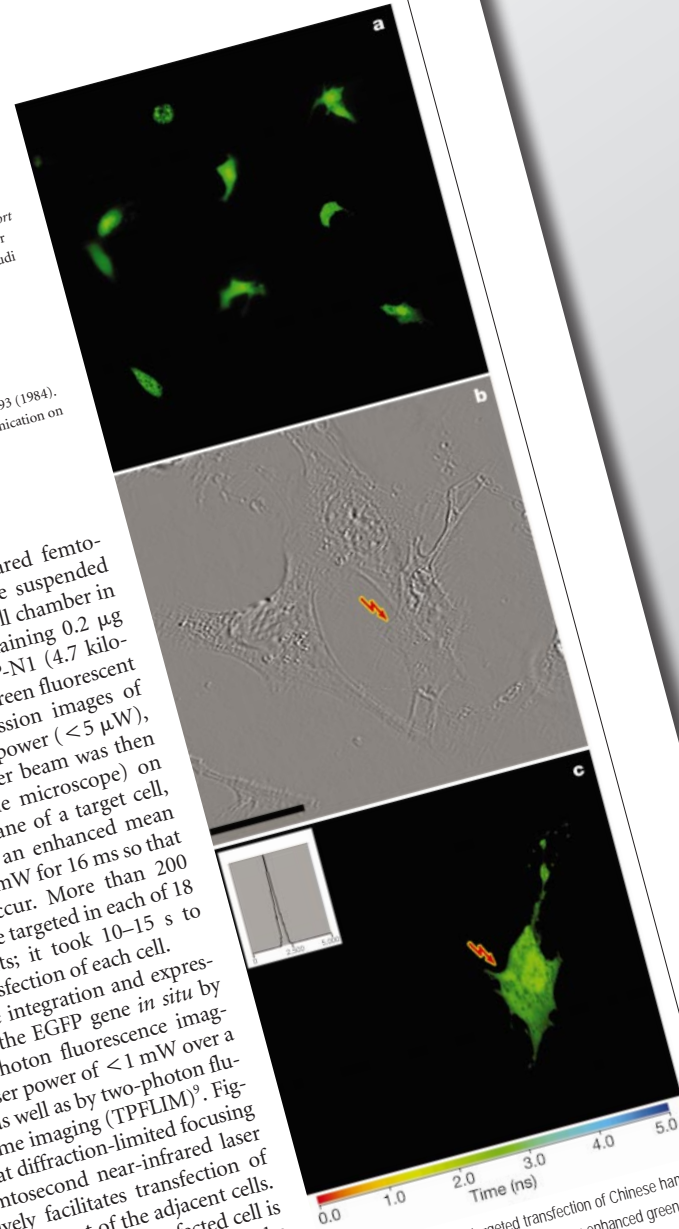
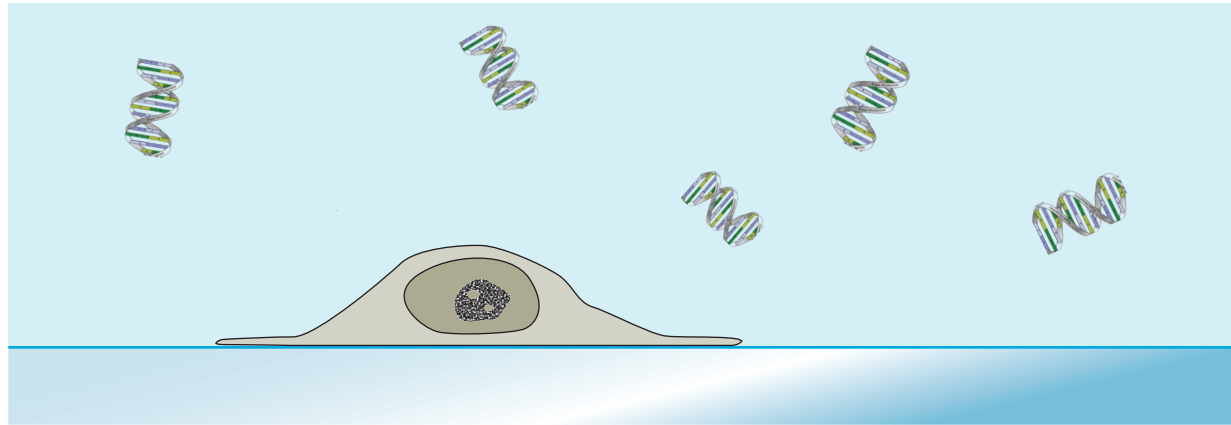


Figure 1 Analysis of the targeted transfection of Chinese hamster ovarian (CHO) cells with a plasmid encoding enhanced green fluorescent protein (EGFP) by *in situ* visualization, and measurement of its expression by near-infrared, two-photon-excitation-evolved fluorescence-lifetime imaging (TPFLIM). **a**, Time-lapse two-photon fluorescence image of several CHO cells expressing EGFP. **b**, Transmission image of the same cells. **c**, Time-lapse two-photon fluorescence image of the target cell, showing the fluorescence lifetime of the EGFP gene. The color scale indicates time in nanoseconds (ns).

Cell transfection



Tirlapur, et al., *Nature* 418, 290 (2002)

leaving the... we show that... cells can be directly transfected... without perturbing their structure in the... creating a tiny, localized perforation in the... membrane using ultrashort (femtosecond),... high-intensity, near-infrared laser pulses. Not only does this superior optical technique give high transfection efficiency and cell survival, but it also allows simultaneous... of the integration and expression of the introduced gene.

Previous techniques that have been developed for transfection of cells with DNA¹ include carrier-mediated transfer² and transfer by plasma-mediated permeabilization³, all as direct transfer⁴, but the efficiency of all these methods is low. Moreover, none allows... cells... replicate... We assessed the integration and... We assessed the integration and... expression efficiency of the EGFP gene *in situ* by... time-lapse two-photon fluorescence imaging⁸ at a mean laser power of < 1 mW over a... period of 72 h, as well as by two-photon fluorescence-lifetime imaging (TPFLIM)⁹. Figure 1 shows that diffraction-limited focusing of intense femtosecond near-infrared laser pulses selectively facilitates transfection of the target cells, but not of the adjacent cells. Expression of EGFP in the transfected cell is also demonstrated by TPFLIM, and the measured fluorescence lifetime of about 2.4 ns is consistent with that reported for... cells expressing EGFP¹⁰.

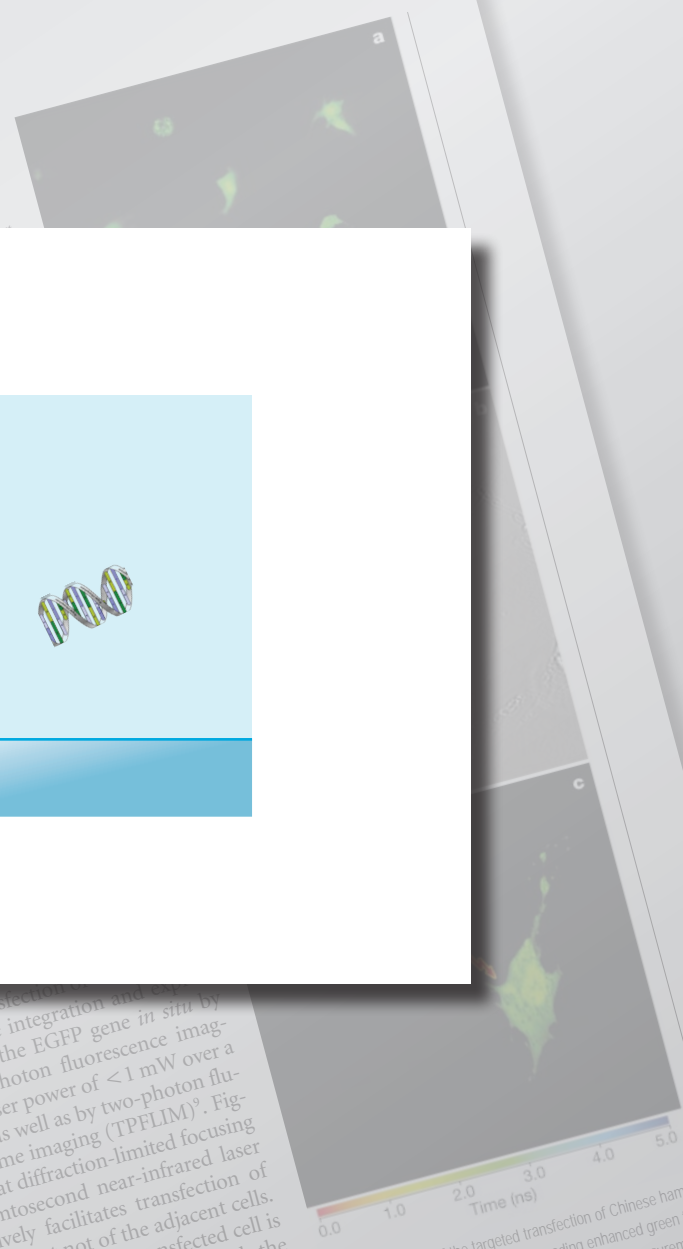
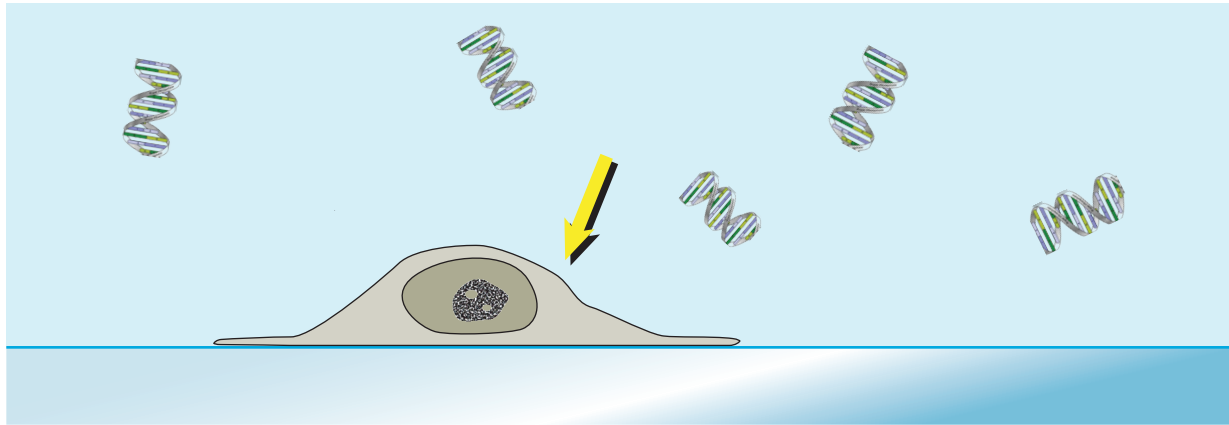


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Cell transfection



Tirlapur, et al., *Nature* 418, 290 (2002)

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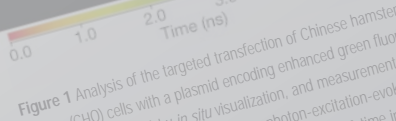
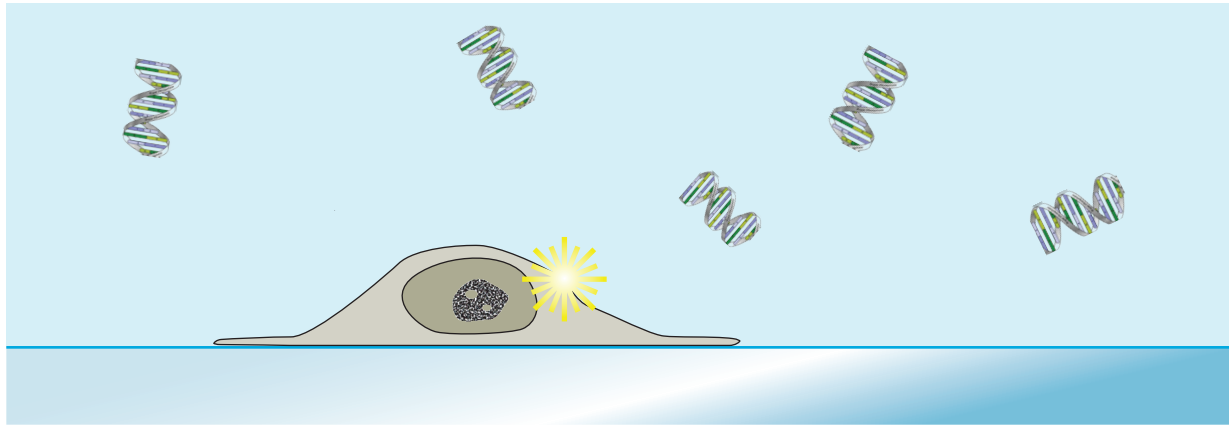


Figure 1 Analysis of the targeted transfection of Chinese hamster ovarian (CHO) cells with a plasmid encoding enhanced green fluorescent protein (EGFP) by *in situ* visualization, and measurement of its expression by near-infrared, two-photon-excitation-evolved fluorescence-lifetime imaging (TPFLIM). **a**, Transmission image of the cells. **b**, Two-photon fluorescence image of the cells. **c**, TPFLIM image of the cells. Scale bars, 10 μ m.

Cell transfection



Tirlapur, et al., *Nature* 418, 290 (2002)

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Cell transfection

	Toxicity	Efficiency	Throughput	Specificity
Goal	VL	H	H	L

Cell transfection

	Toxicity	Efficiency	Throughput	Specificity
Goal	VL	H	H	L
Naked DNA	VL	L	H	L
Polymer/lipid	M	M	H	H
Viral transfection	M	H	H	H
Electroporation	H	H	H	L

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Viral transfection	M	H	H	H
Electroporation	H	H	H	L
Laser poration	VL	H	VL	L

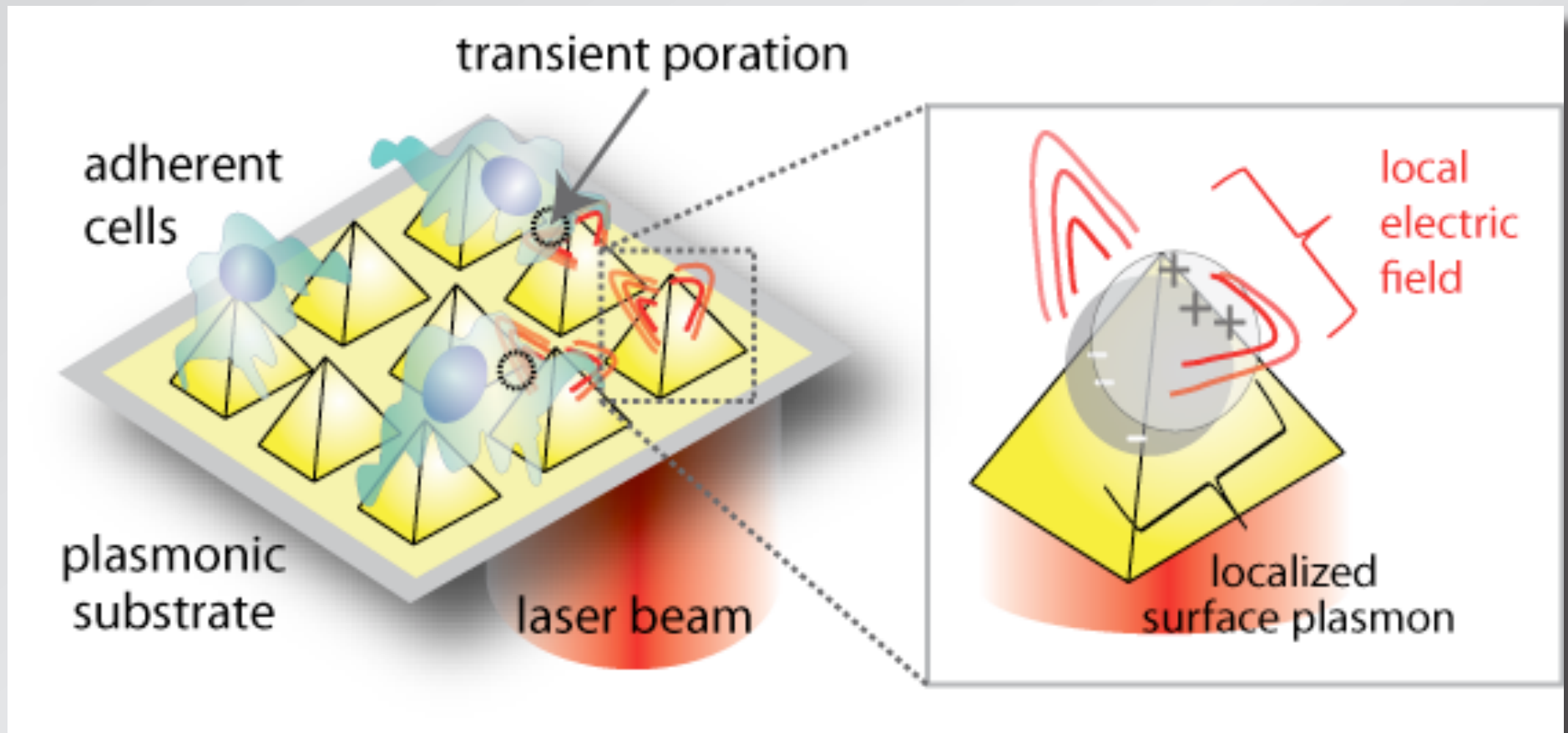
Cell transfection

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Cell transfection

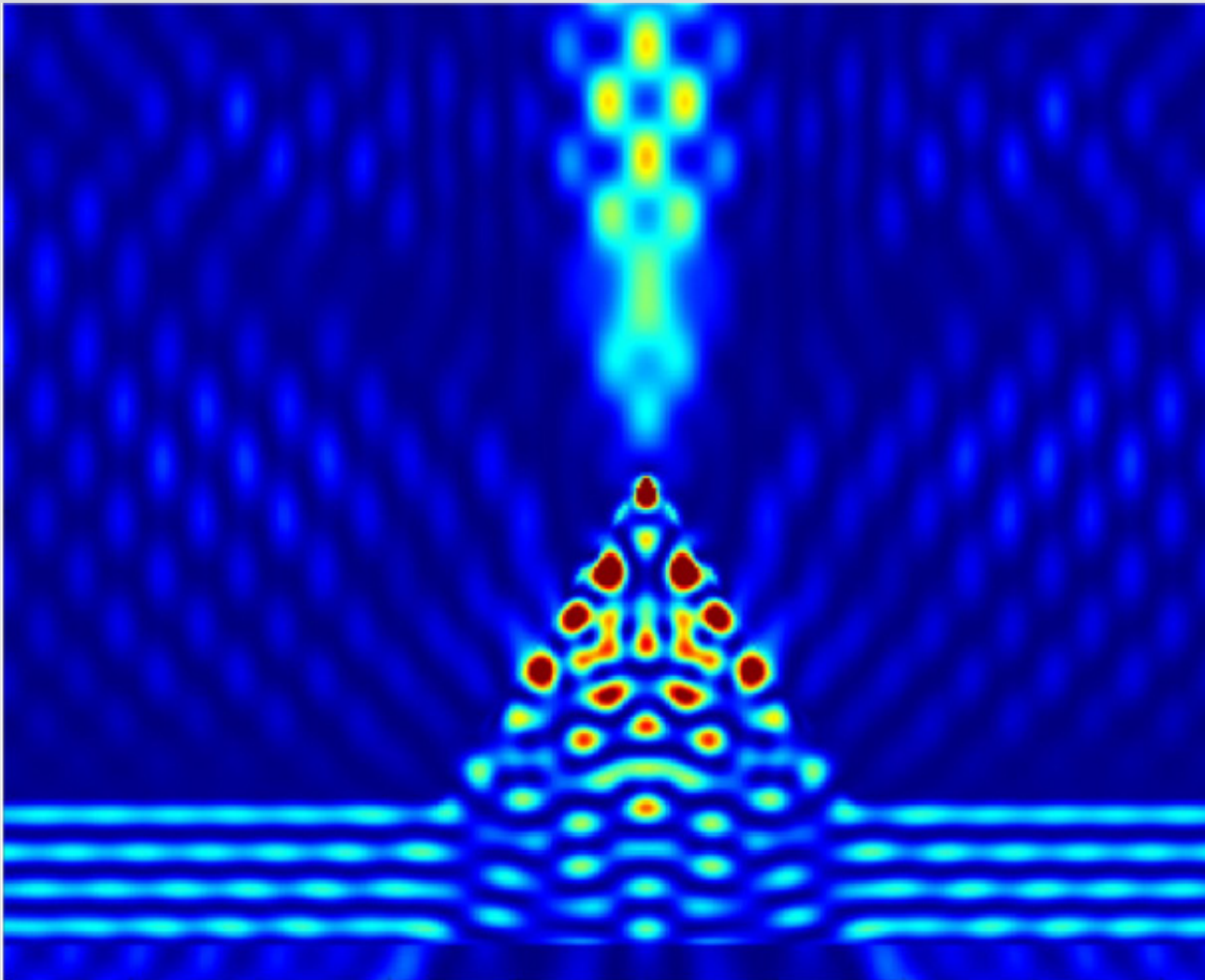
use structured plasmonic substrate

Cell transfection

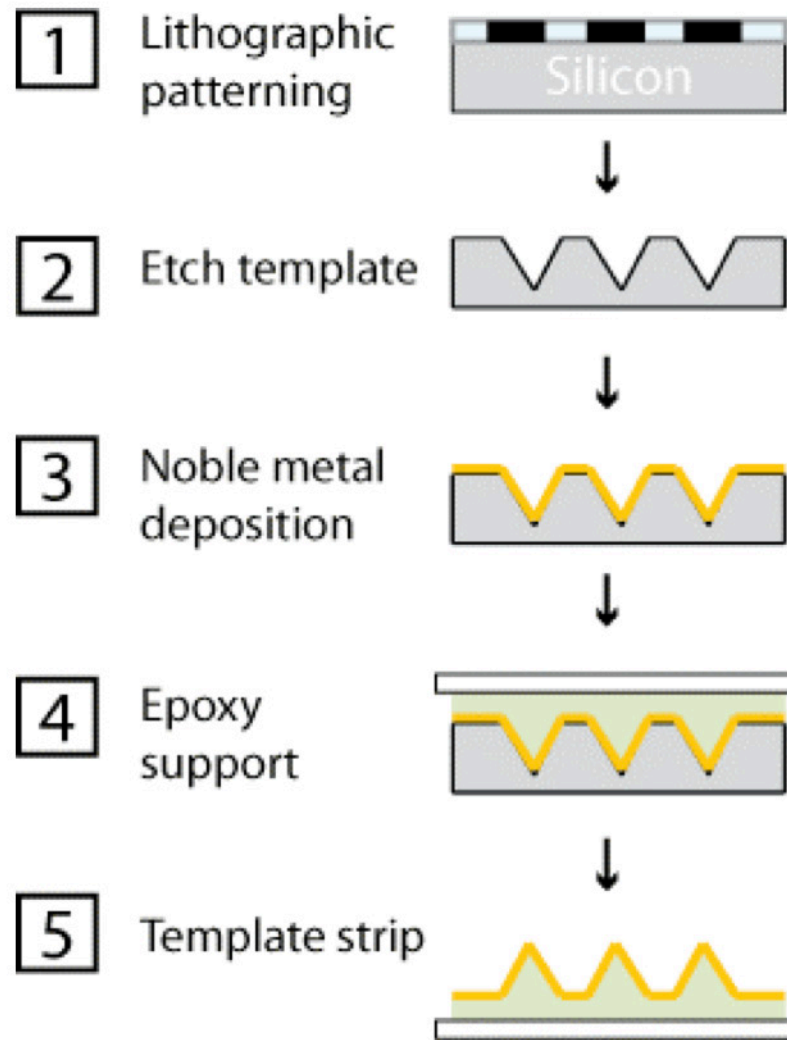


Cell transfection

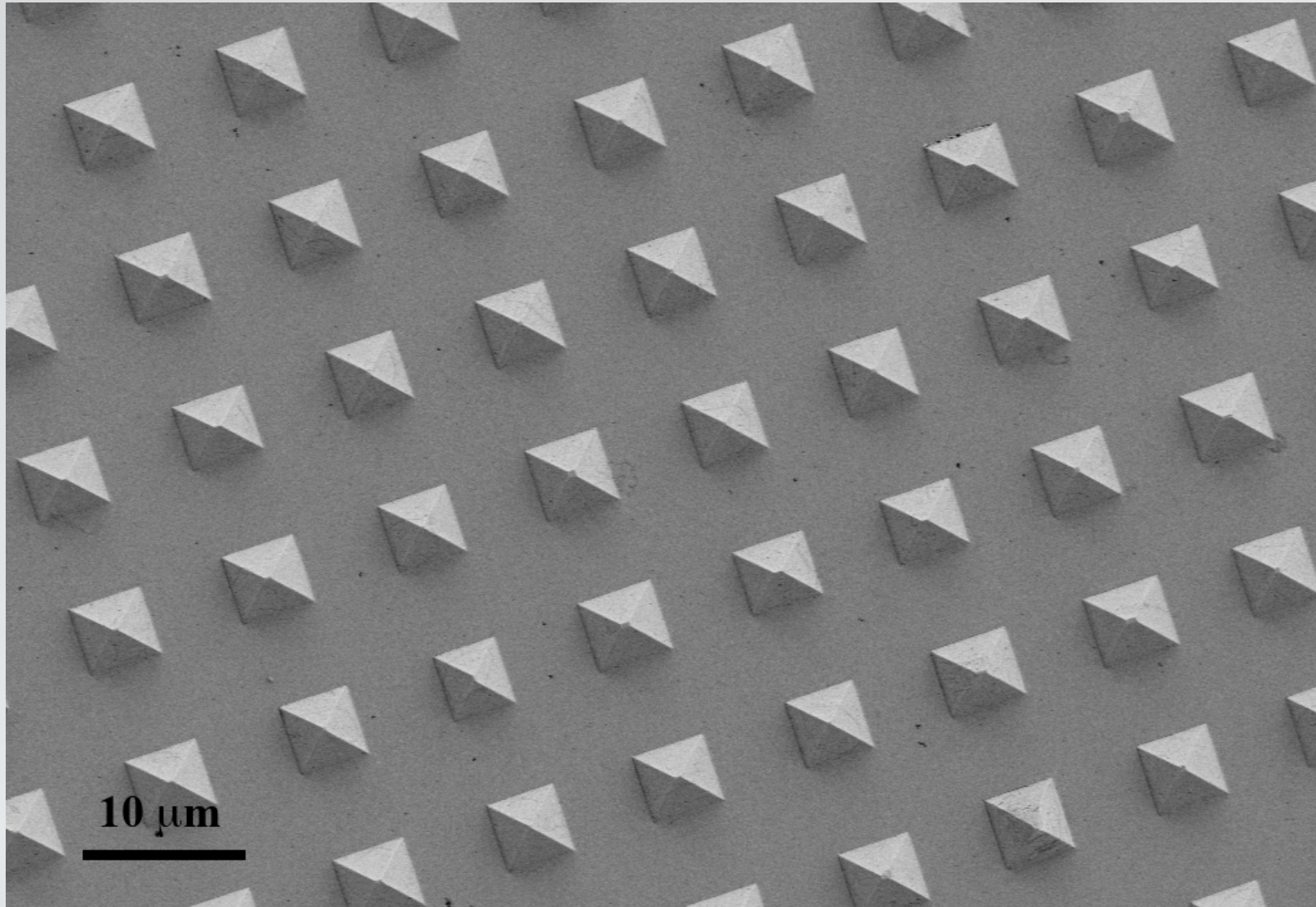
field enhancement at tip



Cell transfection

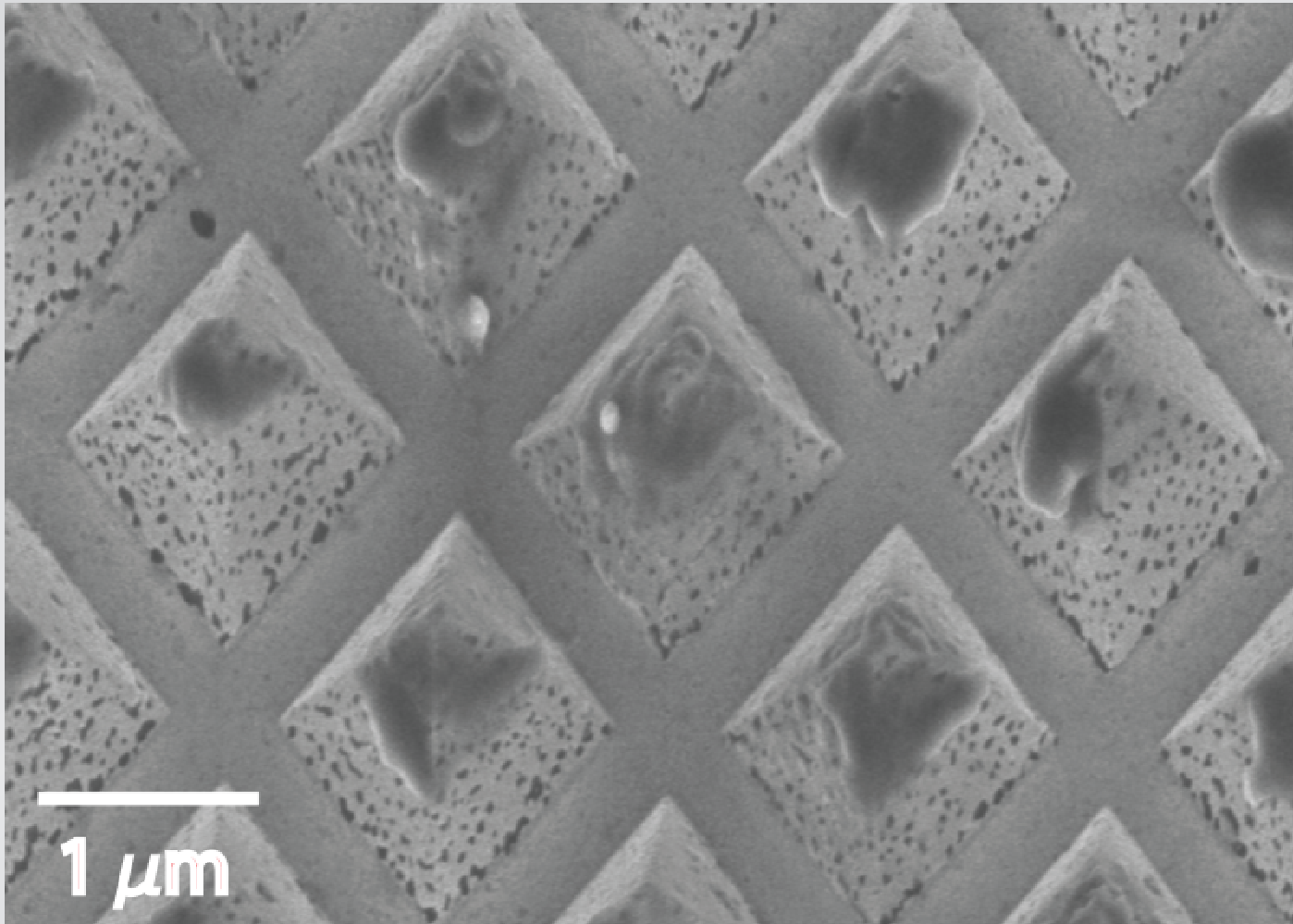


Cell transfection



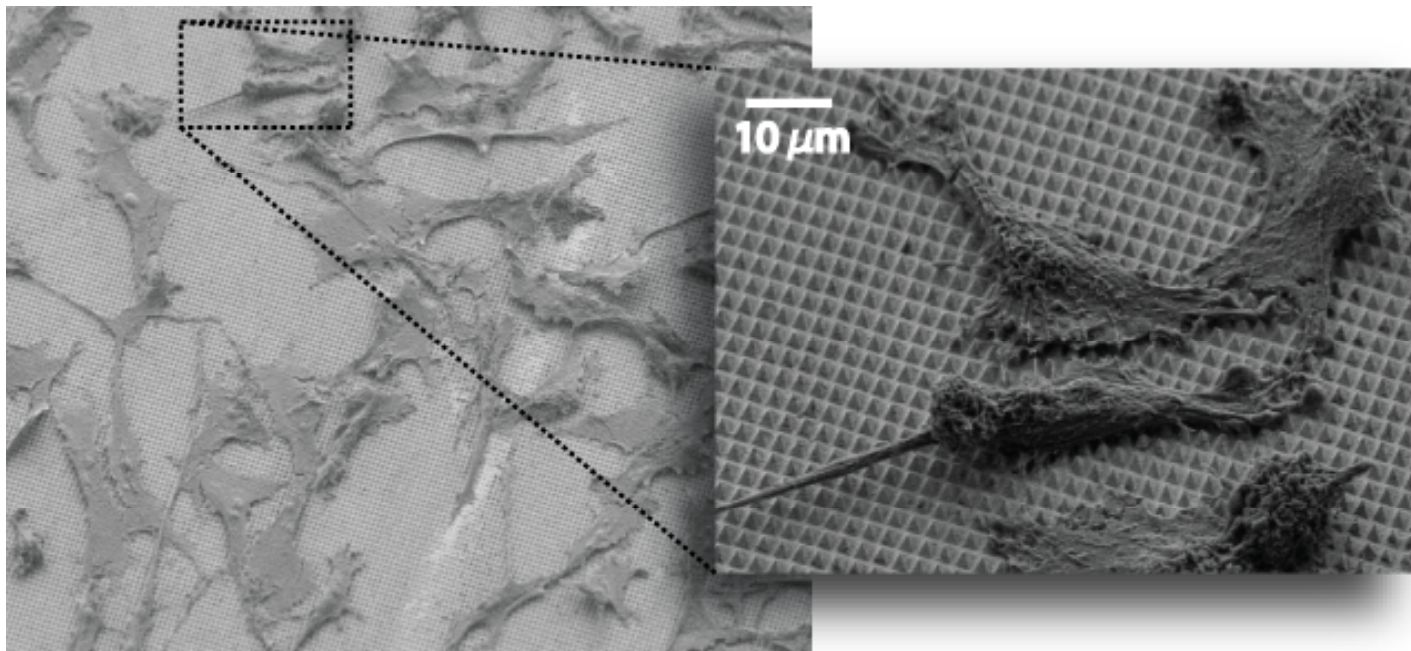
Cell transfection

two-photon polymerization enhancement



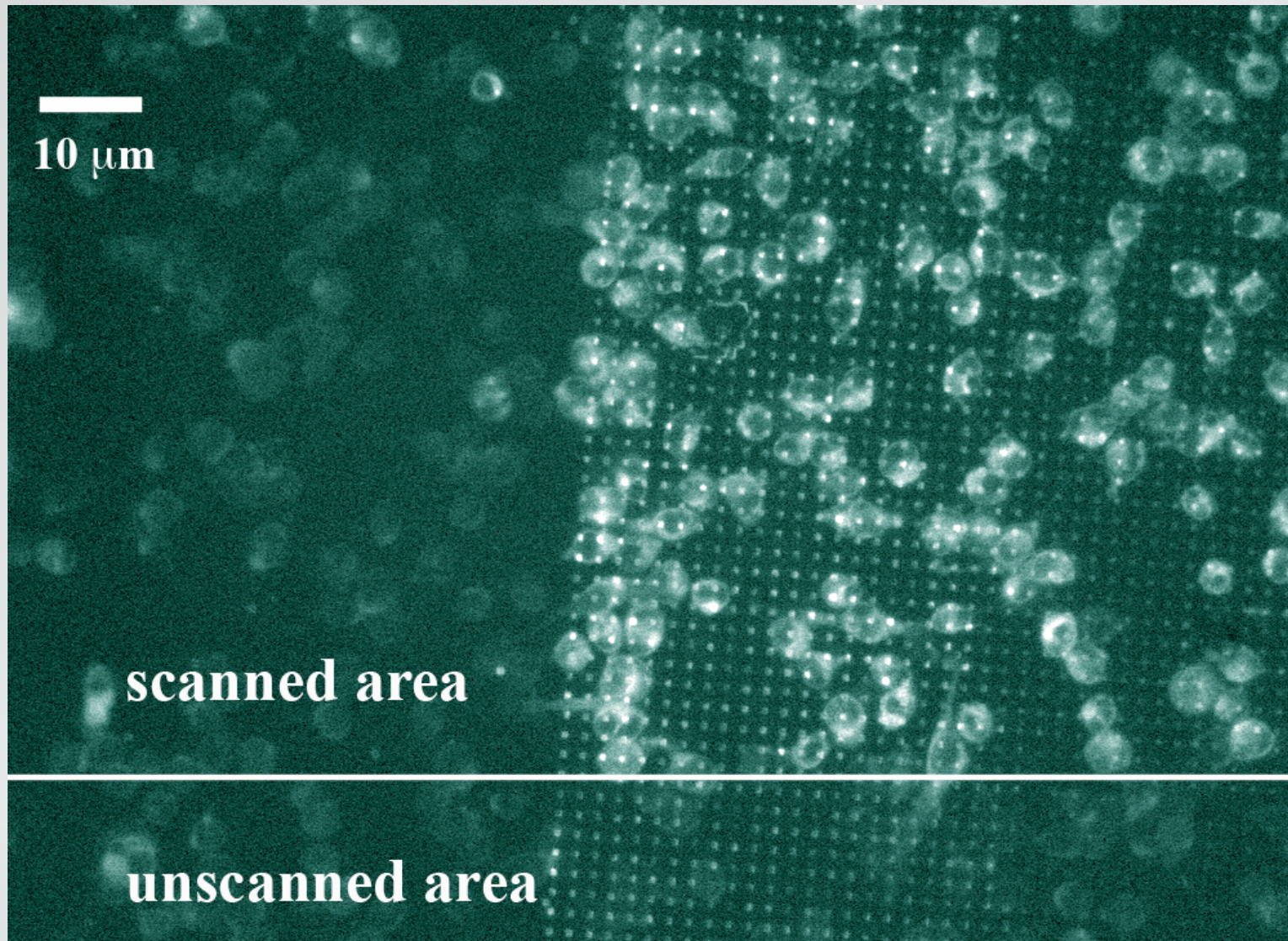
Cell transfection

attachment of TE cells on pyramid arrays



Cell transfection

only exposed cells on pyramids take up dye



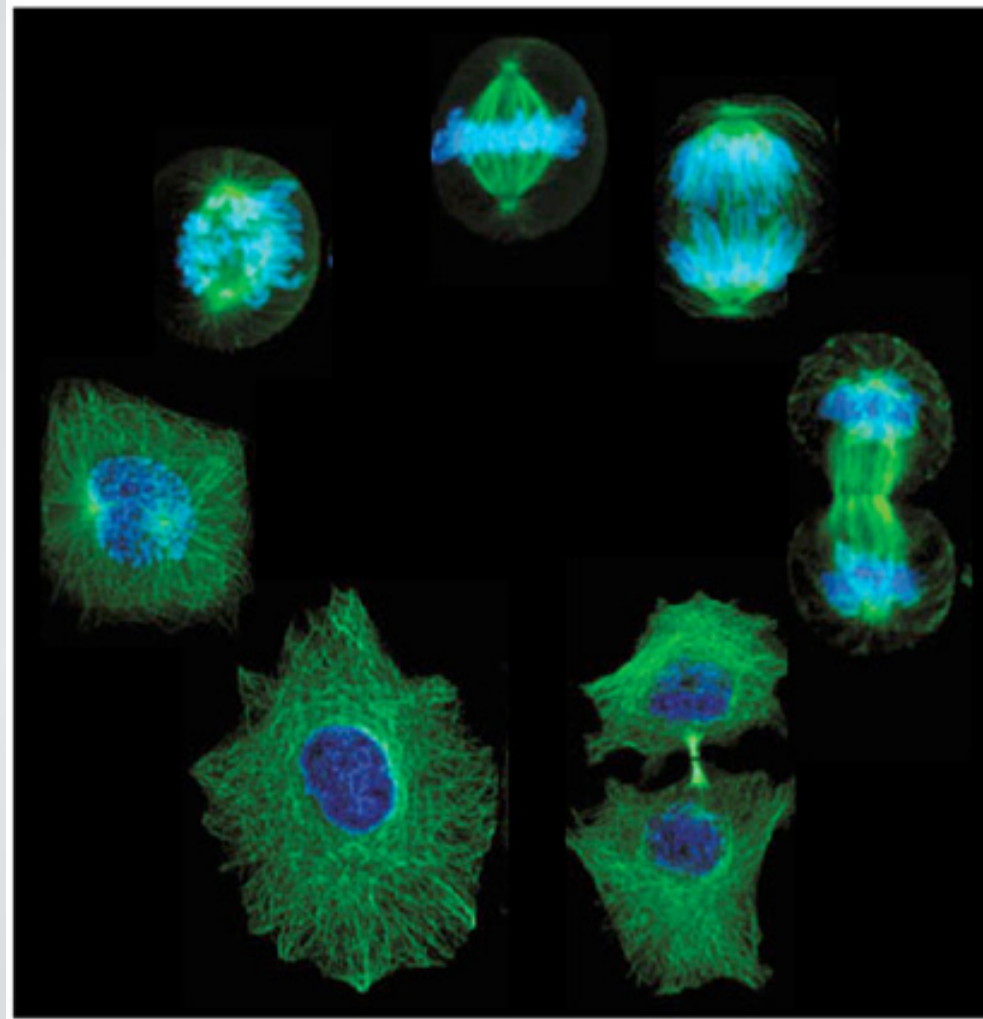
Spindle mechanics

study dynamics of microtubules in mytotic spindle

A fluorescence microscopy image of a mitotic spindle. The spindle is composed of microtubules that are organized into two asters at opposite poles. The microtubules are stained with different fluorescent dyes: red, green, and blue. The red microtubules form the outer layer of the asters, while the green and blue microtubules form the inner layer. The blue microtubules are concentrated in the region where the chromosomes are located, indicating their attachment to the kinetochores. The overall structure is a bipolar spindle that is responsible for the segregation of chromosomes during cell division.

Spindle mechanics

spindle forms during cell division



Spindle mechanics

spindle forms during cell division

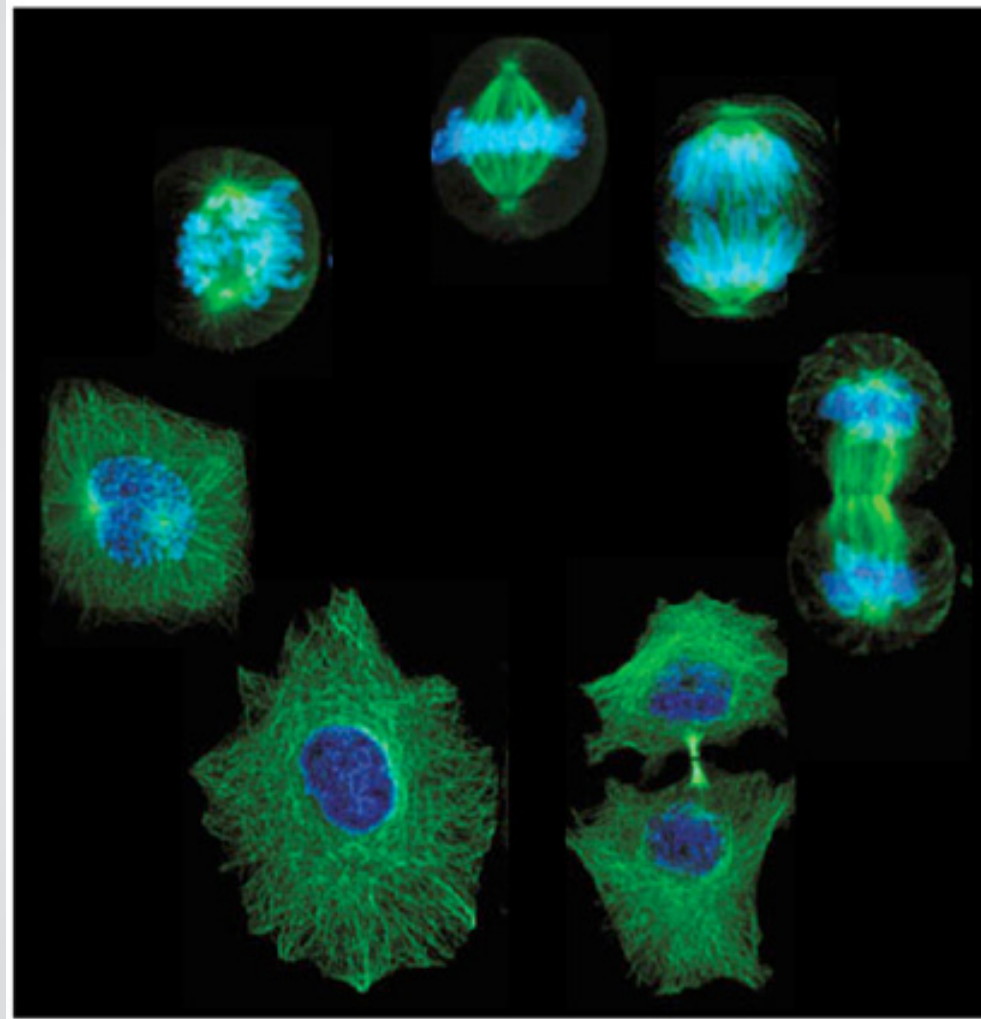
metaphase

prophase

anaphase

telophase

interphase

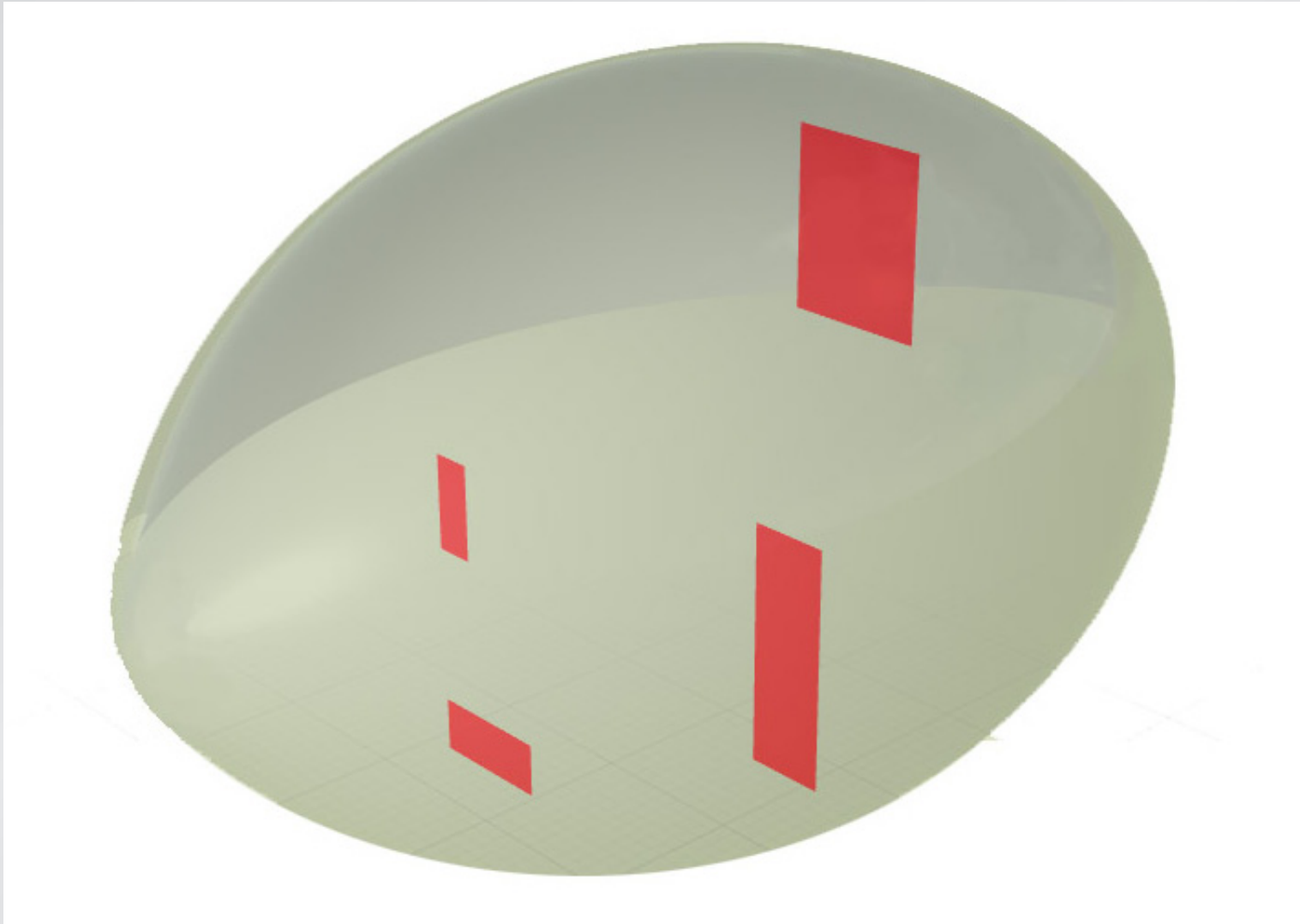


Spindle mechanics

can we determine polarity and length of microtubules?

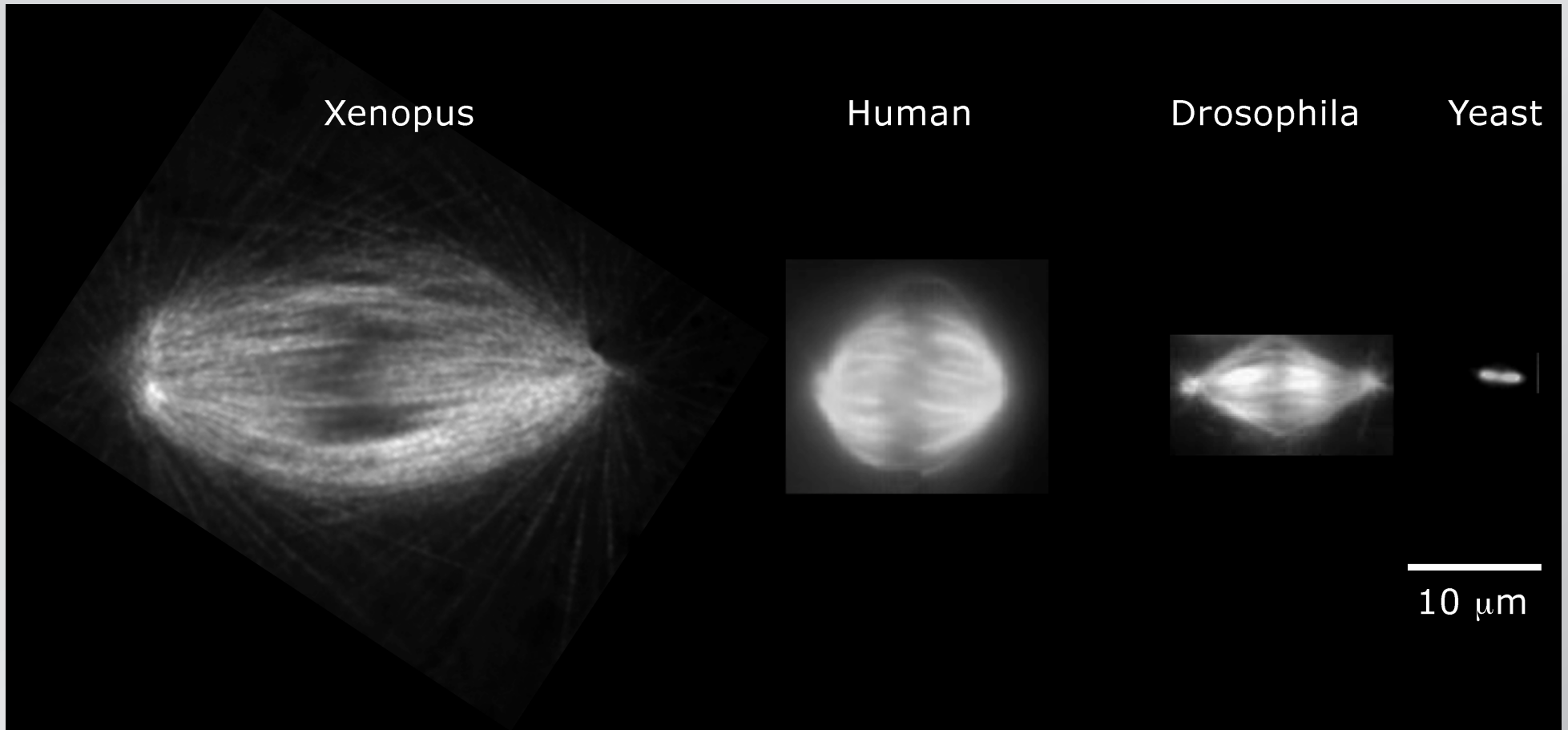
Spindle mechanics

observe depolymerization dynamics after planar cut(s)



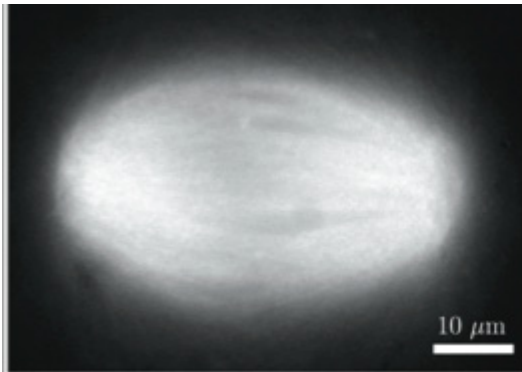
Spindle mechanics

spindles from frog egg extract



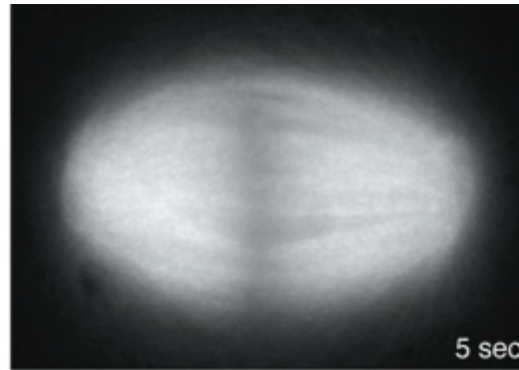
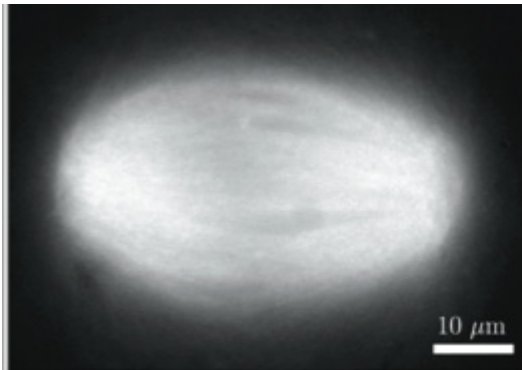
Spindle mechanics

direct observation of depolymerization wave



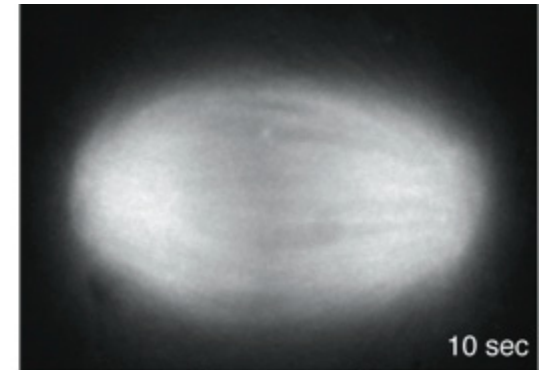
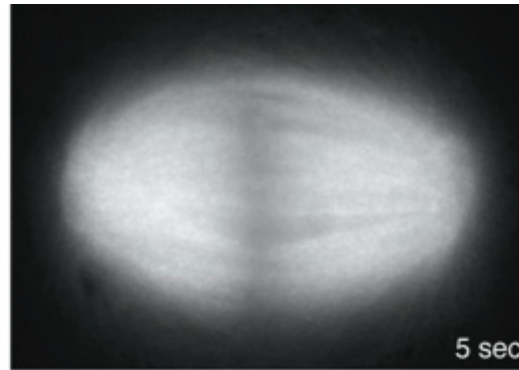
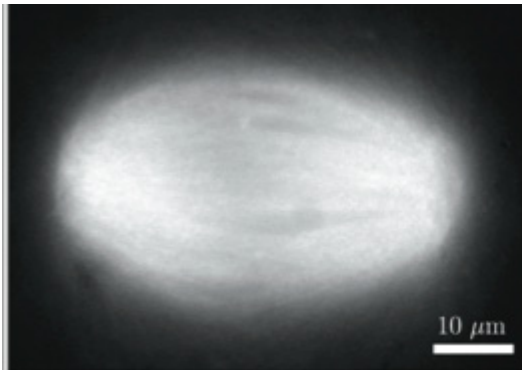
Spindle mechanics

direct observation of depolymerization wave



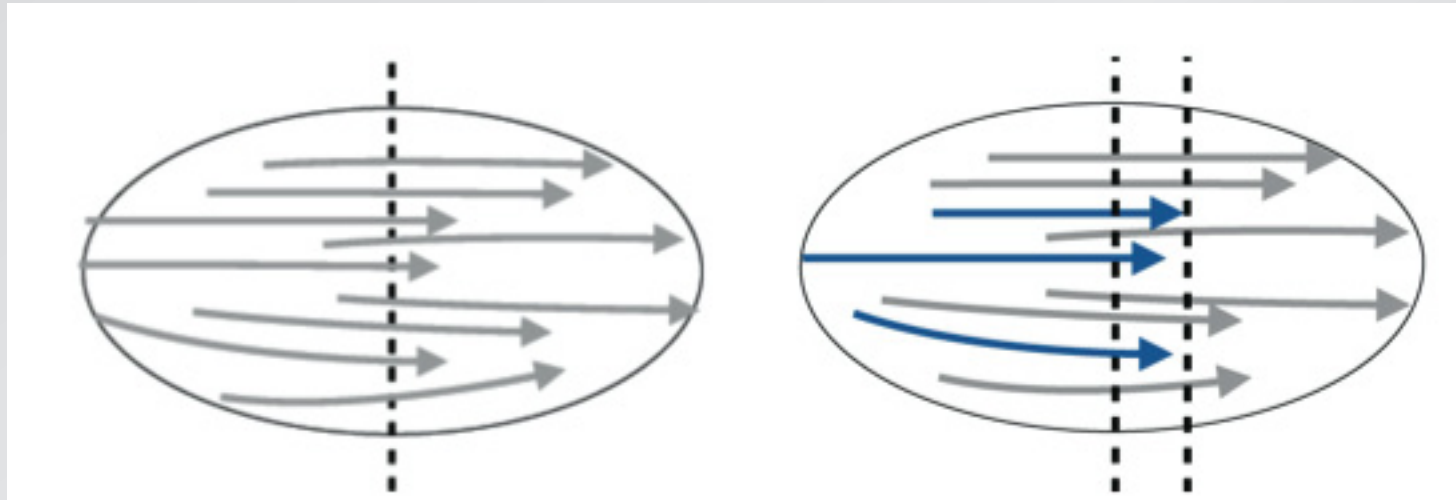
Spindle mechanics

direct observation of depolymerization wave



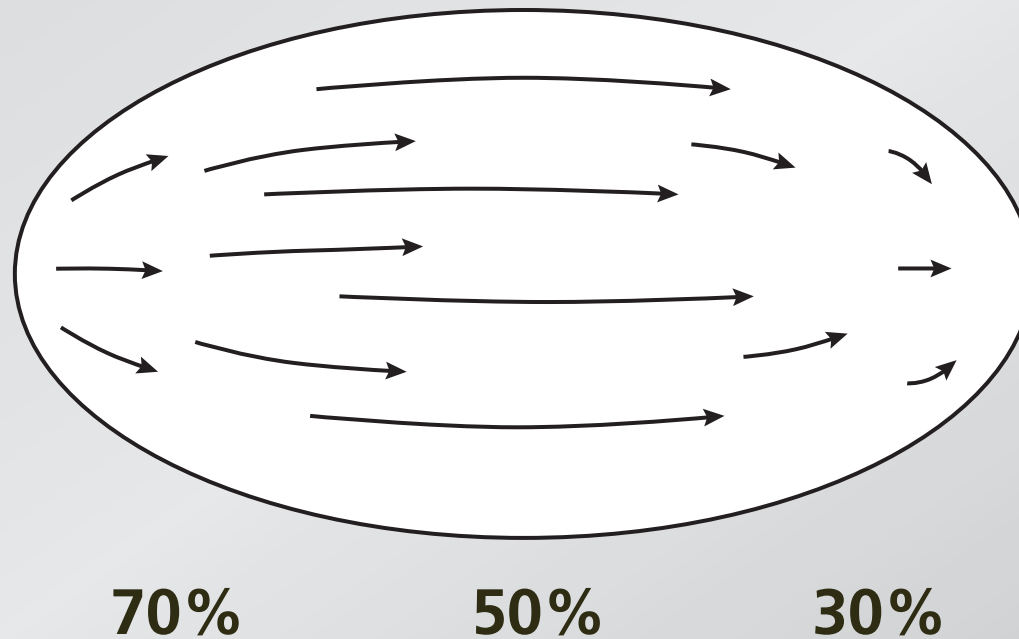
Spindle mechanics

double cuts provide information on mean length



Spindle mechanics

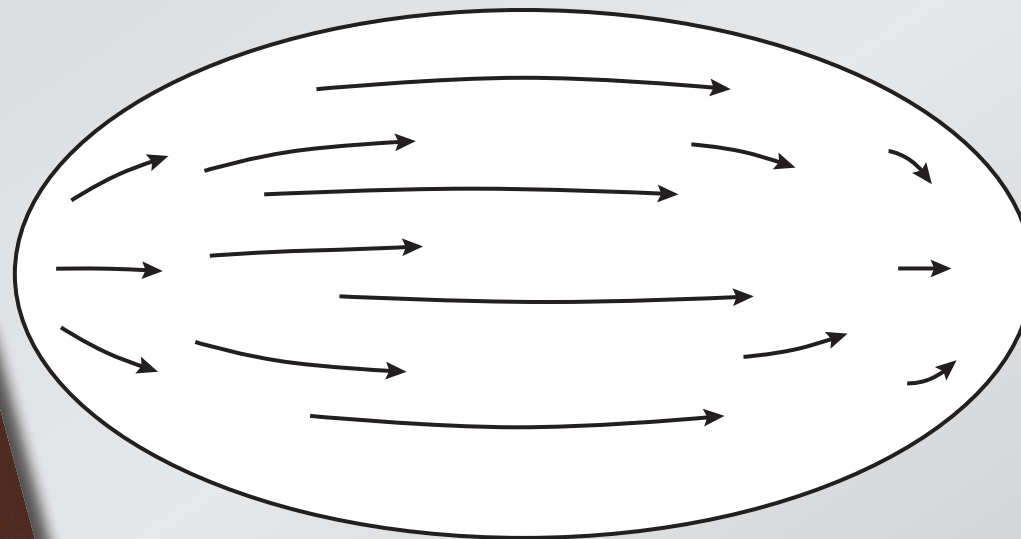
spindle organization



polarity & length distributions varies across cell

Spindle mechanics

spindle organization



70%

50%

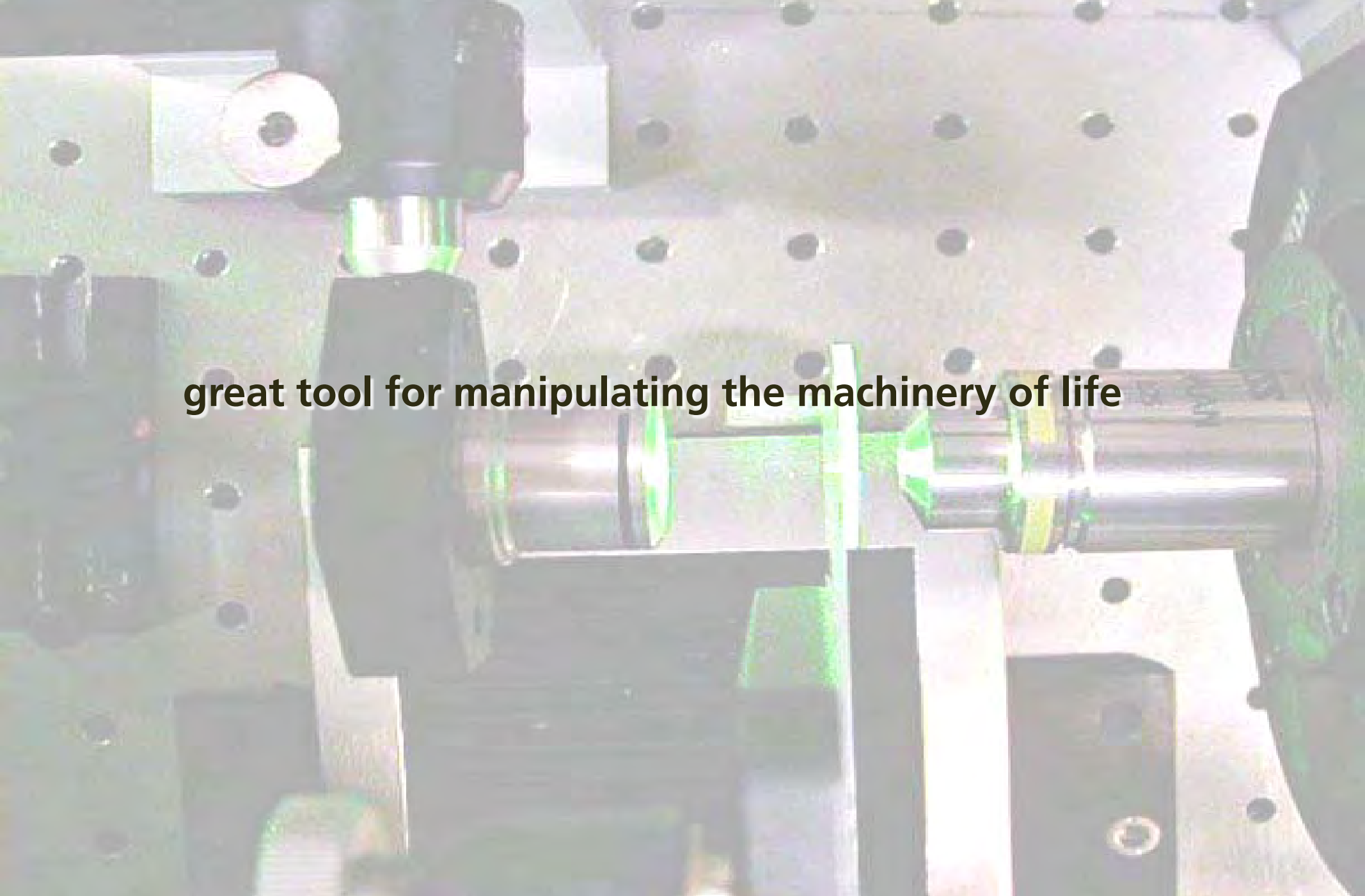
30%

length distributions varies across cell



Conclusion

great tool for manipulating the machinery of life



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**Harvard Center for Imaging and Mesoscopic Structures
National Science Foundation
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