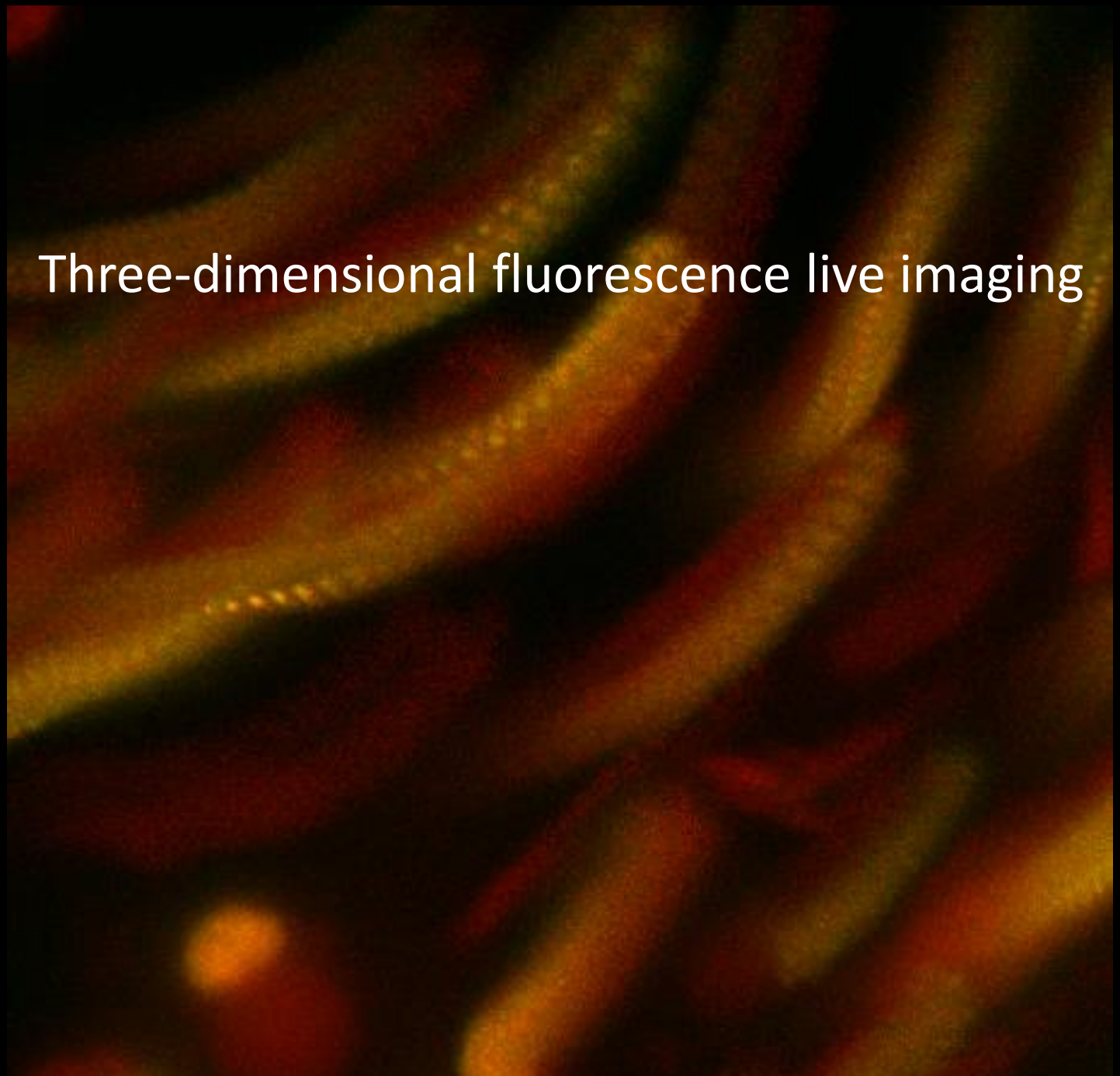
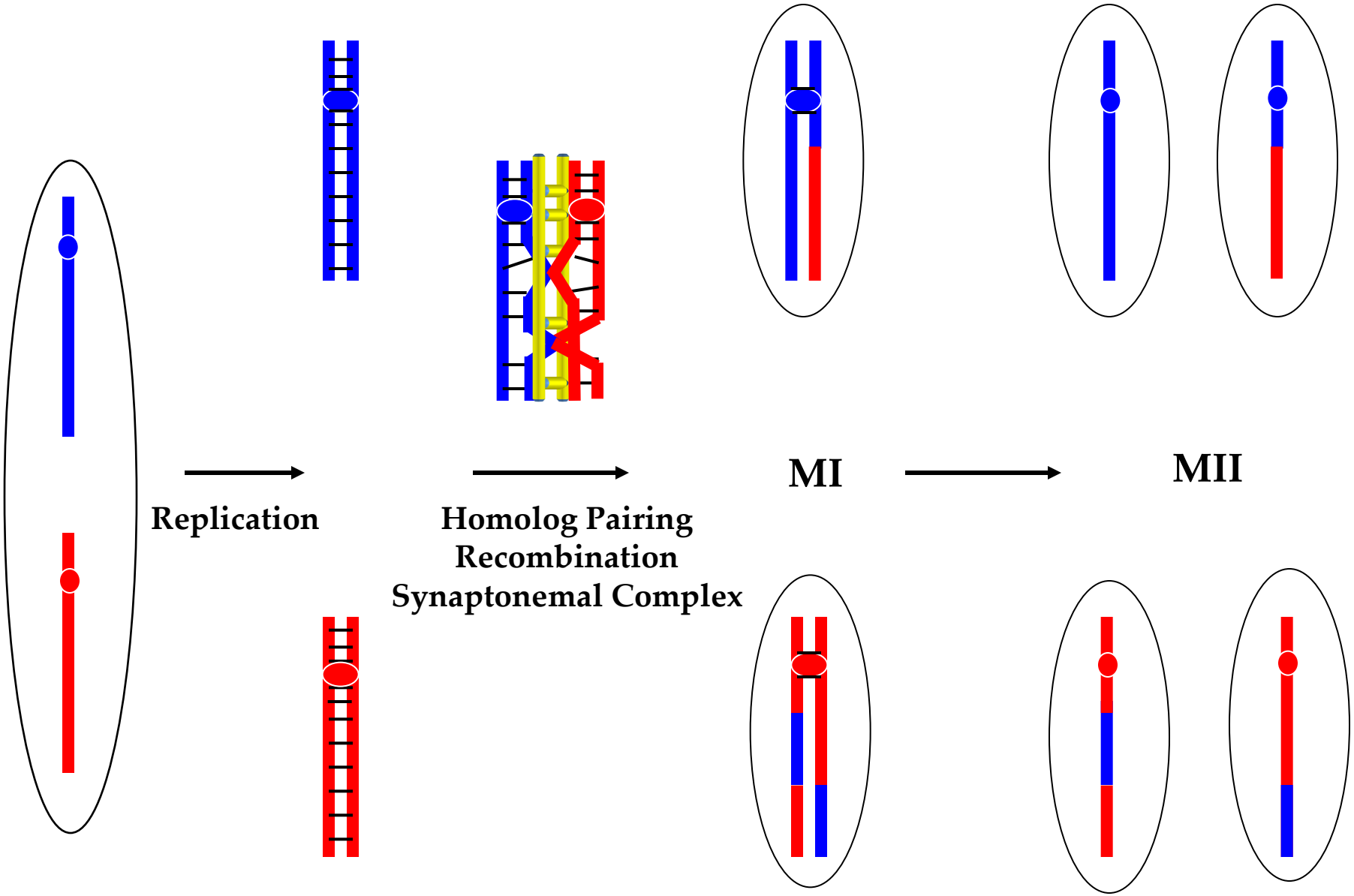

Three-dimensional fluorescence live imaging

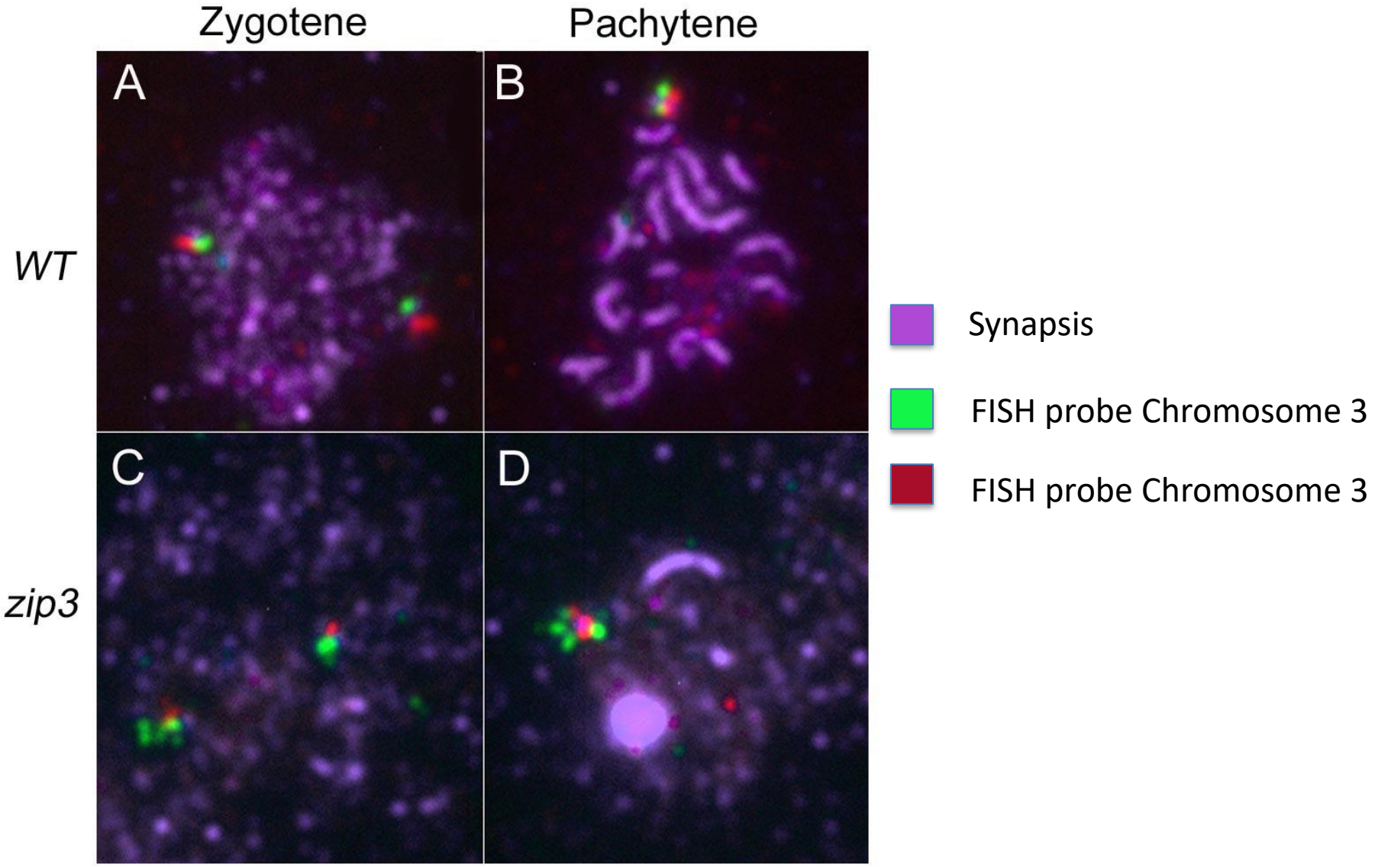
Key Elements for Successful Live Imaging

1. Choice of biological specimen
2. Validation
3. Reducing photoxicity and photobleaching
1. Close preservation of normal environment

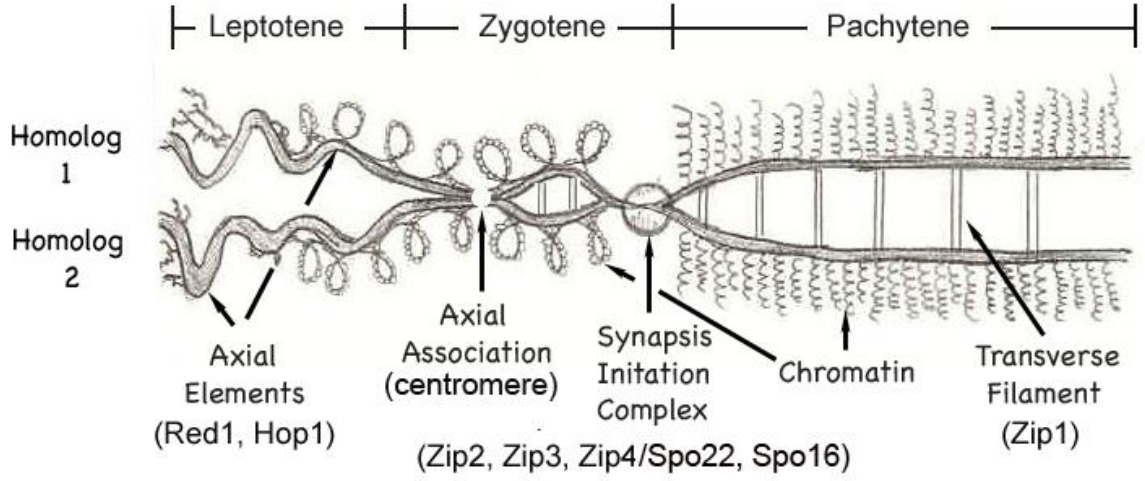
Meiosis


Replication
Homolog Pairing
Recombination
Synaptonemal Complex
MI
MII

Progression of Synapsis in Fixed Budding Yeast Spreads



Open Questions about Synaptonemal Complex Assembly



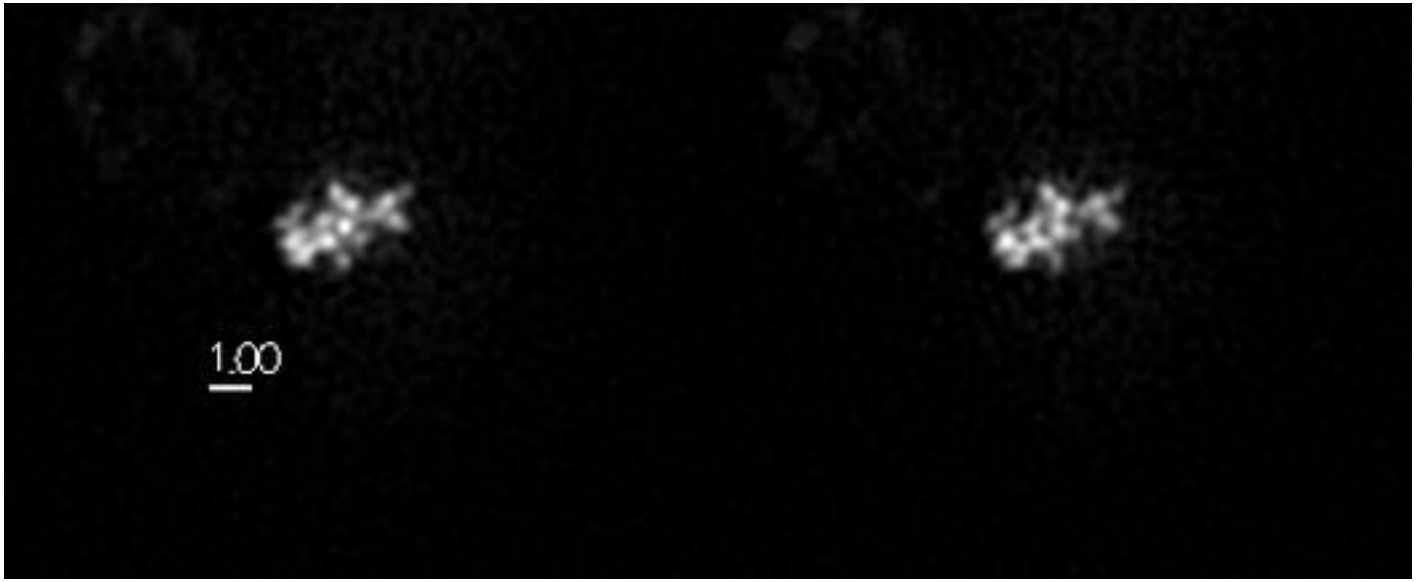
1. What is the rate, extent and direction of synapsis formation?
2. Is synapsis continuous, stepwise or dynamically assembled and disassembled?
3. Does the kinetics of synapsis nucleation differ at centromeric vs. noncentromeric sites?
4. Do the kinetics of synapsis play a role in controlling the distribution of crossovers?

1. How is synapsis disassembled?
2. How long does the whole process take?
3. When is synapsis established relative to pairing?

Choosing the Appropriate Organism to Answer your Question

1. Easily transfected/transformed or easily take up labeled proteins or dyes
2. Biological process can be found at sufficient frequency
3. The biological process completes in a reasonable amount of time
4. The process can be sustained over the time it takes to image
5. Not imaging a process that occurs too deep into the tissue
6. The time resolution isn't beyond the imaging capabilities
7. Ways to validate your results?

Imaging Synapsis in WT Budding Yeast Cells

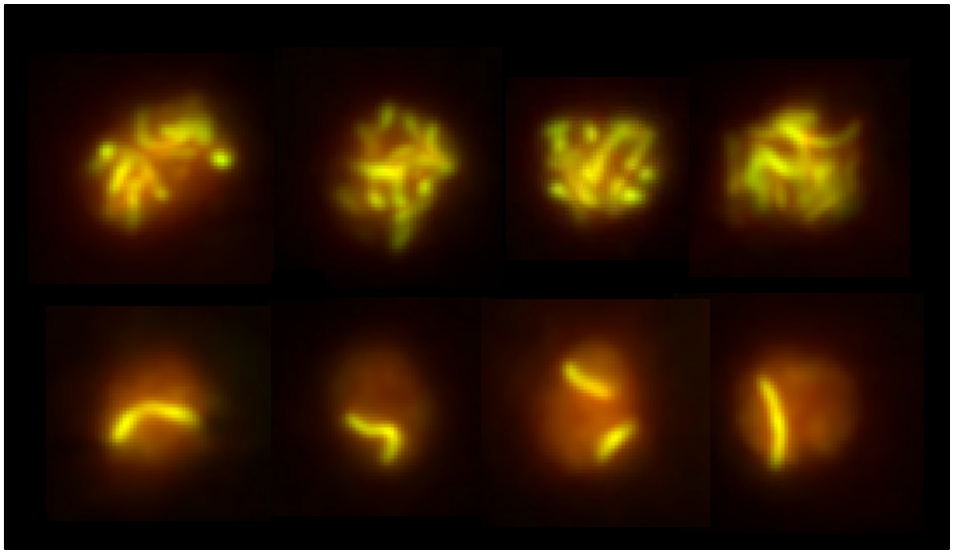


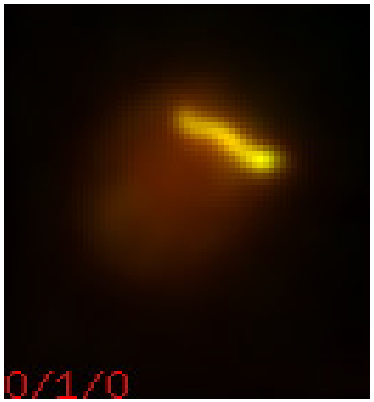
1. 16 chromosomes are synapsing within a confined space
2. Moving rapidly (1 micron/sec)
3. Nucleation is occurring at 50-70 sites, potentially simultaneously

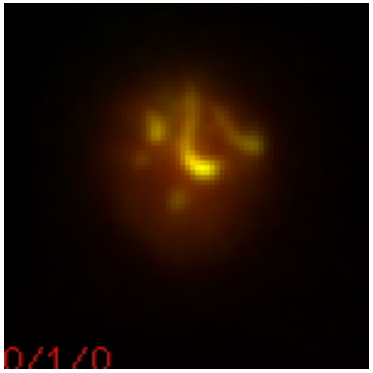
Synapsed Chromosomes Imaged in a Δzip3 Mutant

ZIP3+

Δzip3

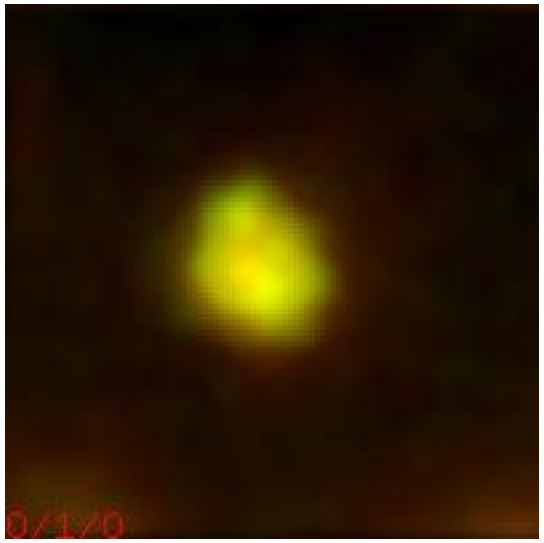


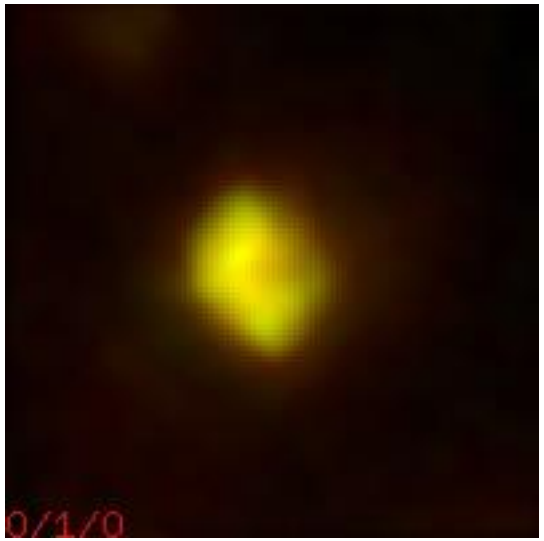


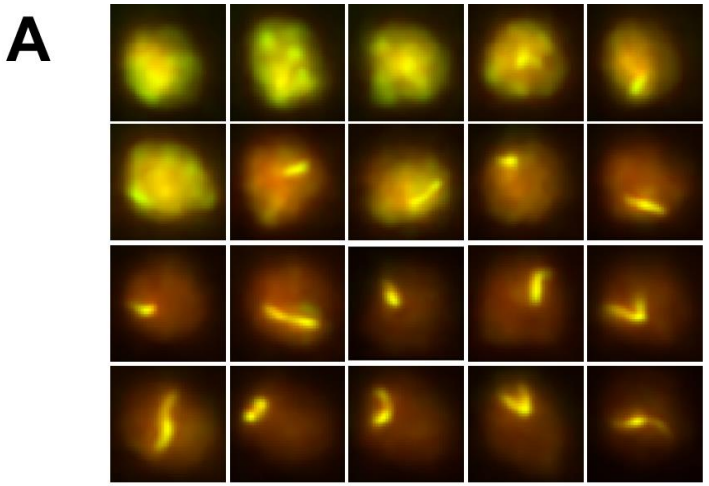


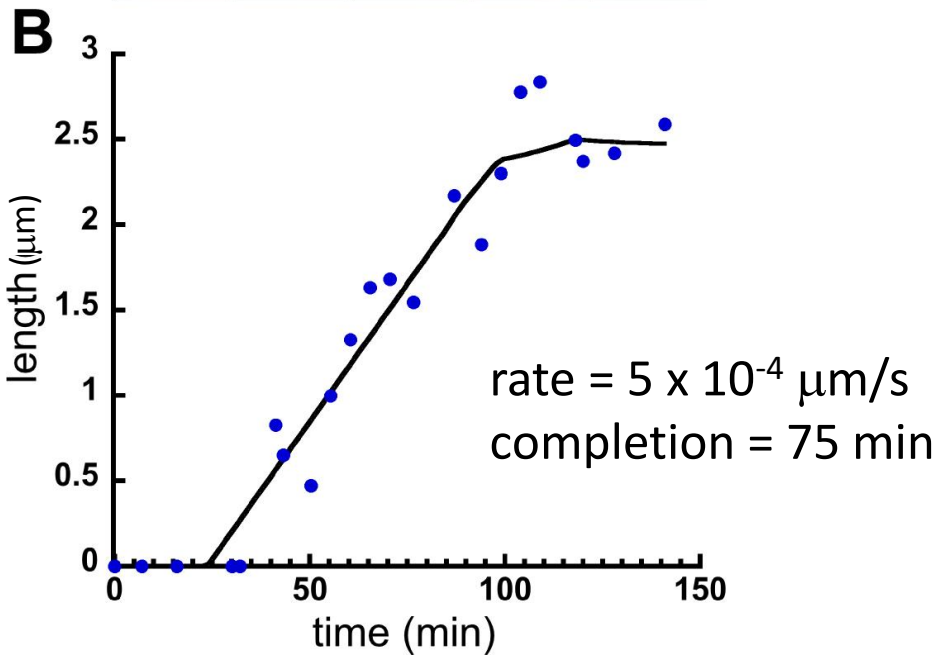
zip1::LEU2	*ura3-1*	*Δzip3::LYS2*
zip1::LEU2	*Zip1-GFP(Δ3'UTR)@URA3*	*Δzip3::LYS2*

Synapsis Formation is Progressive

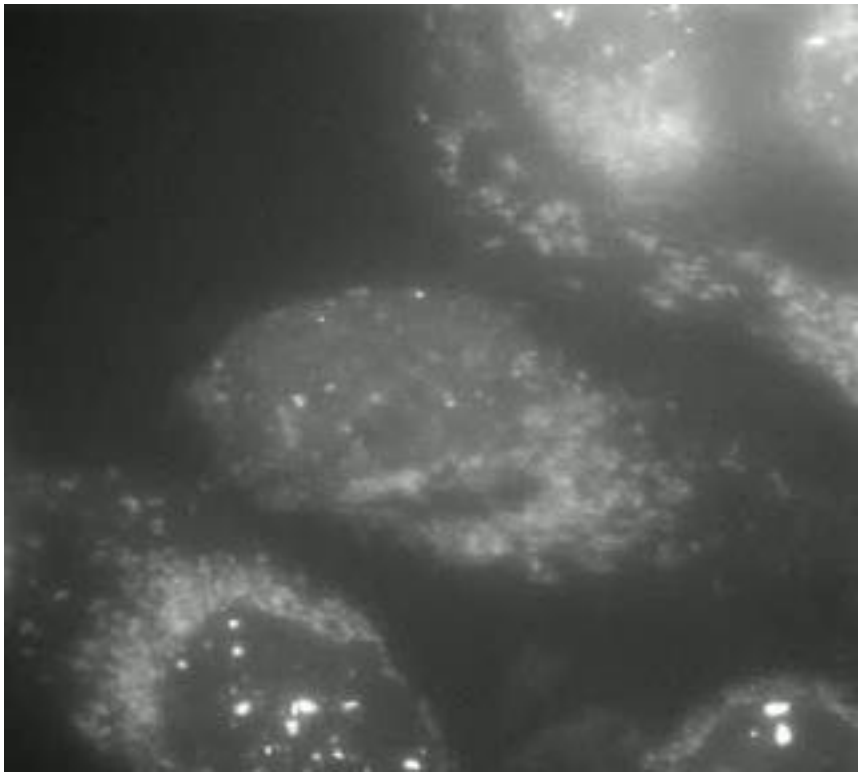








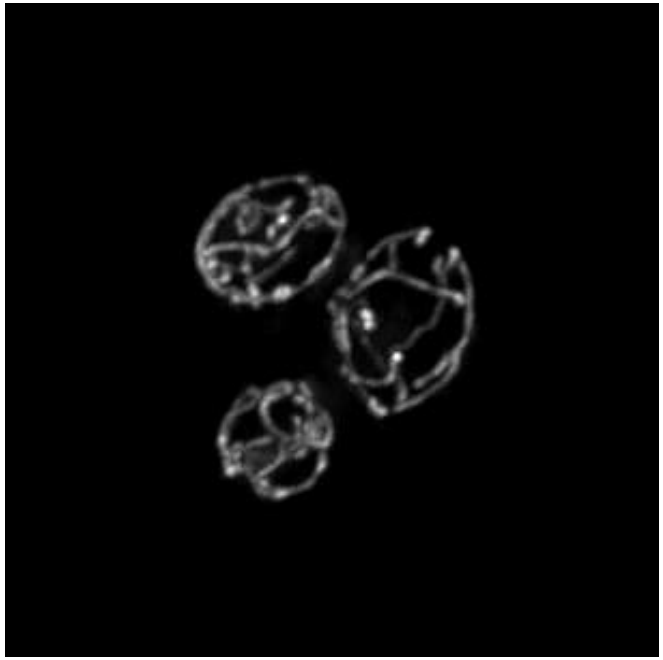
Imaging Under NonOptimal Conditions



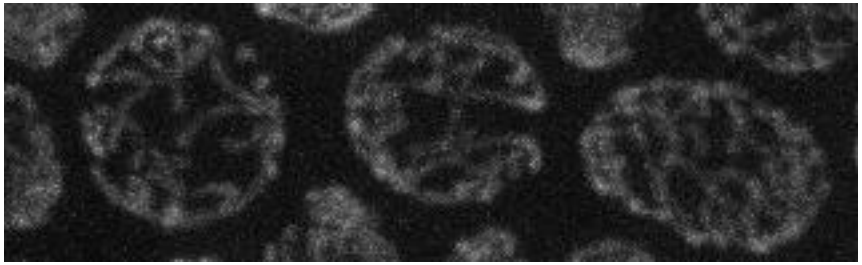
UMUC3 cancer cells expressing a mTagBFP labeled 53BP1 (DNA damage sensor) fragment
(405nm arc lamp exposure every minute for about an hour, no heat or CO2)

Images courtesy of Beth Cimini

GFP targeted to the mitochondrial matrix imaged under nonoptimal conditions



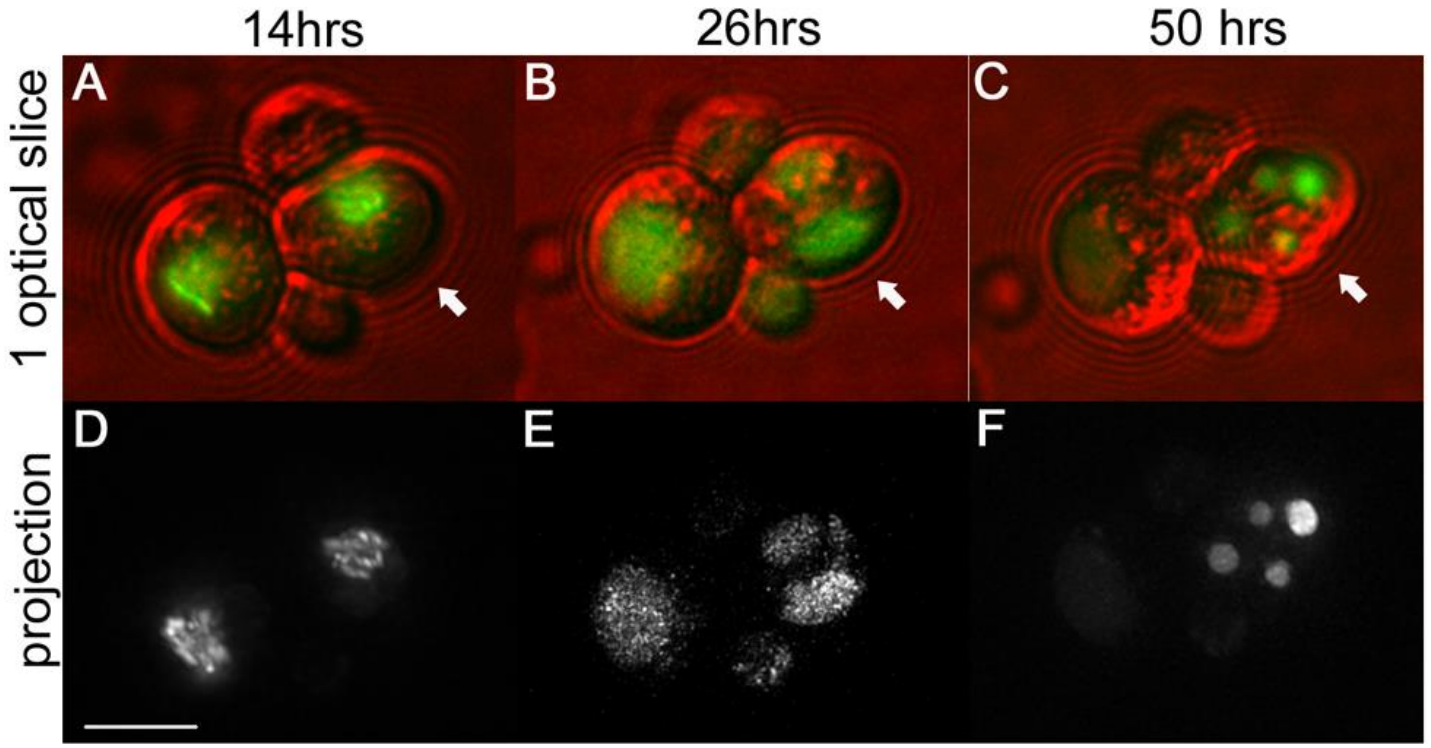
Z-stack taking every 20 secs for 200 secs
(wide-field deconvolution – high light levels)



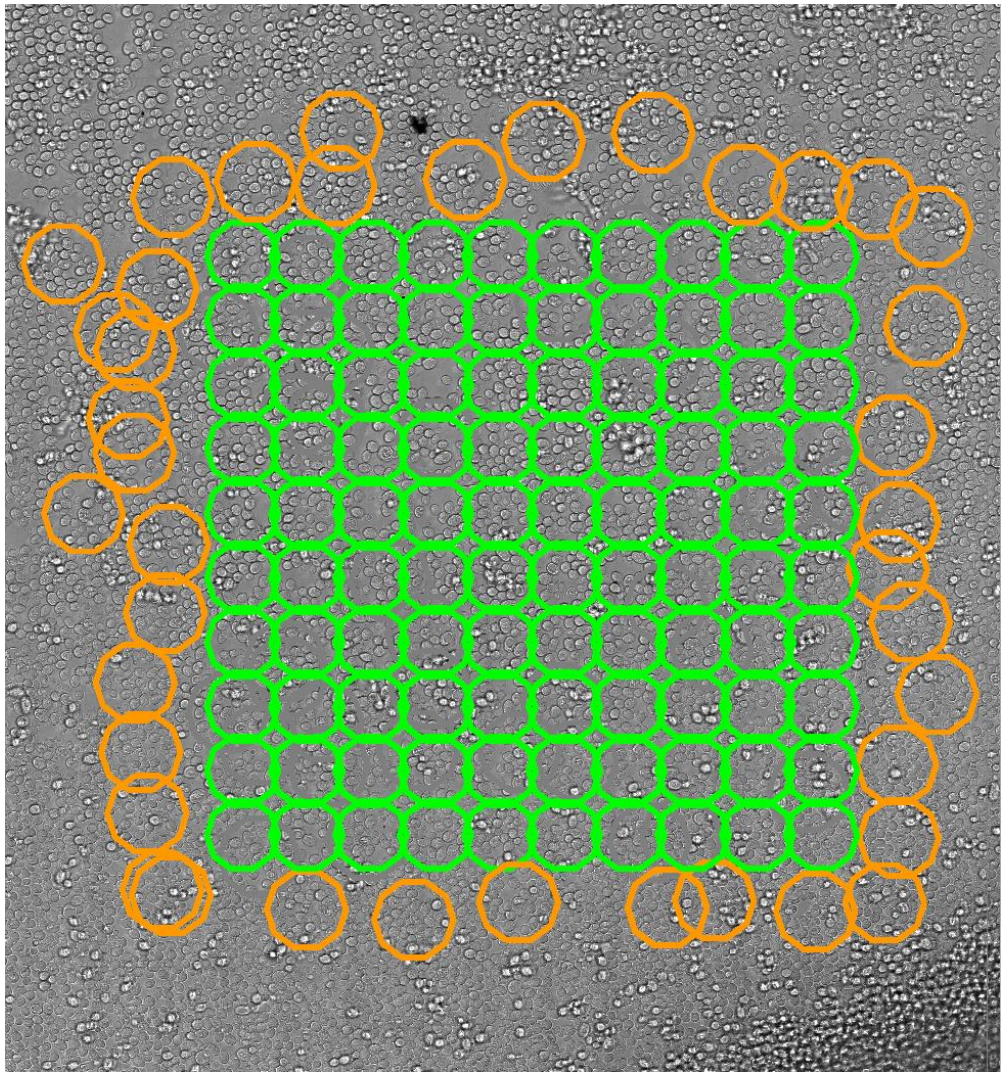
Z-stack taking every 1 secs for 200 secs
(spinning disk confocal – low light levels

Images courtesy of Susanne Rafelski

Using Meiotic Progression to Evaluate Phototoxicity



Tetrad Formation 3 fold decreased in imaged areas



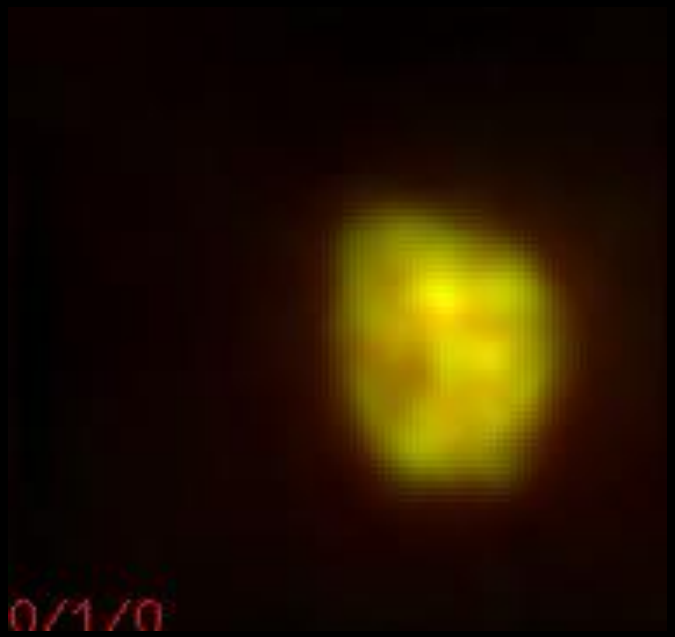
Imaging Area

Not Imaged Area

Tetrads

3X > 1X

Photoxicity Affects Chromosome Synapsis



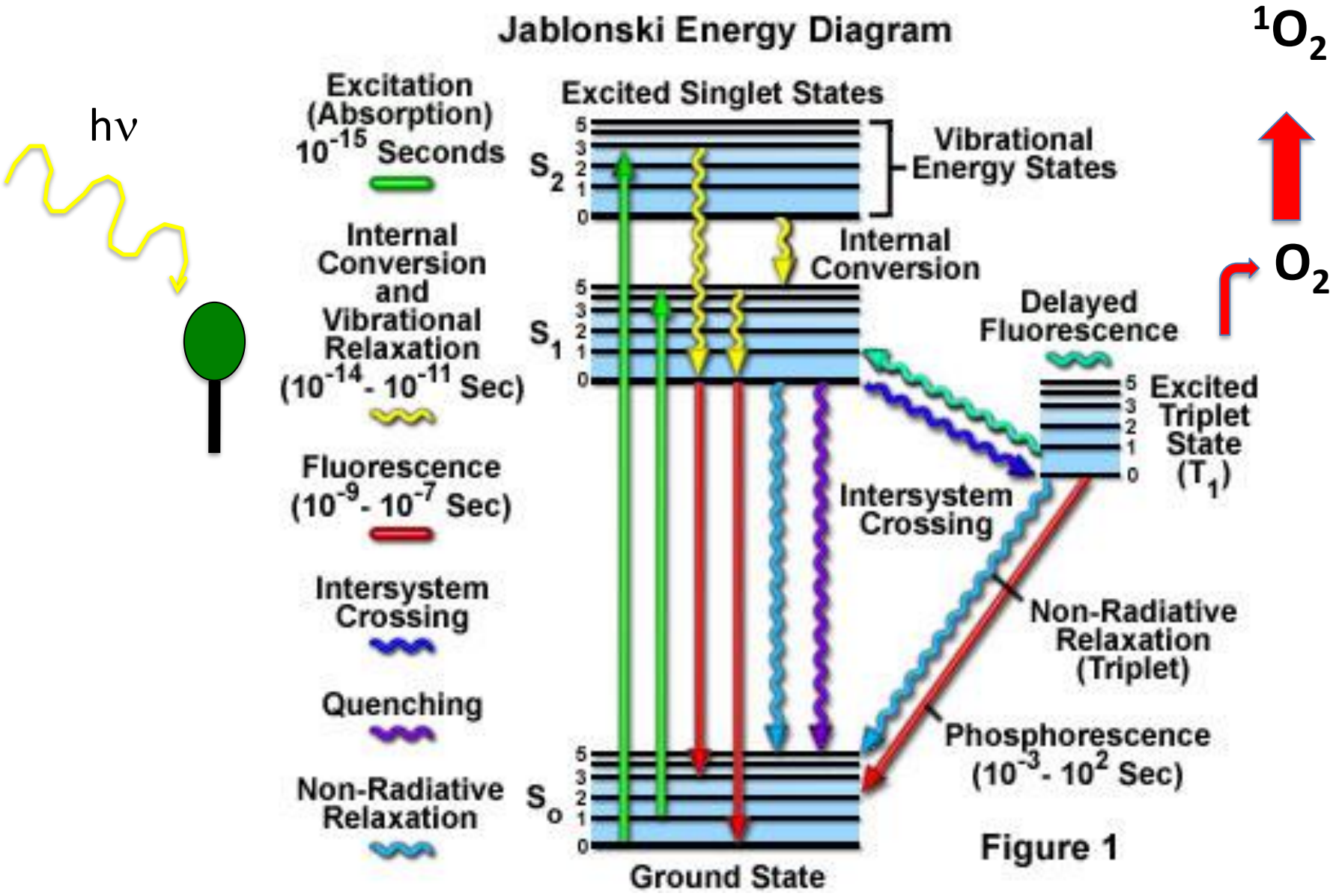

Jablonski Energy Diagram
hν
Excitation (Absorption) 10-15 Seconds
Internal Conversion and Vibrational Relaxation (10-14 - 10-11 Sec)
Fluorescence (10-9 - 10-7 Sec)
Intersystem Crossing
Quenching
Non-Radiative Relaxation
Excited Singlet States
S2
S1
So
Vibrational Energy States
Internal Conversion
Delayed Fluorescence
Excited Triplet State (T1)
Intersystem Crossing
Non-Radiative Relaxation (Triplet)
Phosphorescence (10-3 - 102 Sec)
Ground State
1O2
O2

Figure 1

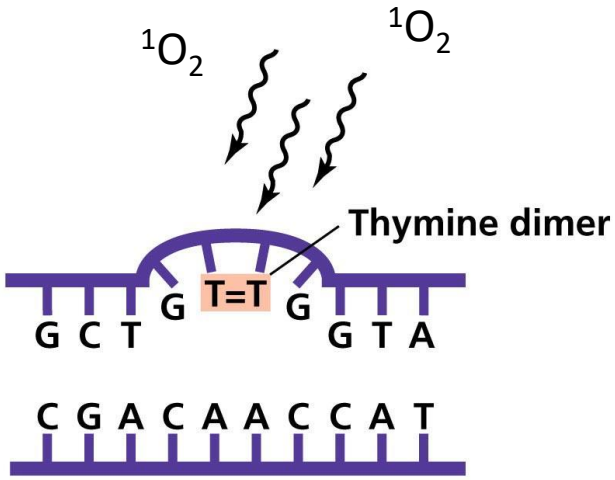
Biological Harmful Effects of Free Radicals

Because of its highly reactive nature (unpaired electron) singlet O_2 will attack sites of high electron density -e.g. C=C, N atoms.

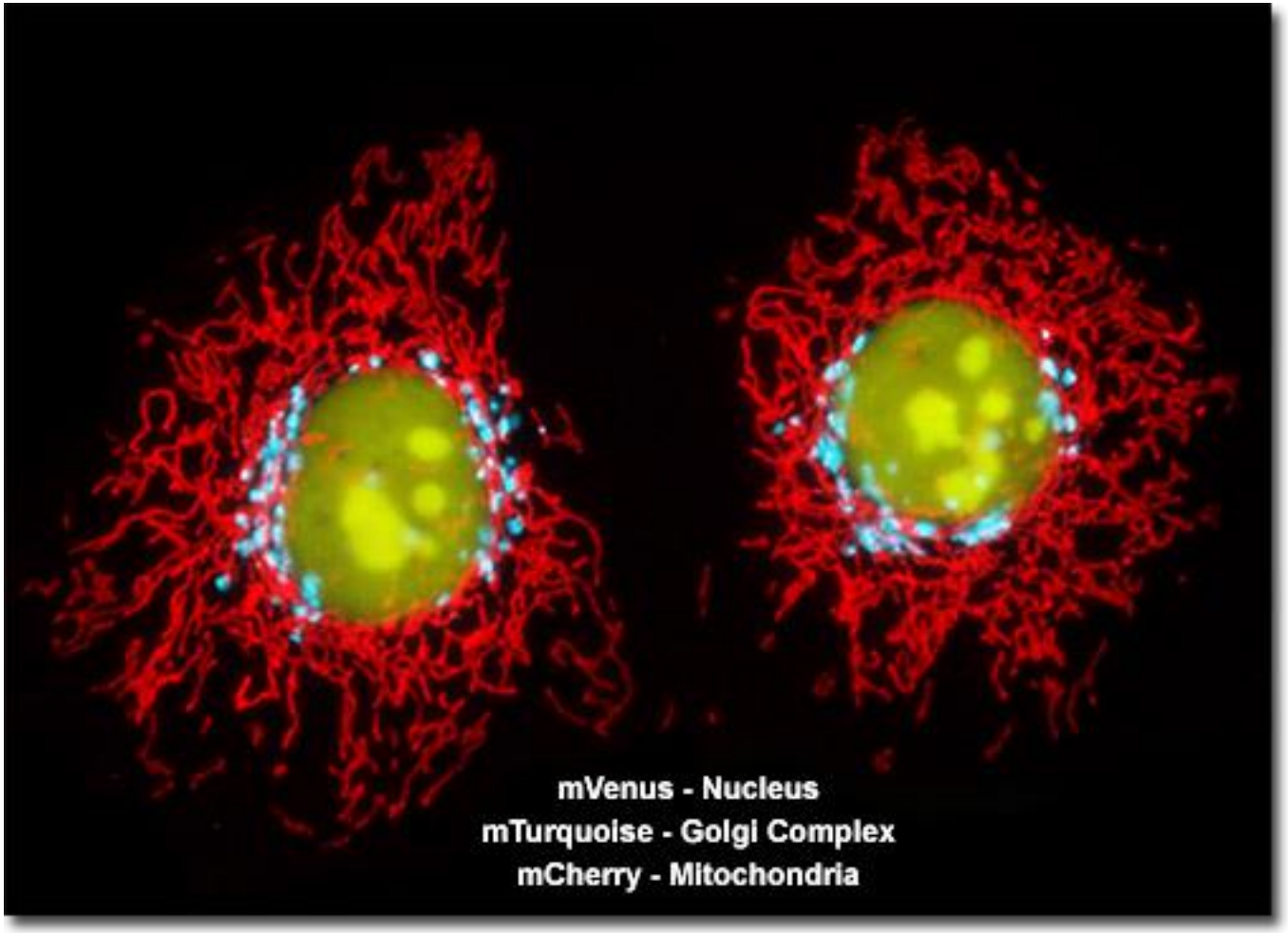
Lipid peroxidation

Protein modification

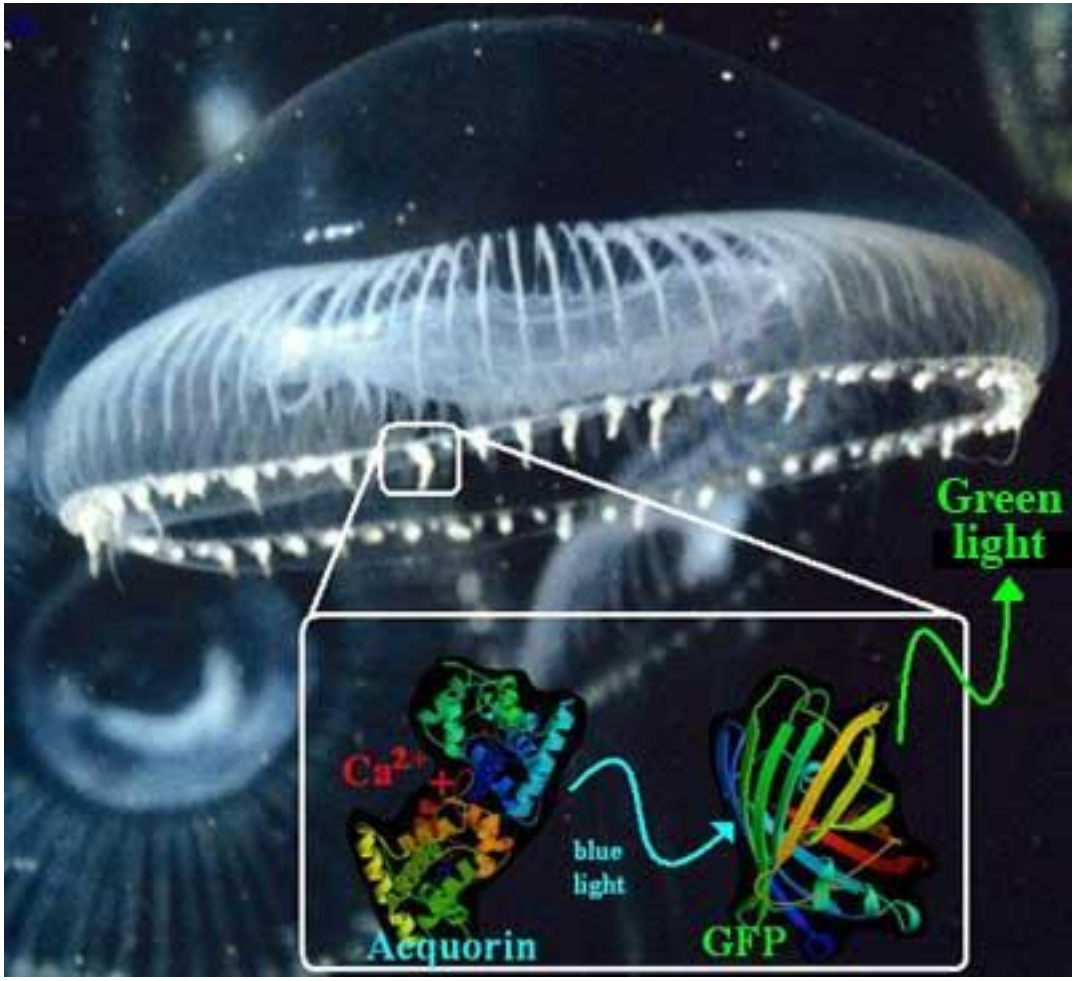
DNA modification



Multi-wavelength Imaging Increases Photoxicity



Green Fluorescent Protein



Genetically Encoded Fluorescent Fusion Proteins

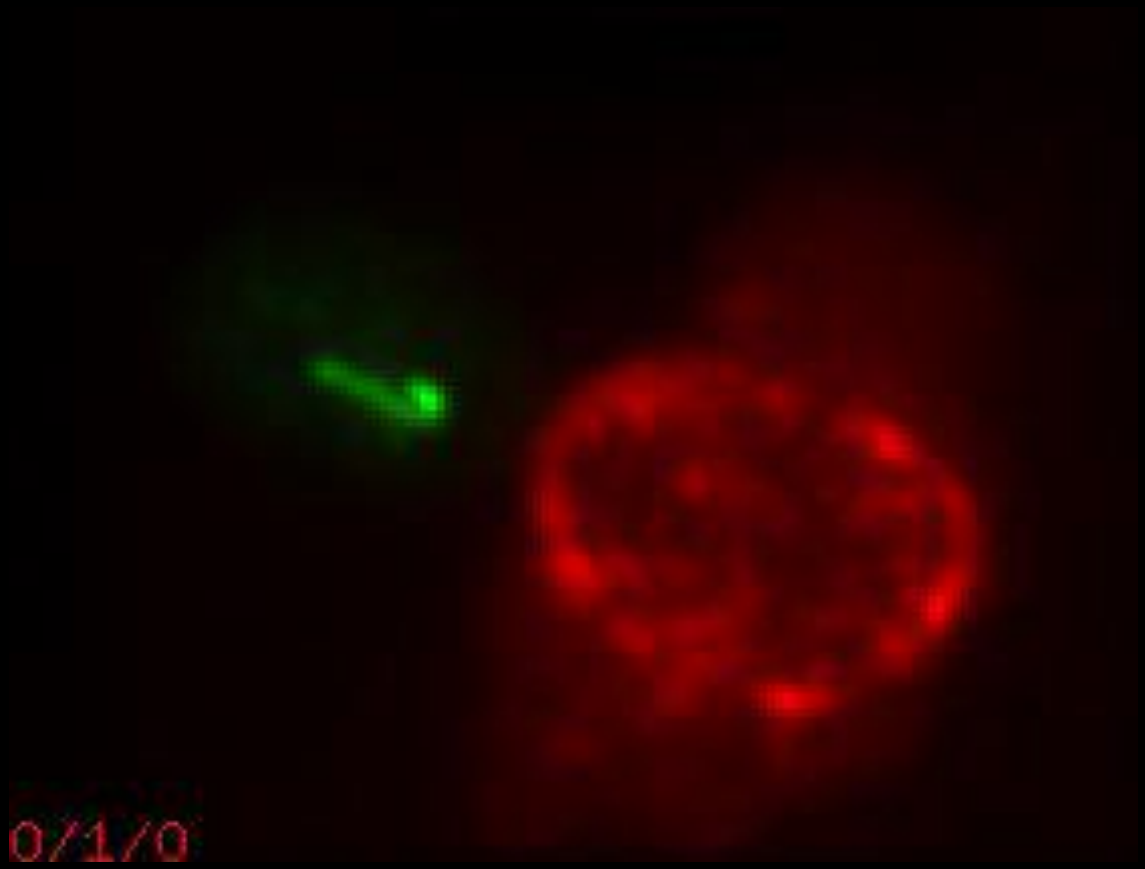
Protein (Acronym)	Excitation Maximum (nm)	Emission Maximum (nm)	Molar Extinction Coefficient	Quantum Yield	*in vivo* Structure	Relative Brightness (% of EGFP)
GFP (wt)	395/475	509	21,000	0.77	Monomer*	48
Green Fluorescent Proteins						
EGFP	484	507	56,000	0.6	Monomer*	100
Emerald	487	509	57,500	0.68	Monomer*	116
Superfolder GFP	485	510	83,300	0.65	Monomer*	160
Blue Fluorescent Proteins						
EBFP	383	445	29,000	0.31	Monomer*	27
EBFP2	383	448	32,000	0.56	Monomer*	53
Azurite	384	450	26,200	0.55	Monomer*	43
mTagBFP	399	456	52,000	0.63	Monomer	98
Cyan Fluorescent Proteins						
ECFP	439	476	32,500	0.4	Monomer*	39
mECFP	433	475	32,500	0.4	Monomer	39
Cerulean	433	475	43,000	0.62	Monomer*	79
mTurquoise	434	474	30,000	0.84	Monomer*	75
Yellow Fluorescent Proteins						
EYFP	514	527	83,400	0.61	Monomer*	151
Topaz	514	527	94,500	0.6	Monomer*	169
Venus	515	528	92,200	0.57	Monomer*	156
mCitrine	516	529	77,000	0.76	Monomer	174

Genetically Encoded Fluorescent Fusion Proteins

Protein (Acronym)	Excitation Maximum (nm)	Emission Maximum (nm)	Molar Extinction Coefficient	Quantum Yield	*in vivo* Structure	Relative Brightness (% of EGFP)
Orange Fluorescent Proteins						
Kusabira Orange	548	559	51,600	0.6	Monomer	92
Kusabira Orange2	551	565	63,800	0.62	Monomer	118
mOrange	548	562	71,000	0.69	Monomer	146
mOrange2	549	565	58,000	0.6	Monomer	104
dTomato	554	581	69,000	0.69	Dimer	142
dTomato-Tandem	554	581	138,000	0.69	Monomer	283
TagRFP	555	584	100,000	0.48	Monomer	142
TagRFP-T	555	584	81,000	0.41	Monomer	99
DsRed	558	583	75,000	0.79	Tetramer	176
Red Fluorescent Proteins						
mRuby	558	605	112,000	0.35	Monomer	117
mApple	568	592	75,000	0.49	Monomer	109
mStrawberry	574	596	90,000	0.29	Monomer	78
AsRed2	576	592	56,200	0.05	Tetramer	8
mRFP1	584	607	50,000	0.25	Monomer	37
JRed	584	610	44,000	0.2	Dimer	26
mCherry	587	610	72,000	0.22	Monomer	47

Photobleaching is also a problem

Green – Synapsed chromosome (GFP) Red – Nuclear pore (mcherry)



Ways to Lessen Phototoxicity and Photobleaching Imaging

- Remove oxygen and singlet oxygen
- Improve detection
- Reduce signal
- Reduce frequency of exposures
- Lower noise

Removing Oxygen and Free Radicals

Removing Oxygen

- Oxyrase - enzyme that removes oxygen (shown not to be effective with GFP)

Free radical scavengers

- Trolox – derivative of Vitamin E
- ascorbic acid – Vitamin C
- n-Propyl Gallate

Reduce signal

- Lowering exposure time
- Increasing neutral density filters

Deepstar pulsed lasers – infinite modulation

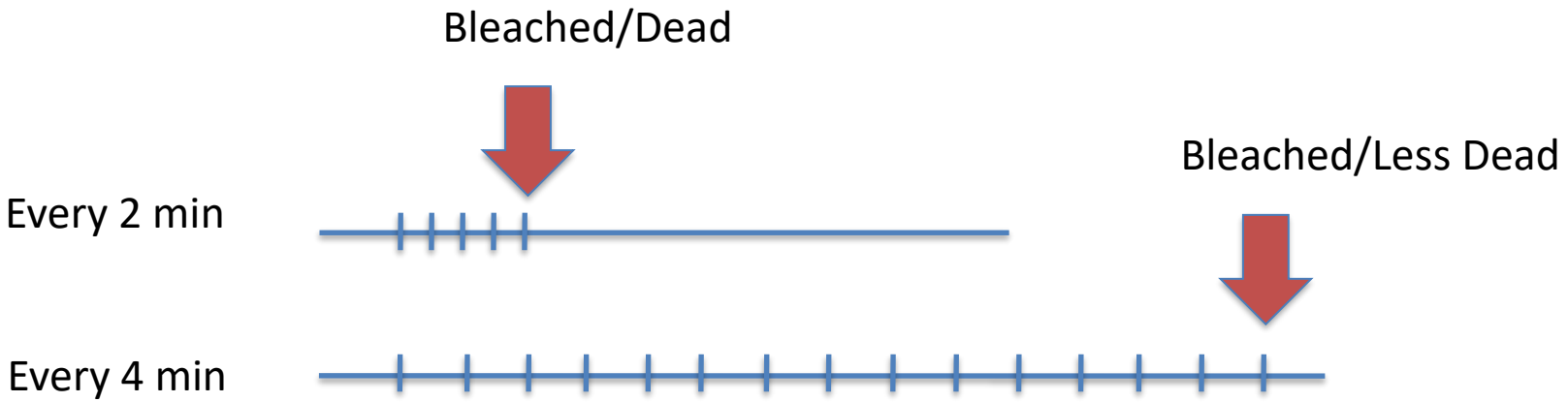
accurately attain short exposures in nano - microsecond time frame

Improve Detection using EMCCD or sCMOS camera

EMCCD – **e**lectron **m**ultiplying **c**harge **c**oupled **d**evice
- low readout noise – 1 electron/pixel
- fast – 30 - 60 Hz (33 – 16 ms per frame)
- quantum efficiency 90-95%

sCMOS – **s**cientific **C**omplementary **M**etal–**O**xide–**S**emiconductor
- low readout noise – 1 electron/pixel
- faster – 100 Hz (2-3 ms per frame)
- quantum efficiency 50%

Reduce frequency of exposures

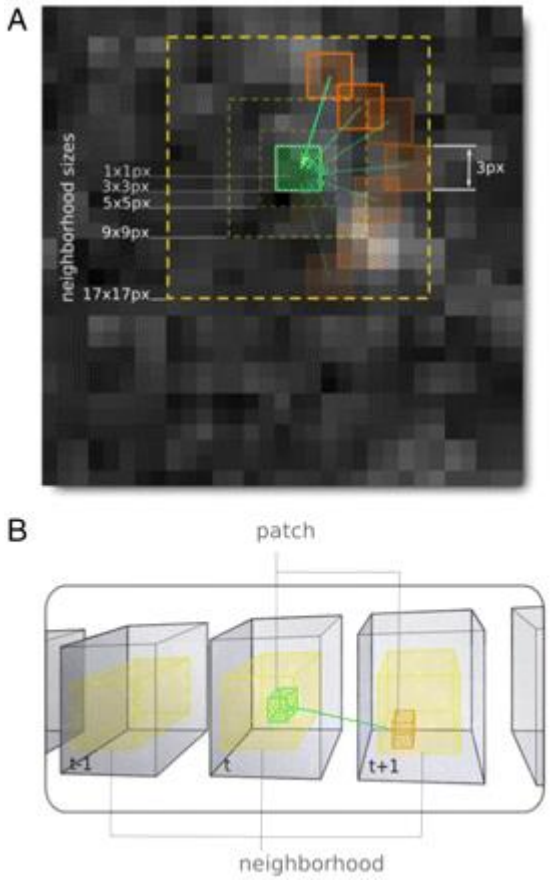


Instead of 2x longer before bleaching, you can go 3x as long

1. Don’t overwhelm endogenous cell antioxidant enzymes
2. Allow longer time for repair

Lowering noise by denoising algorithms

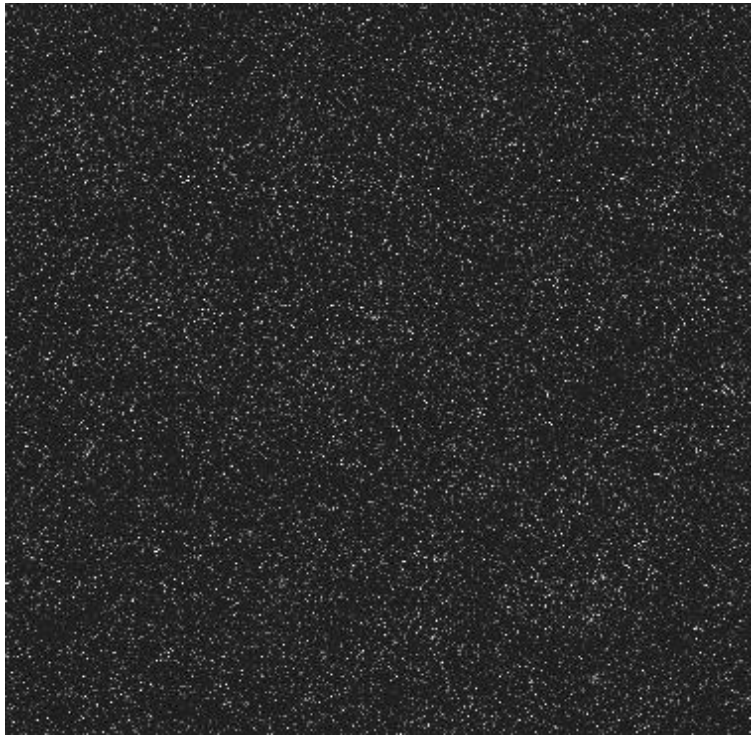
Denoising - The overall strategy is to find regions of the image, which by virtue of similar statistical behavior, are likely to have the same underlying intensity distribution and then average them to reduce noise.



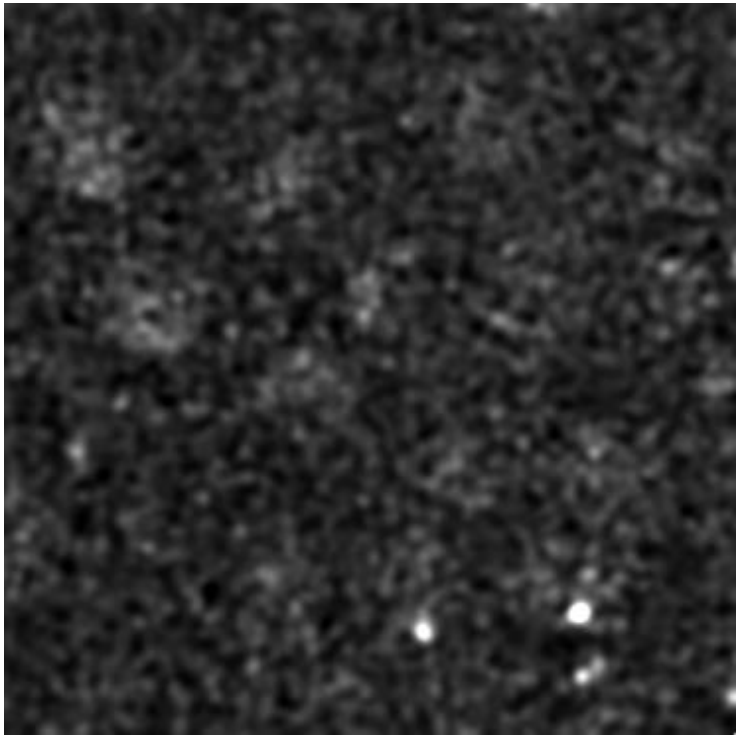
Charles Kervrann

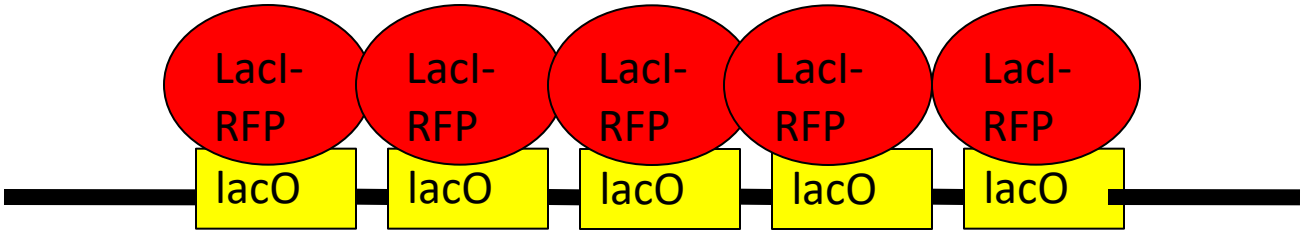
Using denoising to reduce photoxicity

Raw data

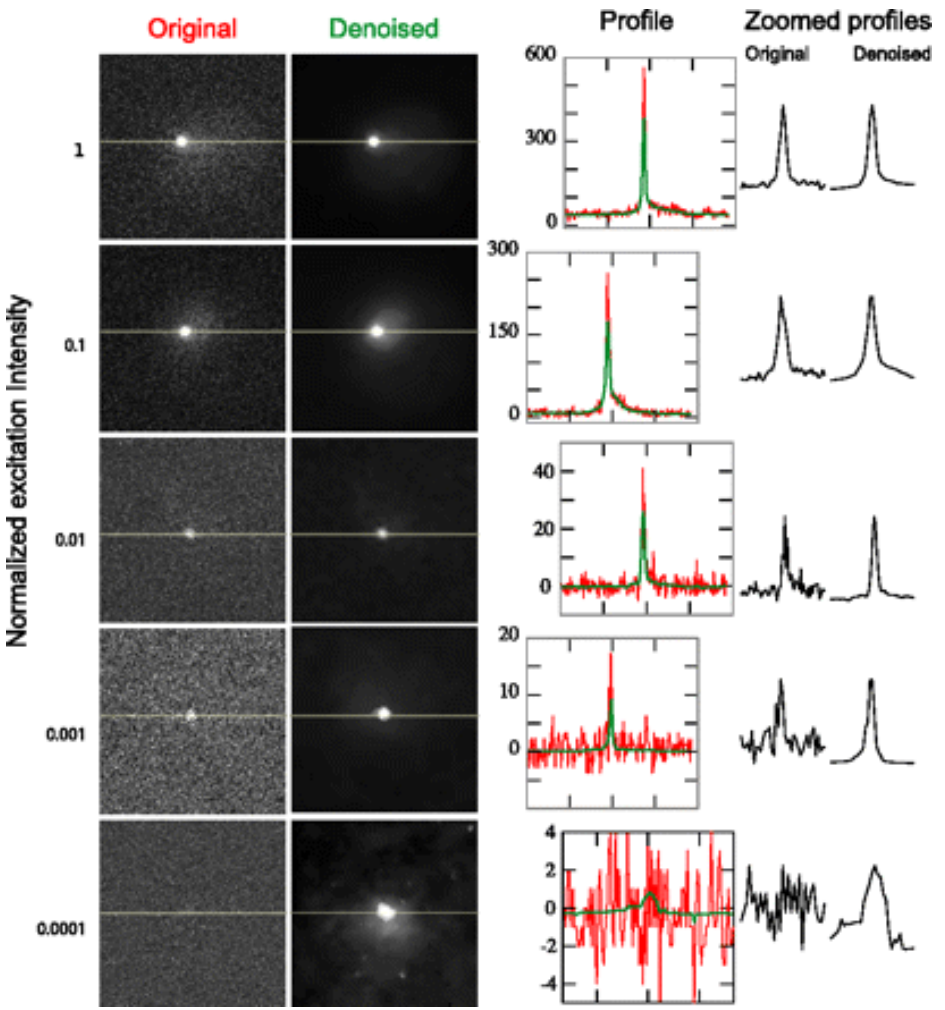


Denoised and Deconvolved



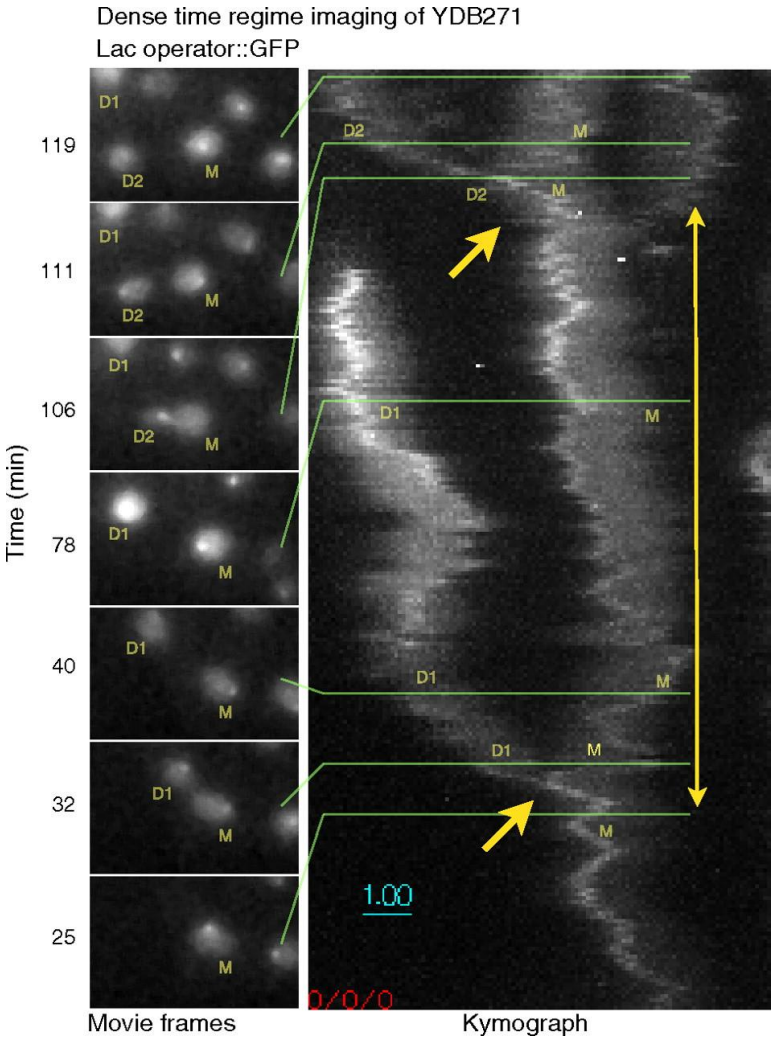


Quantitative Analysis of Improvements Through Denoising



Denoising can improve both phototoxicity and photobleaching

At low light levels (I = 5 × 10-4I0), dense-regime imaging can continue for 2 h, encompassing a whole cell cycle, without fading or loss of viability.



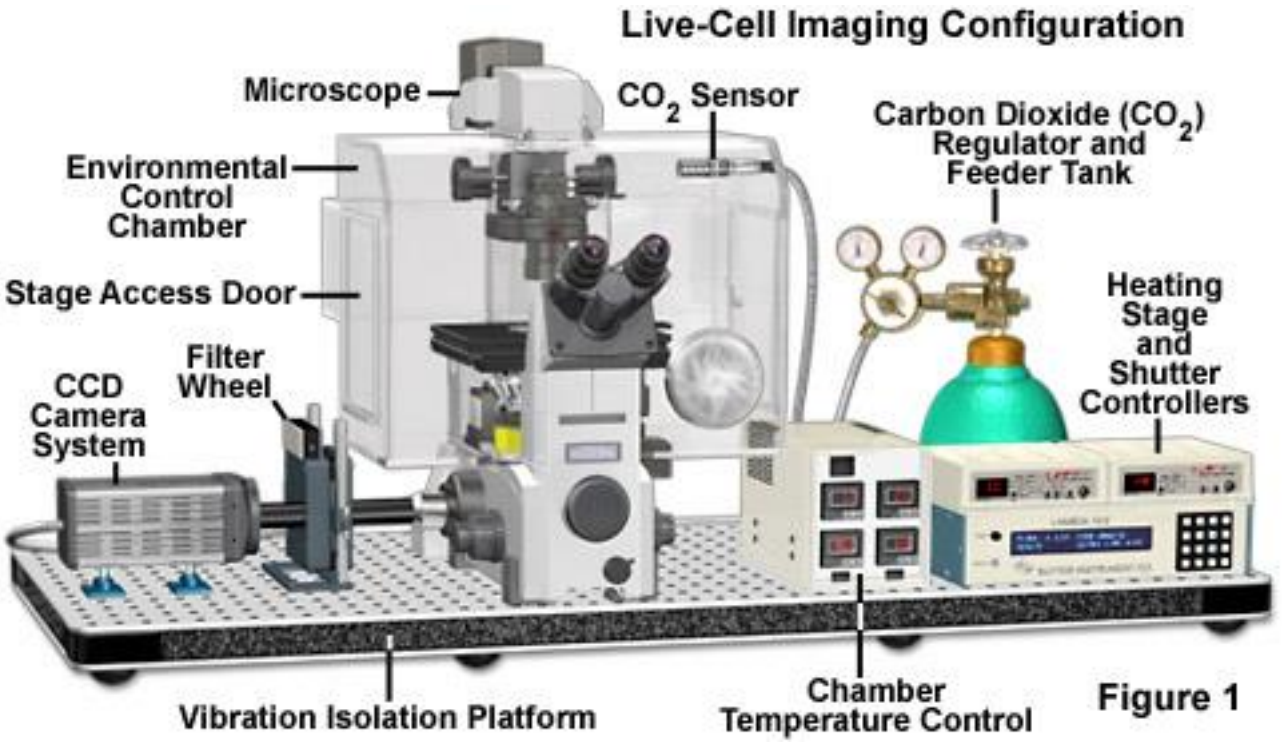
Carlton P M et al. PNAS 2010;107:16016-16022

Preserving a normal environment during imaging

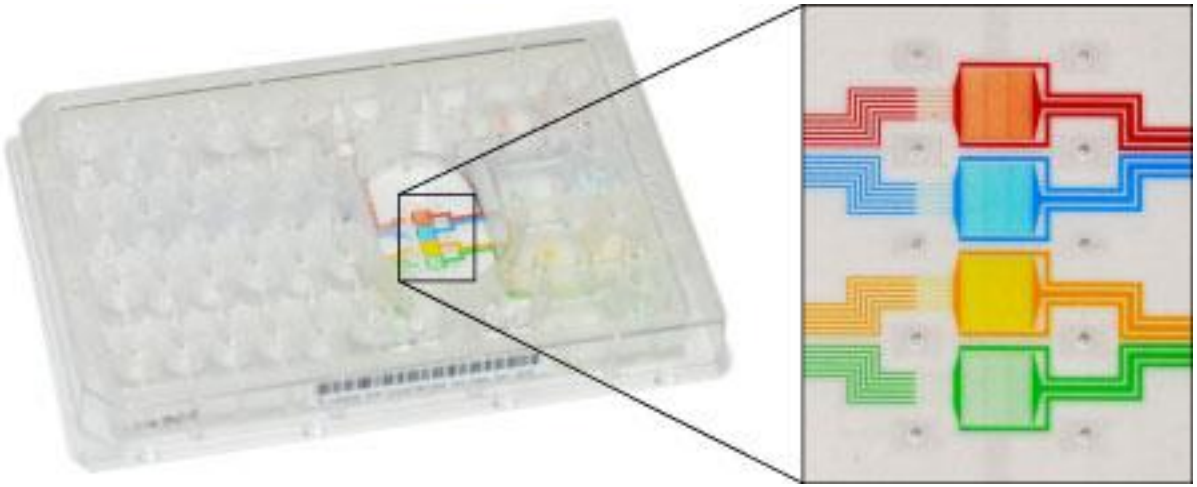
Environmental Variables for Mammalian Cell Lines

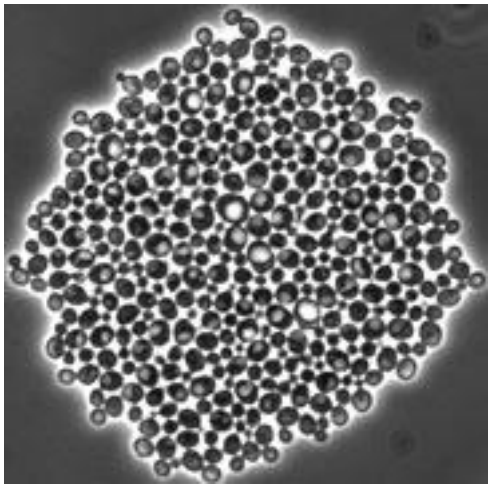
Variable	Optimum Range	Comments
Temperature	28-37ºC	Control with Specimen Chamber Heaters Inline Perfusion Heaters Objective Lens Heaters Environmental Control Boxes
Oxygenation	Variable	Perfuse or Change Media Regularly Use Large Chamber Volume
Humidity	97-100%	Closed chamber, humidified environmental chamber
pH	7.0 -7.7	Use Buffered Media, Perfuse or change media, no phenol red indicator
Osmolarity	260-320 mosM	Avoid evaporation, sealed chamber
Atmosphere	Air or 5-7% C02	Used Buffered media, closed chamber
Media buffer	Bicarbonate or Synthetic buffers	Beware of phototoxicity, closed & open chambers, atmosphere controlled chamber

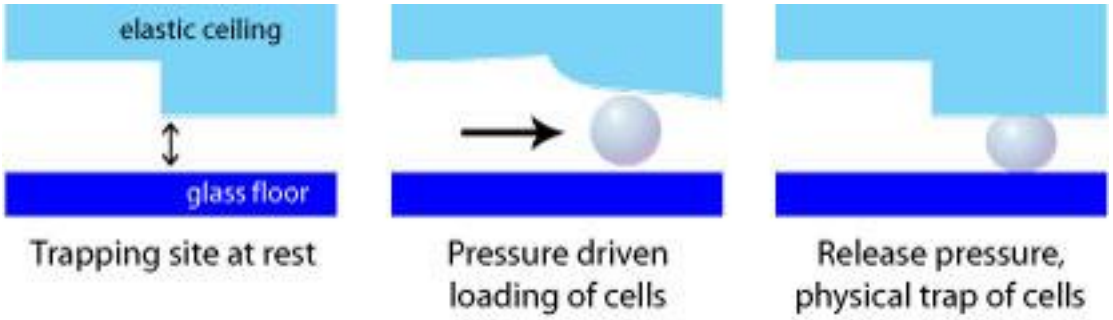
Environmental Chamber



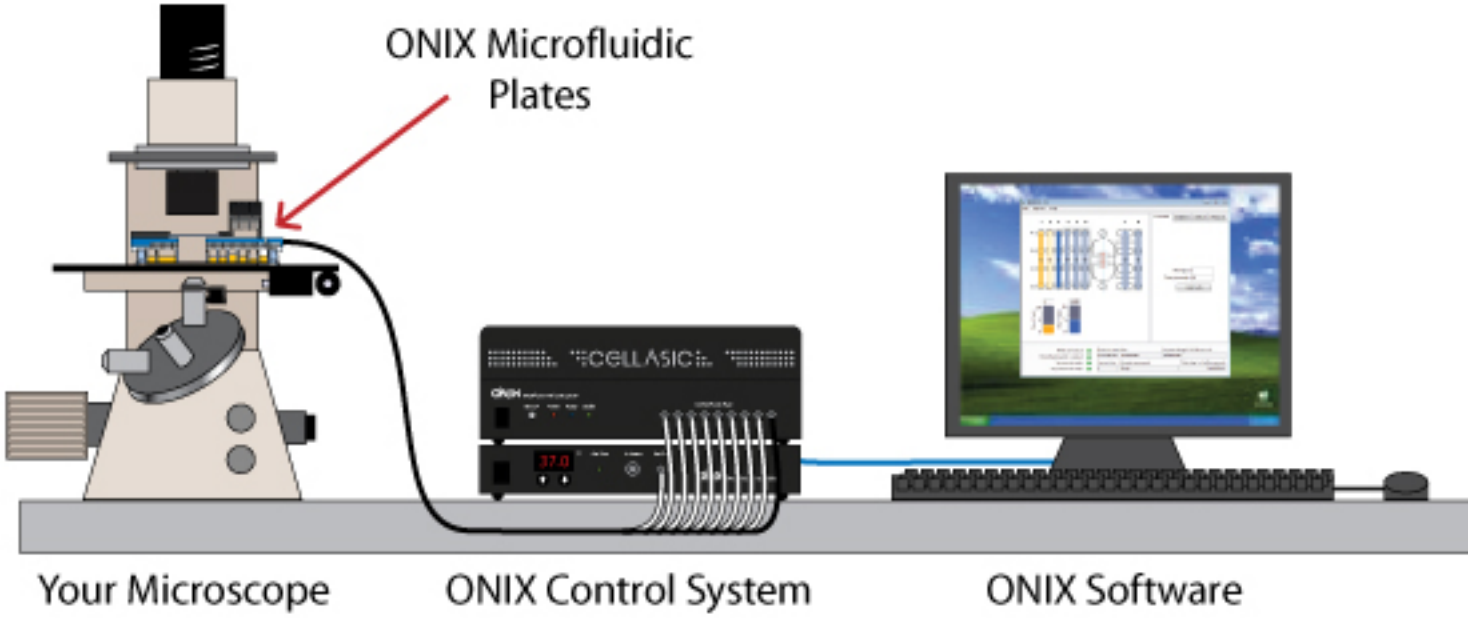
CellASIC – Microfluidic Systems



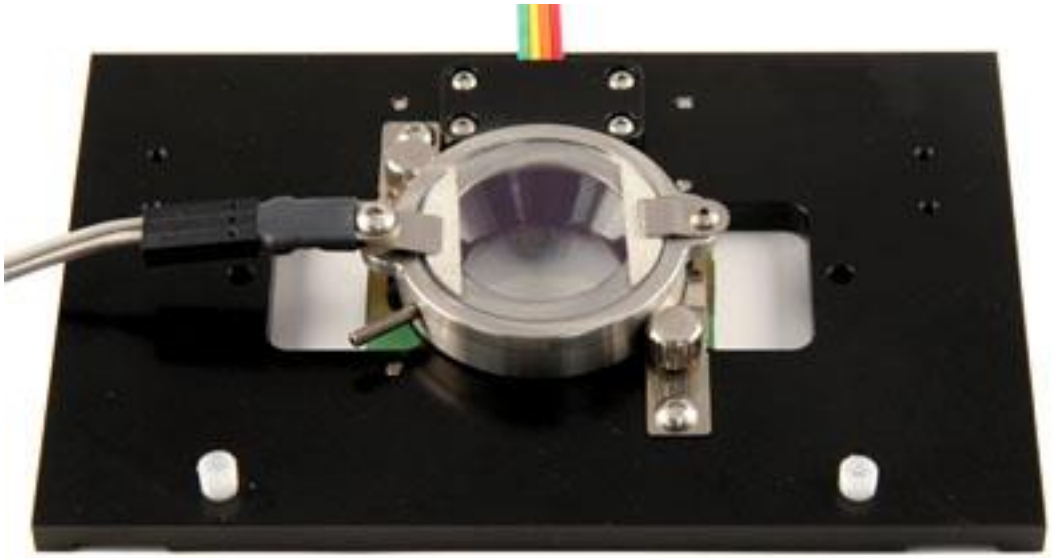


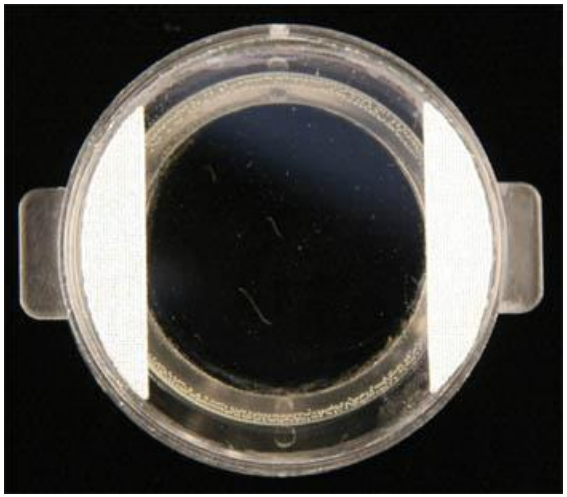


CellASIC – Microfluidic Systems

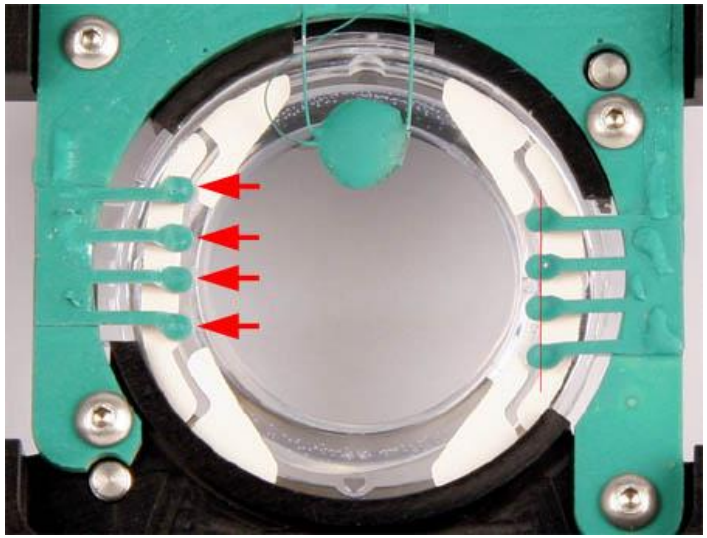


Bioptechs – Open/Closed Dish System

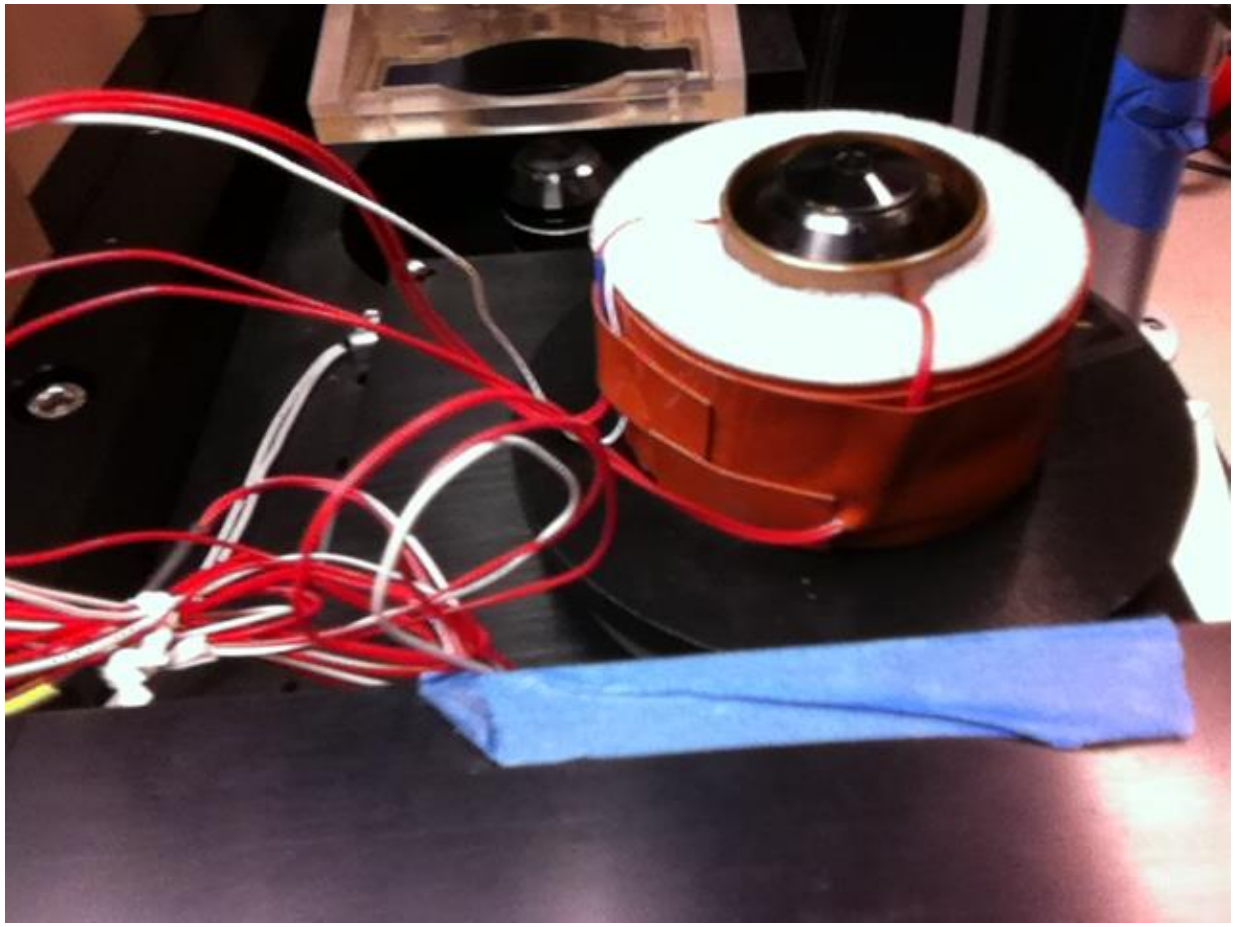




Delta T Dish



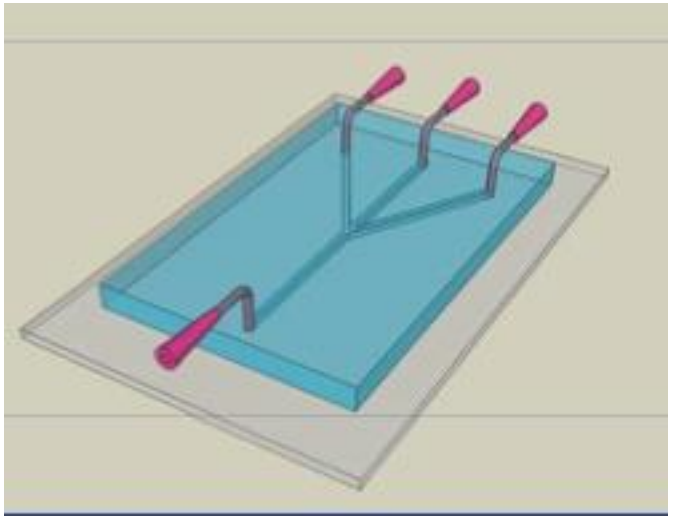
Heated Objective



Custom-made PDMS microfluidics

PDMS - Poly(dimethylsiloxane)

Relatively inexpensive
Easy to use Chemically inert/non-hazardous
Optically clear Flexible and fairly tough when cured
Easily bonded to itself or other materials
Permeable to air and liquids (but can be coated to prevent this).



Future Directions

Better reconstruction and denoising algorithms

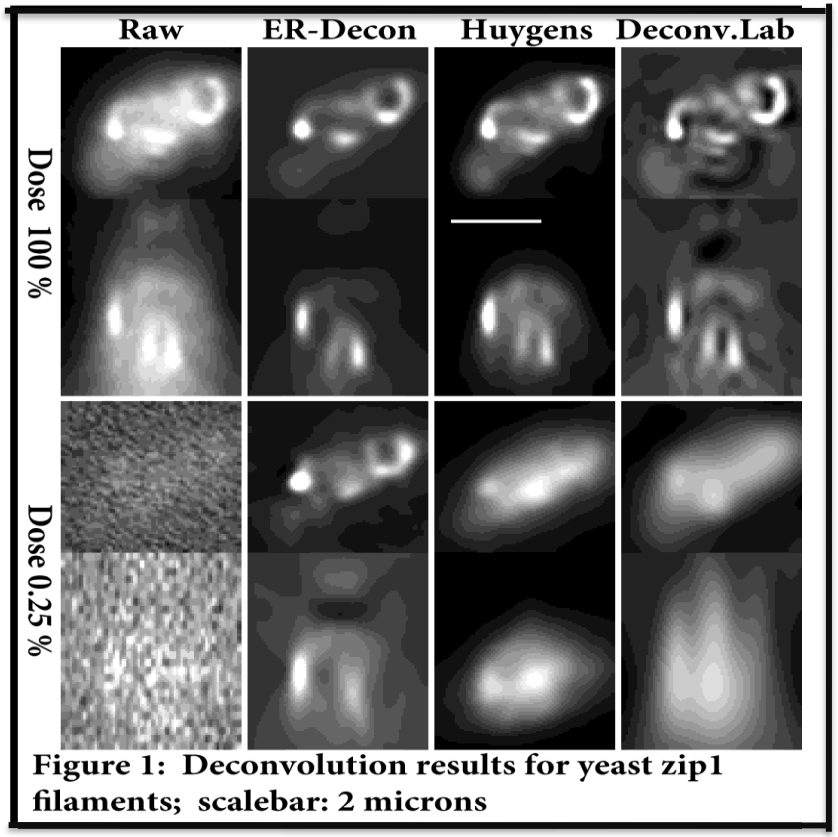
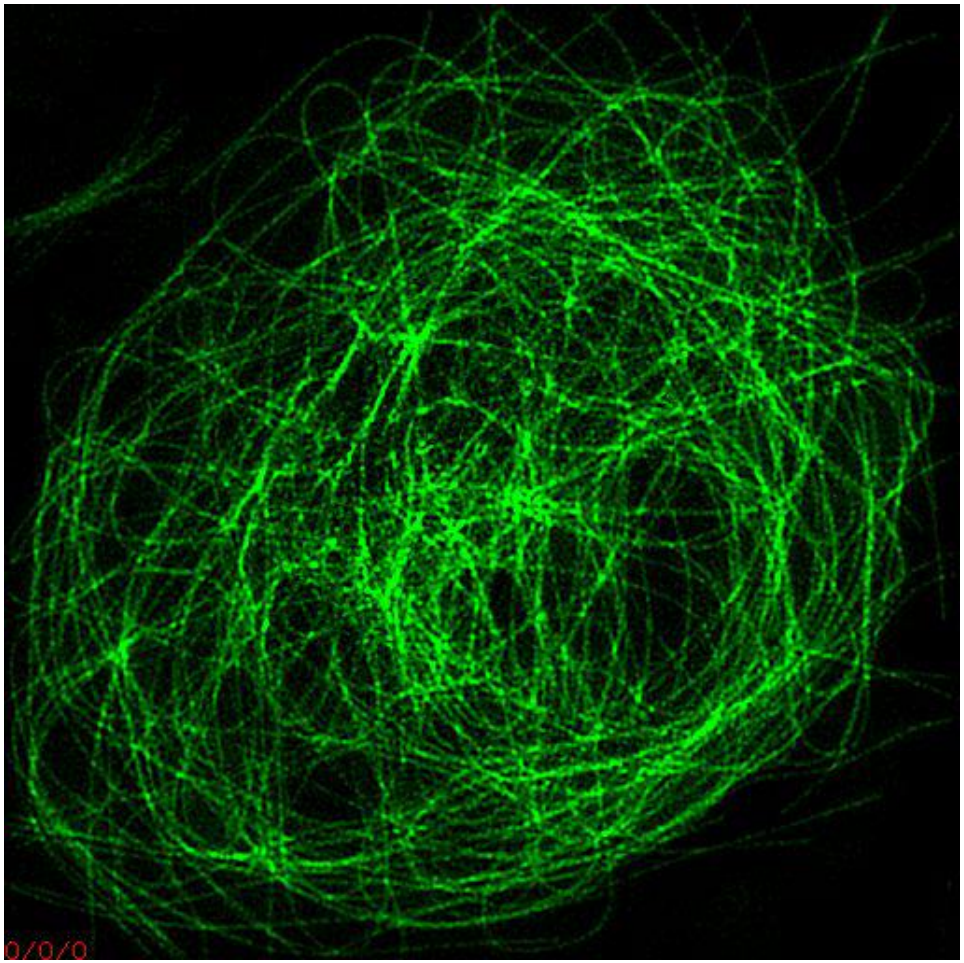


Figure 1: Deconvolution results for yeast zip1 filaments; scalebar: 2 microns

ER-deconvolution – based on filling CTF with constraints other than positivity (Entropy regularized deconvolution)

Stay Tuned

In vivo structured illumination microscopy



Tubulin-GFP in Drosophila S2 Cells

Different Goals for Fixed vs. Live

Fixed specimens: Optimize the signal/noise

Live specimens: Optimize the signal/noise but without perturbing your biological process

Things to consider for live imaging

Speed – how fast can you take the data, z projections
Detection – how sensitive is you camera, how bright and stable are your fluorophores, level of noise, resolution, stage stability
Environment – microfluidics devices, temperature, phototoxicity,oxygen
Pulsed lasers, chambers, matching objectives
Proper sample – not too deep, not too crowded, synchronized – plenty of examples of biological process, limited duration of biological process, sensitivity to DNA damage
Validation
Data storage and analysis
Denoising, combination of denoising and deconvolution – new directions
Pulsed lasers

Typical ways to improve S/N in fixed samples

- Increase exposure time
- Amplify signal
- Brighter fluorophores/dyes
- Decrease photobleaching with antioxidants

>>>> Increase signal

CellASIC – Microfluidic Systems