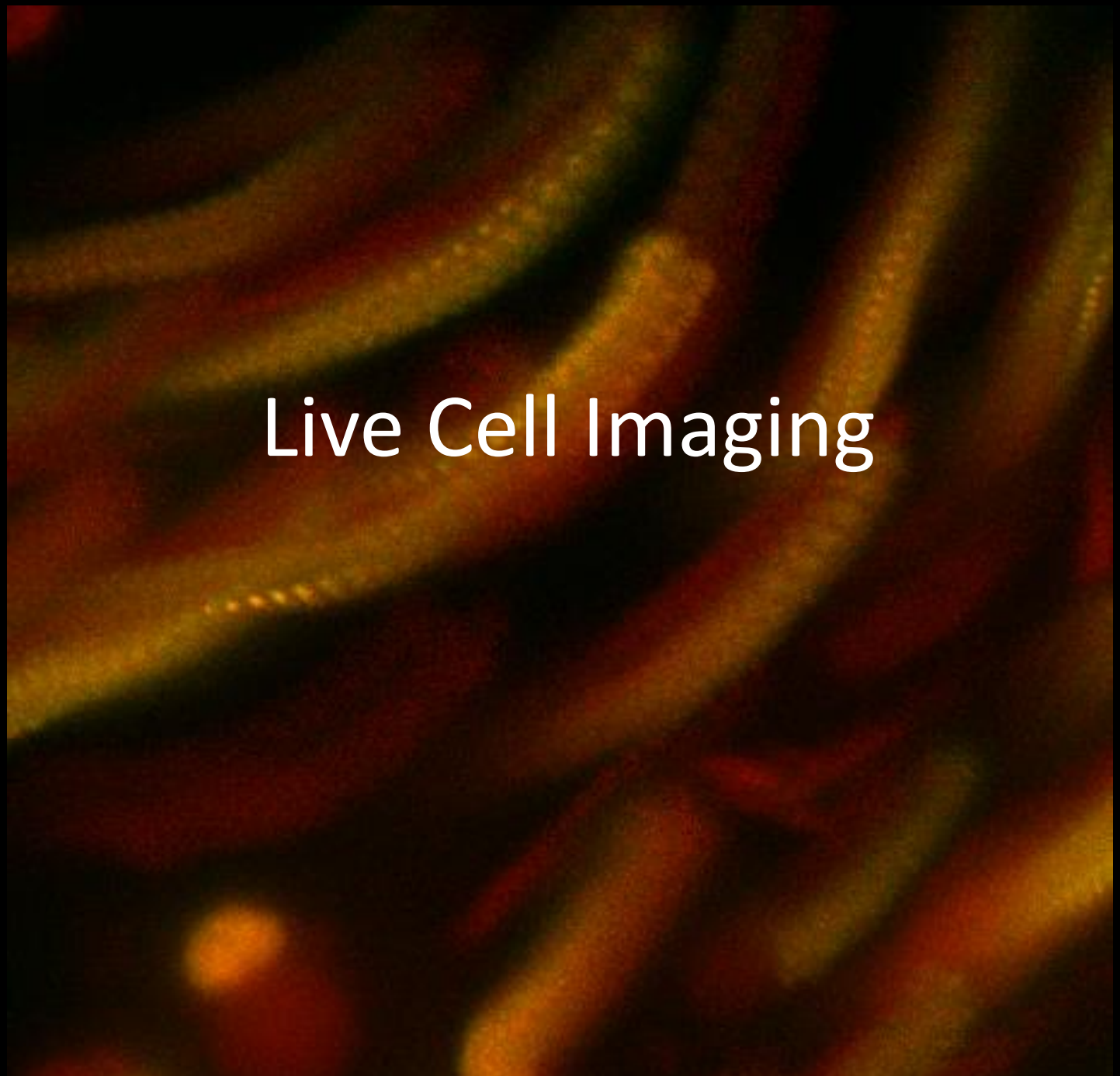

Live Cell Imaging

Key Elements for Successful Live Imaging

1. Choice of biological system

2. Validation

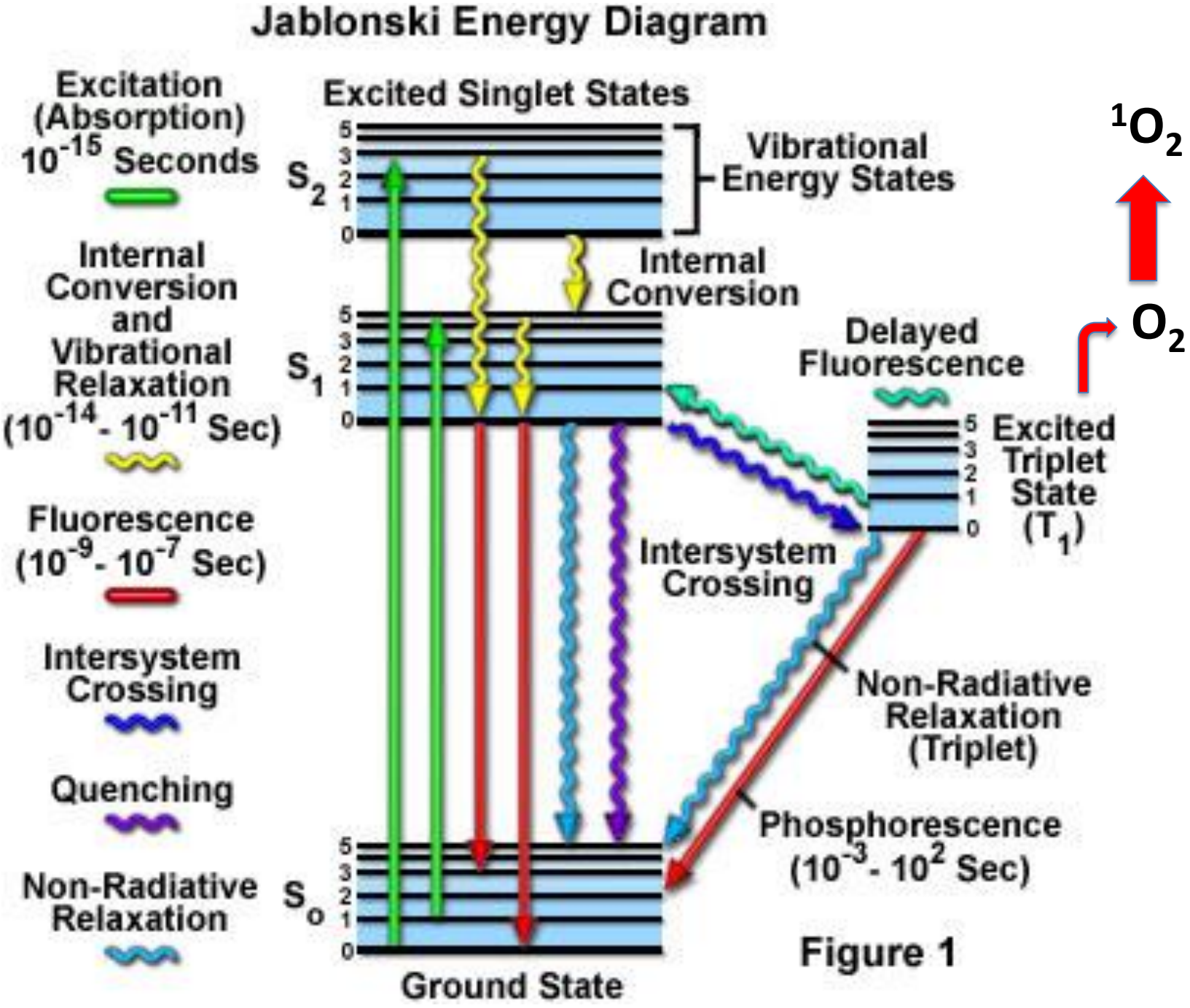
3. Reducing photoxicity and photobleaching

4. Close preservation of normal environment

Choosing the Appropriate Organism to Answer your Question

1. Easy labeling of your target of interest
2. Biological process can be found at sufficient frequency
3. 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?

Phototoxicity


Jablonski Energy Diagram
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
S_2
S_1
S_o
5
3
2
1
0
Vibrational Energy States
Internal Conversion
Delayed Fluorescence
Excited Triplet State (T_1)
Intersystem Crossing
Non-Radiative Relaxation (Triplet)
Phosphorescence (10^{-3} - 10^{2} Sec)
Ground State
$^1O_2$
O_2
Figure 1

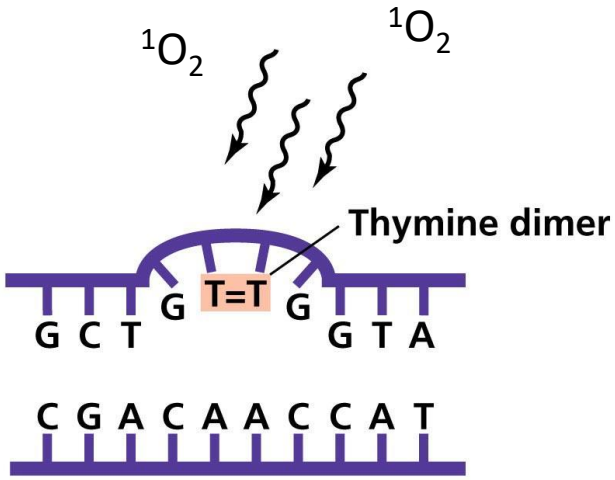
Biological Harmful Effects of Free Radicals

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

Lipid peroxidation

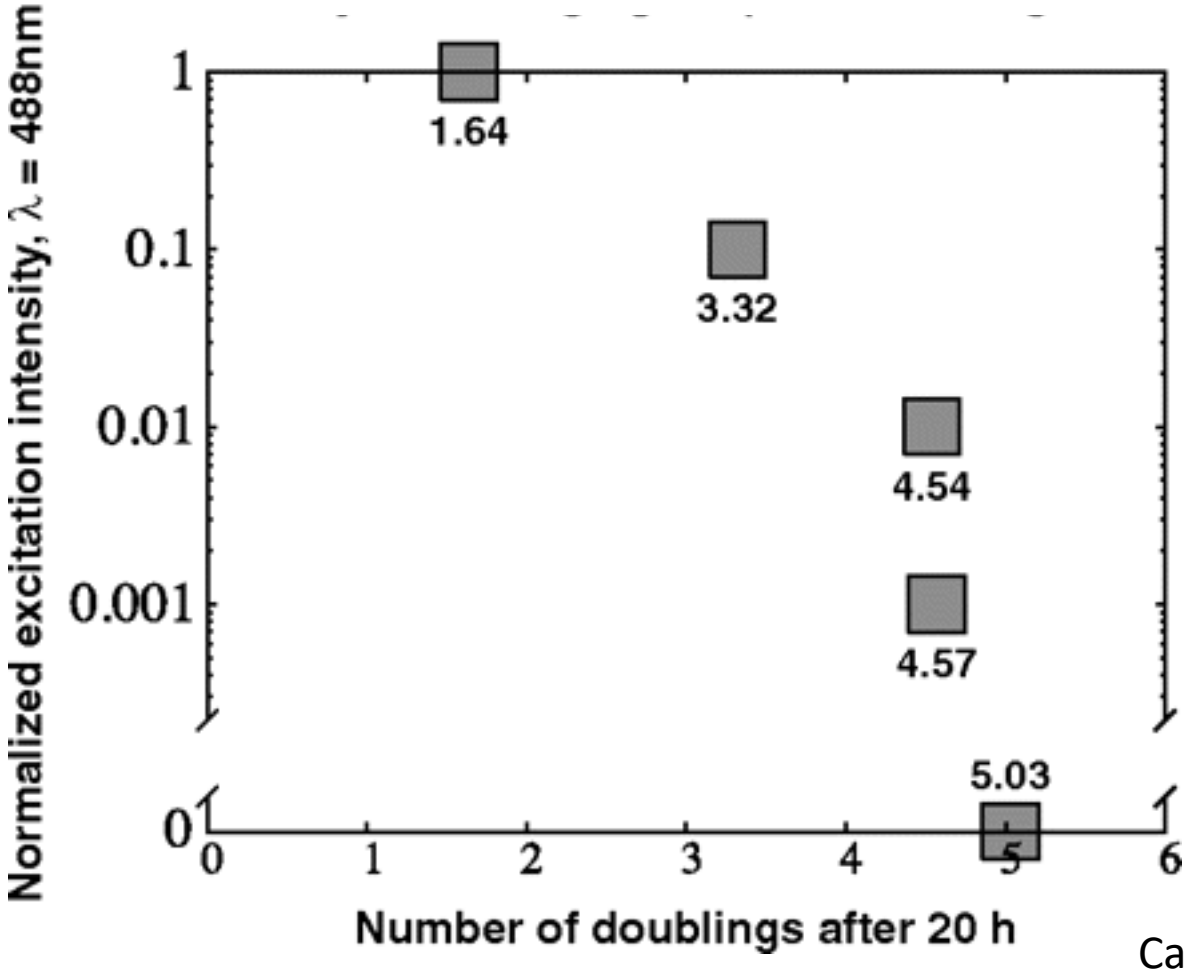
Protein modification

DNA modification



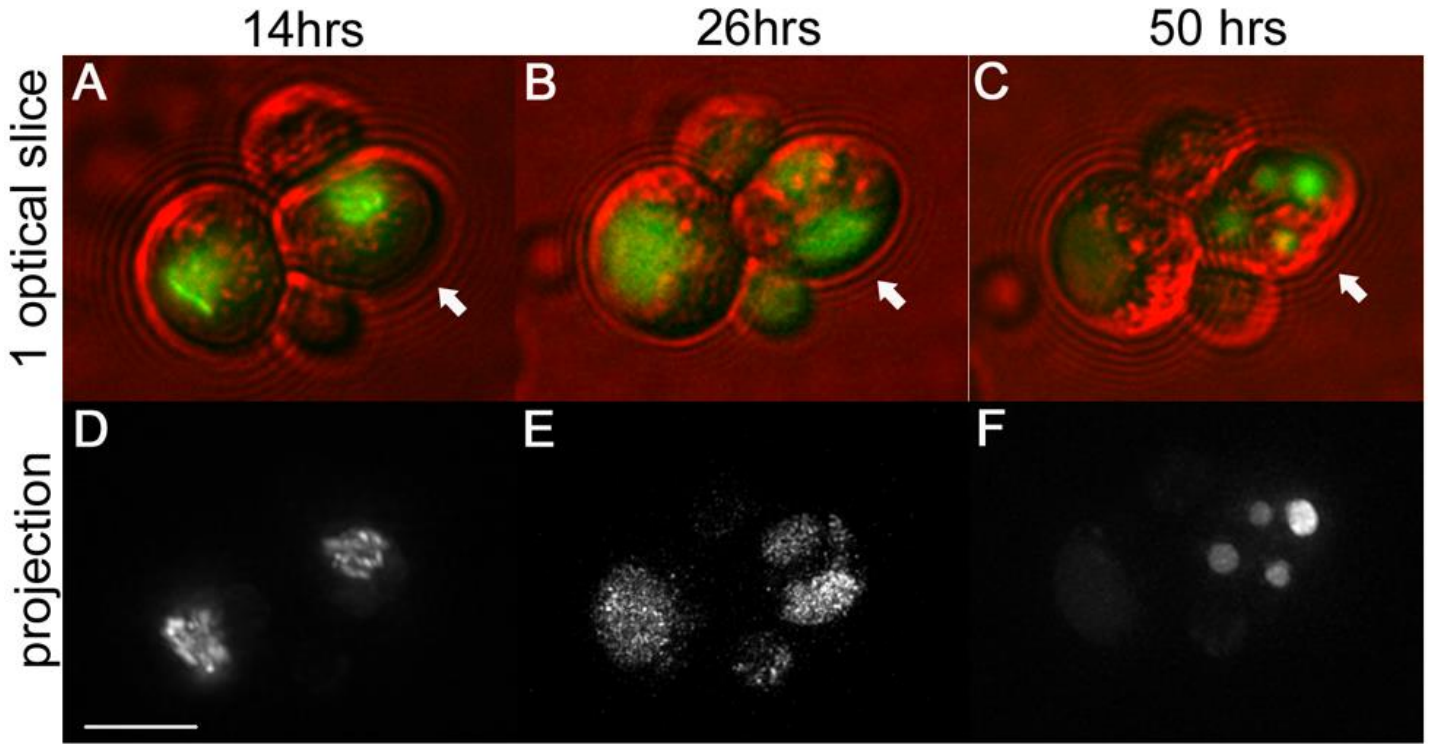
Yeast Viability After Imaging

10 msec exposures, 25 Z slices every 15 sec for 20 min



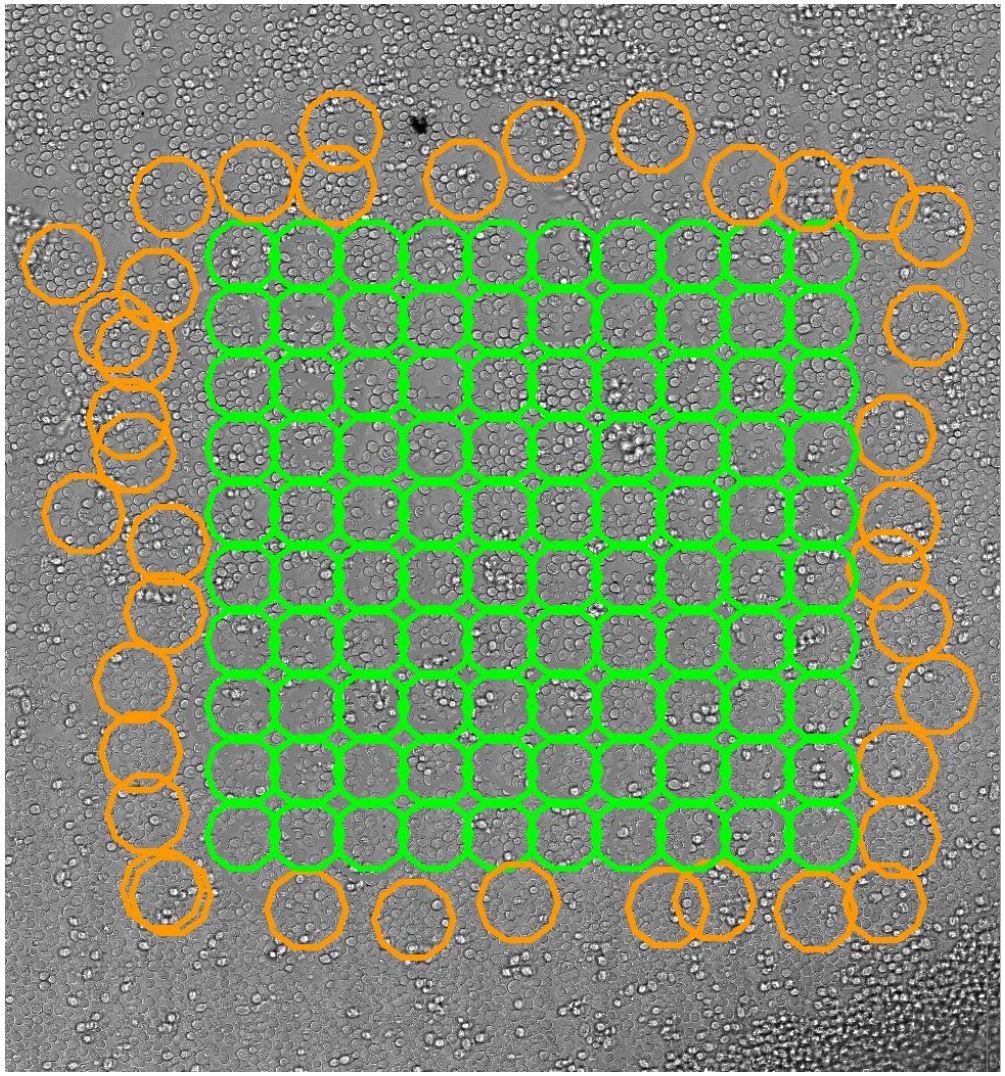
Carlton et al. PNAS 107(37): 16016 (2010)

Using Meiotic Progression to Evaluate Phototoxicity



Jennifer Fung

Tetrad Formation 3 fold decreased in imaged areas



Imaged Area

Not Imaged Area

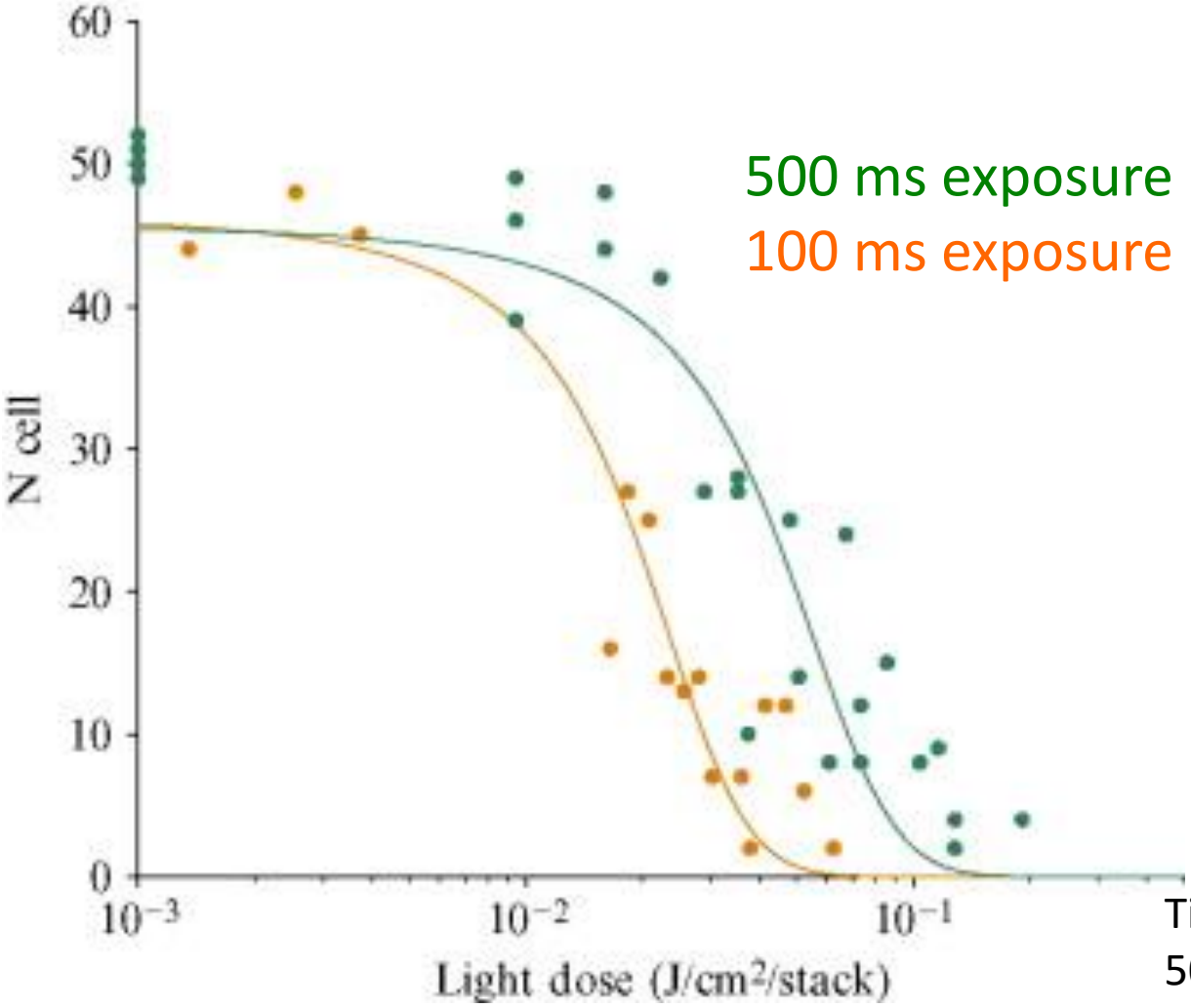
Tetrads

3X > 1X

Jennifer Fung

Quantifying Phototoxicity in C. elegans Embryos

Image: 41 Z-slices every 2 min for 2 hours
Count number of nuclei



Tinevez et al. Meth. Enz. 506: 291 (2012)

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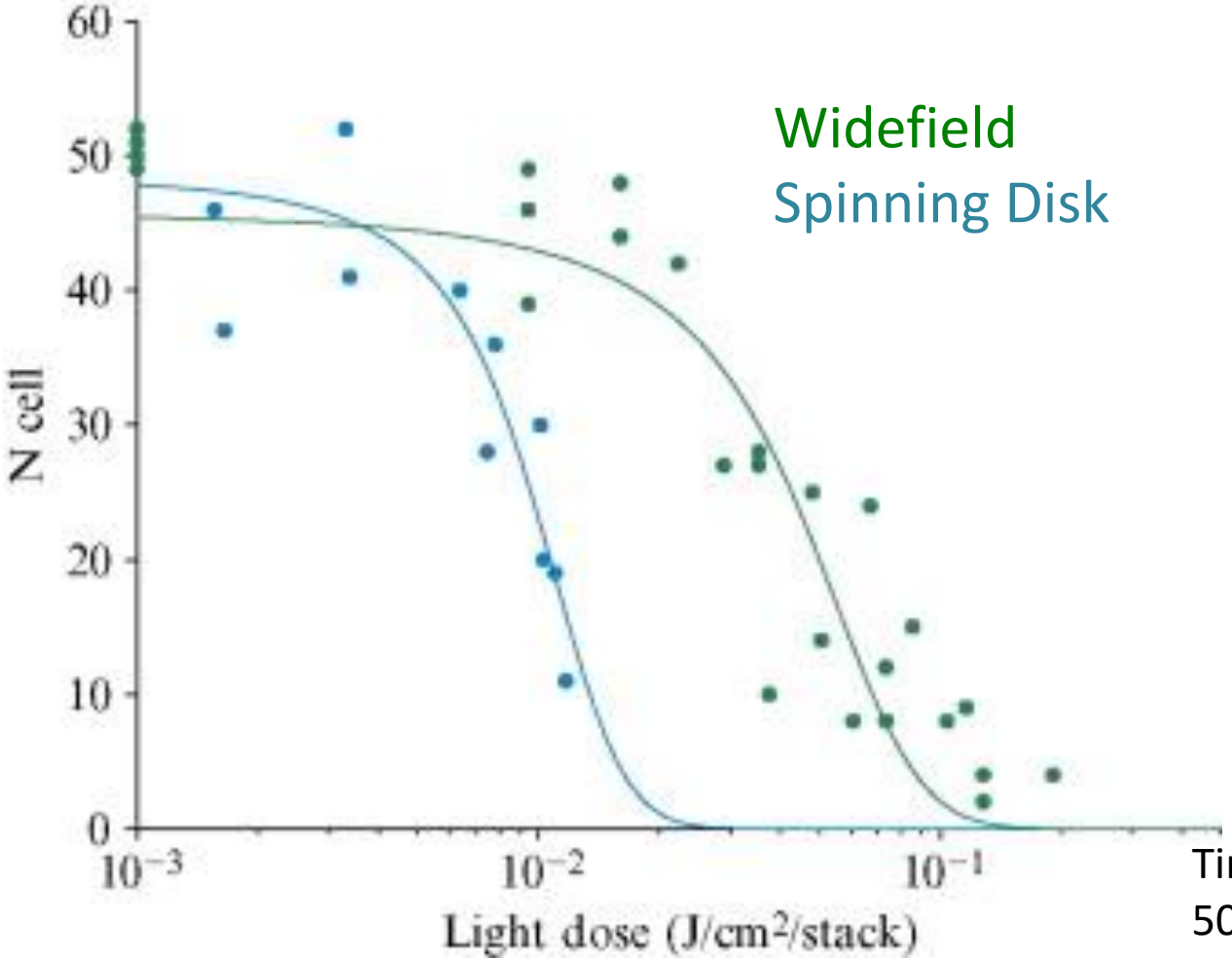


Tinevez et al. Meth. Enz. 506: 291 (2012)

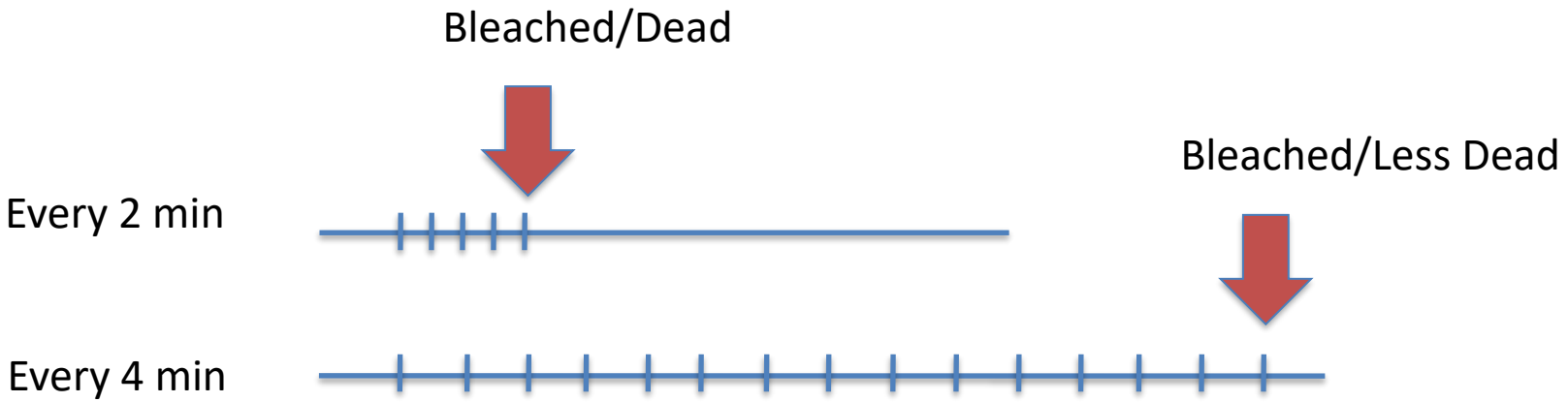
Ways to Lessen Phototoxicity and Photobleaching

- Reduce signal (shorter exposures, lower intensity)
- Reduce frequency of exposures
- Remove oxygen and singlet oxygen
- Lower noise
- Improve detection

Dose Fractionation

- If cells can tolerate X amount of light:
- Can do 1000 exposures at X/1000
- 10 Z slices × 100 time points
- 10 Z slices × 4 colors × 25 time points
- Etc.

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

Minimize Exposure

- Gate illumination by camera expose signal so that light is only on while camera is exposing
- Requires fast light source (laser or LED)

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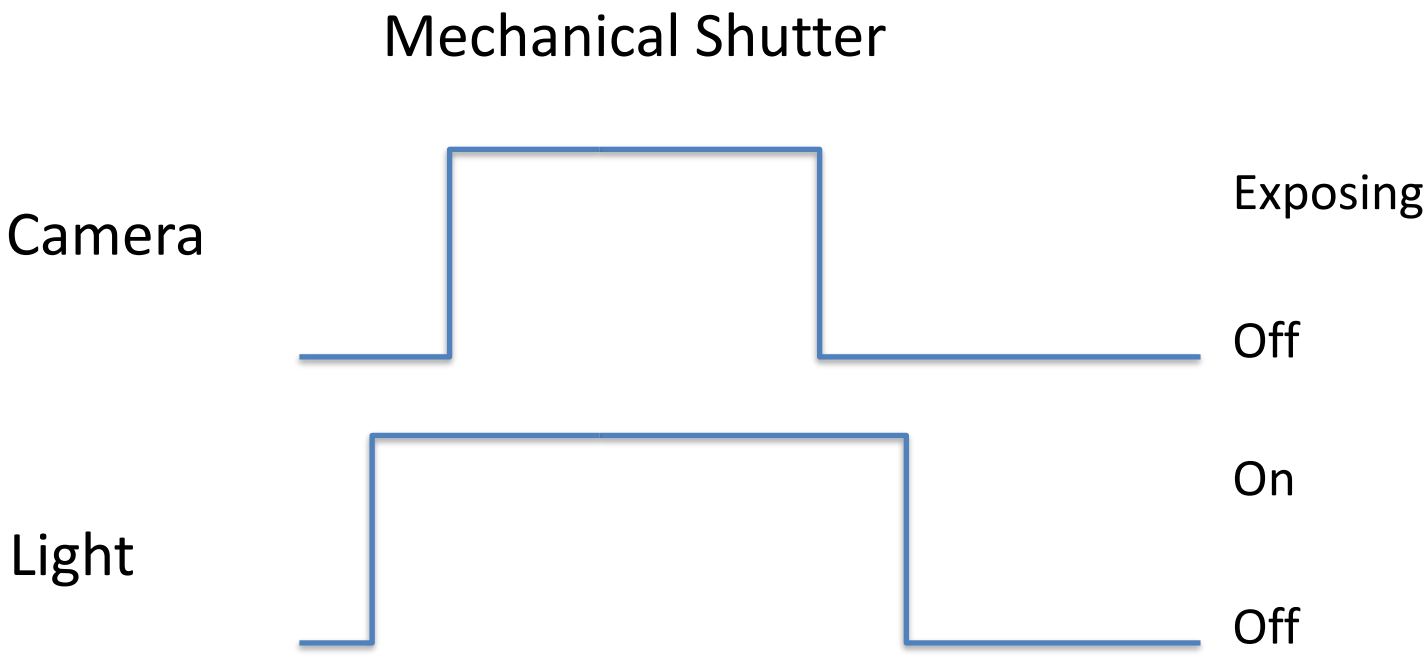

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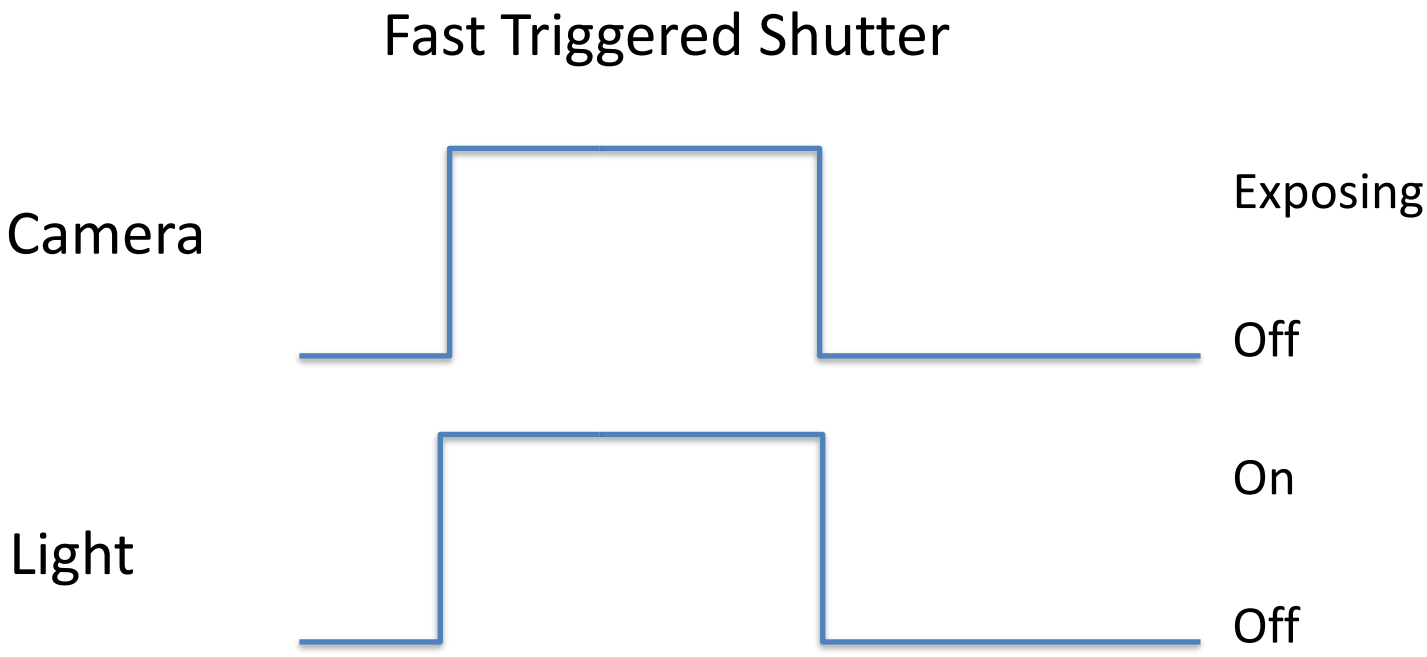


Removing Oxygen and Free Radicals

Oxygen scavengers

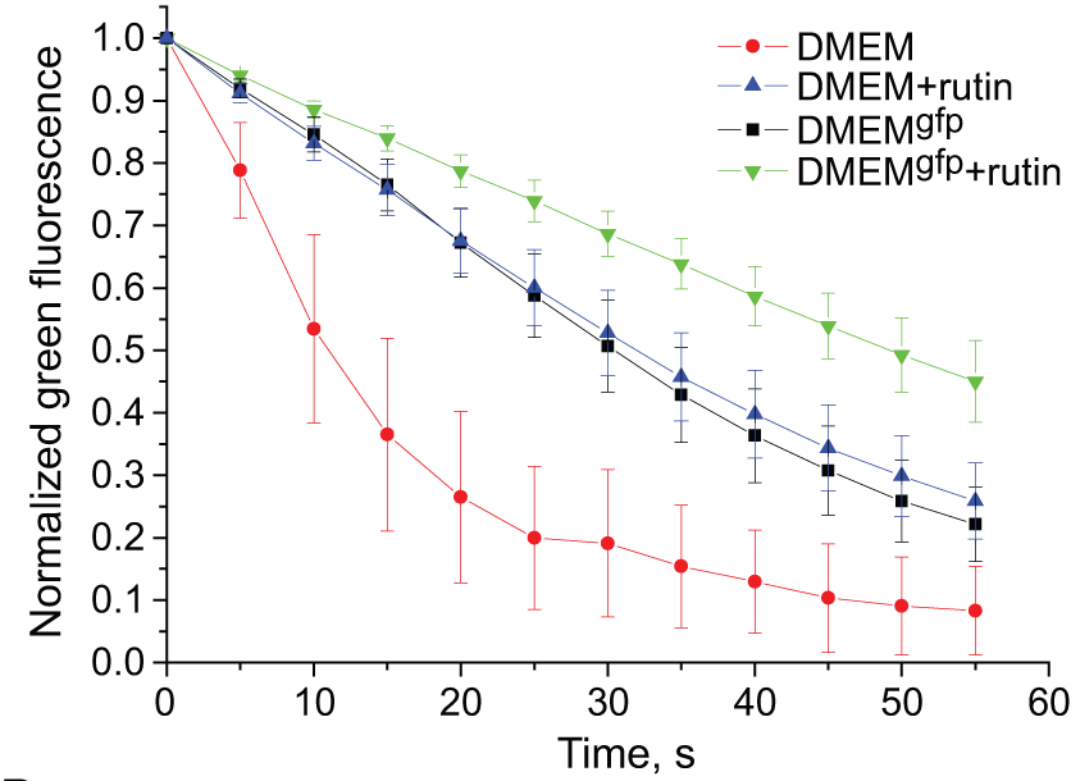
- Glucose oxidase / Catalase
- Protocatechuic acid/protocatechuate-3,4-dioxygenase
- Oxyrase (E. coli membrane particles)

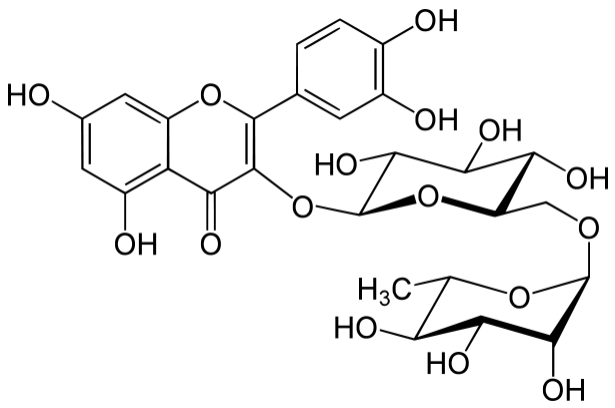
Free radical scavengers

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

GFP Bleaching in Live Cells

Incubation of cells with rutin 30 minutes prior to imaging increases photostability

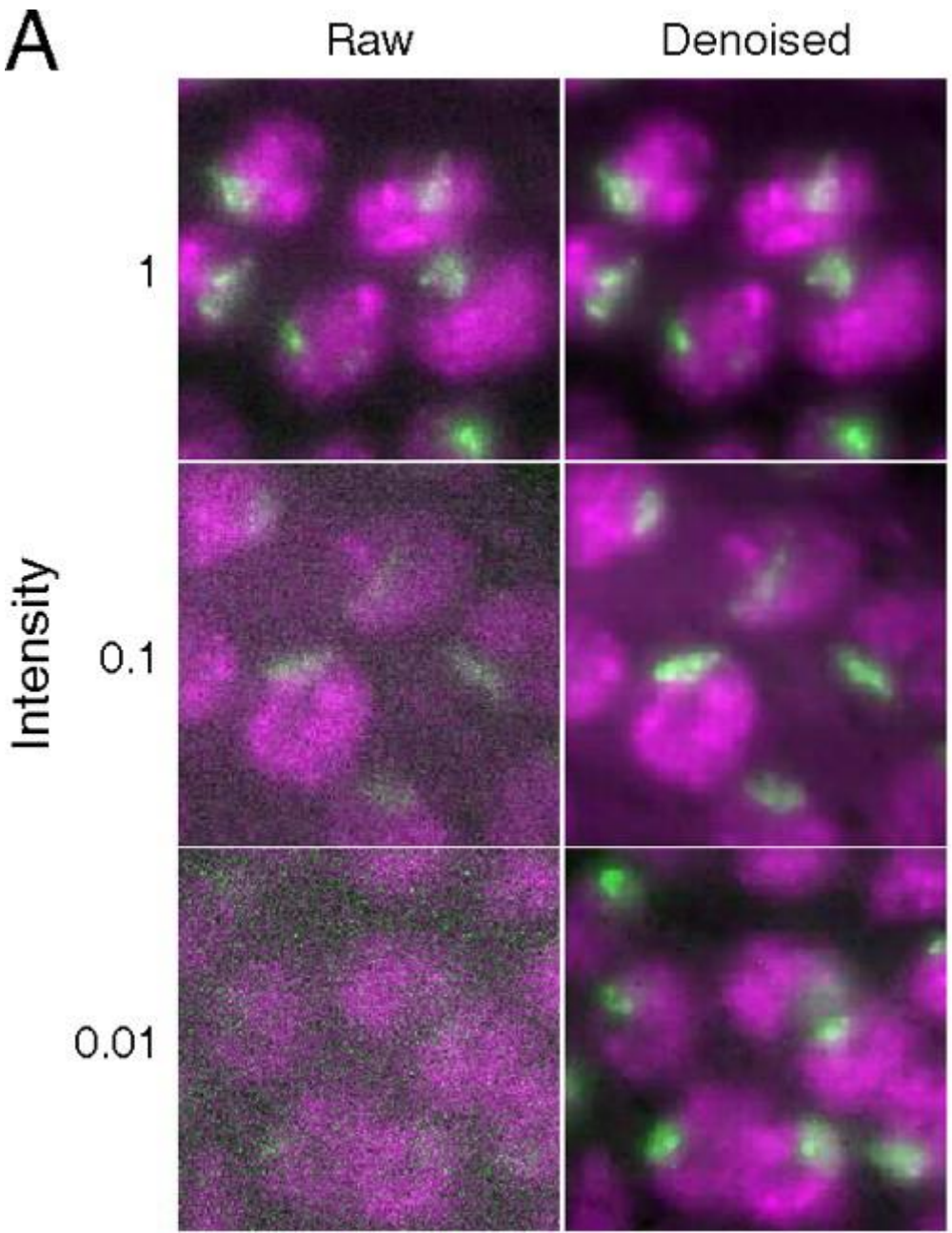




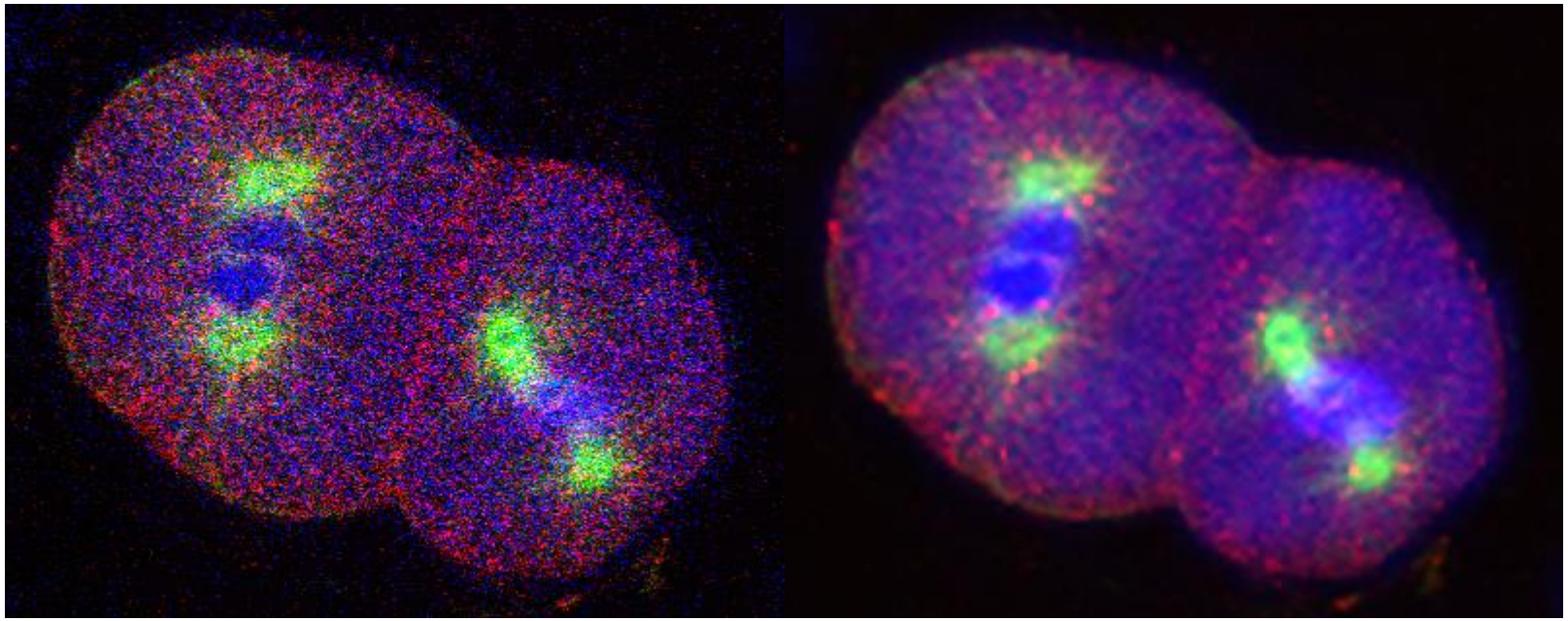
Denoising Algorithms

- Kervrann – Patch based denoising (ND-SAFIR)
  - http://serpico.rennes.inria.fr/doku.php?id=software:nd-safir:index
- Luisier et al. wavelet denoising (SURE-LET)
  - http://bigwww.epfl.ch/algorithms/denoise/
- Also deconvolution

Patch-based denoising



SURE-LET denoising

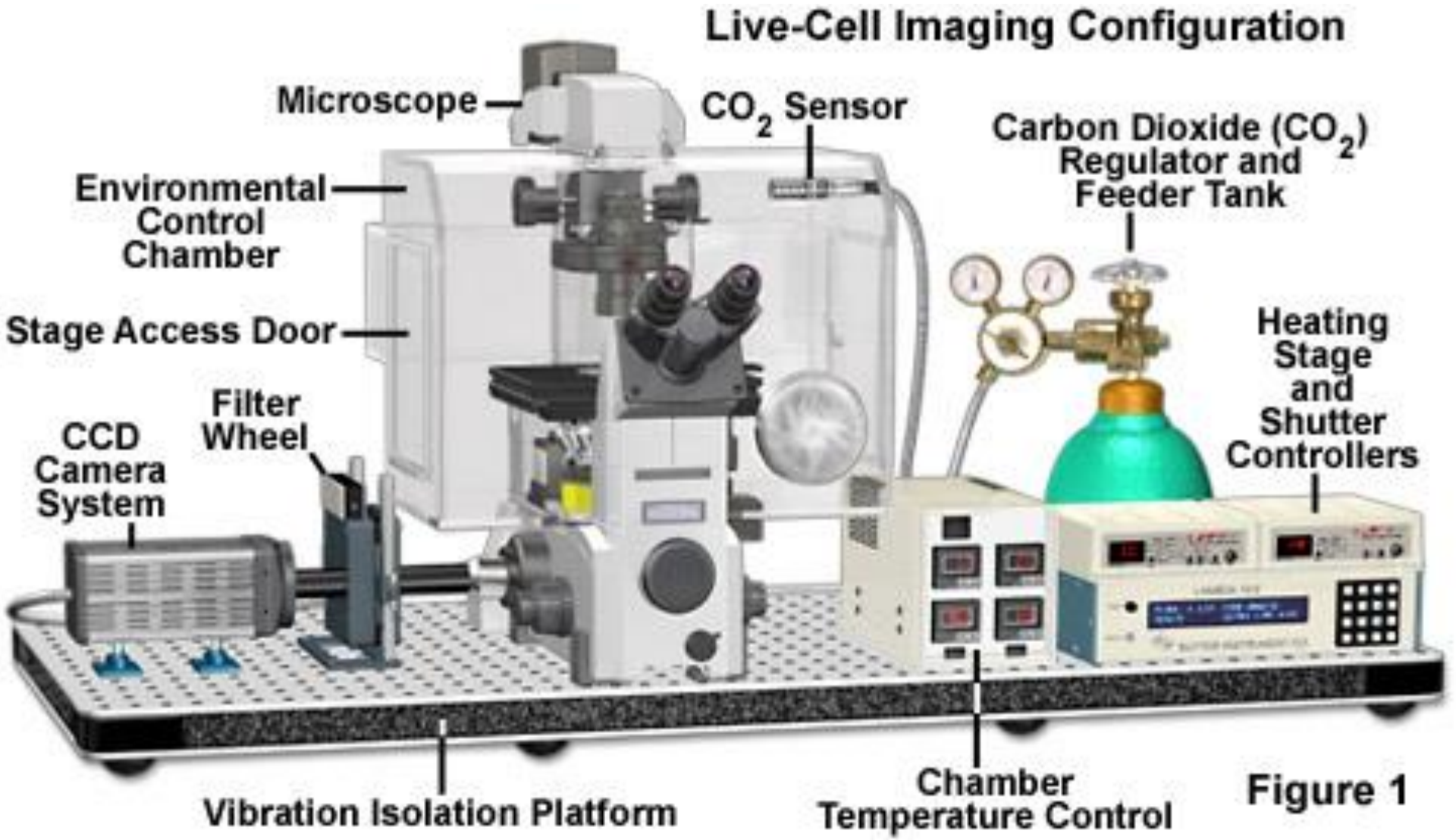


Preserving a normal environment during imaging

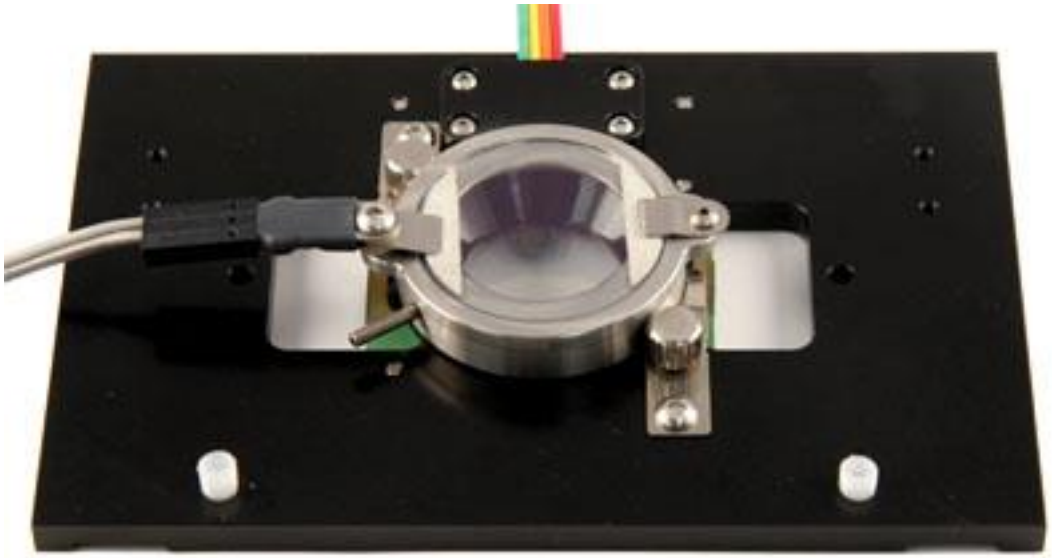
Environmental Variables for Mammalian Cell Lines

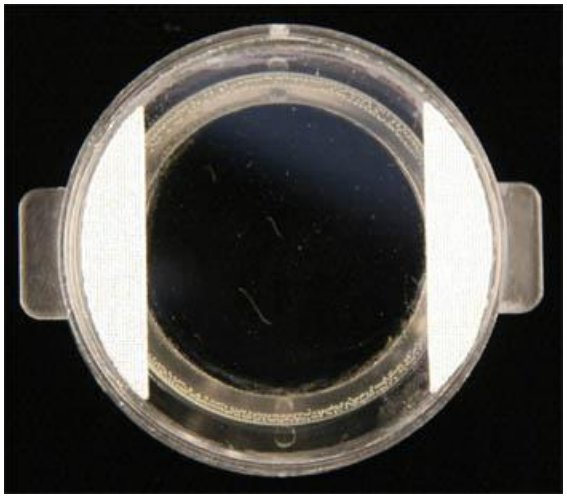
Variable	Optimum Range	Comments
Temperature	28-37ºC	Control with Specimen Chamber Heaters Inline Perfusion Heaters Objective Lens Heaters Environmental Control Boxes
Oxygenation	Variable	Perfuse or Change Media Regularly Use Large Chamber Volume
Humidity	97-100%	Closed chamber, humidified environmental 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	Use buffered media, closed chamber
Media buffer	Bicarbonate or Synthetic buffers	Beware of phototoxicity, closed & open chambers, atmosphere controlled chamber

Environmental Chamber

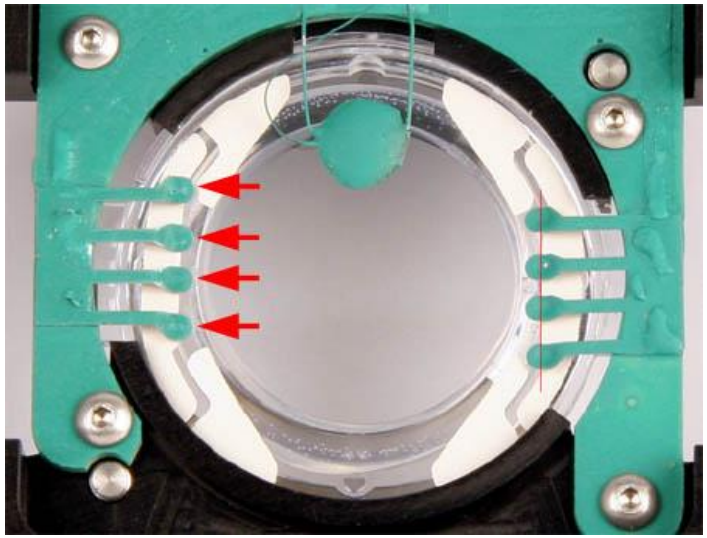


Bioptechs – Open/Closed Dish System

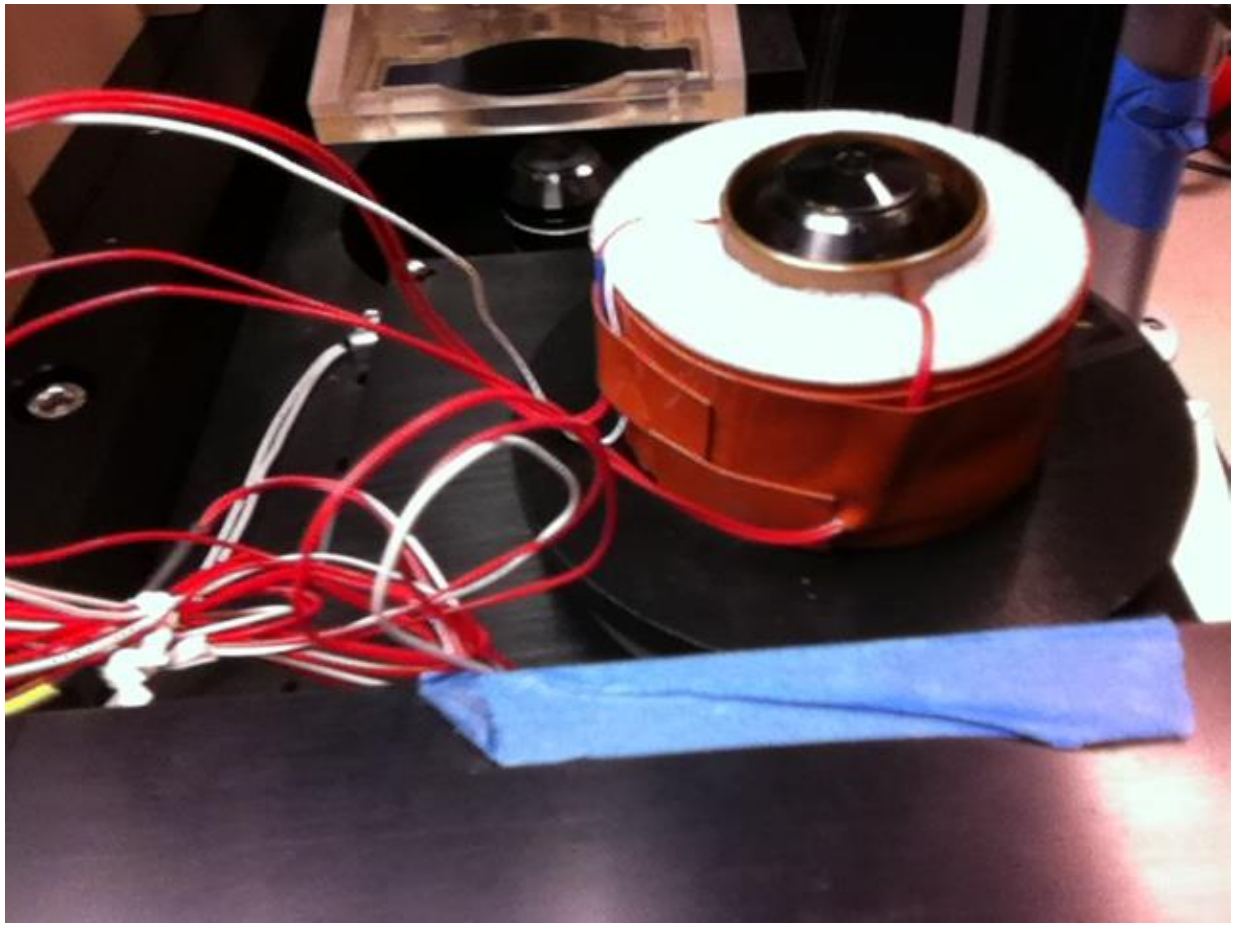




Delta T Dish



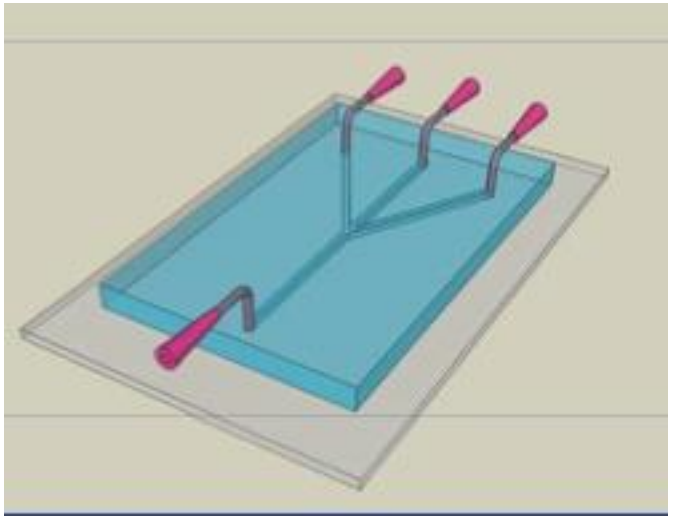
Heated Objective



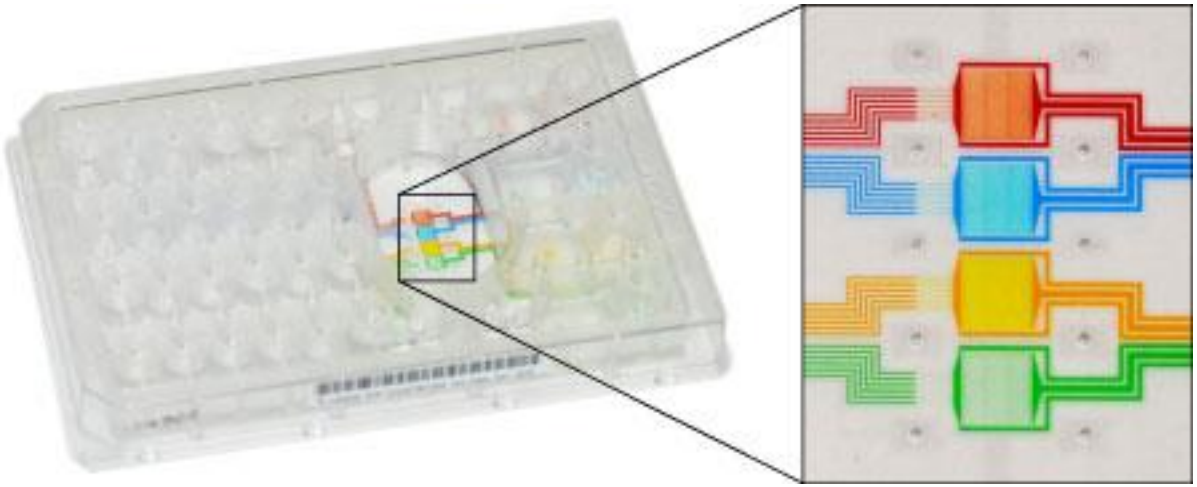
Custom-made PDMS microfluidics

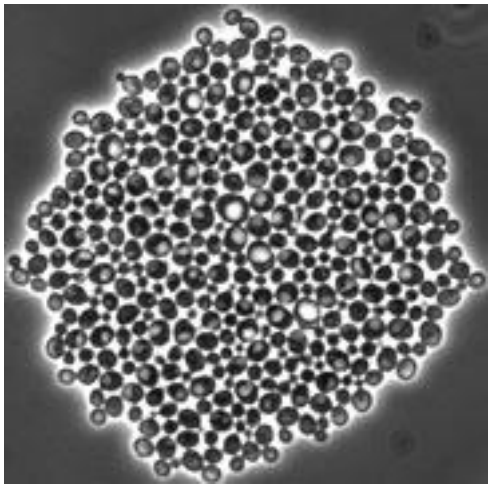
PDMS - Poly(dimethylsiloxane)

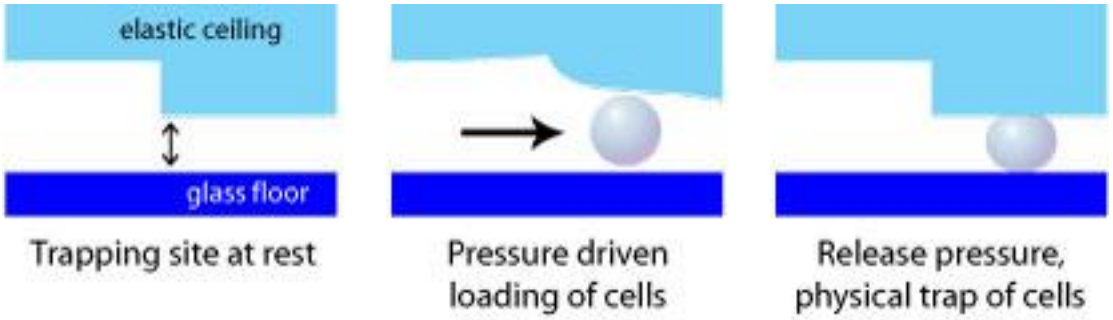
Relatively inexpensive
Easy to use Chemically inert/non-hazardous
Optically clear Flexible and fairly tough when cured
Easily bonded to itself or other materials
Permeable to air and liquids (but can be coated to prevent this).



CellASIC – Microfluidic Systems







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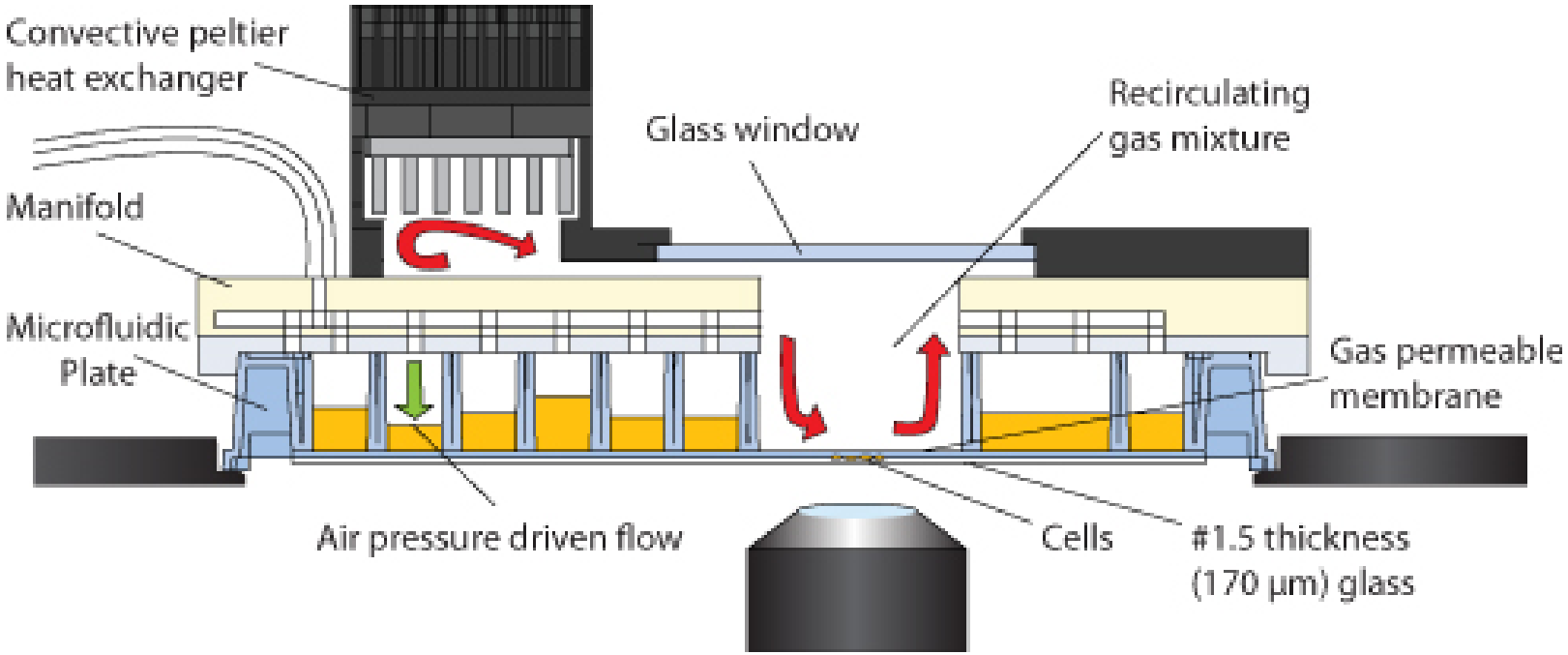

Different Goals for Fixed vs. Live

Fixed specimens: Optimize the signal/noise

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

Typical ways to improve S/N in fixed samples

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

>>>> Increase signal