

# Microscopy

Brightfield, Confocal, and Multi-Photon Microscopy

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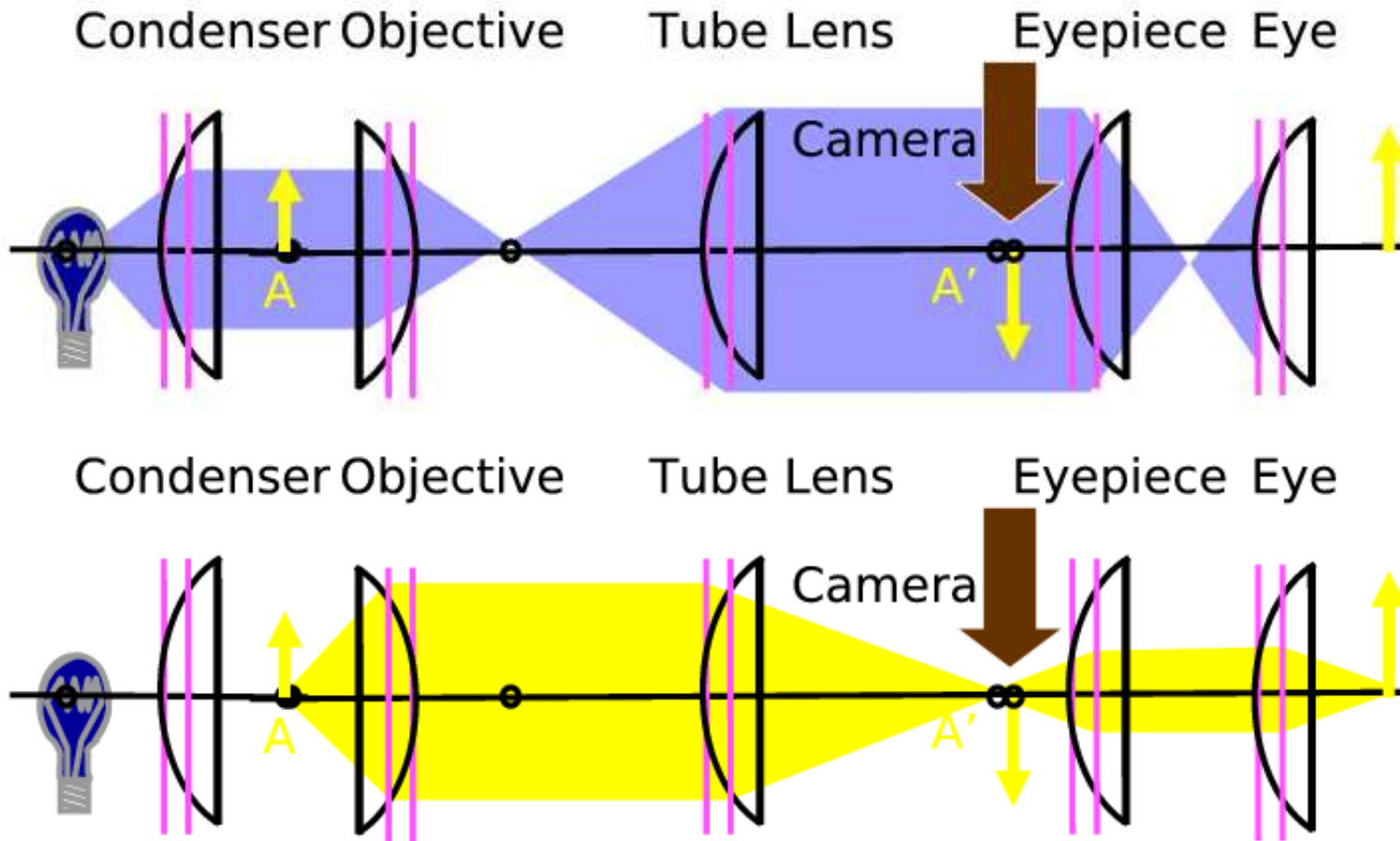
This work was supported in part by the Gordon Center for Subsurface Sensing and Imaging Systems, CenSSIS, under the Engineering Research Centers Program of the National Science Foundation (award number EEC-9986821).

# Topics

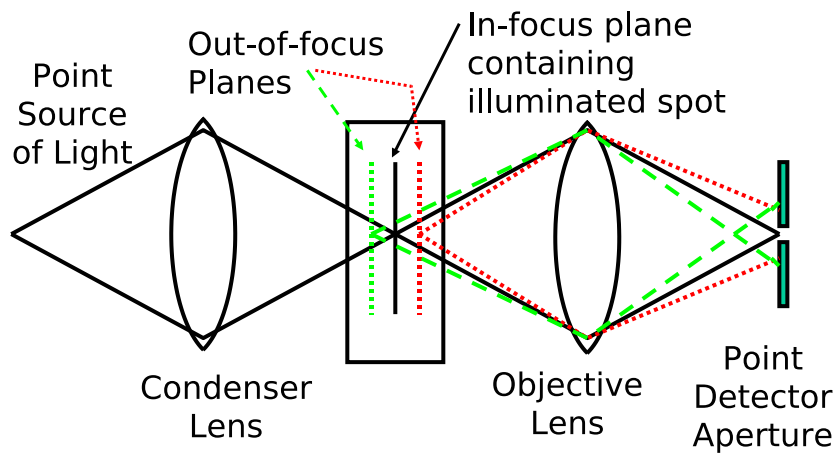


- Brightfield Microscopy
- Confocal Microscopy
  - Reflectance
  - Fluorescence
- 2-Photon-Induced Fluorescence Microscopy
- Second-Harmonic-Generation Microscopy

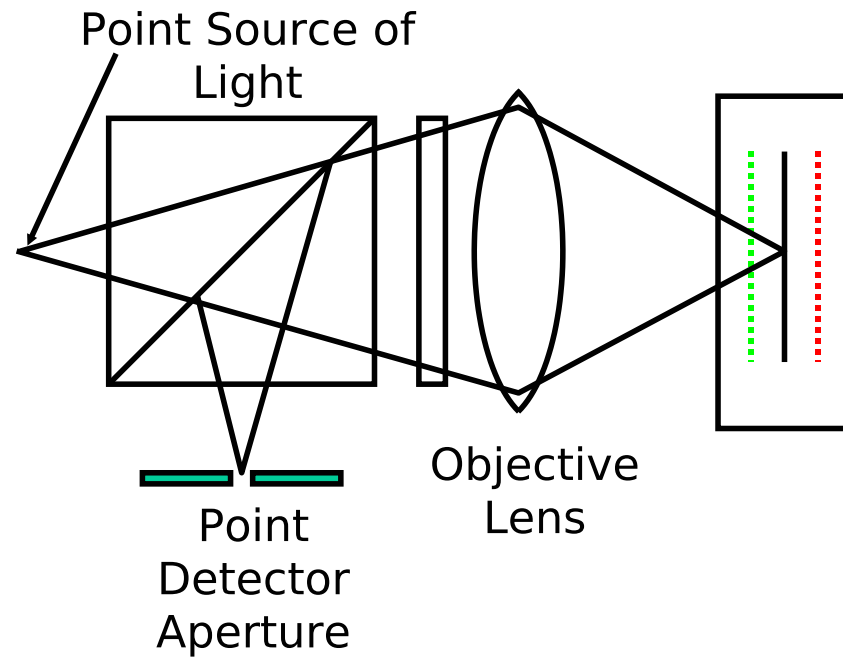
# Brightfield Microscopy



# Confocal Microscopy



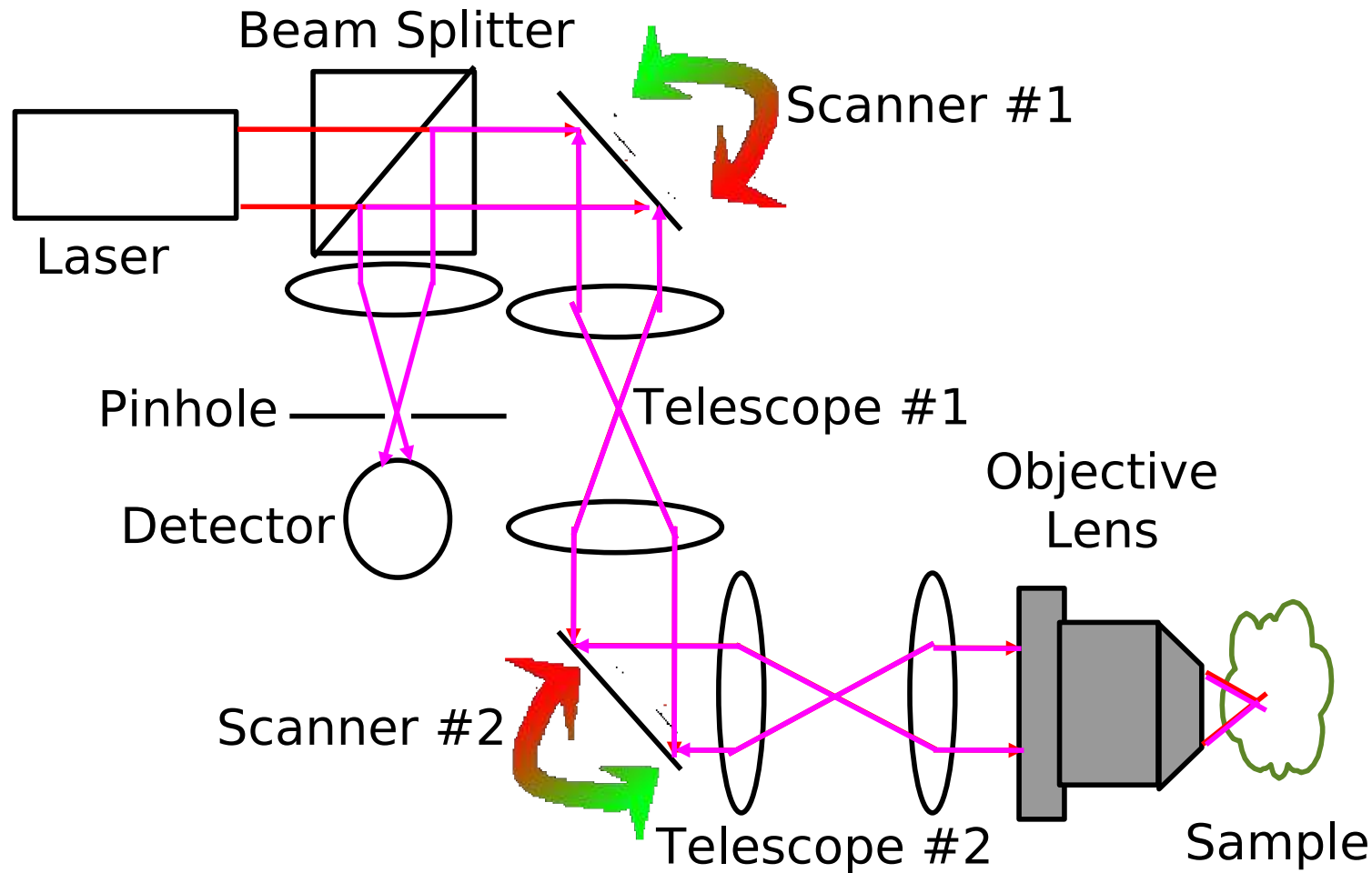
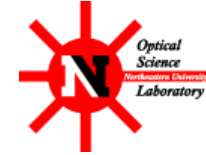
Transmission



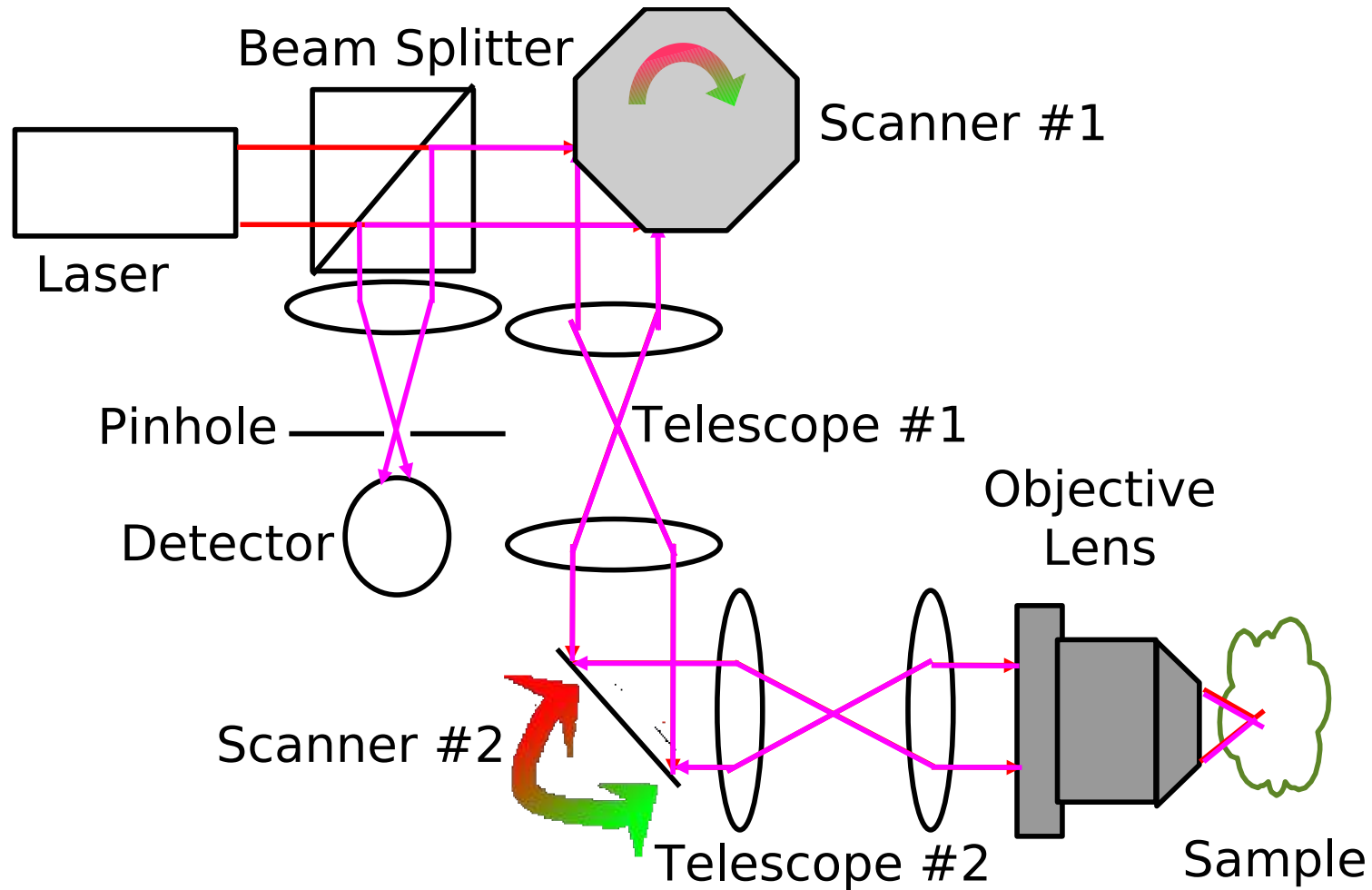
Reflection (Usual)

Adapted from Milind Rajadhyaksha

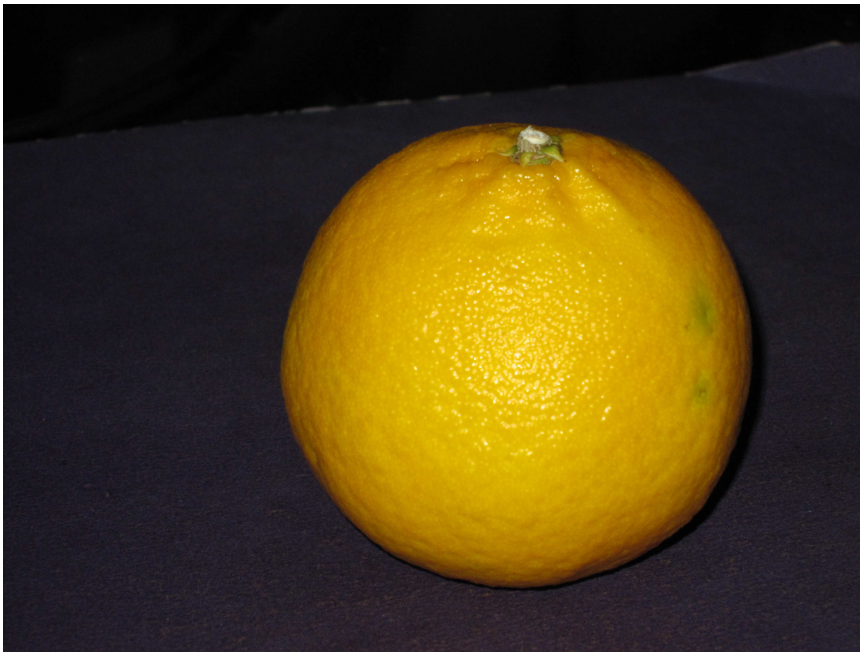
# CRM 2-Galvo System



# CRM Polygon



# Brightfield Focusing

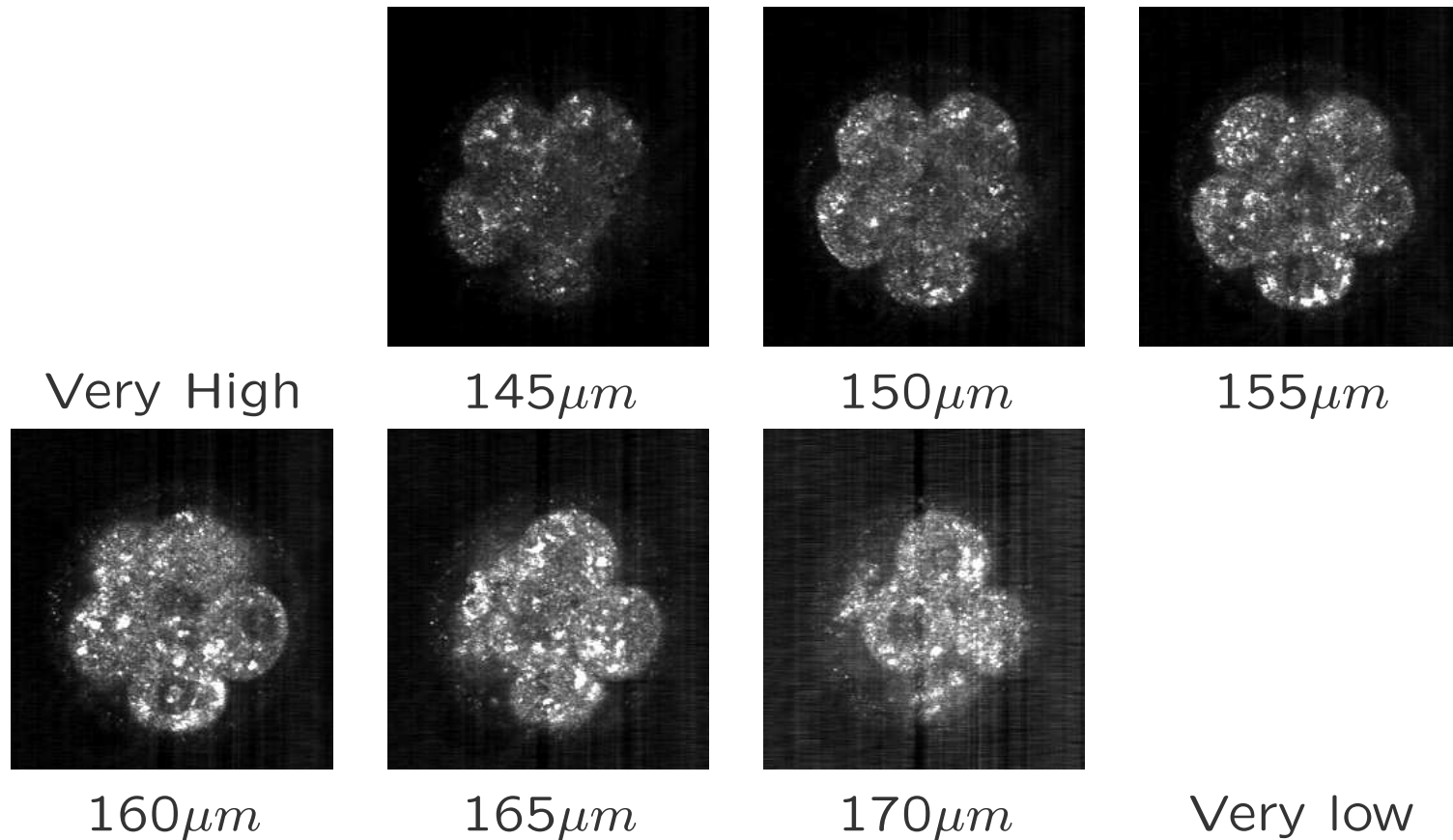


In-Focus Image



Out-Of-Focus Image

# Confocal Focusing



Judy Newmark (Warner Group), Bill Warger



# Optical Sectioning



1/9	1/9	1/9
1/9	1/9	1/9
1/9	1/9	1/9

Broad Source

0	0	0
0	1	0
0	0	0

Pixel In Focus

0	0	0
0	1/9	0
0	0	0

Brightfield in Focus

1/9

1/9	1/9	1/9
1/9	1/9	1/9
1/9	1/9	1/9

Broad Source

1/9	1/9	1/9
1/9	1/9	1/9
1/9	1/9	1/9

Pixel Out of Focus

1/81	1/81	1/81
1/81	1/81	1/81
1/81	1/81	1/81

Brightfield Out of Focus

1/9

0	0	0
0	1	0
0	0	0

Source In Focus

0	0	0
0	1	0
0	0	0

Pixel In Focus

0	0	0
0	1	0
0	0	0

Confocal In Focus

1

1/9	1/9	1/9
1/9	1/9	1/9
1/9	1/9	1/9

Source Out of Focus

1/9	1/9	1/9
1/9	1/9	1/9
1/9	1/9	1/9

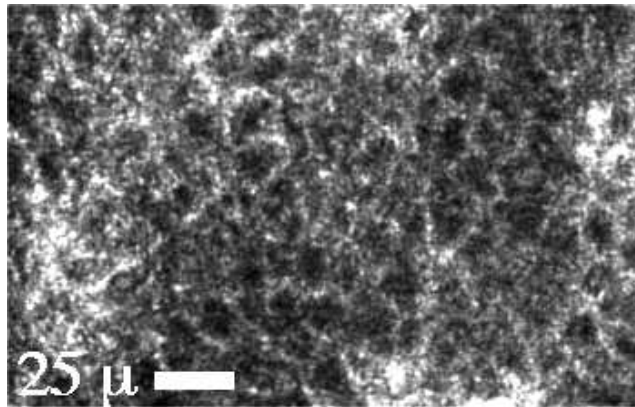
Pixel Out of Focus

1/81	1/81	1/81
1/81	1/81	1/81
1/81	1/81	1/81

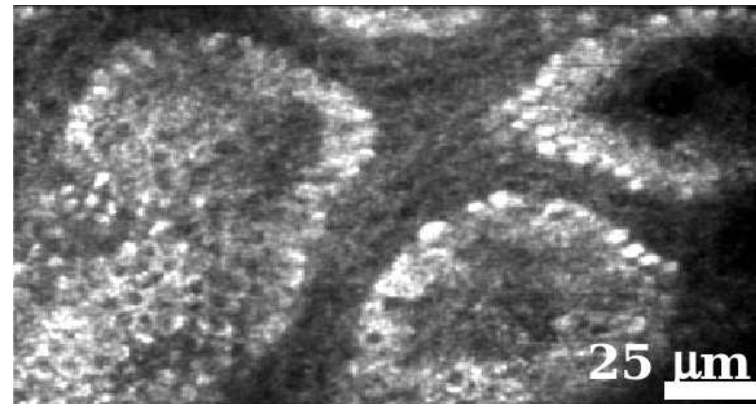
Confocal Out of Focus

1/9

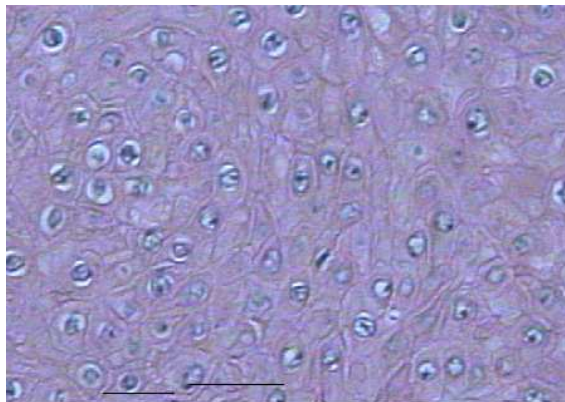
# Normal Skin



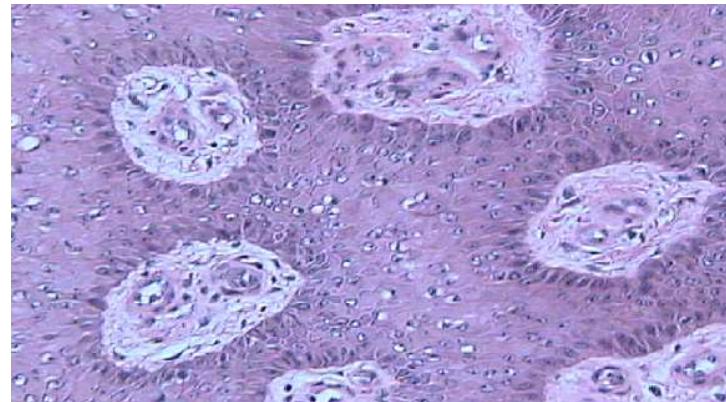
CRM, Spinous Layer



Basal Layer



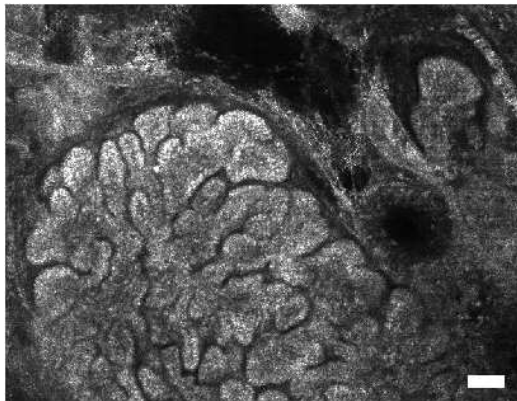
H&E, Spinous Layer



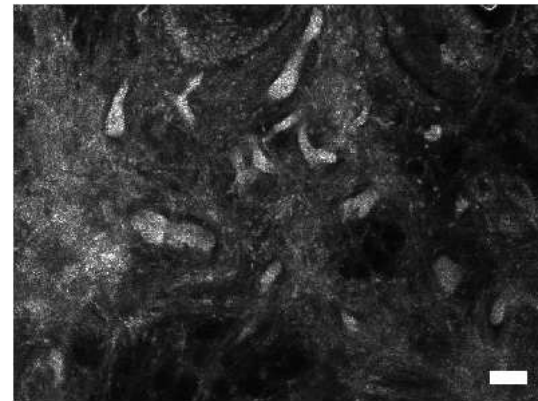
Basal Layer

Milind Rajadhyaksha

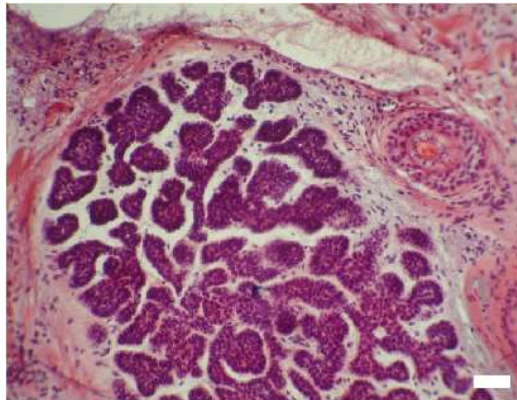
# Skin Cancers



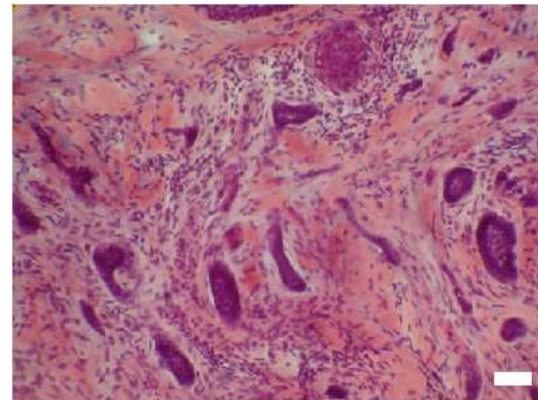
CRM, Nodular BCC



Infiltrative BCC



H&E, Nodular BCC



Infiltrative BCC

Milind Rajadhyaksha



# Large 3-D Mosaics

Mouse Embryo at Day 9  
Z-Stack from Confocal Reflectance Microscopy



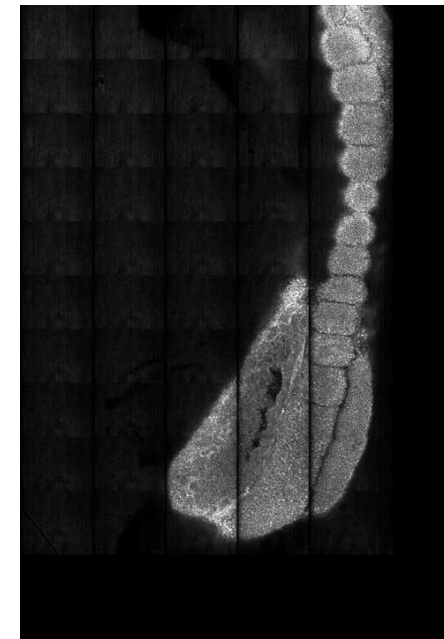
$-3\mu m$



$27\mu m$



$84\mu m$



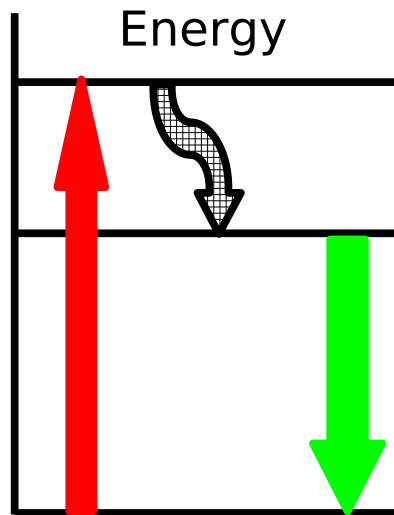
$114\mu m$

Selected Sample Z Locations from Mosaic

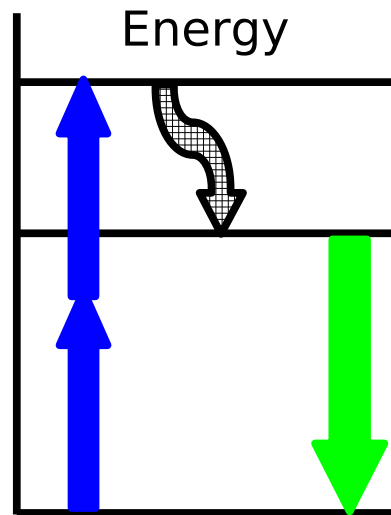
3200 wide by 4800 high by 160 deep, Decimated for Display

Irina Larina (Baylor), Kirill Larin (Houston), Joe Kerimo

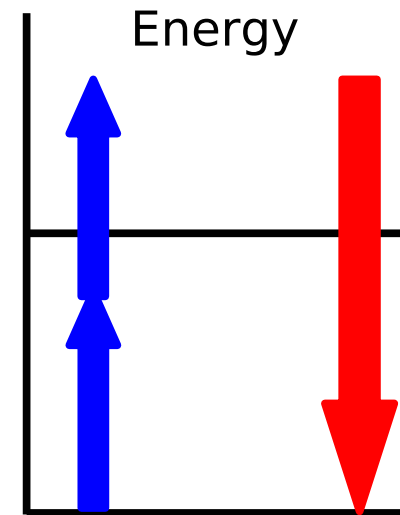
# Energy Diagrams



Fluorescence

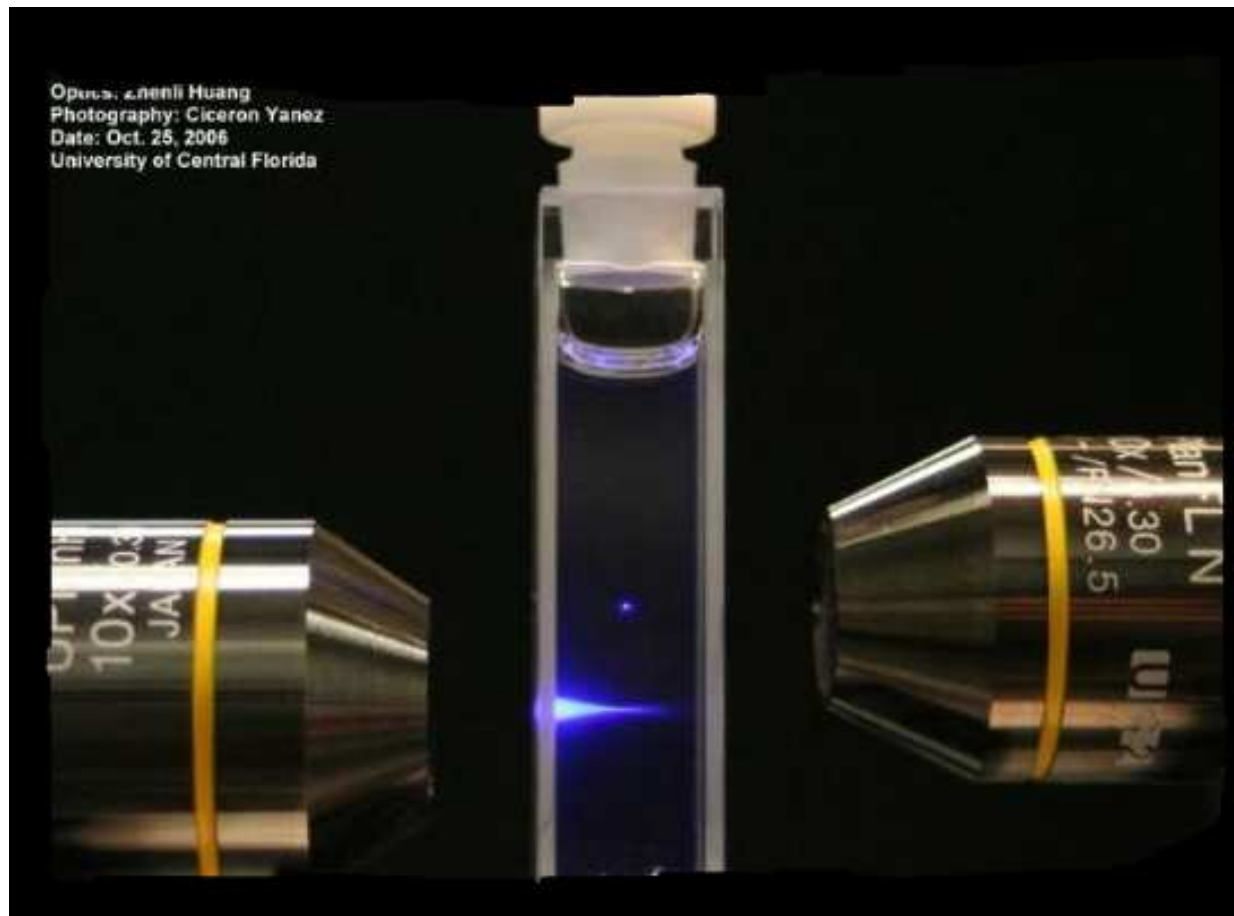


2-Photon Fluorescence



Second Harmonic

# 2-Photon Microscopy



Huang, UCF

# 2-P Sectioning



- 2-Photon Excitation Proportional to Square of Irradiance
- Sectioning Without Pinhole
- High-Power, Short Pulse, Tight Focus

0	0	0
0	$1^2$	0
0	0	0

Source Squared

$1/9$	$1/9$	$1/9$
$1/9$	$1/9$	$1/9$
$1/9$	$1/9$	$1/9$

Wide Detector

0	0	0
0	$1/9$	0
0	0	0

2-P in Focus

$1/9$

$1/9^2$	$1/9^2$	$1/9^2$
$1/9^2$	$1/9^2$	$1/9^2$
$1/9^2$	$1/9^2$	$1/9^2$

Source Out of Focus

$1/9$	$1/9$	$1/9$
$1/9$	$1/9$	$1/9$
$1/9$	$1/9$	$1/9$

Wide Detector

$1/9^3$	$1/9^3$	$1/9^3$
$1/9^3$	$1/9^3$	$1/9^3$
$1/9^3$	$1/9^3$	$1/9^3$

2-P Out of Focus

$1/81$

# 2-P Advantages



- IR Light to Reduce Photodamage
- Nonlinearity to Reduce Photodamage
- IR Light to Increase Penetration
- No Pinhole (Better Alignment, Better Sectioning)
- Wide Detector (Collects All Light, including Scattered)
- Easier Filtering

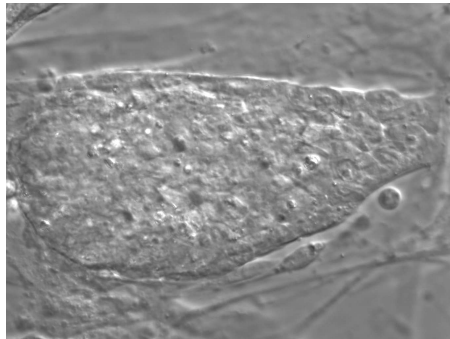


# Embryonic Stem Cells

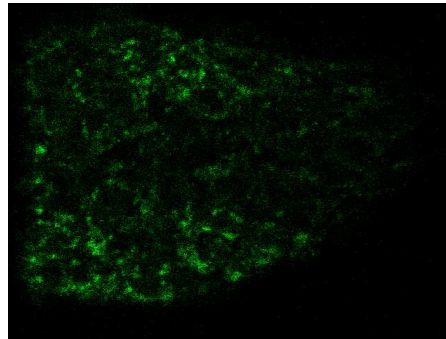
C57BL/6 Embryonic Stem Cells



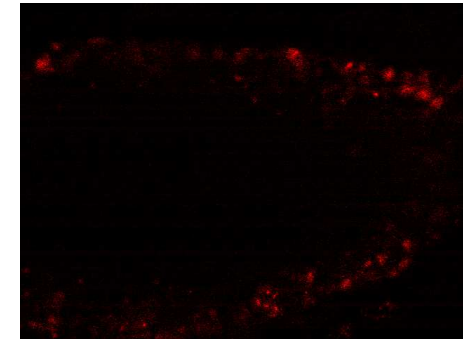
DIC



CFM of JC1 Green (1P)  
(Inactive Mitochondria)

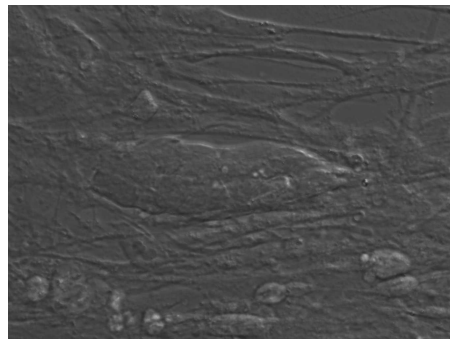


fCFM of JC1 Red (1P)  
(Active Mitochondria)

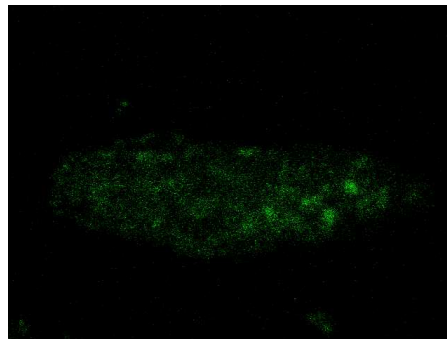


mtGFP–tg Embryonic Stem Cells

DIC



2-Photon of GFP  
(All Mitochondria)



Embryoid Bodies  
on Next Page

Note:

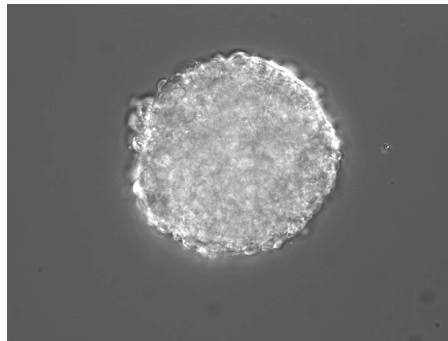
GFP is brighter and more  
stable than the standard  
mitochondrial stains.

Judy Newmark (Warner Group)

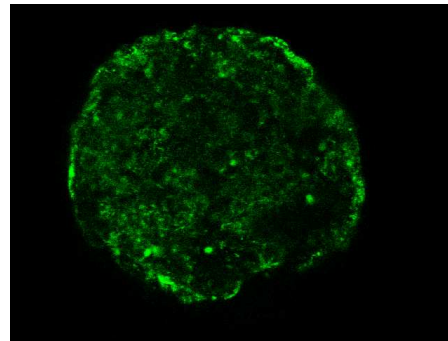
# Embryoid Bodies

mtGFP-tg Embryoid Body

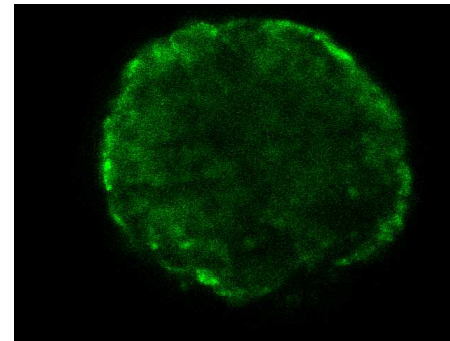
DIC



CFM of GFP (1P)  
(All Mitochondria)

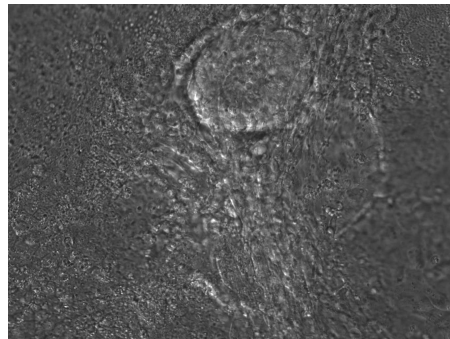


2P of GFP  
(All Mitochondria)

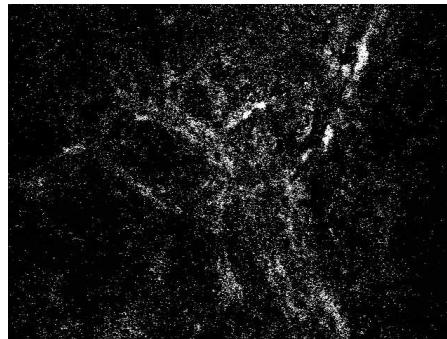


mtGFP-tg Plated Embryoid Body

DIC



2-Photon of GFP  
(All Mitochondria)

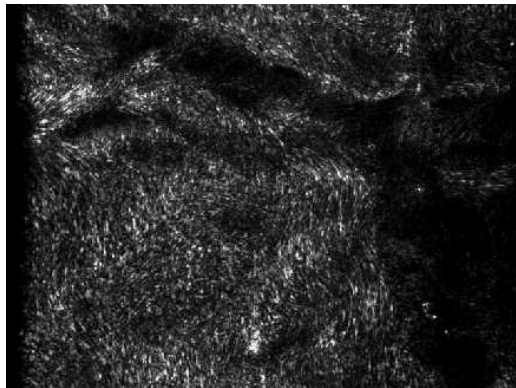


Note:

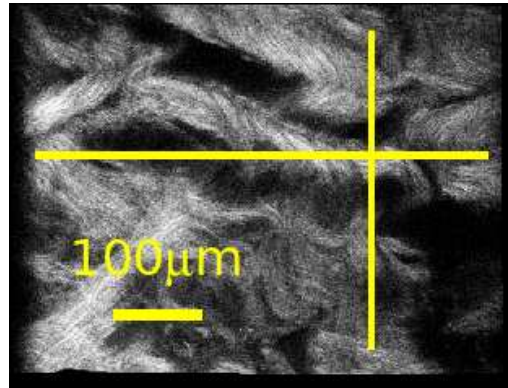
After two weeks of differentiation, beating cells are observed.

Judy Newmark (Warner Group)

# Sclera in SHG



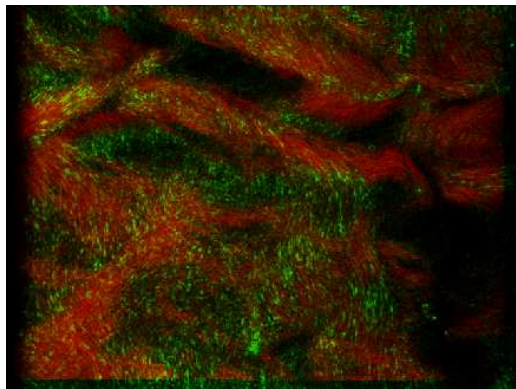
CRM Slice 9



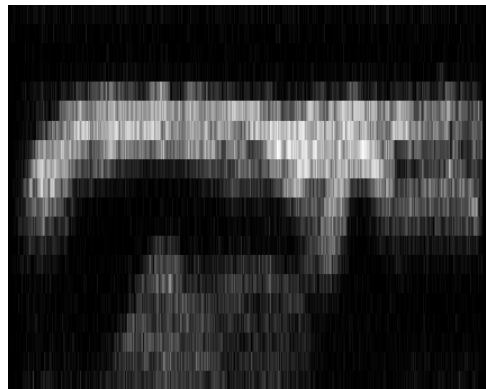
SHG Slice 9



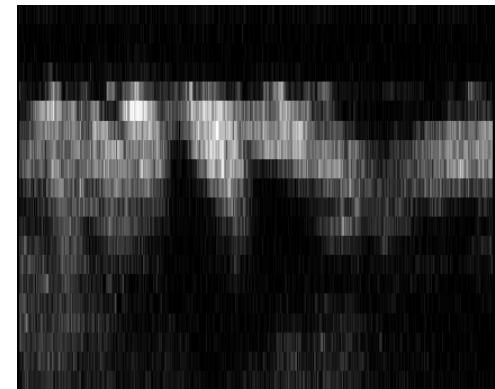
SHG MIP



SHG (R) + CRM (G)



X-Z



Y-Z

Nima Saeidi (Ruberti Group), Bill Warger