Reproducibility & Rigor in Imaging Experiments

Open MIC 4/20/17

CANCER RESEARCH LABORATORY
MOLECULAR IMAGING CENTER

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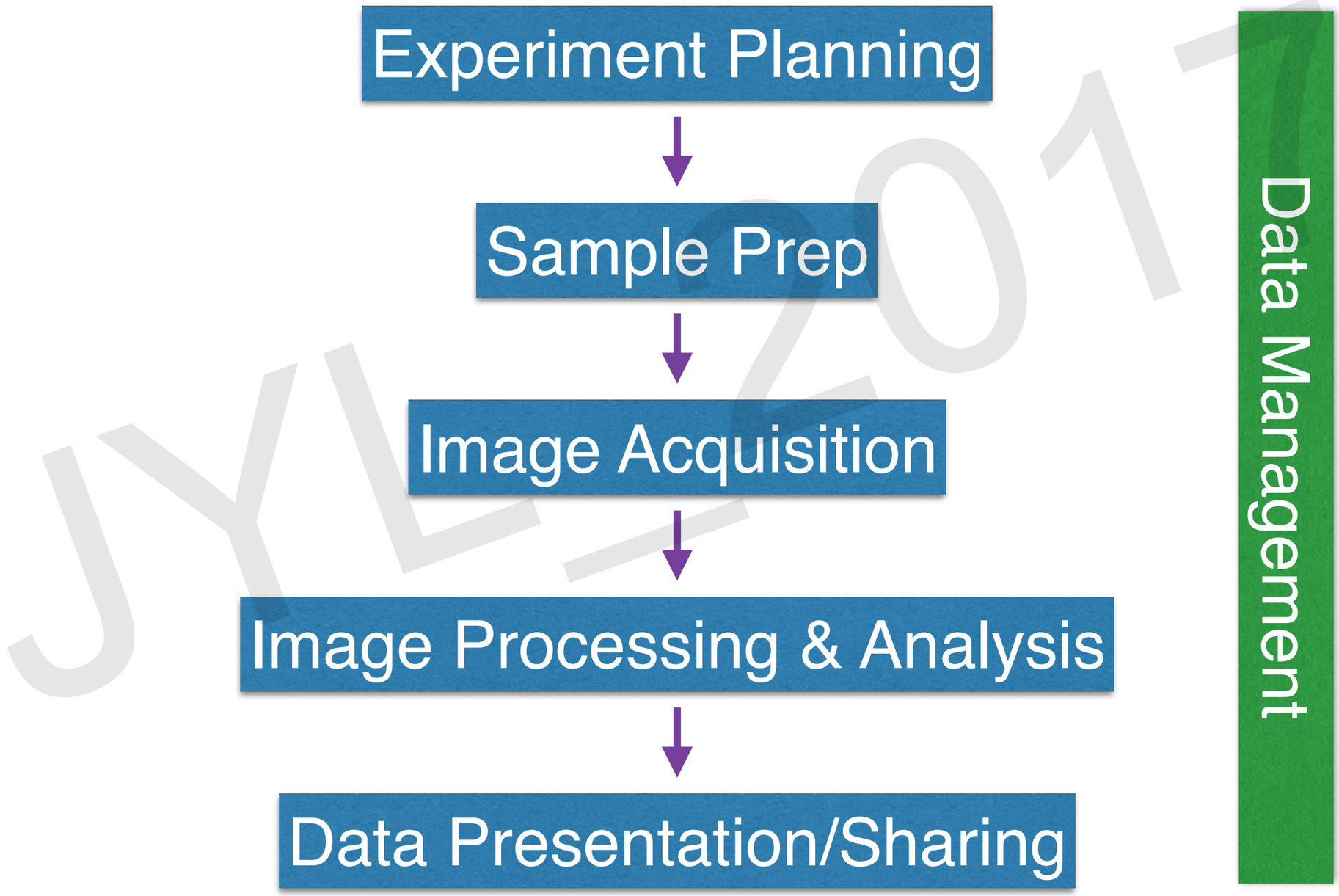
Lecture Goals

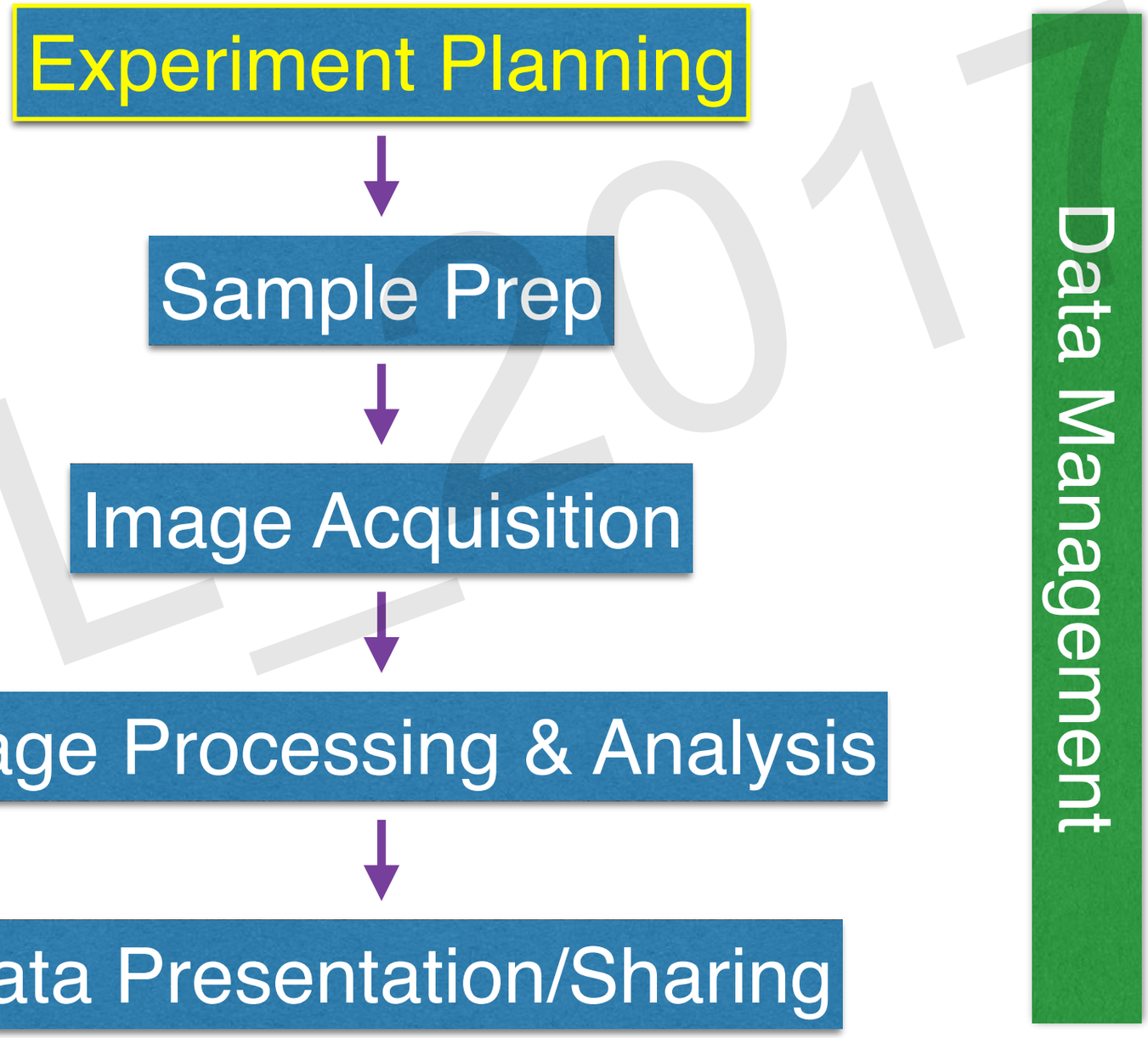
- To give a broad overview of most common pitfalls in imaging experiments
- To outline best practices for reproducibility in microscopy
- To provide general guidelines for the presentation and evaluation of microscopic images

i.e., "What I wish someone would've told me as a first year graduate student"

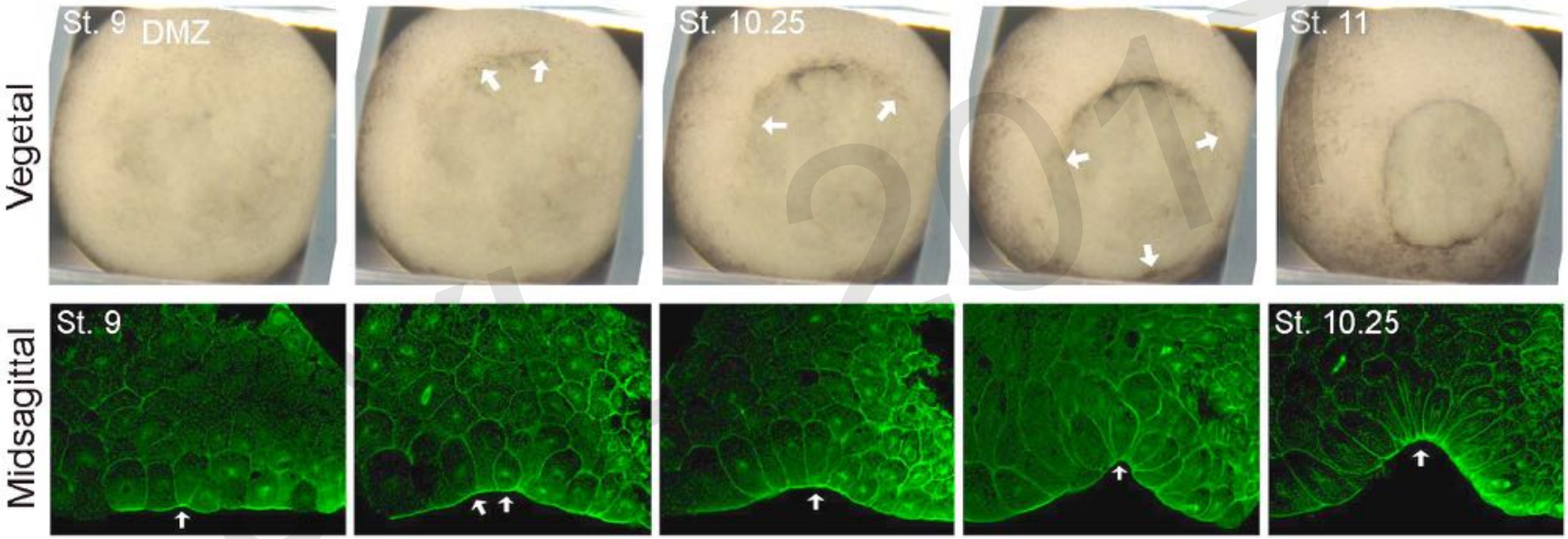
Typical Imaging Experiment Workflow



Typical Imaging Experiment Workflow



Experiment Planning: An Example



Lee & Harland, *Dev Biol* 2007

What factors need to be considered when setting up this experiment?

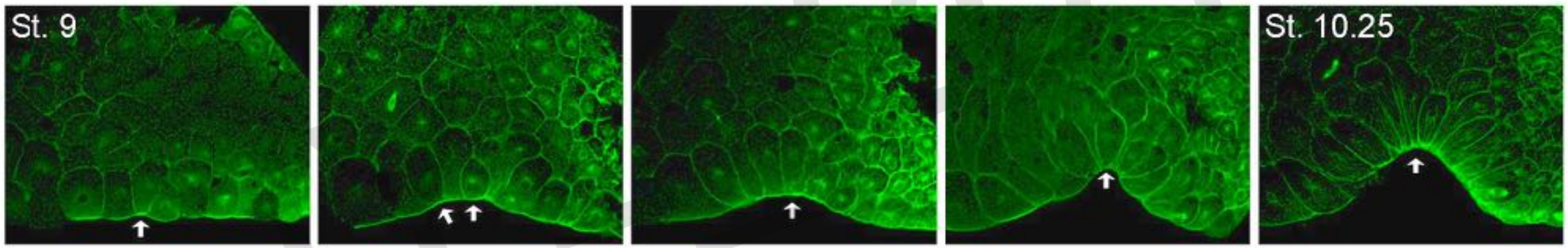
Experiment Planning: An Example

Hypothesis

Protocol(s)

How to interpret results?

Sampling & statistical power (n)

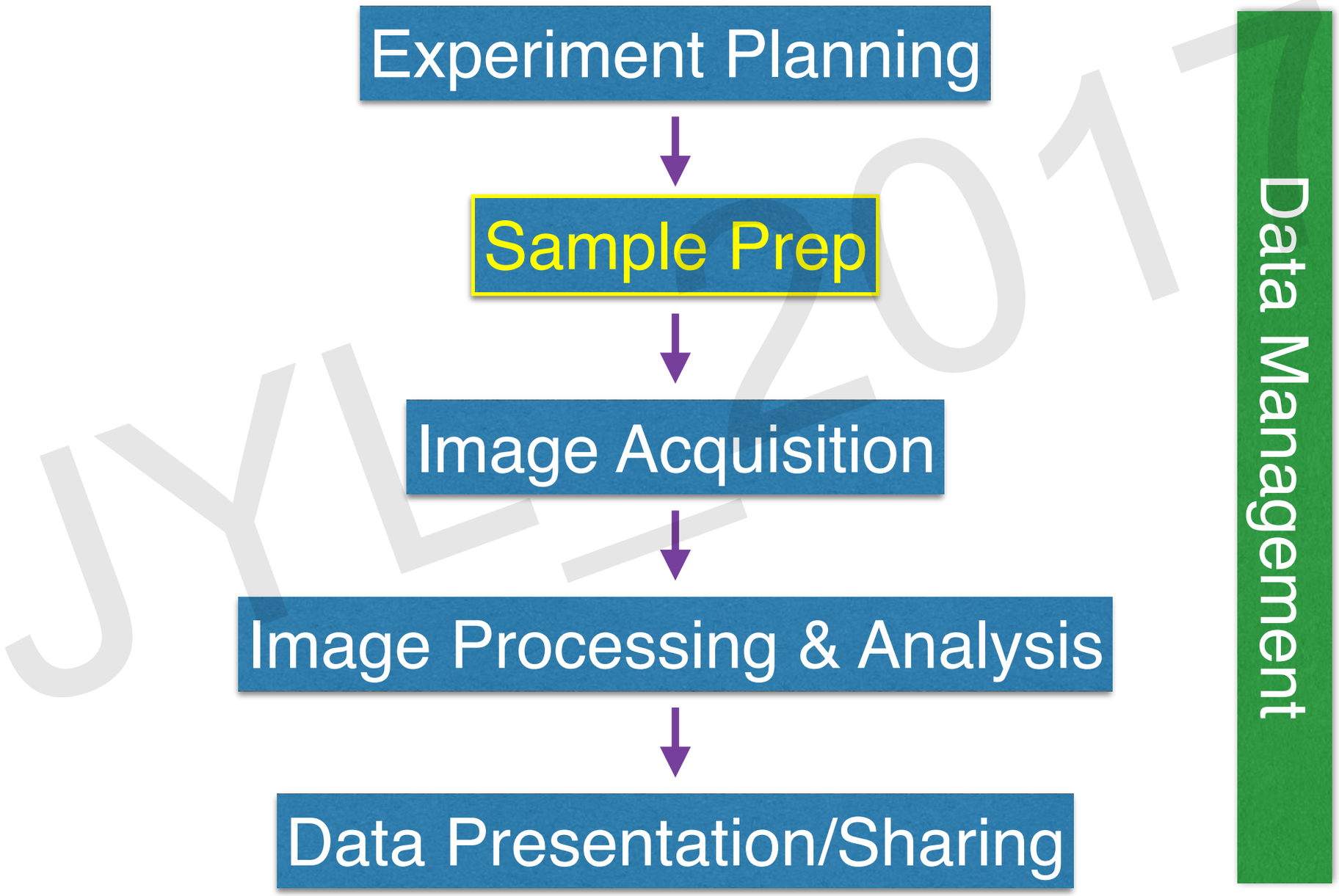


Appropriate fluorophores

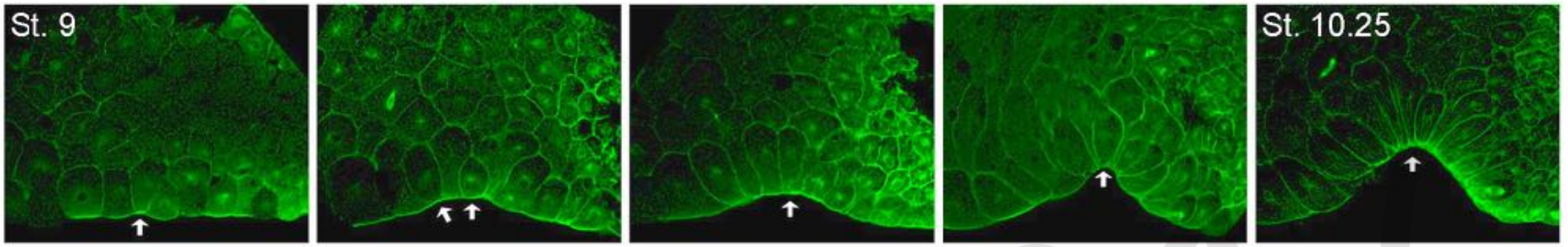
Appropriate microscope/ hardware

Imaging Parameters

Typical Imaging Experiment Workflow



Sample Prep: An Example



- Controls for antibody staining, transfection
- Use materials best suited for microscope set-up (e.g., coverslip, glass bottom dishes)
- Customize mounting
- Optimize staining and transfection protocols before you get to the scope
- Reproducibility:
  - write down protocols with specific notes, lot numbers, part numbers
  - example: Phalloidin fixation

Typical Imaging Experiment Workflow

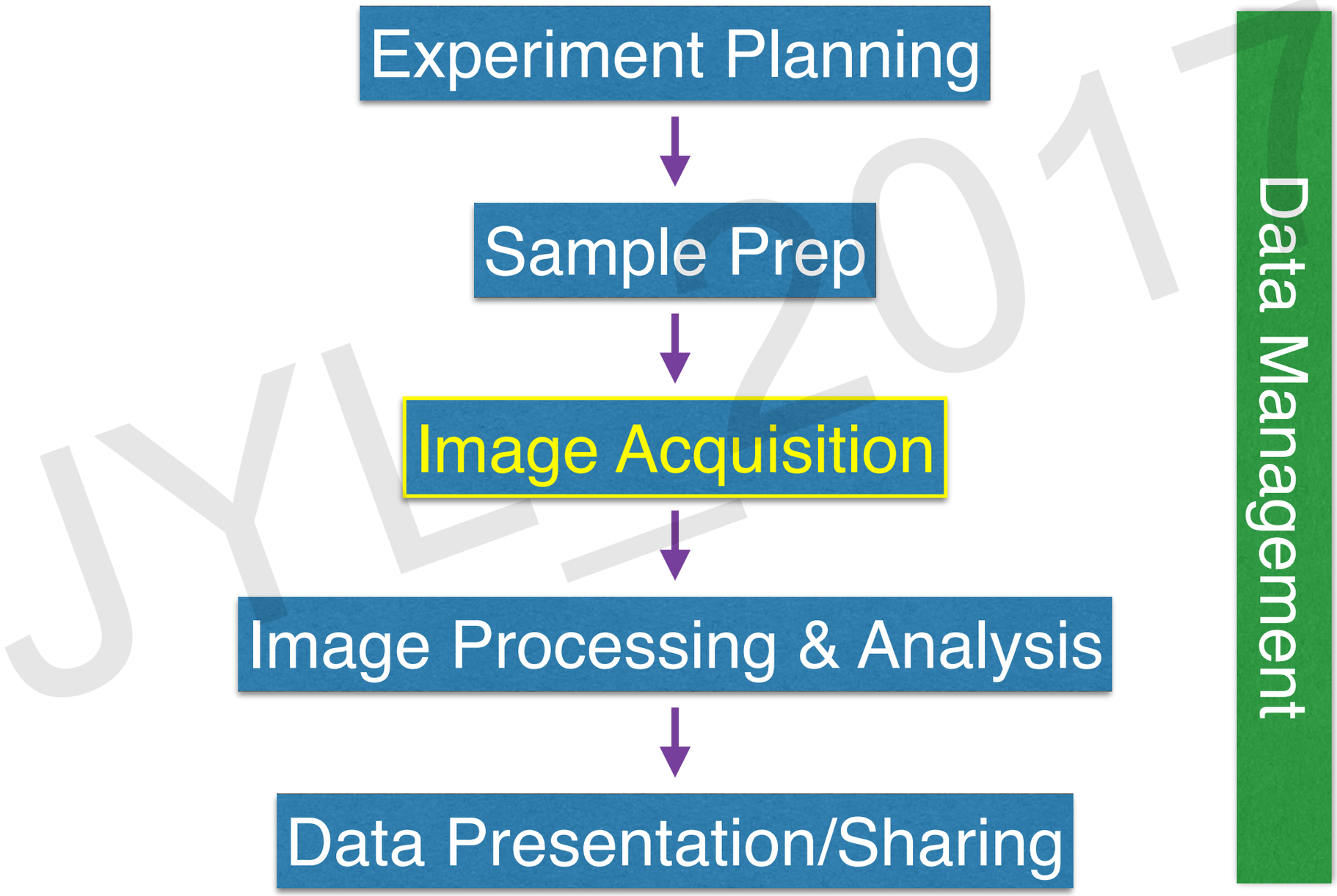
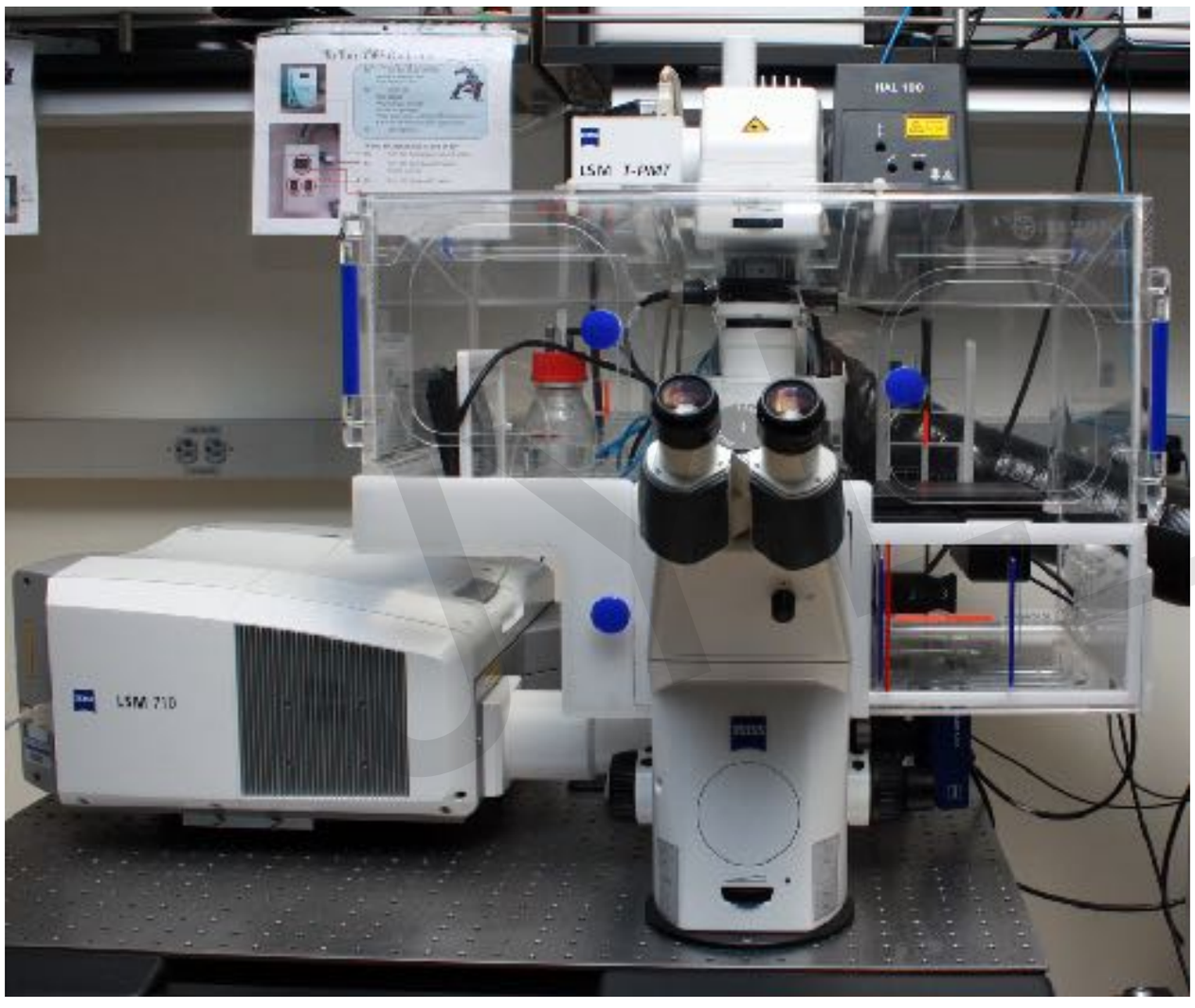
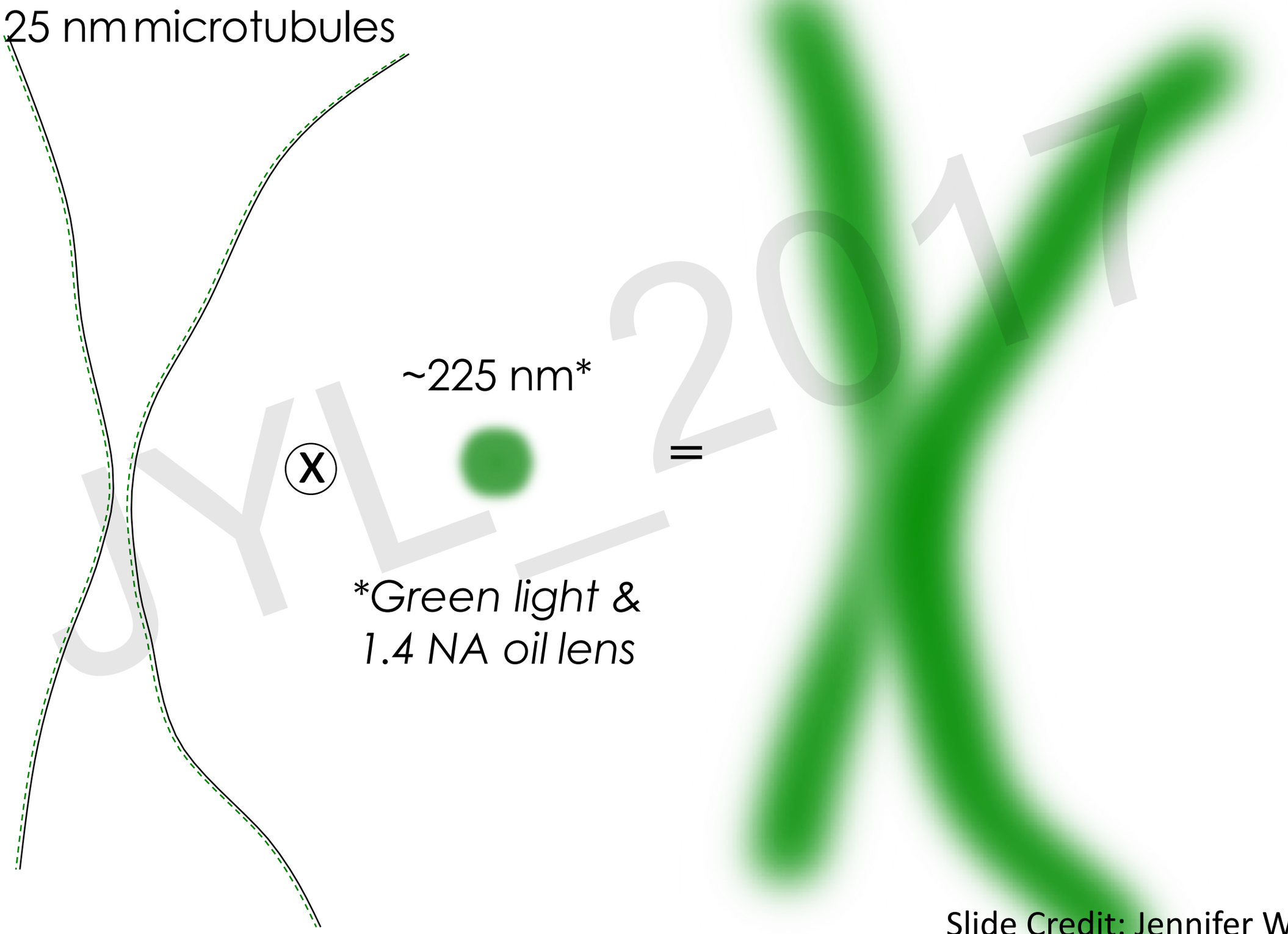


Image Acquisition



- Resolution & Sampling
- Digital Image Formation
- Histograms/Saturation
- Calibrating Hardware
- Reproducibility

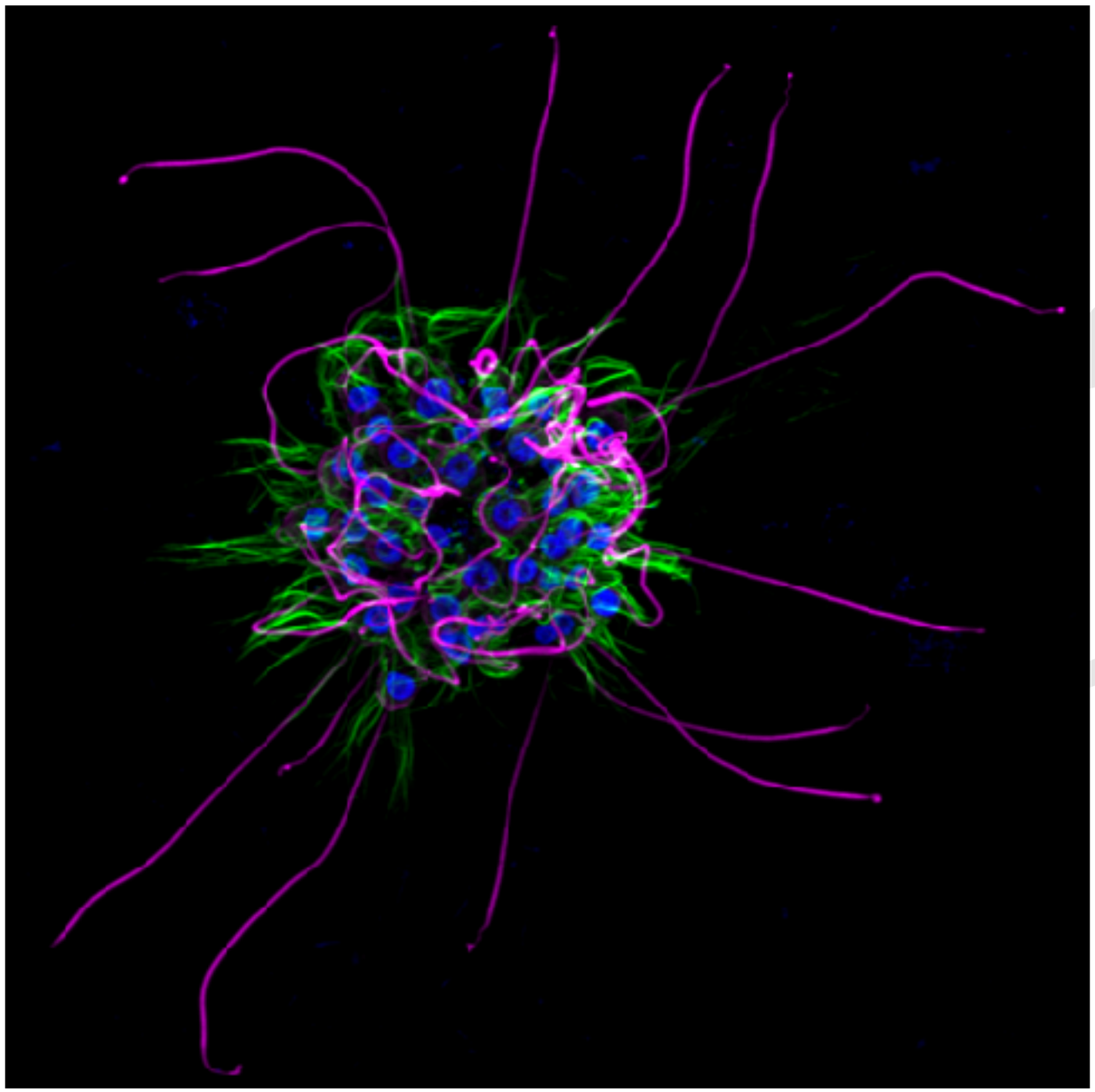
Images from light microscopes are convoluted



Slide Credit: Jennifer Waters

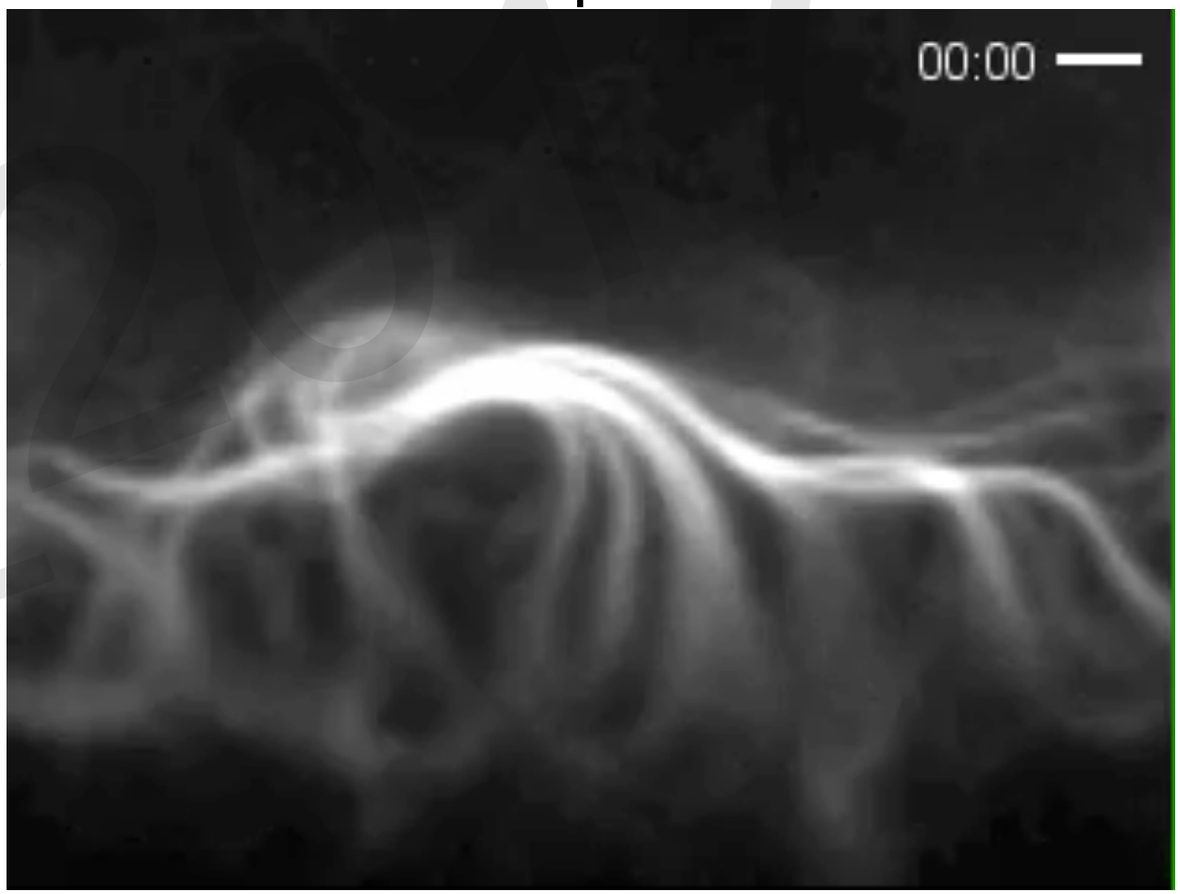
Resolution

Spatial



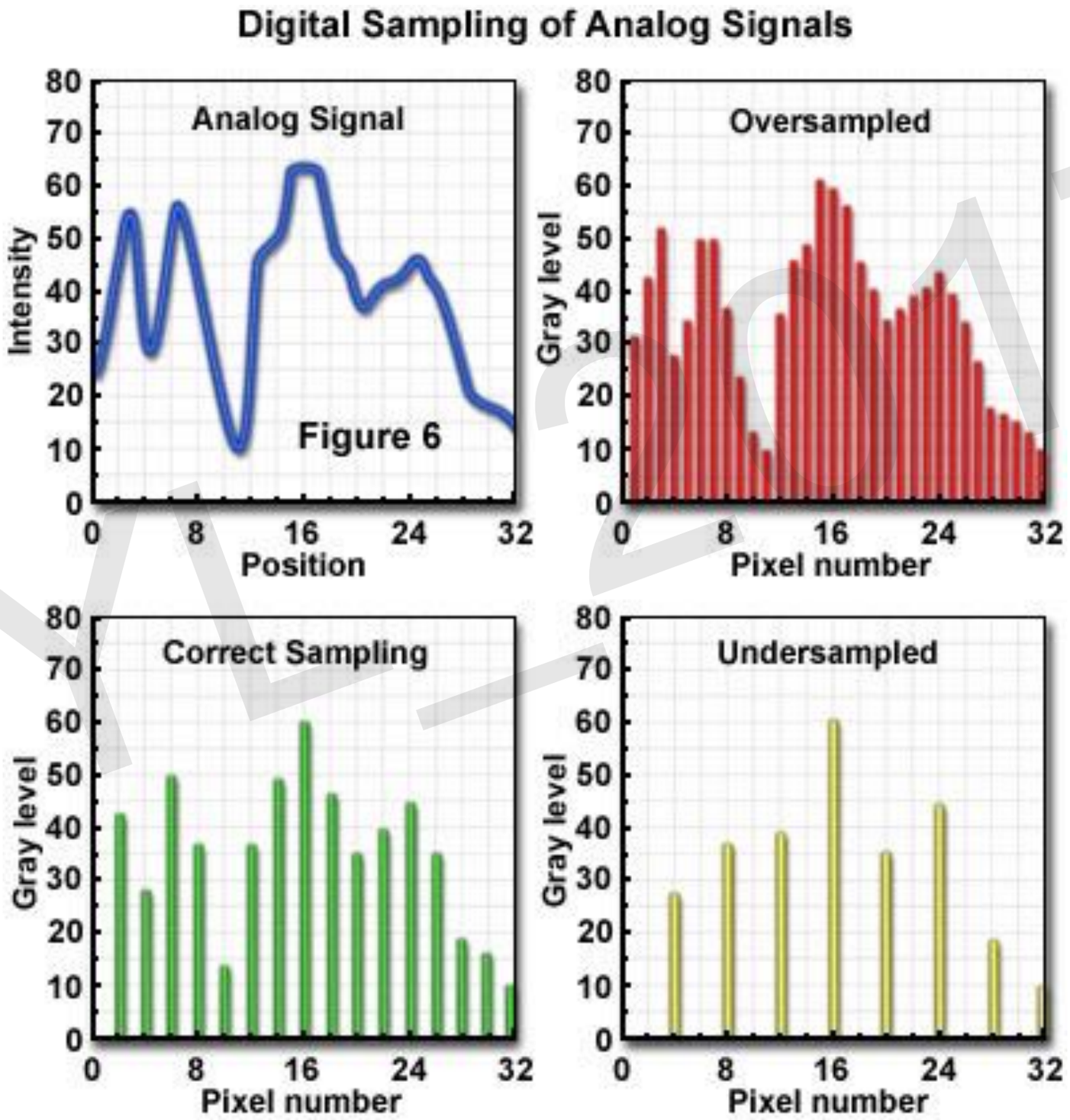
Kayley Hake, King Lab

Temporal



Sanchez et. al., *Science* 2011

Proper Sampling is Essential in Microscopy



http://www.olympusconfocal.com/theory/resolutionintro.html

Shannon-Nyquist Sampling Criterion

a.k.a., how to avoid undersampling/aliasing

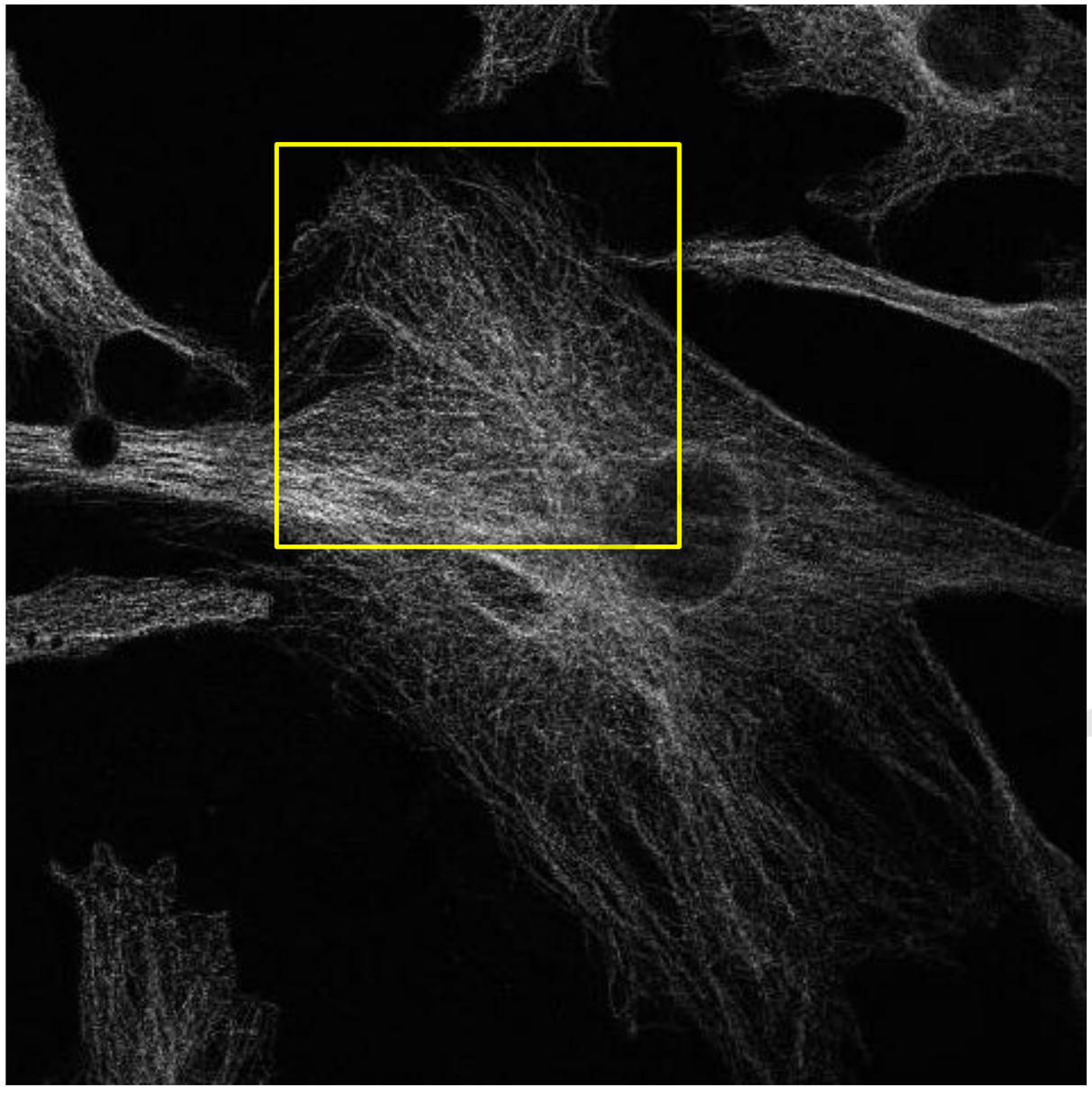
$$\Delta s \leq \Delta x,y,z,t \, / \, 2$$

Take your limit of resolution & divide it by 2
(Some people divide by 2.3 to be safe)

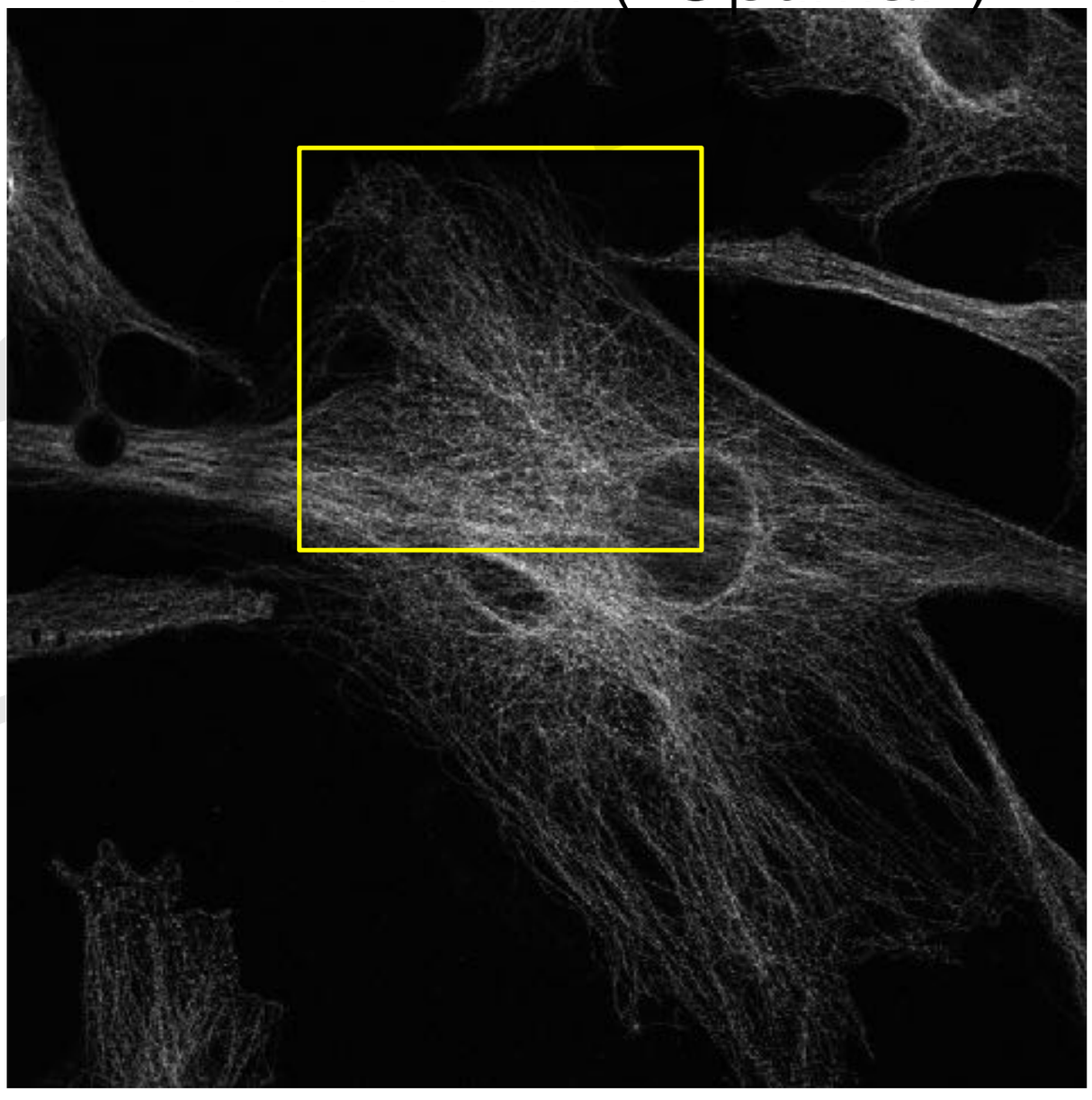
OR, press “optimal” button on confocal software
(“Sampling for Dummies”)

Nyquist Sampling - Lateral

512 x 512

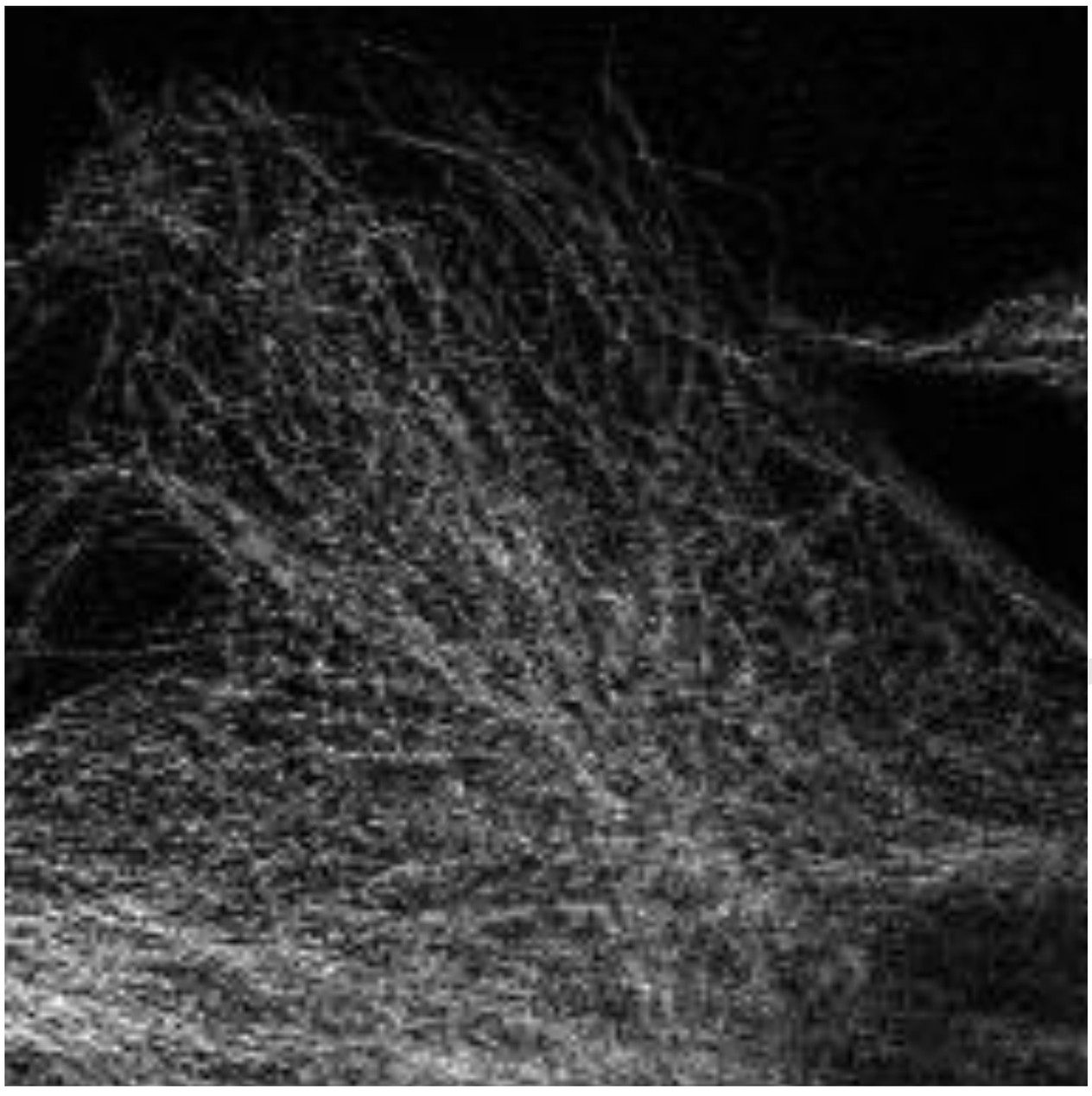


1444 x 1444 (“Optimal”)

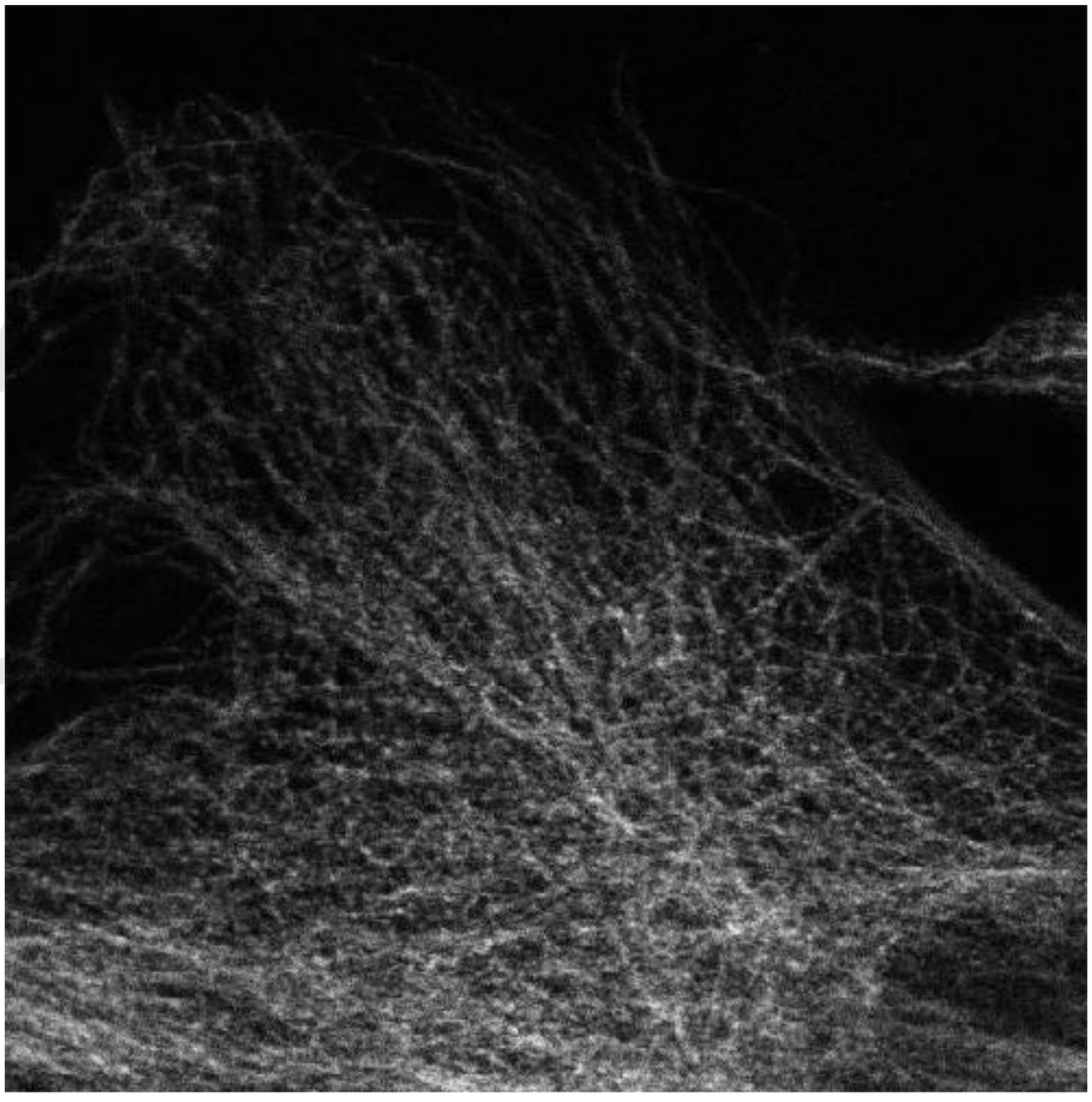


Nyquist Sampling - Lateral

512 x 512



1444 x 1444 (“Optimal”)



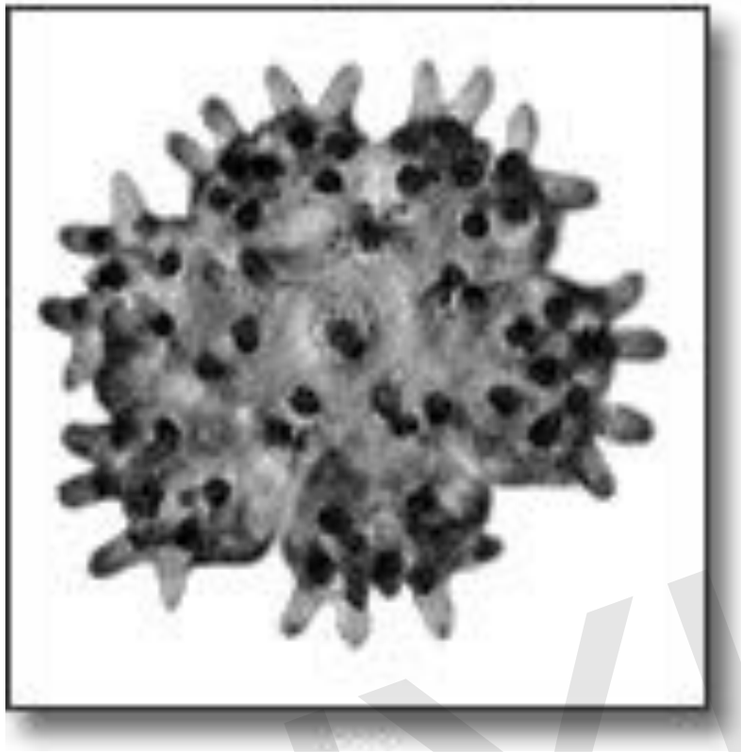
Improper Temporal Sampling

Helicopter blade frequency = frame rate

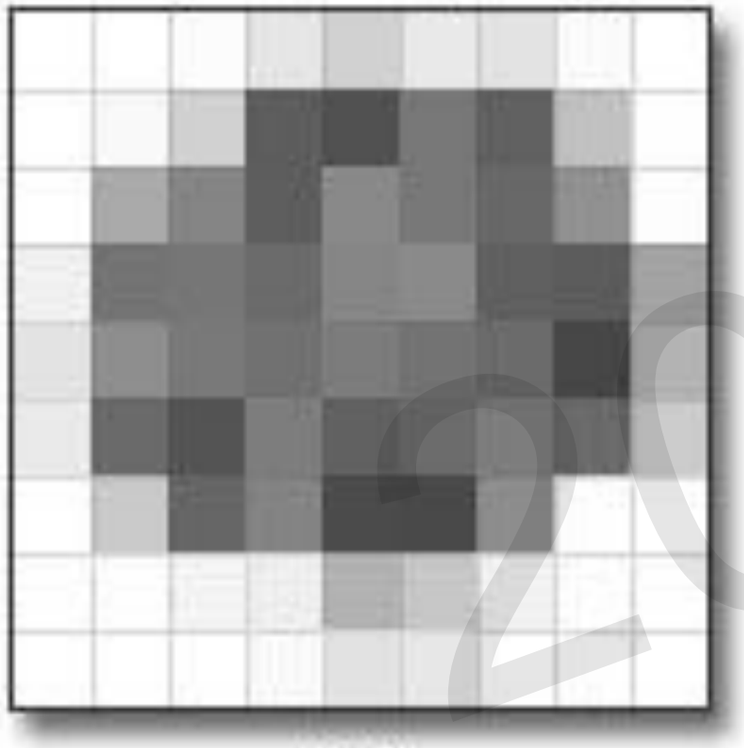
https://www.chrisfay.de

Digital Image Formation

Analog Image



Digital Sampling



Pixel Quantization

249	244	240	230	209	233	227	251	255
248	245	210	93	81	120	97	193	254
250	170	133	94	137	120	104	145	253
241	116	118	107	134	138	96	92	163
277	142	121	113	124	115	107	71	179
234	106	84	125	97	108	125	106	204
241	202	102	132	75	73	141	246	252
253	252	244	239	178	199	242	250	245
255	249	244	250	226	231	240	251	253

http://hamamatsu.magnet.fsu.edu/articles/digitalimagebasics.html

<u>Bit depth</u> = digital readout of intensity levels

8-bit = 2^8 = 256 levels of gray

12-bit = 2^{12} = 4096 levels of gray

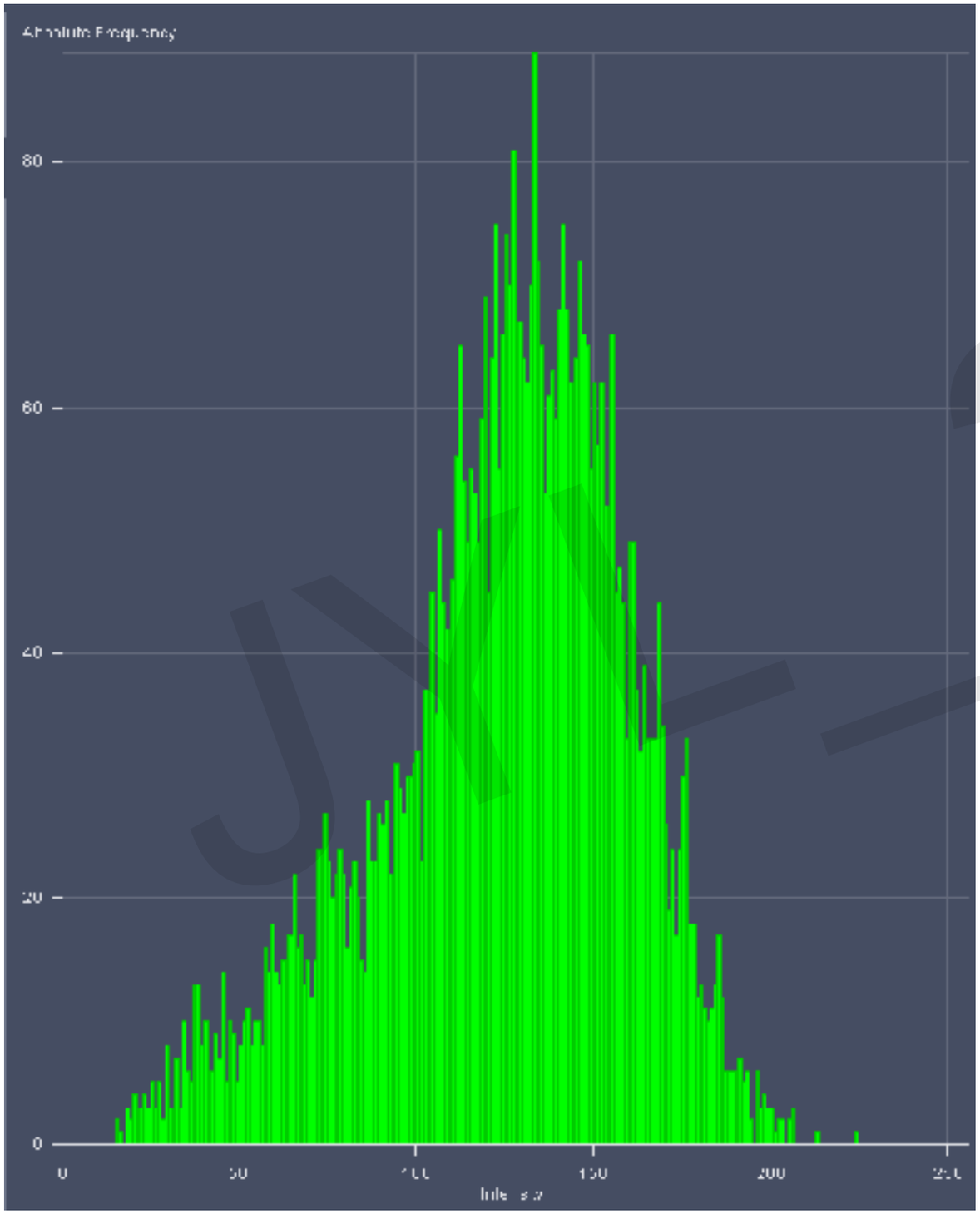
16-bit = 2^{16} = 65,536 levels of gray

The human eye can only detect between 32-64 levels of gray!

Histograms are a Microscopist's Best Friend

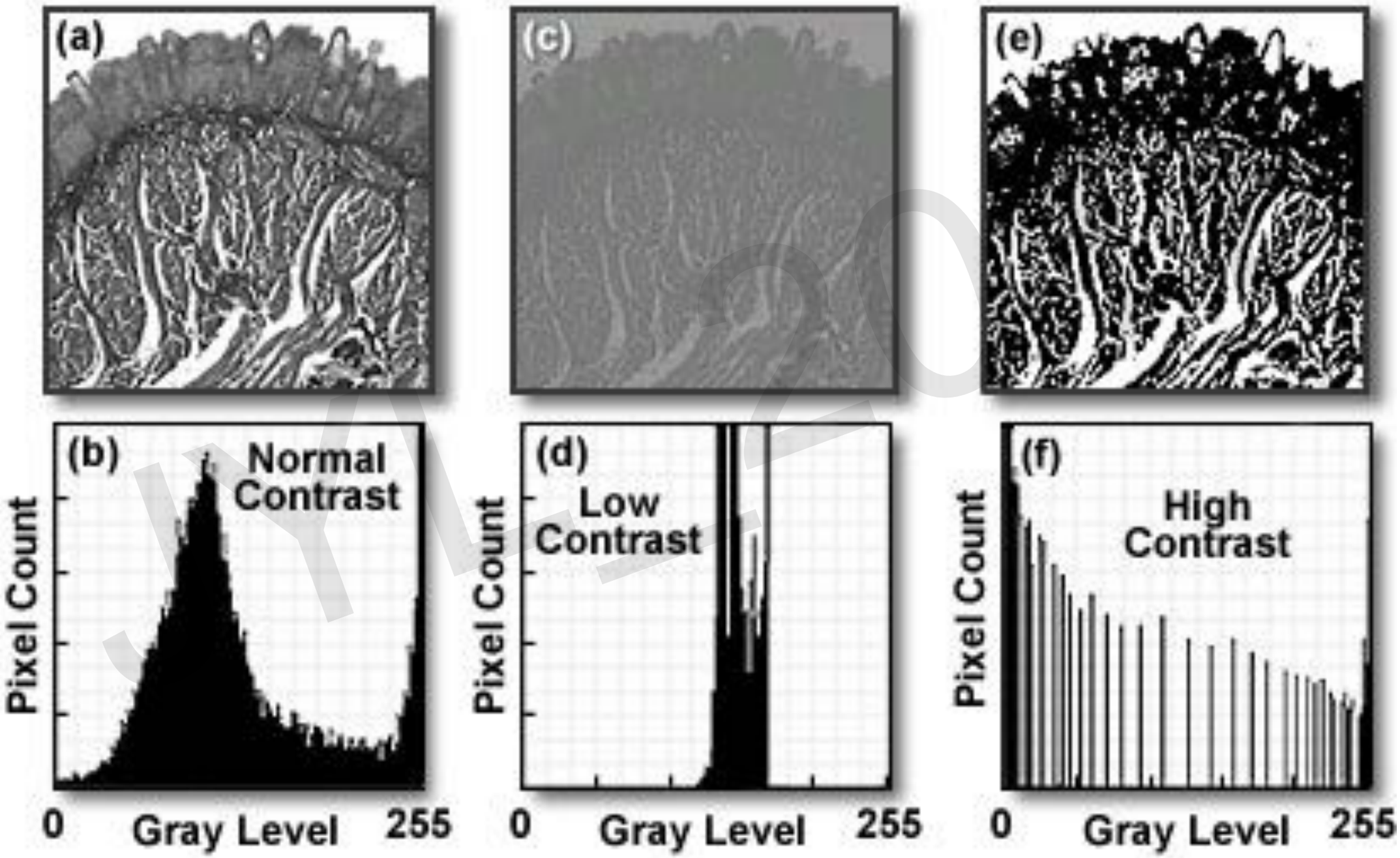
Best way to optimize settings for maximum contrast and dynamic range without saturation (i.e., information loss)

What are histograms?



- Graph of intensity vs. frequency
- Quick way to assess contrast, saturation

Dynamic Range = Contrast



http://hamamatsu.magnet.fsu.edu/articles/digitalimagebasics.html

Histograms are a Microscopist's Best Friend

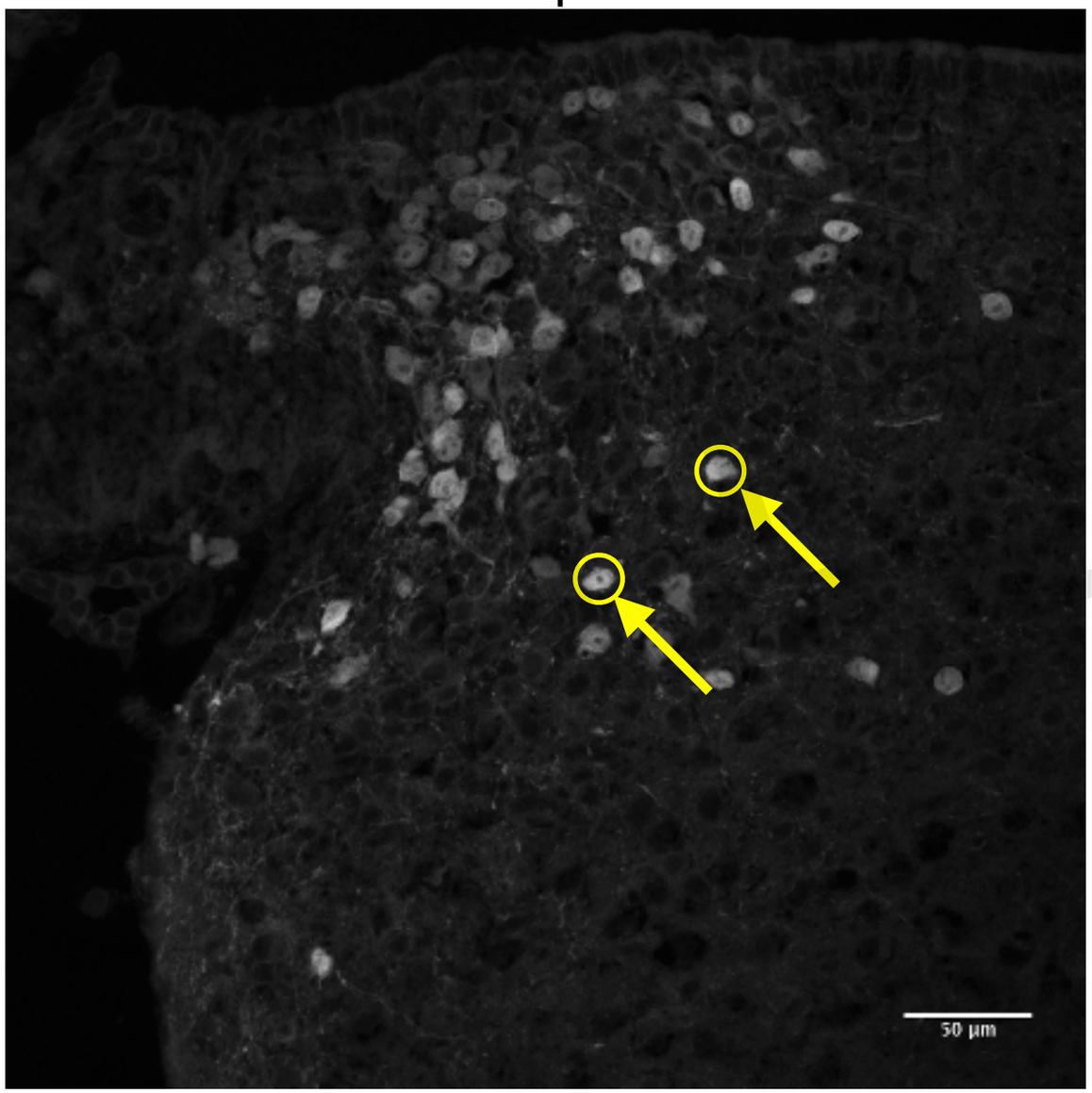
Larger dynamic range = higher signal-to-noise ratio

However, you want to avoid saturation because saturation = data loss

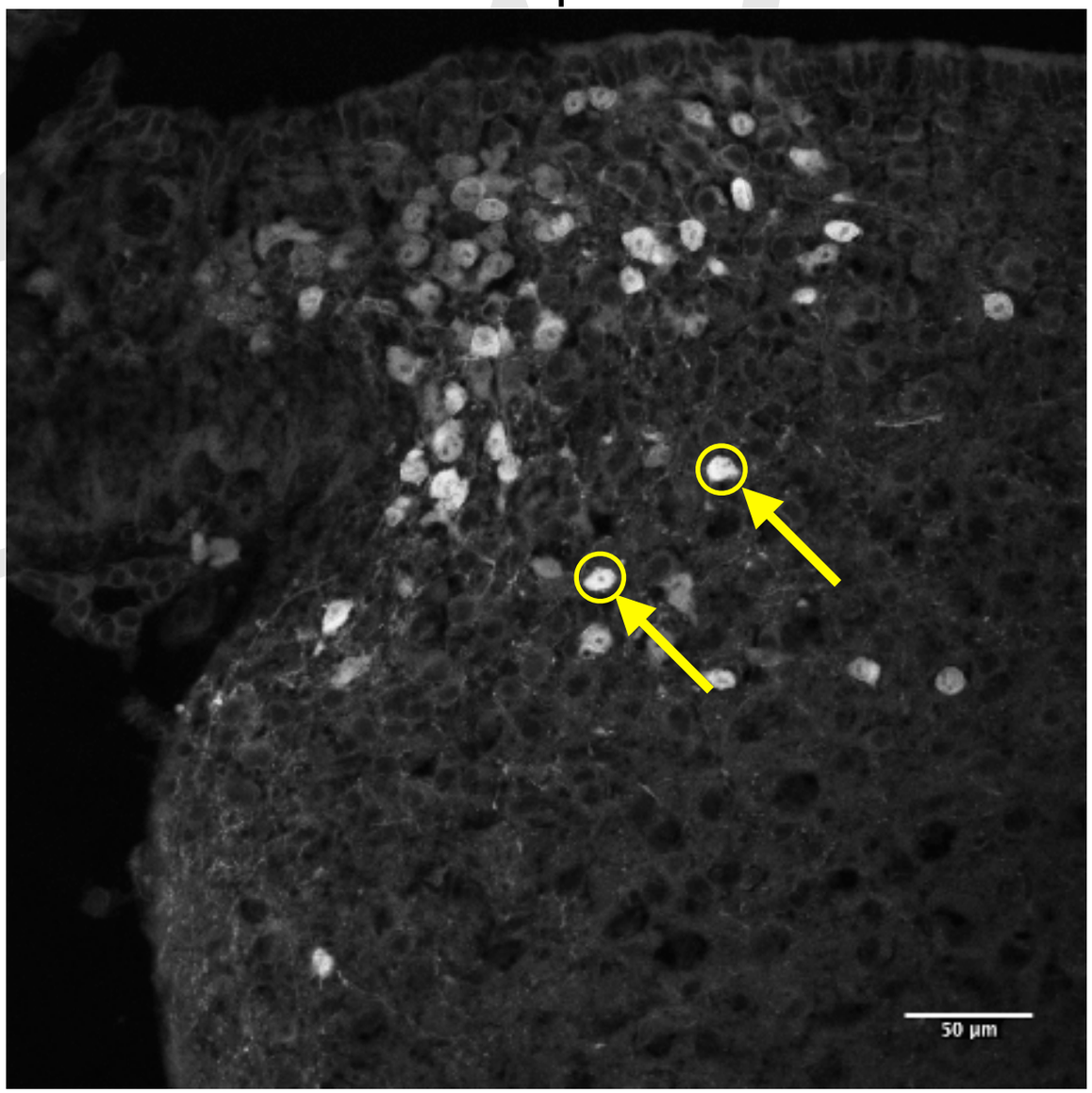
Avoiding Saturation

Mouse brain section, imaged on the LSM 880 (“Trinity”)
20X W/1.0 NA, 1 AU

Sample A

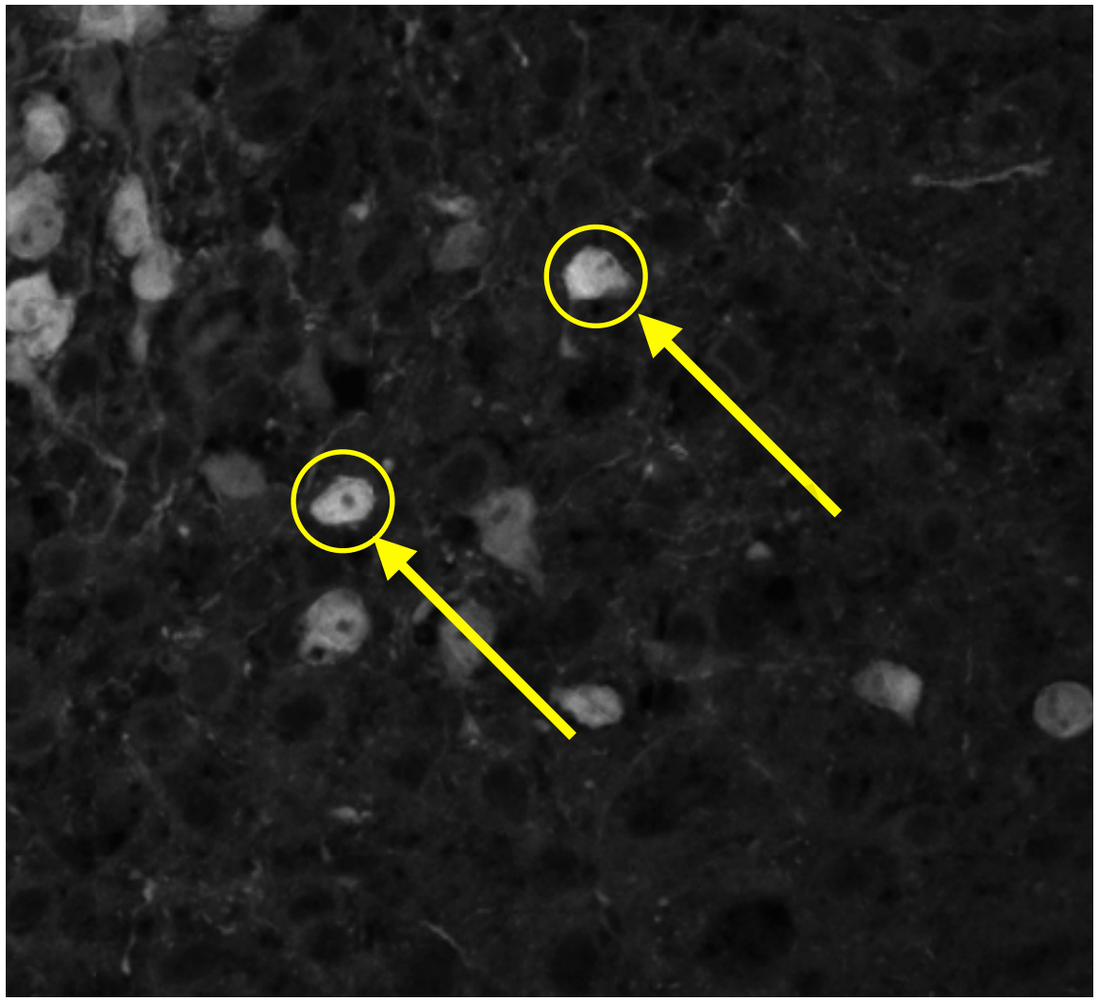


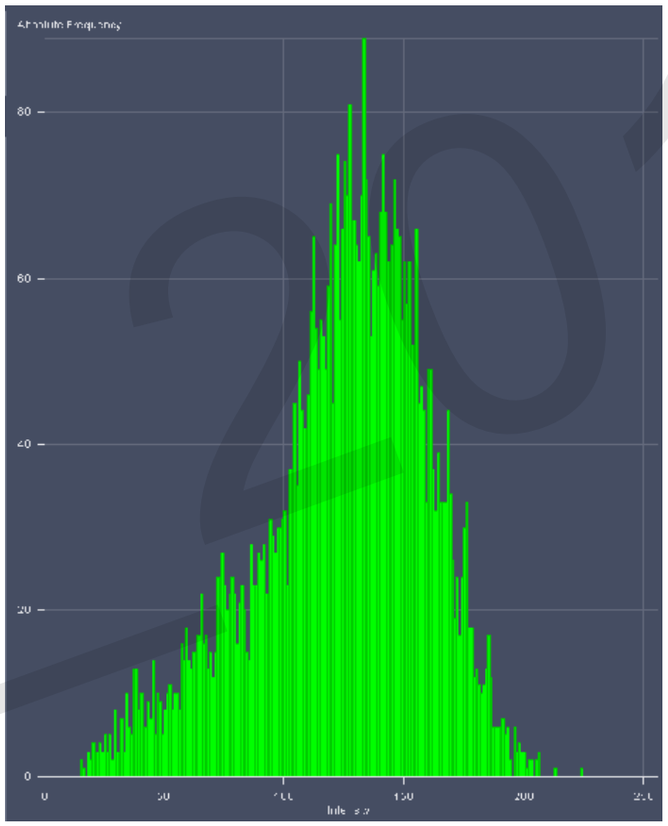
Sample B

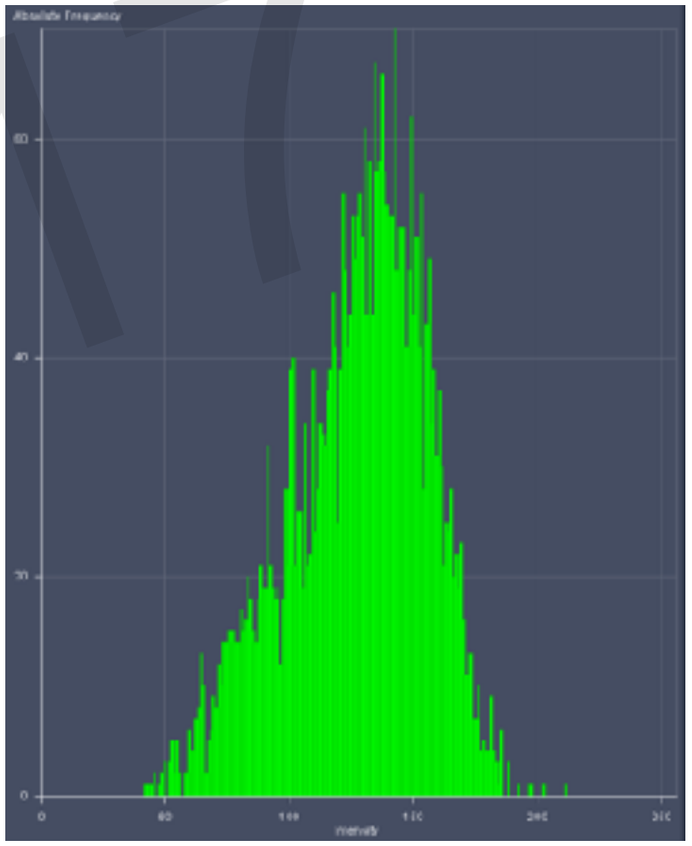


Sample courtesy of George Stratigopoulos, Columbia University

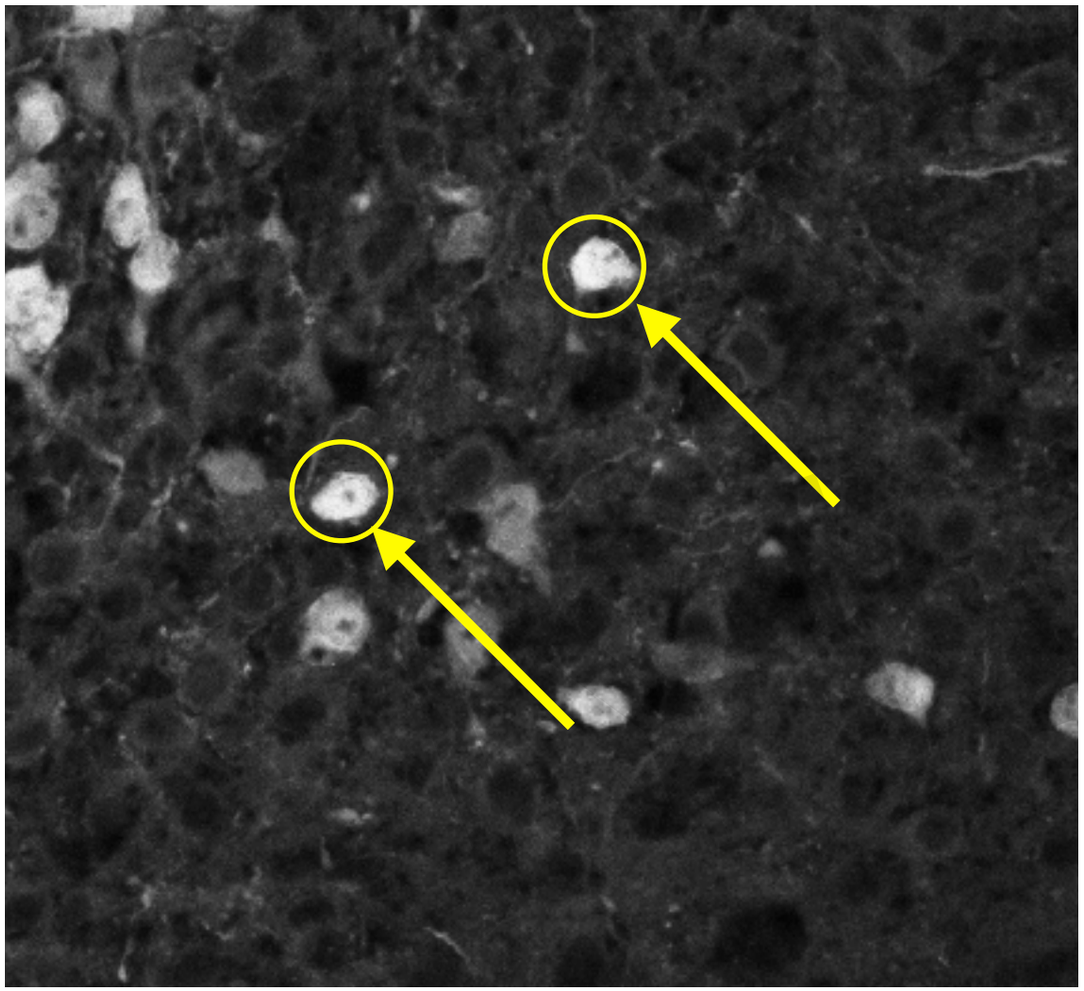
Sample A

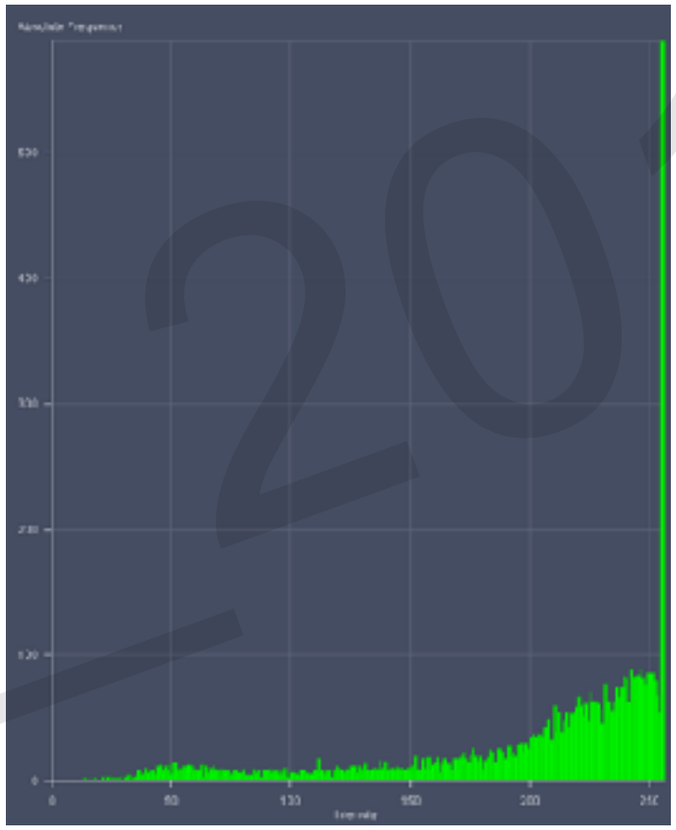


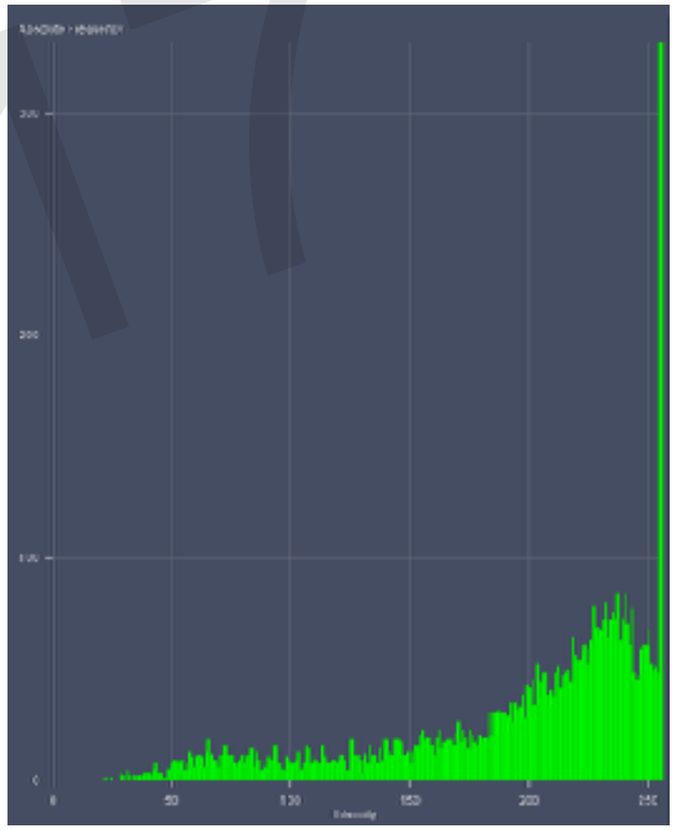




Sample B







Saturation = Potential Data Loss, Skewed Distribution

	Sample A (Not Saturated)	Sample B (Saturated)
Cell 1- mean intensity	124 (+/- 36)	202 (+/- 57)
Cell 2- mean intensity	127 (+/- 27)	195 (+/- 55)
Cell 1 - saturated pixels	0	590
Cell 2- saturated pixels	0	332

Remember you can always change contrast/levels for presentations/figures.

Calibrating Hardware

- Hardware can fluctuate, go out of alignment
- Calibration should be done at beginning of experiments
- Especially important if measuring intensities, colocalization

Calibrating Hardware

Example 1: Light Source Intensity

Hartley, *Photonics Media* 2016

Calibrating Hardware

Example 2: Colocalization

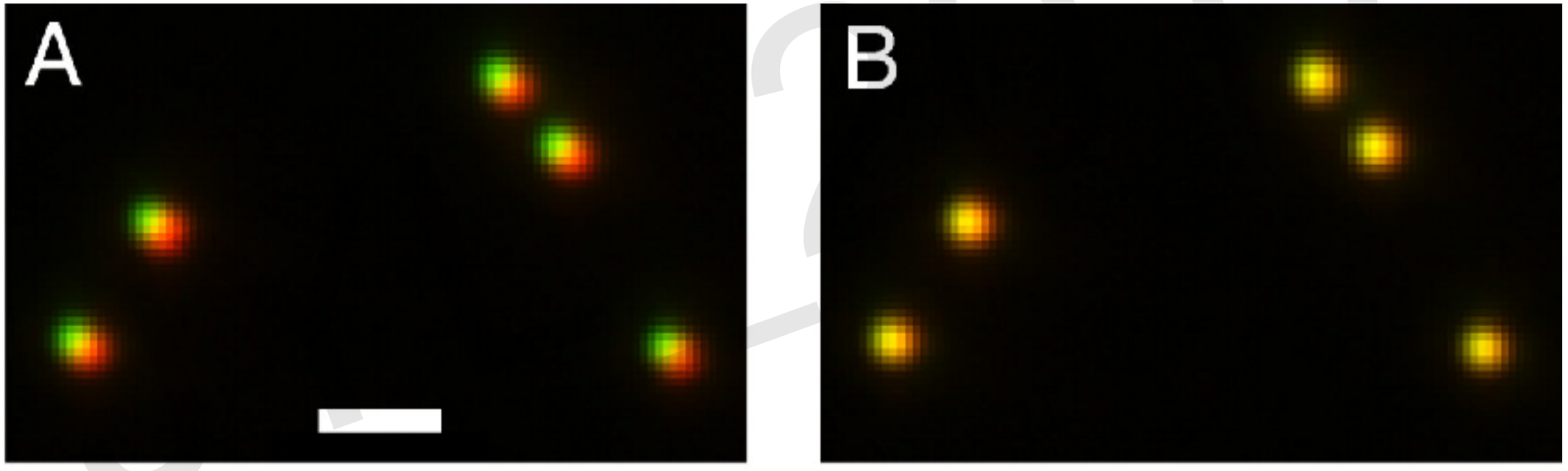
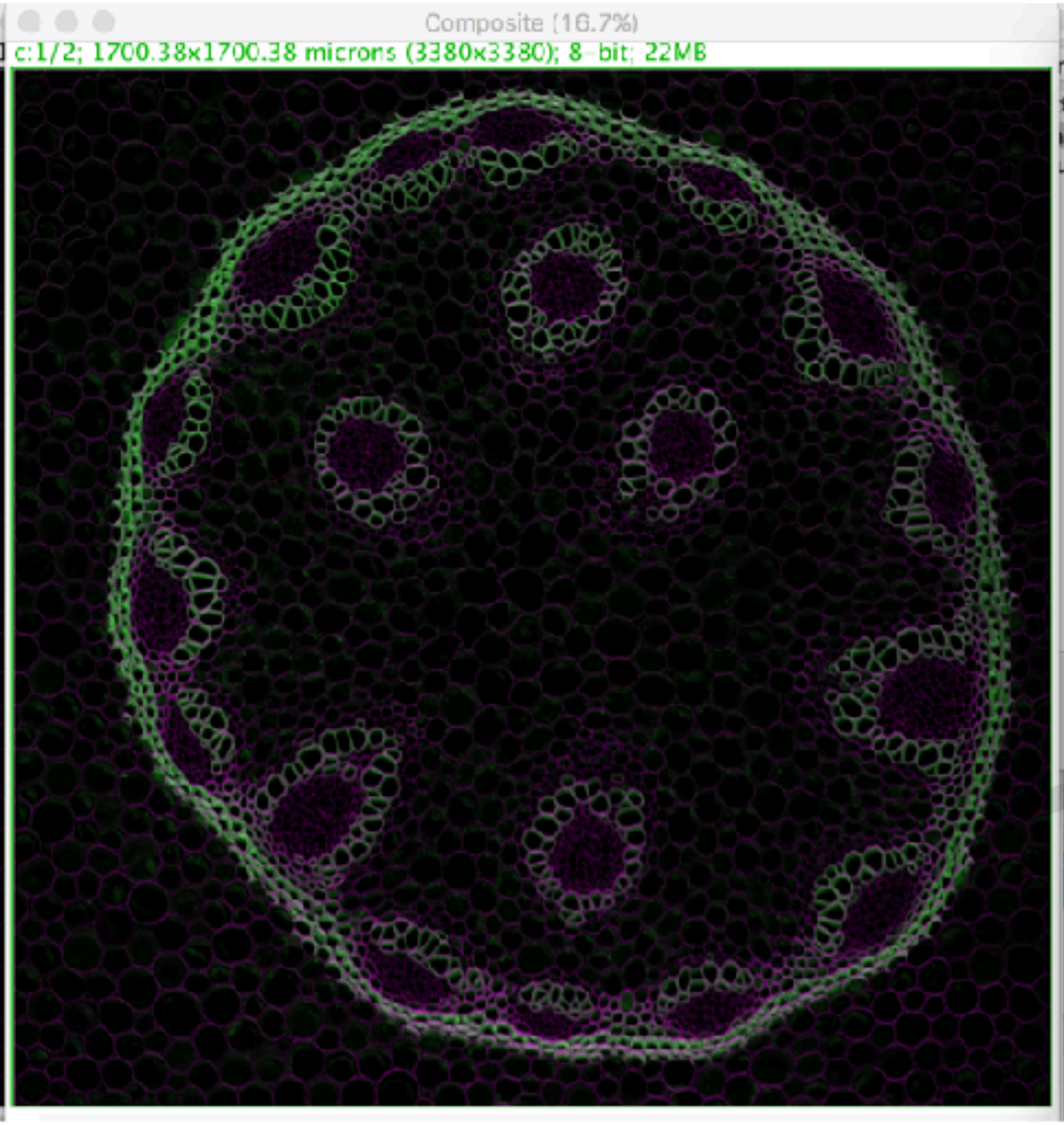


Image Acquisition: Reproducibility

- **Metadata**! Contains most of the essential acquisition information
- On a home-built scope, may need to take more notes about hardware setup, imaging conditions
- Most rigorous/best practices:
  - Measuring laser power before every session
  - Having internal controls or calibration samples
  - Noting environmental conditions (temperature, humidity)

Metadata: Raw File Format



Original Metadata - Image 1.czi

Key	Value
BitsPerPixel	8
DimensionOrder	XYCZT
IsInterleaved	false
IsRGB	false
LittleEndian	true
PixelType	uint8
Series 0 Name	Image 1 #1
SizeC	2
SizeT	1
SizeX	3380
SizeY	3380
SizeZ	1
Appliance|Data|ShuttleAndFindData|Calibration|Marker|FocusPosition #1	0
Appliance|Data|ShuttleAndFindData|Calibration|Marker|FocusPosition #2	0
Appliance|Data|ShuttleAndFindData|Calibration|Marker|FocusPosition #3	0
Appliance|Data|ShuttleAndFindData|Calibration|Marker|Id #1	Marker:1
Appliance|Data|ShuttleAndFindData|Calibration|Marker|Id #2	Marker:2
Appliance|Data|ShuttleAndFindData|Calibration|Marker|Id #3	Marker:3
Appliance|Data|ShuttleAndFindData|Calibration|Marker|StageXPosition #1	0
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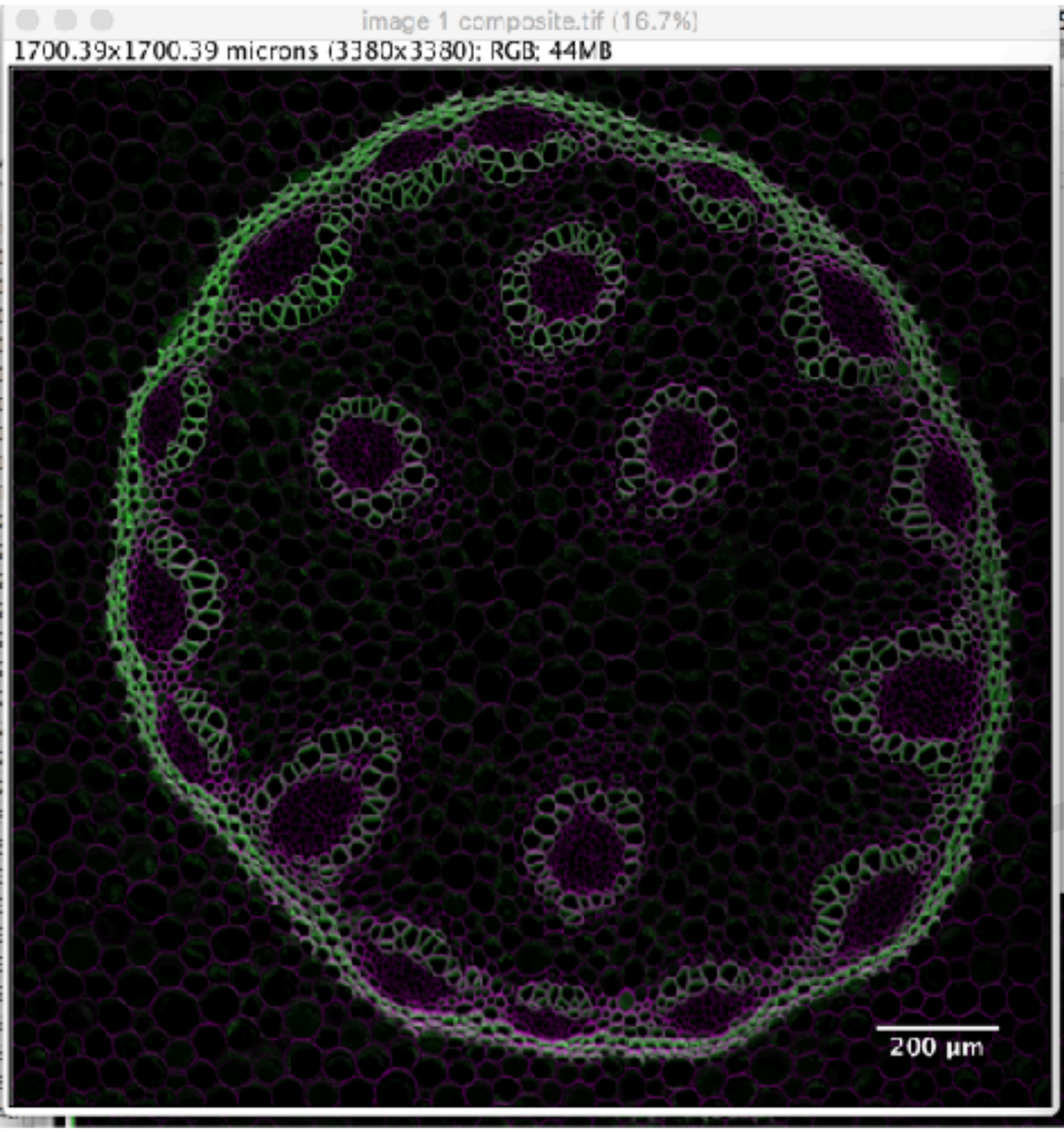
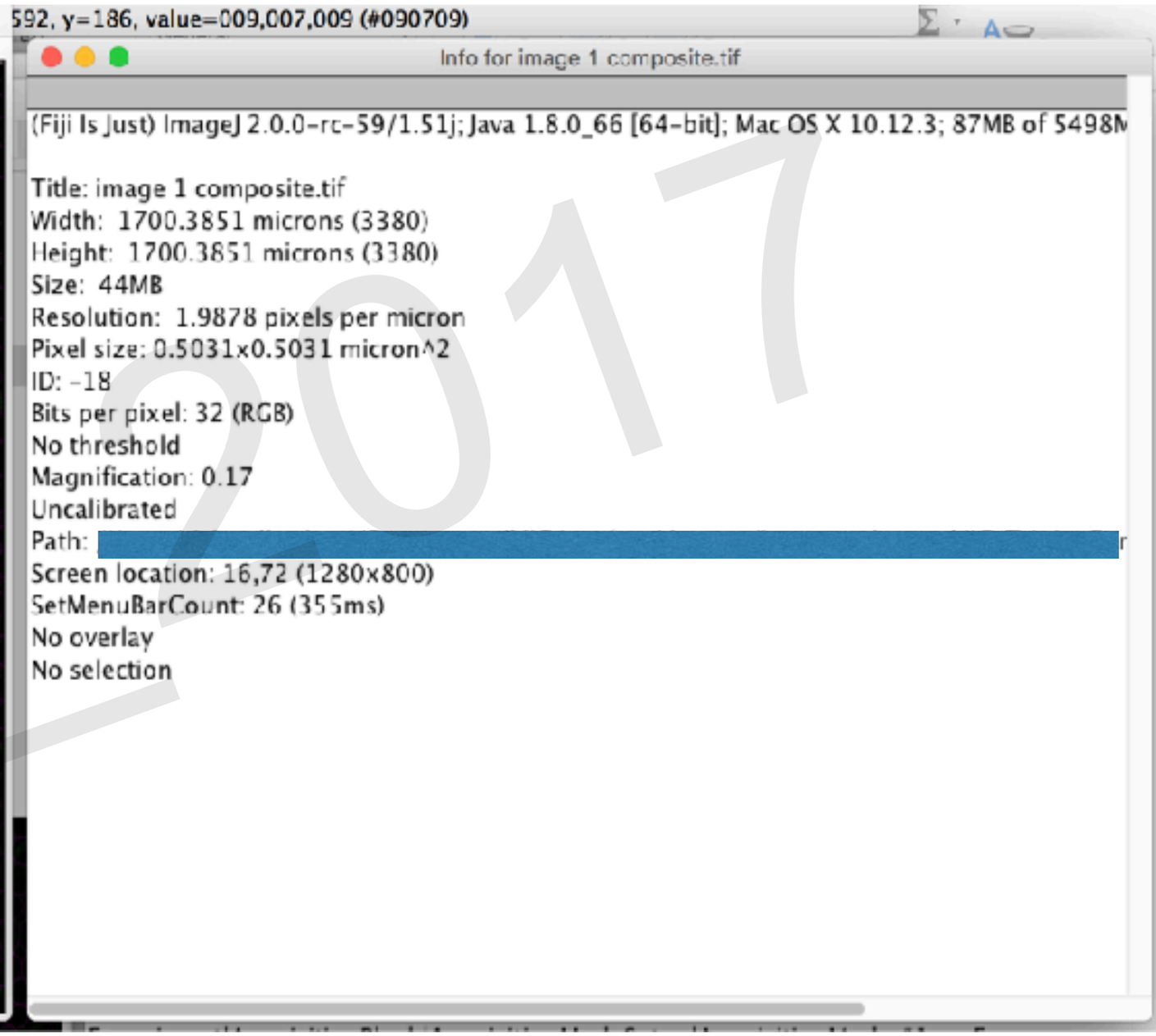
Appliance|Data|ShuttleAndFindData|Calibration|Marker|StageXPosition #3	0
Appliance|Data|ShuttleAndFindData|Calibration|Marker|StageYPosition #1	0
Appliance|Data|ShuttleAndFindData|Calibration|Marker|StageYPosition #2	0
Appliance|Data|ShuttleAndFindData|Calibration|Marker|StageYPosition #3	0
Appliance|Data|ShuttleAndFindData|Calibration|MicroscopeType #1	LM
Appliance|Data|ShuttleAndFindData|Calibration|StageOrientation|X #1	1
Appliance|Data|ShuttleAndFindData|Calibration|StageOrientation|Y #1	-1
Appliance|Id #1	ShuttleAndFir
Experiment|AcquisitionBlock|AcquisitionModeSetup|AcquisitionMode #1	Frame

399 pieces of metadata

Metadata: Exported Tiff





Lost metadata!

Typical Imaging Experiment Workflow

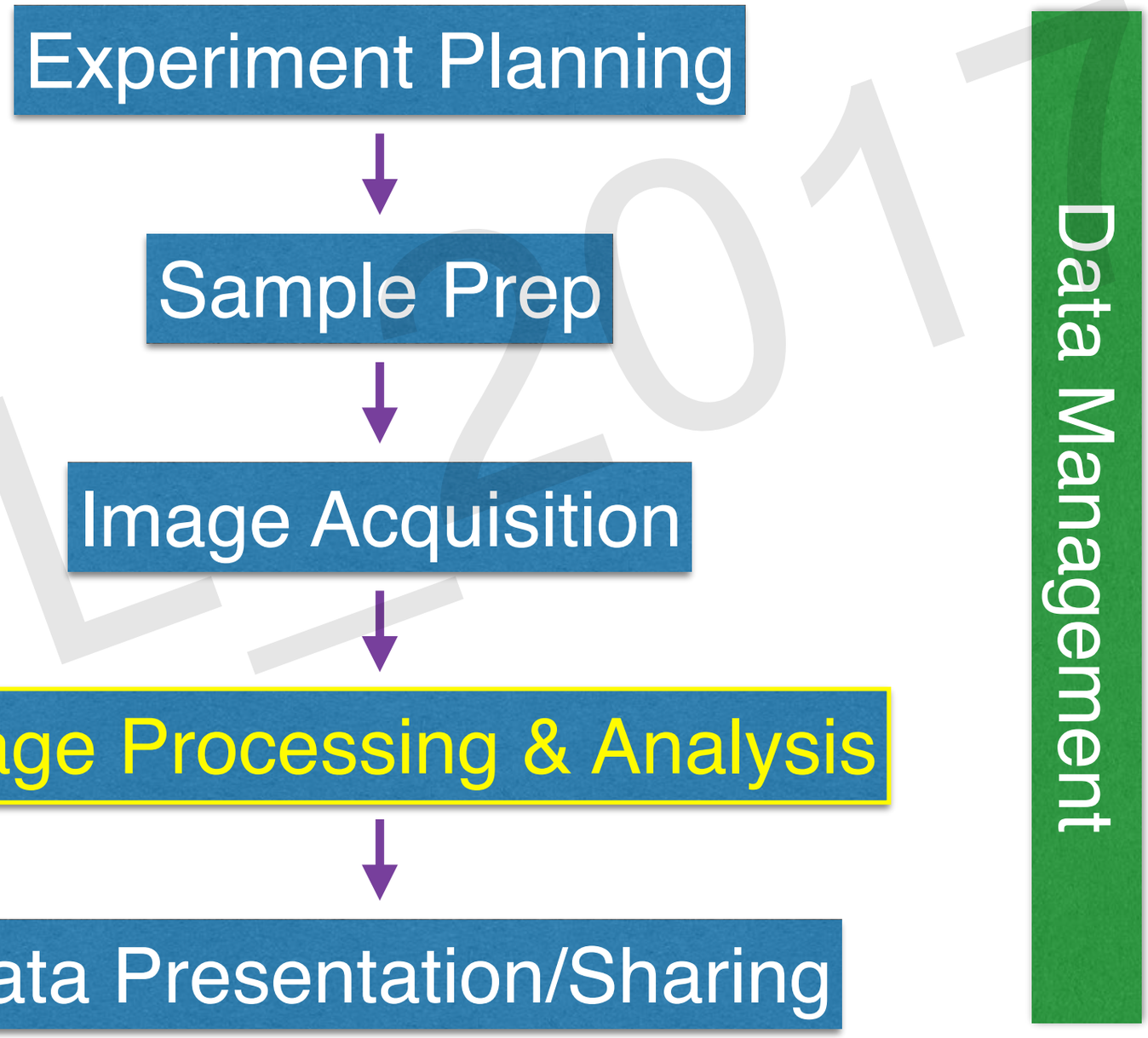
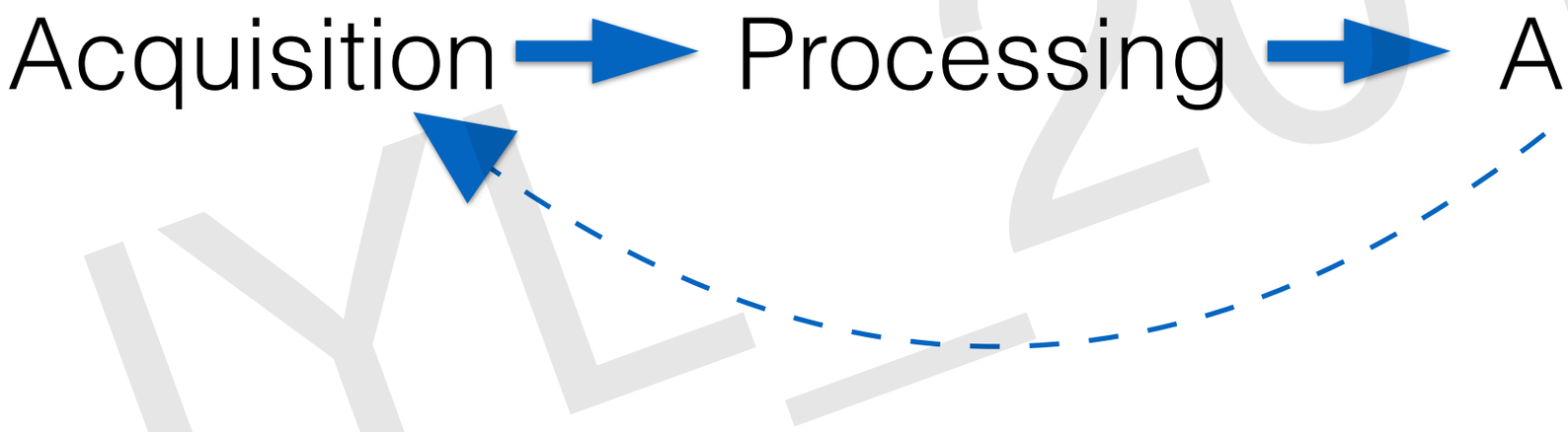


Image Processing & Analysis

- Usually requires several iterations to know how to best process & analyze data

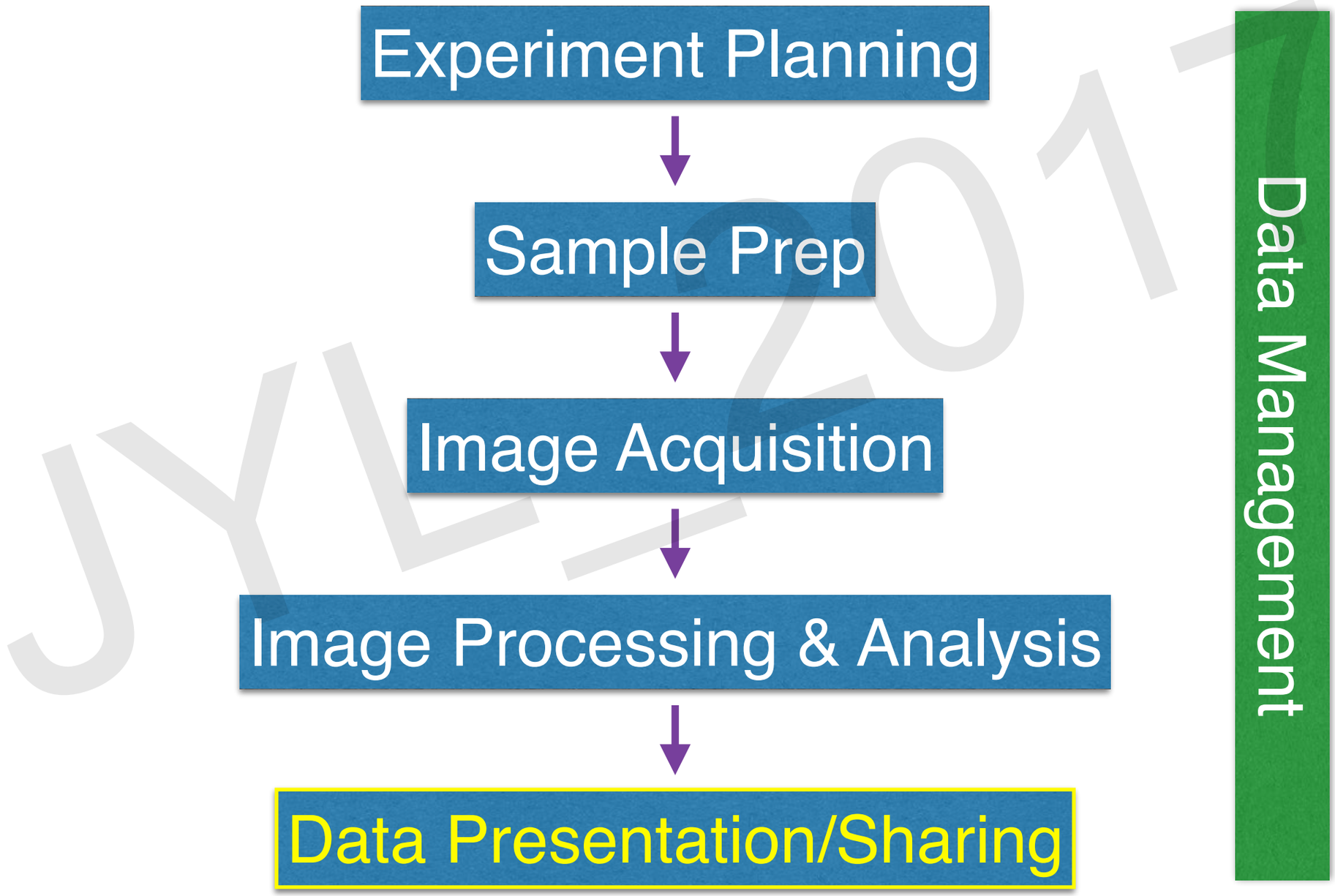


- Software tools: ImageJ, Imaris, Arivis, etc.
- Don’t use Photoshop for processing!

Image Processing & Analysis: Reproducibility

- Keep log/text file of processing steps in same folder as files
- Can write macros in ImageJ, Matlab to track processing steps
- Beware of:
  - Saving as jpegs (compression)
  - Saving as tiffs (metadata loss)
  - Accidentally changing bit-depth, levels
- Version control: keep multiple versions of the same file with identifiable file names
- Have others try to replicate your results using your processing and analysis workflow, or do double-blind tests

Typical Imaging Experiment Workflow



Data Presentation & Sharing

- Communication of results: making figures for publication and presentation
- Reproducibility:
  - Methods section - complete documentation
  - Sharing raw data & analysis methods with other scientists

Image Presentation - Tips

- Grayscale = easiest to see contrast
- Include a scale bar
- Avoid red/green overlays if possible (R/G color blindness)
- Always save raw AND processed images! Some journals and reviewers will ask to see raw data. For example, *Neuron* now requires all raw data to be uploaded to their website.

Images for Analysis (Quantitative)

vs.

Images for Presentation (Qualitative)

biowrites

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QUALITY AND INTEGRITY
JCB

« New issue: September 18th 2015 | Main | Everything you need to know about image screening at Rockefeller University Press in 10 posts »

October 05, 2015

Everything you need to know about image screening at Rockefeller University Press in 10 posts

#1: Our guidelines to beautiful, high-quality figures

You may have heard: *JCB* has high image standards. Part of *JCB*'s and many other journals' routine production process involves screening all editorially accepted manuscripts to confirm that images are of sufficiently high resolution for publication and to ensure that they have not been over-adjusted or manipulated in any way that could impact the conclusions of the work. Sometimes, figures fail this screening, or, more often, are not of sufficient quality to allow this screening process. But what are these researchers doing wrong? Not that much, actually. It turns out that, in the vast majority of cases, a few simple fixes can make a figure *JCB*-ready. It doesn't take much time or effort, but it does require that you know a few golden rules about quality image preparation. As scientific editors at Rockefeller University Press journals, we work every day with our production editors to move manuscripts through this screening, and in this series, we'll share with you what you need to know about image data acquisition and storage, as well as figure preparation. We hope it will be useful if you wish to submit your work to a journal that screens figures or if you just want to learn more about the process and earn bragging rights.

New in the JCB
JCB home

Subscribe to biowrites' feed

biosights: March 14, 2016
During *Drosophila* oogenesis, the collective migration of egg chamber follicle cells drives the chambers' rotation and elongation.... (more)

biosights archive

biobytes: February 29, 2016
In the February 29th edition of biobytes, Jean Gautier (Columbia University, New York, NY) describes how cells repair Top2-DNA adducts ... (more)

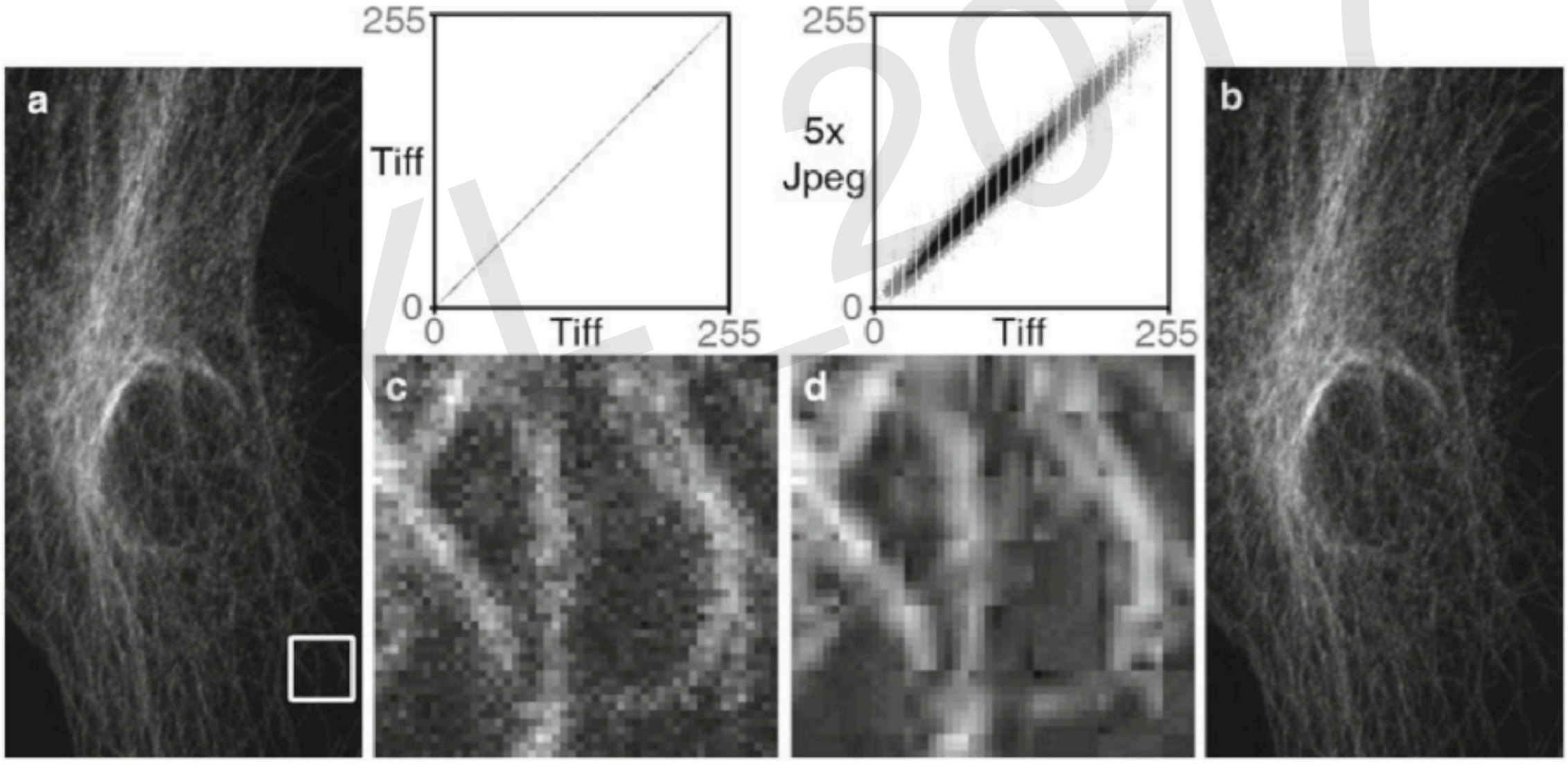
biobytes archive

Figure Resolution

- JCB guidelines:
  - the size of the figure should be comparable to that of the published, printed figure
  - the figure file itself should be at a resolution of 600 dpi
  - each individual image copied or imported into the figure file should also originally be at a resolution of 300 dpi (minimum)
- “It is essential to pay attention to resolution from the start as, unfortunately, ‘resampling’ in Photoshop does not yield true high-resolution images"

How to Get Image Data of Appropriate Quality & Resolution

- Scan/export at the highest quality (300 dpi)
- Export in loss-less file format (TIFF) vs. JPEG



Cromey, *Methods Mol Biol.* 2013

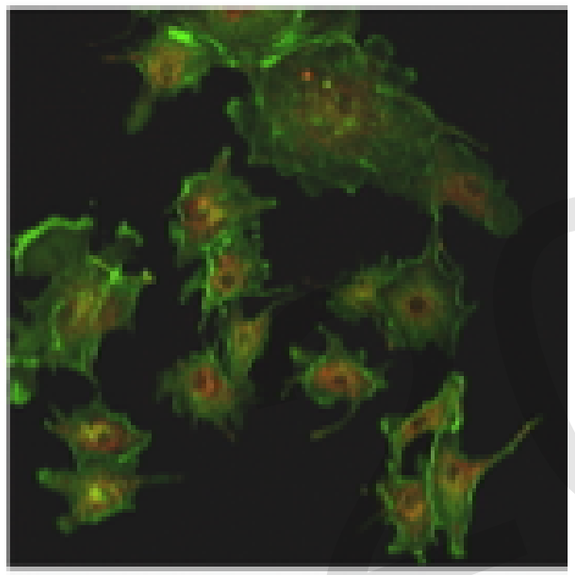
Which Software Programs Should I Use to Make My Figures?

- Recommended:
  - Adobe Creative Suite (Photoshop, Illustrator)
  - CorelDraw
  - Inkscape
  - OMERO.figure
- Not recommended (low quality export):
  - Powerpoint
  - Keynote
  - MS Paint

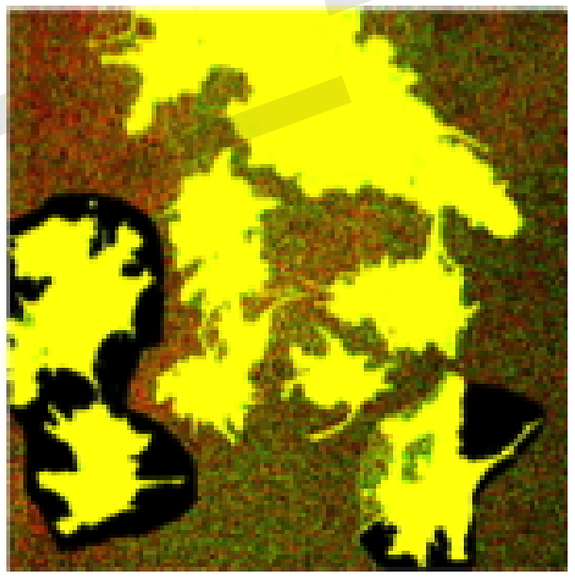
What is Appropriate or Inappropriate Micrograph Manipulation?

Inappropriate Micrograph Manipulation: Misrepresentation of a Microscope Field

Manipulated image

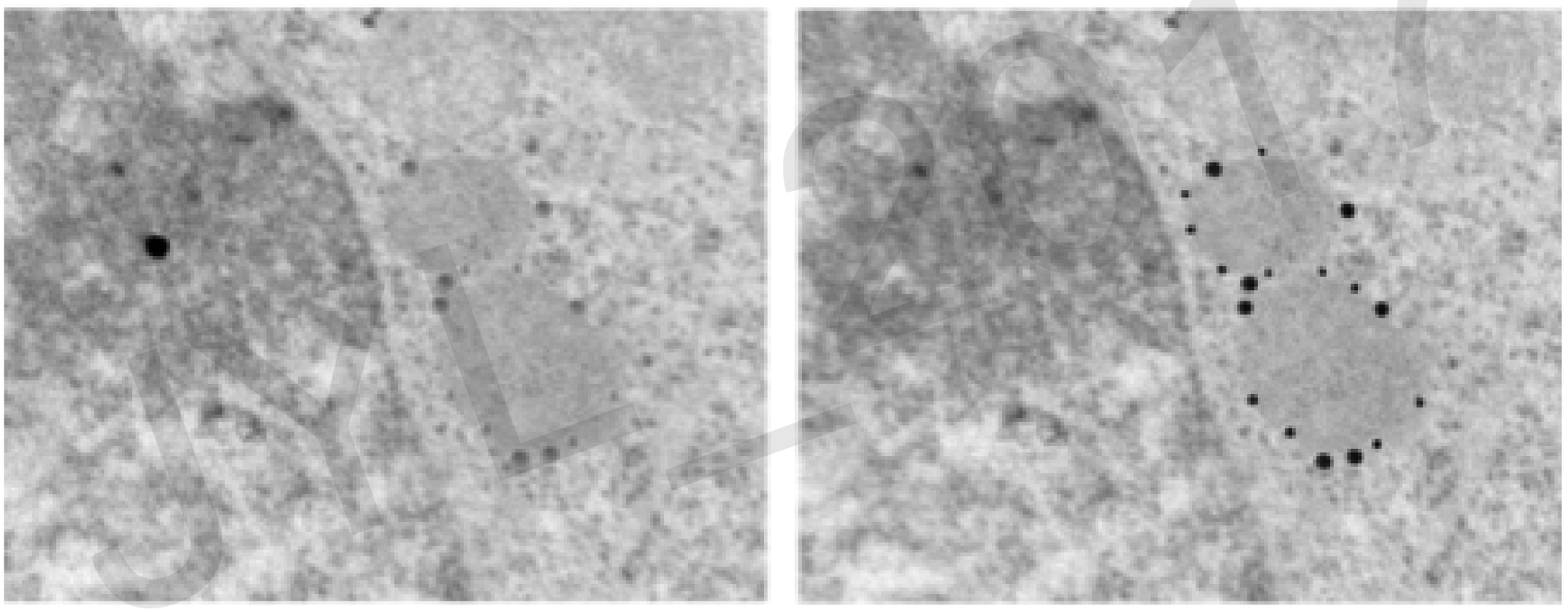


Manipulation revealed by contrast adjustment



Mike Rossner, and Kenneth M. Yamada J Cell Biol 2004;166:11-15

Inappropriate Micrograph Manipulation: Selectively Enhancing Specific Elements of an Image



Mike Rossner, and Kenneth M. Yamada J Cell Biol 2004;166:11-15

JCB

Inappropriate Micrograph Manipulation: Copying & Pasting

The New York Times | SCIENCE

SCIENCE

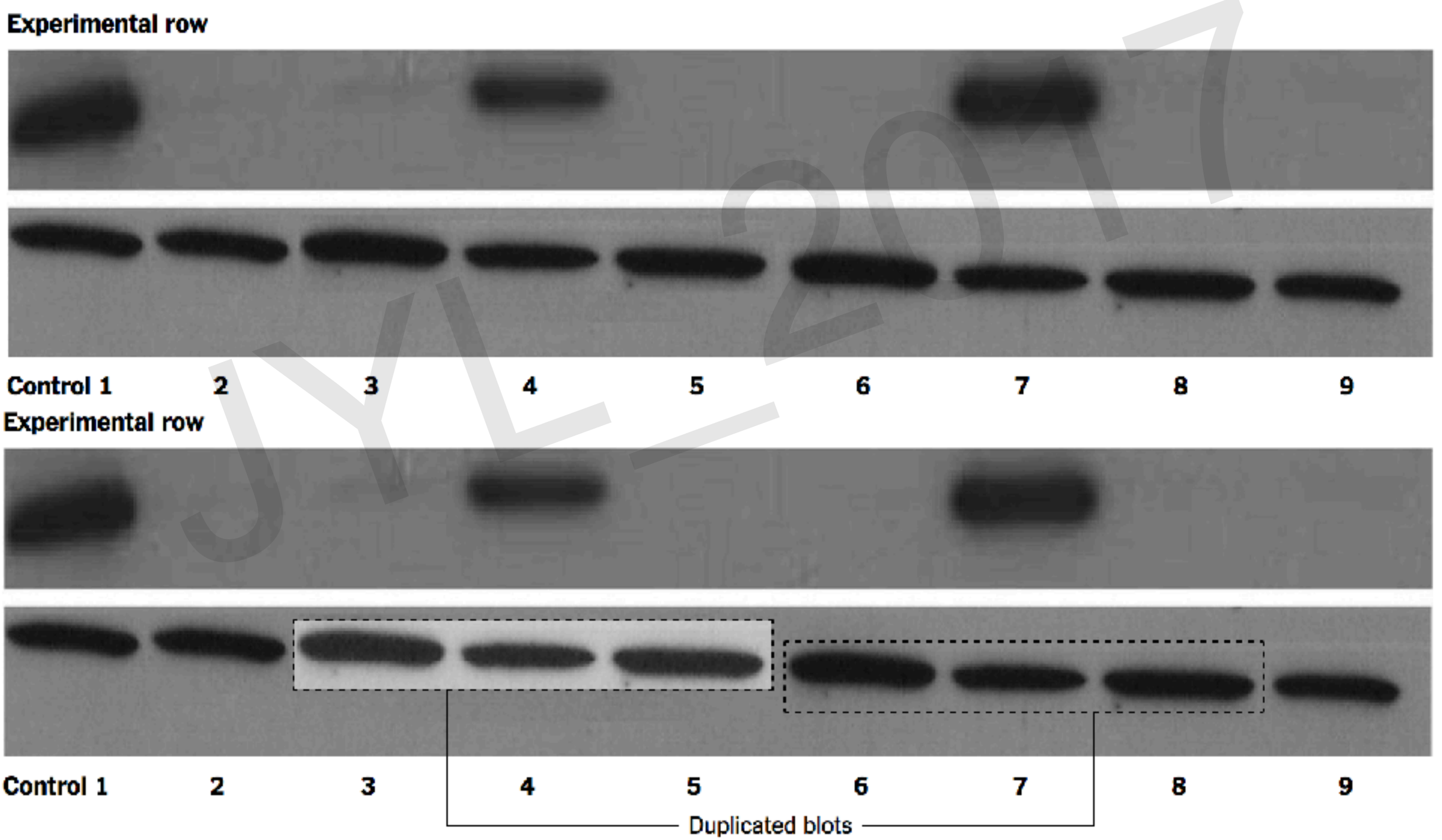
Years of Ethics Charges, but Star Cancer Researcher Gets a Pass

Dr. Carlo Croce was repeatedly cleared by Ohio State University, which reaped millions from his grants. Now, he faces new whistle-blower accusations.

By JAMES GLANZ and AGUSTIN ARMENDARIZ MARCH 8, 2017

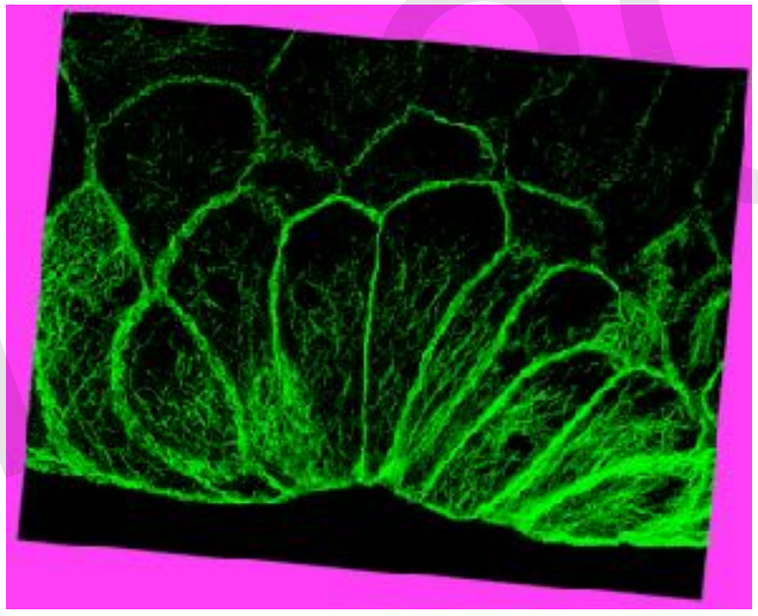
Dr. Carlo Croce has been charged with data falsification and other scientific misconduct.
Alberto Conti/Contrasto/Redux

Inappropriate Micrograph Manipulation: Copying & Pasting



What is <u>Appropriate</u> Micrograph Manipulation?

- Cropping or rotation (false background preferred)



- <u>Uniform</u> image enhancement (levels, brightness, contrast, color)

Data Presentation: Reproducibility

- Methods section - <u>complete</u> documentation (Follow the Golden Rule!)
- Sharing raw data & analysis methods with other scientists:
  - Online depositories/databases
  - Set up cloud server

Methods Section: Sample

From Sanchez et. al., *Science* 2011

Microscope Make & Model

Light Source

Filter Set

Light microscopy

All observations of active microtubule bundles were obtained using standard epi-fluorescence microscopy (Nikon Eclipse Ti microscope). Alexa Fluor 647-labeled microtubules were illuminated with a 120W metal halide light source (X-cite 120) and a Semrock Cy5-4040B-NTE filter set. Image sequences were acquired with an Andor Clara camera. To monitor large areas, the motorized stage of the microscope and the Ti's Perfect Focus hardware were used to acquire adjacent fields of view, which were stitched together using Matlab.

Detector

Relevant Hardware

Image Processing

Missing info:

- Objective (mag, NA, immersion, correction)
- Acquisition software

Evaluating Images: The Good

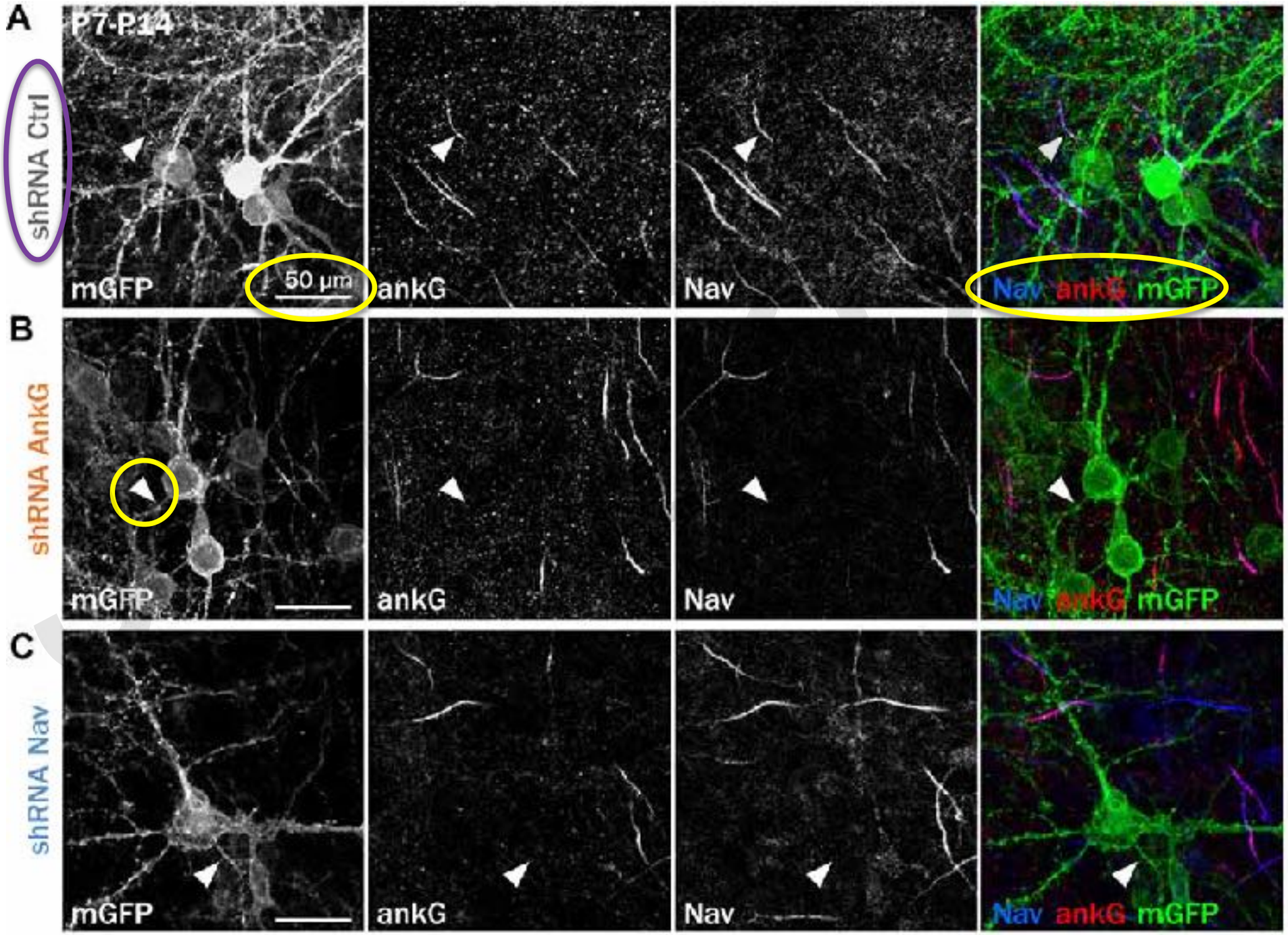
Separate Channels + Merged

Control Panel

Color-Coded Labels

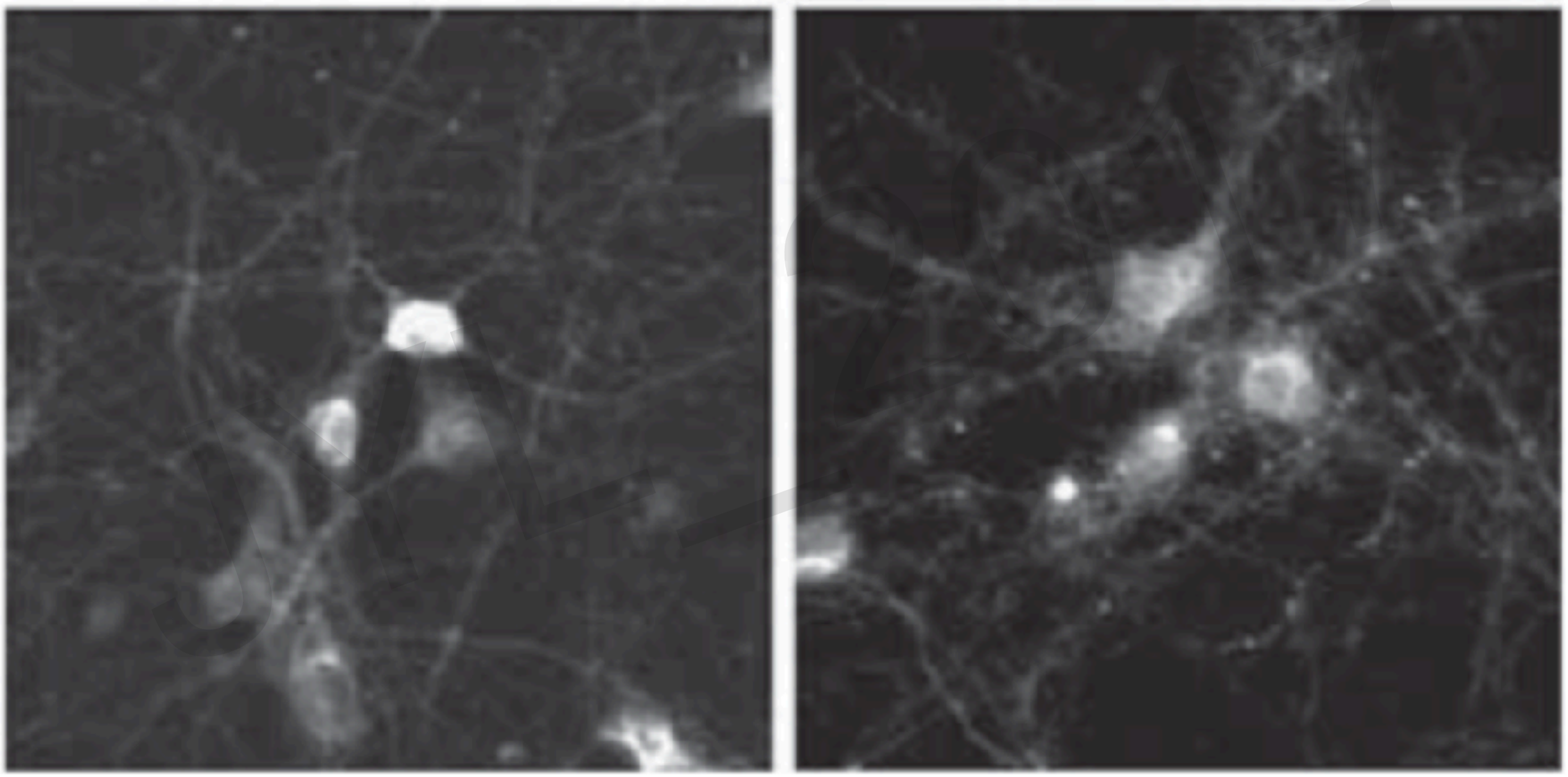
Scale Bars

Arrow-heads



Leterrier et. al., *Front. Cell. Neurosci.*, 2017

Evaluating Images:
The Bad



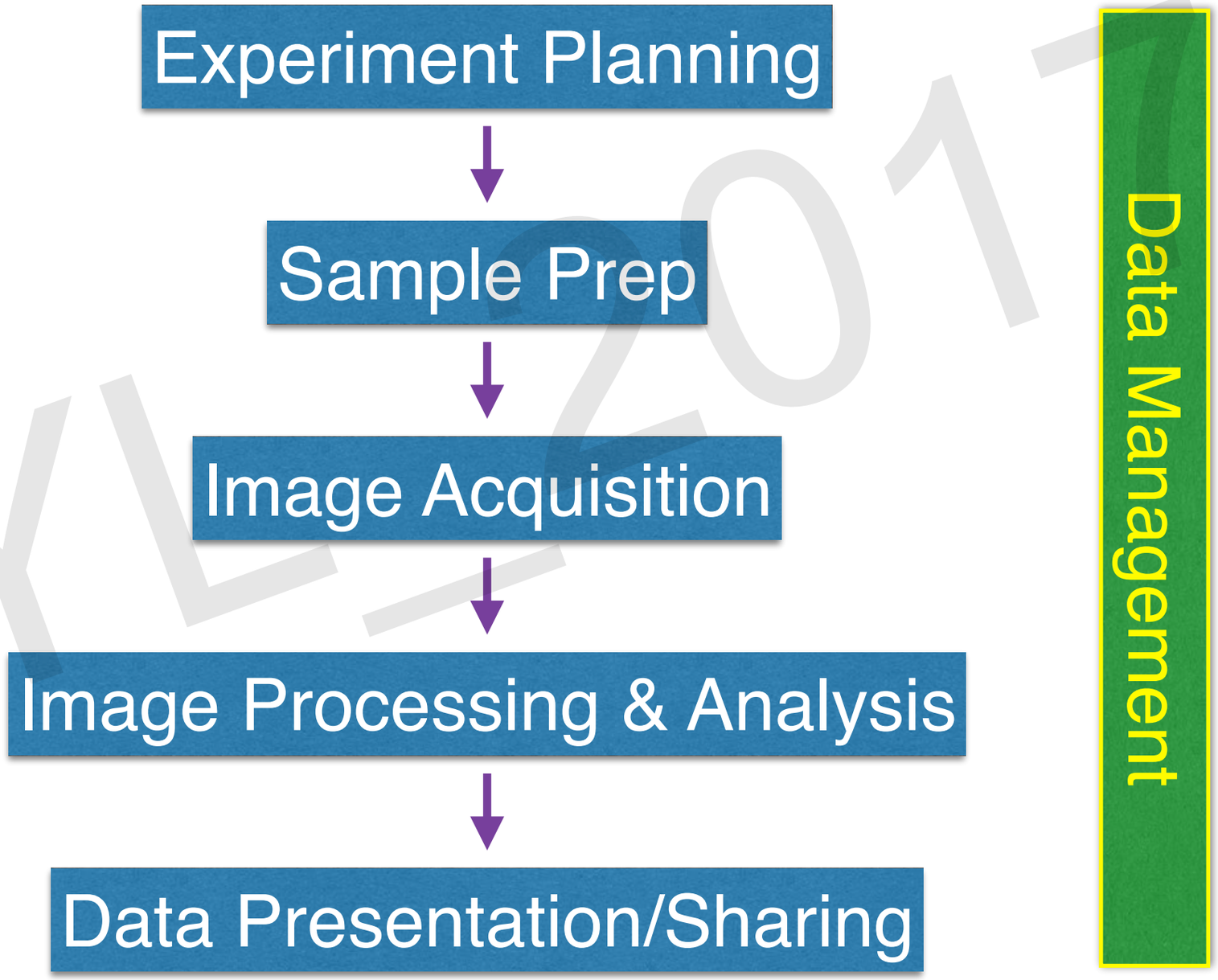
Synaptophysin

PSD95

Evaluating Images: What to Look for

- If reviewing, look for image saturation, appropriate image processing & analysis, statistical methods & power
- Be wary of:
  - Figures & text not matching up
  - Small n
  - “Representative image” without corroboration (stats)
  - Lack of context (too zoomed in or cropped)

Typical Imaging Experiment Workflow



Data Management: Reproducibility

- Version control
- Digital notebooks
- Software: OMERO, ImageJ Bioformats, custom software
- Server/Cloud backup
- Raw files sometimes requested by NIH and by journals
- File naming

Data Management: File Naming Tips

- Names should be consistent, orderly, & informative
  - Good: 2017-03-21_slide1a_image1
  - Bad: slide1a march212017_weird
- Organize images in folders & subfolders in a consistent manner
- Don’t use names to describe quality of image; use readme/text files instead
- Goal is to make things easier to find data (especially in the future)

www.jmu.edu/microscopy/resources/microscopyDataManagement2016-05-19.pdf

Data Management: “readme” files

- Good way to keep track of how images were acquired, processed, analyzed, and organized
- Text files are ideal because they can be read by any computer without proprietary software
- Items to include:
  - Your name!
  - The microscope system used to acquire the images
  - Explanation of the file naming and organization
  - Where to find the experiment in your lab notebook
  - Short description of the experiment
  - Notes about the images
  - Metadata not stored in your image files (e.g. microscope/camera/objective used)

www.jmu.edu/microscopy/resources/microscopyDataManagement2016-05-19.pdf

Data Management:
Always Back Up Your Data!

Theft, fire, & flooding can instantly erase years of work!

“What I wish someone would’ve told me as a first year graduate student”

- Understand that imaging is more than taking a pretty picture
- How you handle the original file can have serious consequences on quantitative analysis
- Having a good data management protocol from the start will make life so much easier
- Never delete original data. Back it up at least two different ways.

Questions?