DRAFT

Image Analysis & Processing of Digital Light Microscopy

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August 2013

Important take-home messages

- Raw Data Sacrosanct
- Save uncompressed
- Keep a back-up in a separate location
- Only change in ways valid for quantification
- GIGO: Measurements not useful without well planned experiments with proper controls

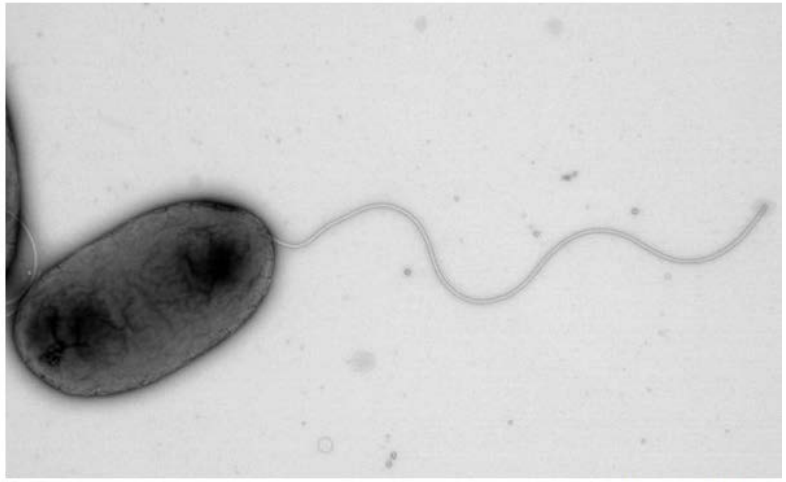
There are two types of operations we can do on images to change the way they appear:

1. Things that do not change the raw data.
2. Things that do change the raw data.

www.nytimes.com/2013/08/15/science/watching-bacteria-evolve-with-predictable-results.html?_r=0

MATTER

Watching Bacteria Evolve, With Predictable Results

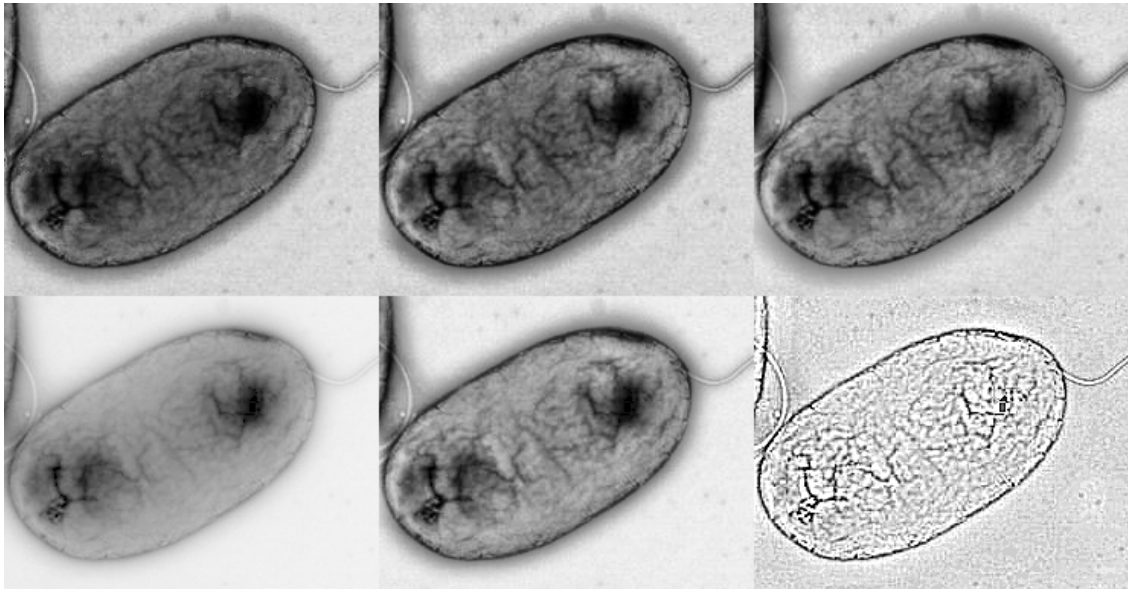


Alice Liang/New York University

Pseudomonas aeruginosa, a common bacterium, normally has a single tail that it uses to move about.

By CARL ZIMMER

Published: August 15, 2013



Photoshop is good at keeping the underlying raw data under adjustment layers. This is especially good for making figures where different experimental conditions may all have the same adjustments applied and changed for different venues (e.g. printed journal, video projection, or web browser).

You can get PhotoShop from IT and use on campus where you have an Internet connection to the keyserver.

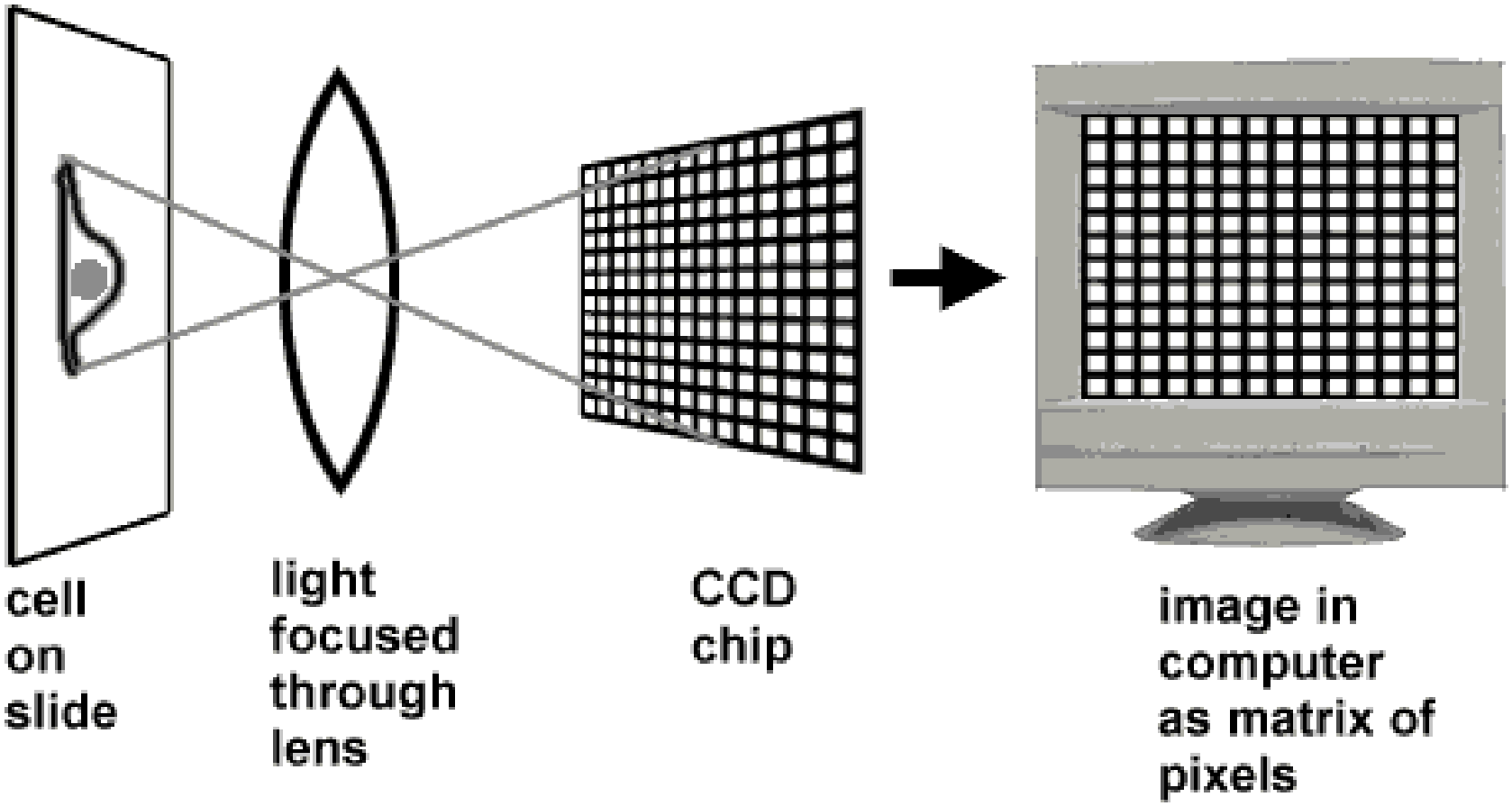
This class uses ImageJ specifically for quantification as well as for examples of mathematical operations of images.

Other similar software:

- Image Pro
- Slidebook
- Nikon Elements
- Metamorph
- Volocity
- Imarus
- MatLab
- IDL and lots more…


What is an image?

What is a digital image?



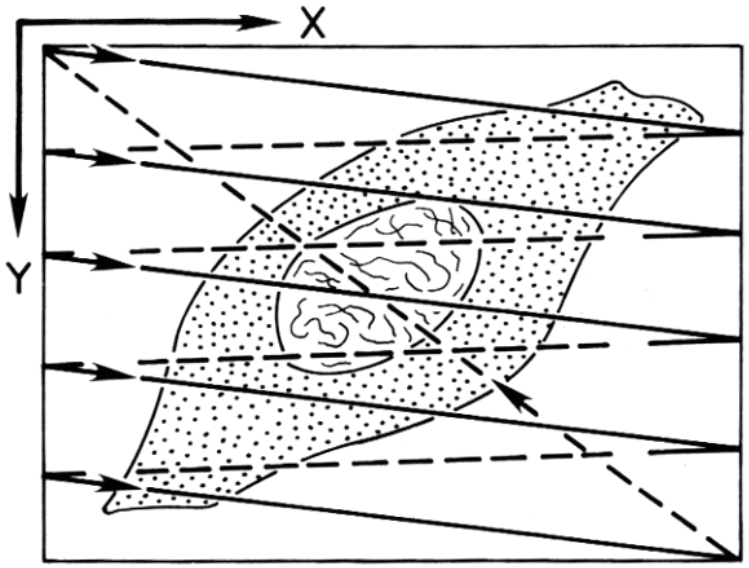
http://www.einstein.yu.edu/aif/instructions/ccd1/why_ccd.htm

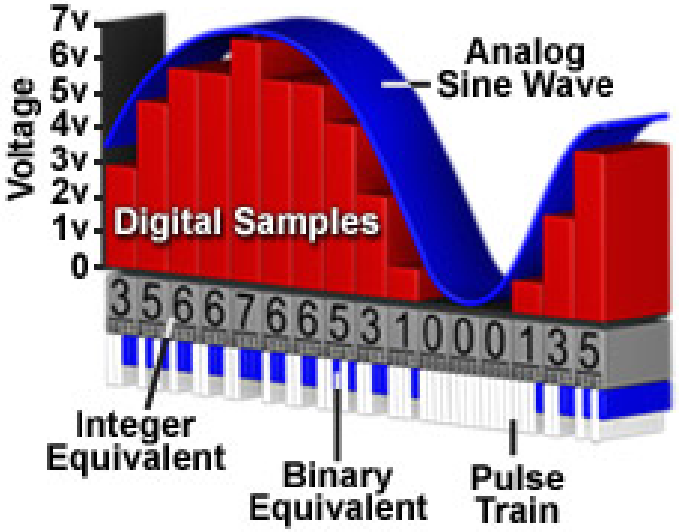
How do we get images?

Most commonly CCD (or CMOS) camera

Raster scanning device, i.e. laser scanning confocal

(Film & video are obsolete)


X
Y


Analog and Digital Signals
7v
6v
5v
4v
3v
2v
1v
0
Voltage
Analog
Sine Wave
Digital Samples
3 5 6 6 7 6 6 5 3 1 0 0 0 1 3 5
Integer
Equivalent
Binary
Equivalent
Pulse
Train

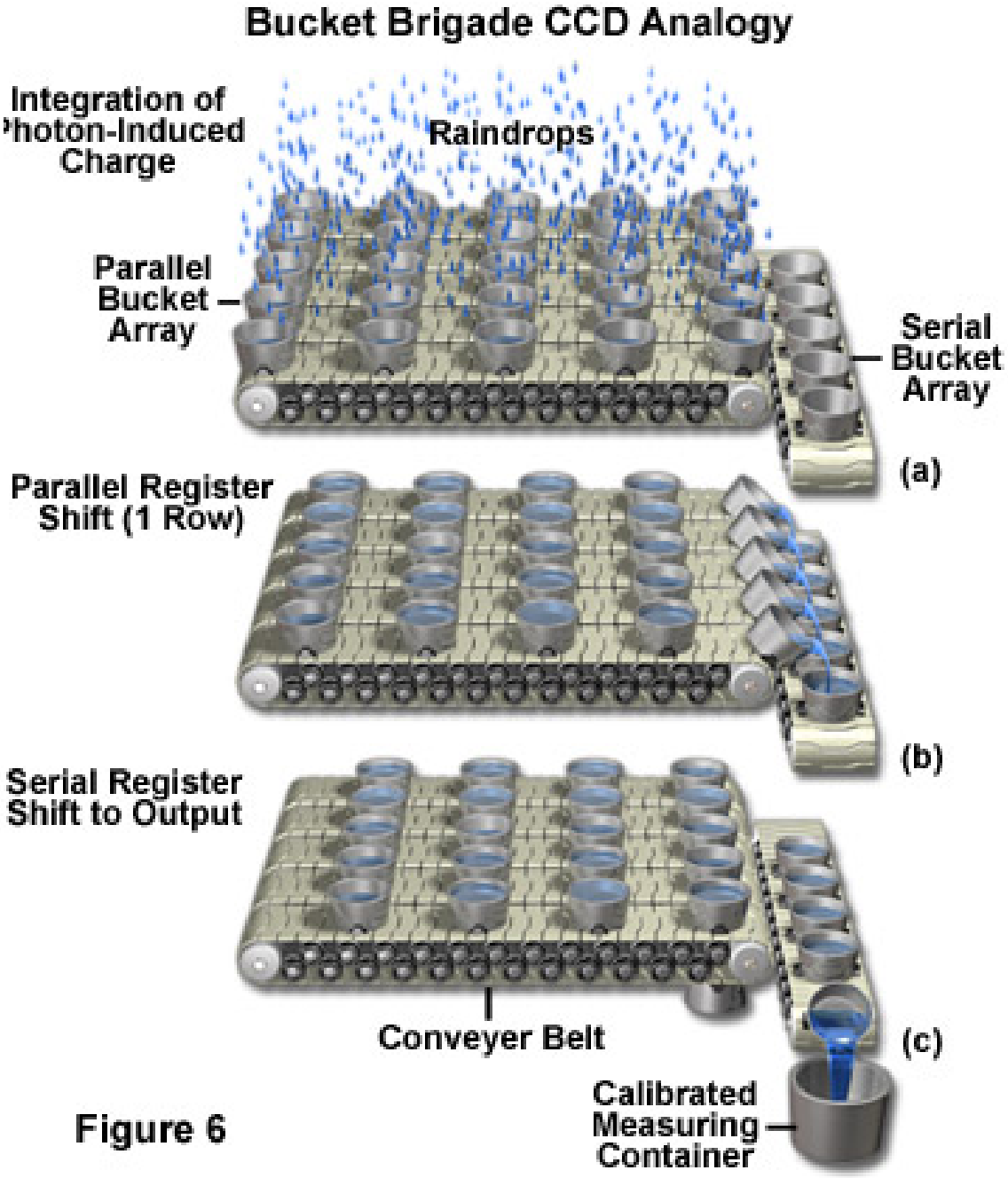
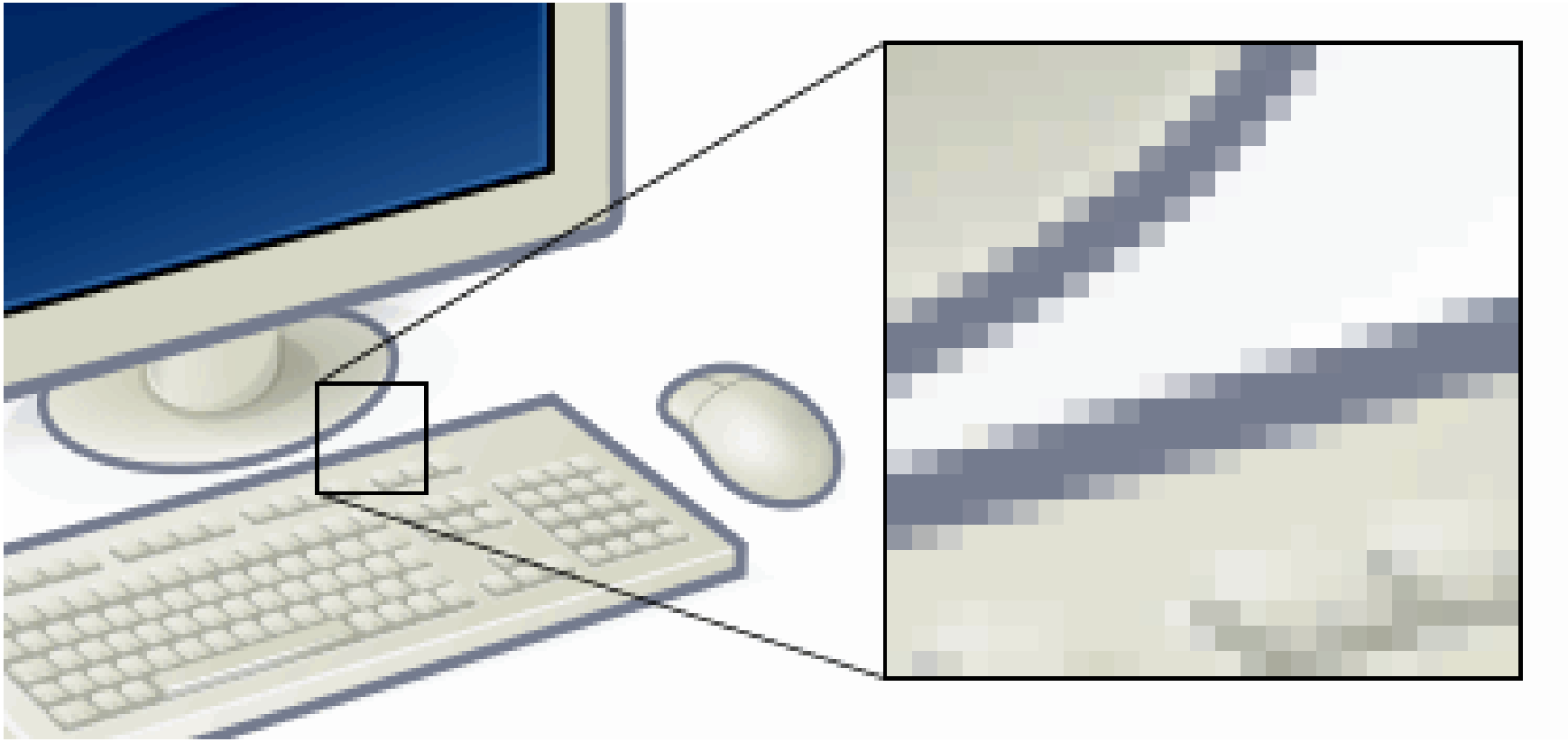

Bucket Brigade CCD Analogy
Integration of
Photon-Induced
Charge
Raindrops
Parallel
Bucket
Array
Serial
Bucket
Array
(a)
Parallel Register
Shift (1 Row)
(b)
Serial Register
Shift to Output
Conveyer Belt
(c)
Calibrated
Measuring
Container

Figure 6

Pixel: “Picture Element”

Smallest unit of a 2D computer image



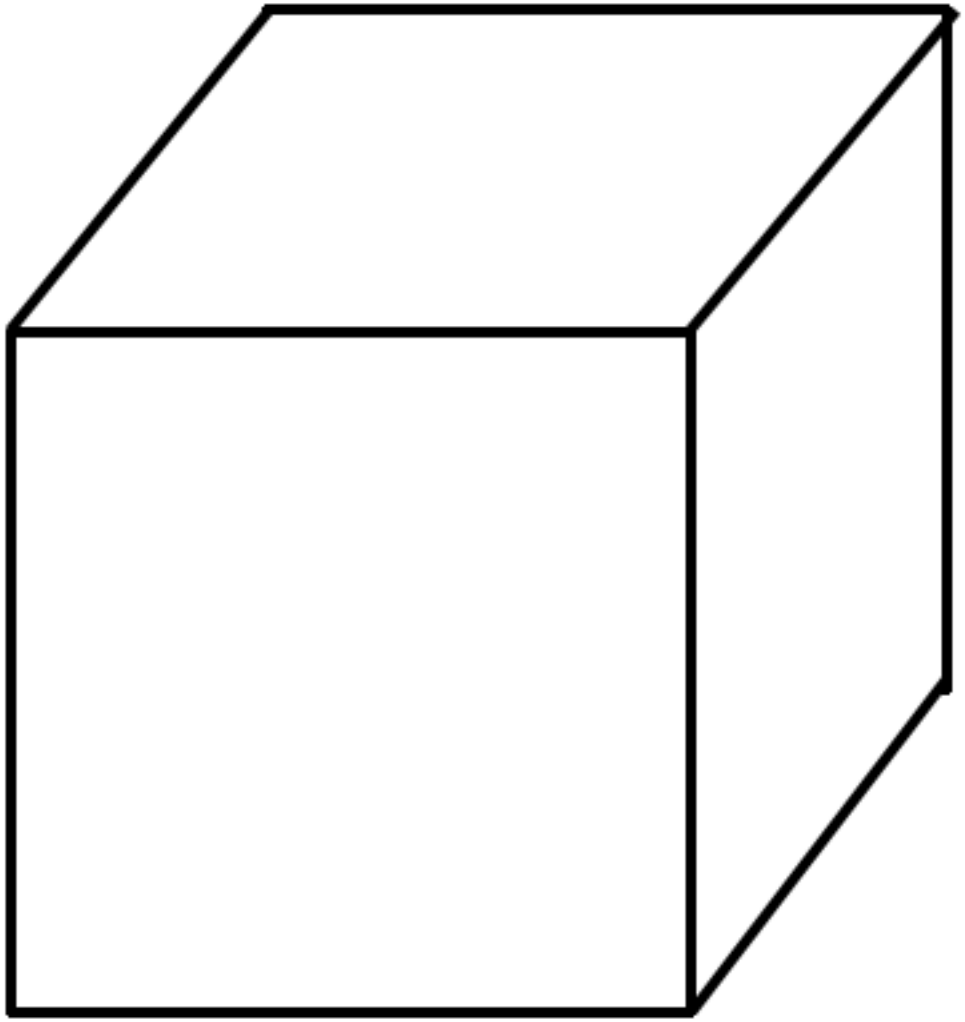
http://en.wikipedia.org/wiki/File:Pixel-example.png

Each pixel is a discrete number which (usually) is intensity or luminosity.

Examples what the numbers represent:

- amount of a protein at a location
- activity of a protein
- bound or free Ca^{++}
- temperature
- population (# of objects at a location)
- decay time of a fluorescent molecule

Voxel



Each pixel or voxel is a discrete number which (usually) is intensity or luminosity.

Examples what the numbers represent:

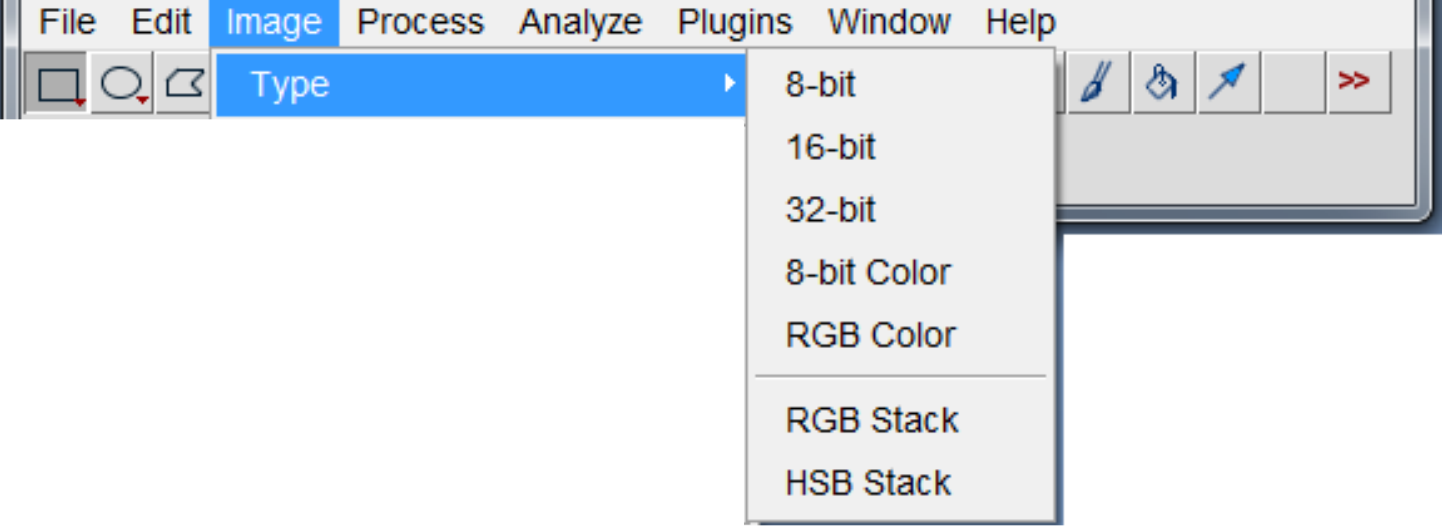
- activity of a protein
- bound or free Ca^{++}
- temperature
- population (# of objects at a location)

Each pixel is a discrete number which (usually) is intensity or luminosity.

Typical integers for digital microscopy:

- 0 – 255
- 0 – 4095
- 0 – 16383
- 0 – 65535

From black to white or from no signal to saturation.



Each pixel is a discrete number which (usually) is intensity or luminosity.

Typical integers for digital microscopy:

- 8-bit 0 – 255
- 12-bit 0 – 4095
- 14-bit 0 – 16383
- 16-bit 0 – 65535

From black to white or from no signal to saturation.

We will talk about 32 bit floating point later…

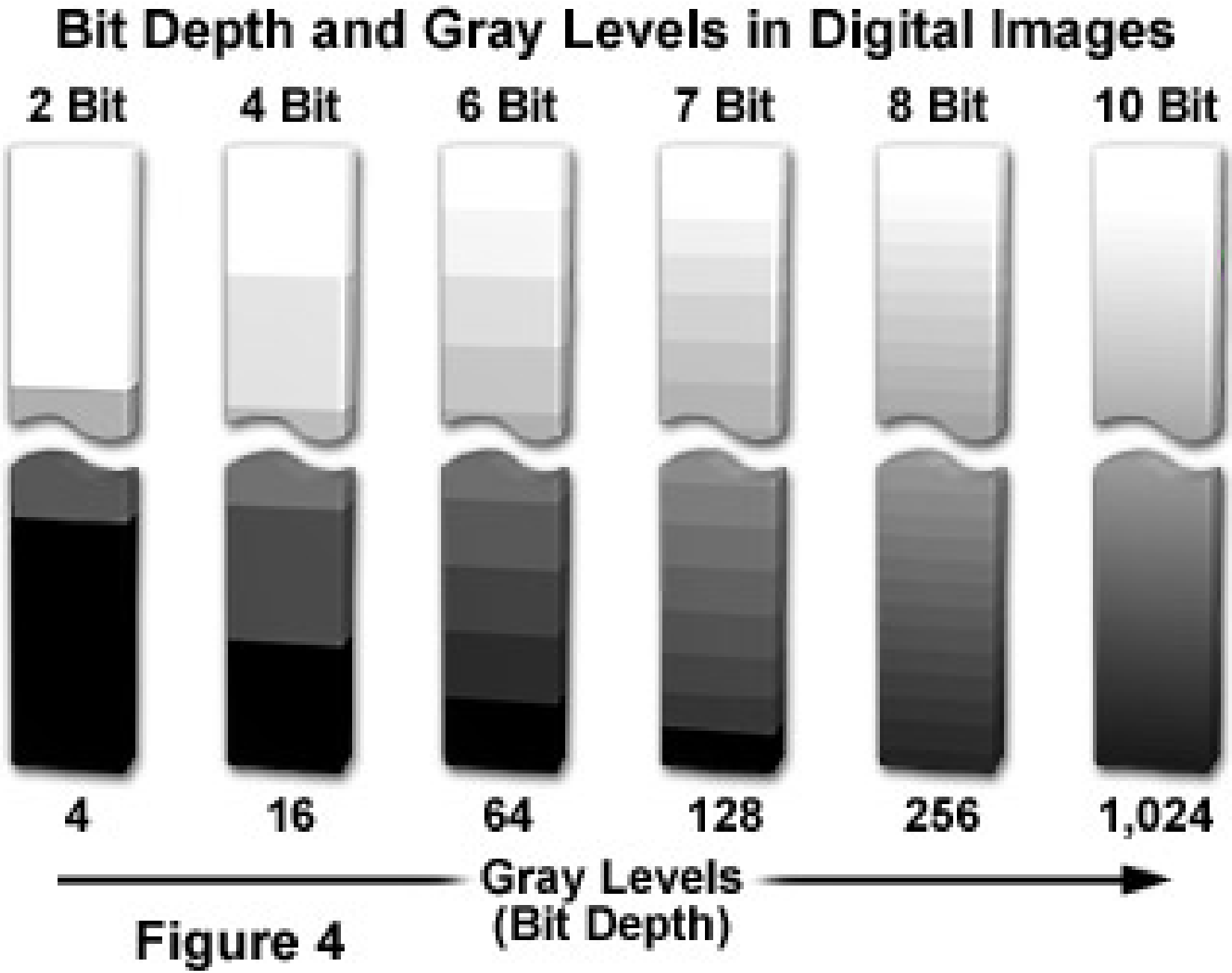
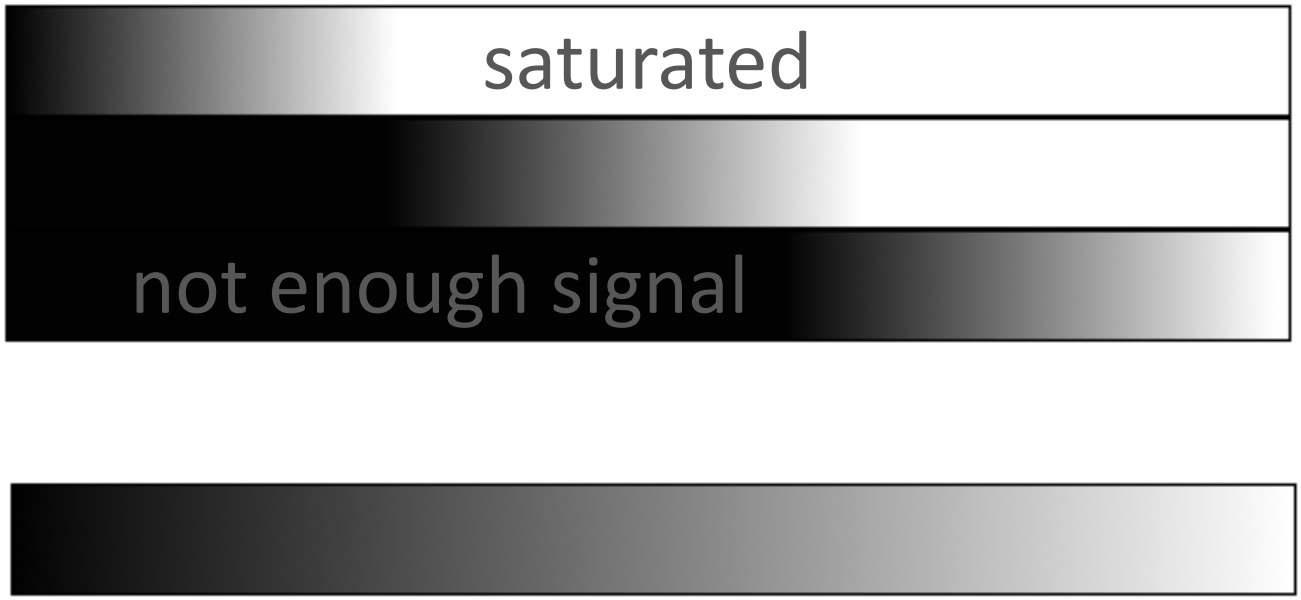

Bit Depth and Gray Levels in Digital Images
2 Bit
4 Bit
6 Bit
7 Bit
8 Bit
10 Bit
4
16
64
128
256
1,024
Gray Levels
(Bit Depth)

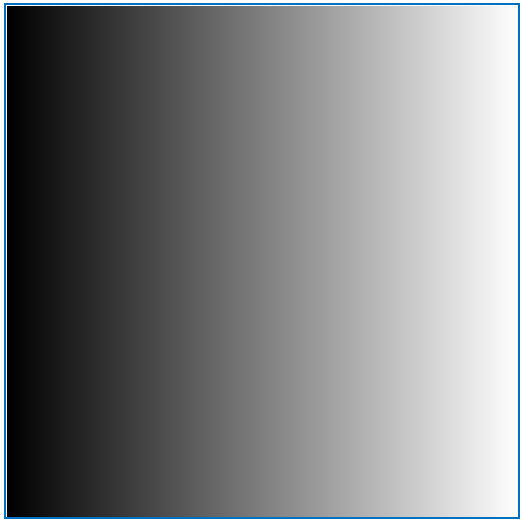
Figure 4

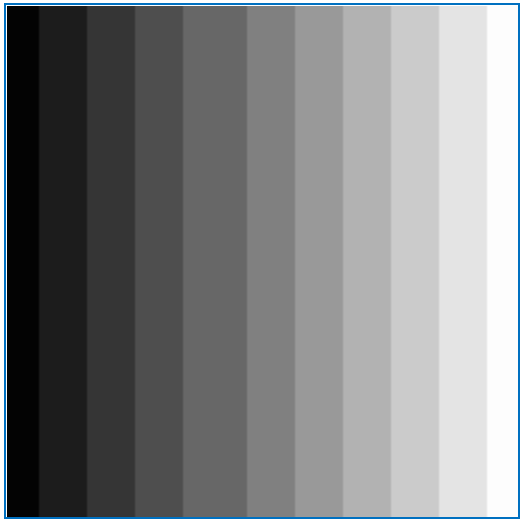
If a scene varies greatly from dark to light, the full dynamic range may need more #s.



Don’t Compress Raw Data!

This can turn into JPEG → this.





To paraphrase Monty Python,

Every pixel is sacred.

Compression Loses Spatial Information

(exercise to see this and learn stacks and calculator)

- Open all the JPG images \exercises and demos\JPG compression\
- Image > Stacks > Images to Stack…
- Play through stack, note name in upper left
- Open original TIFF image
- Process > Image Calculator… to see Difference

  Each pixel (or voxel) has a unique location in X, Y, C, Z, T and may be operated on by a pixel at the same location in another image.

Common Color Spaces

- RGB (Red Green Blue)
- HSV (Hue Saturation Value)
- Overlayed channels (grayscale images with LUTs (Look Up Tables) assigned
- CMYK (Cyan Magenta Yellow Black)

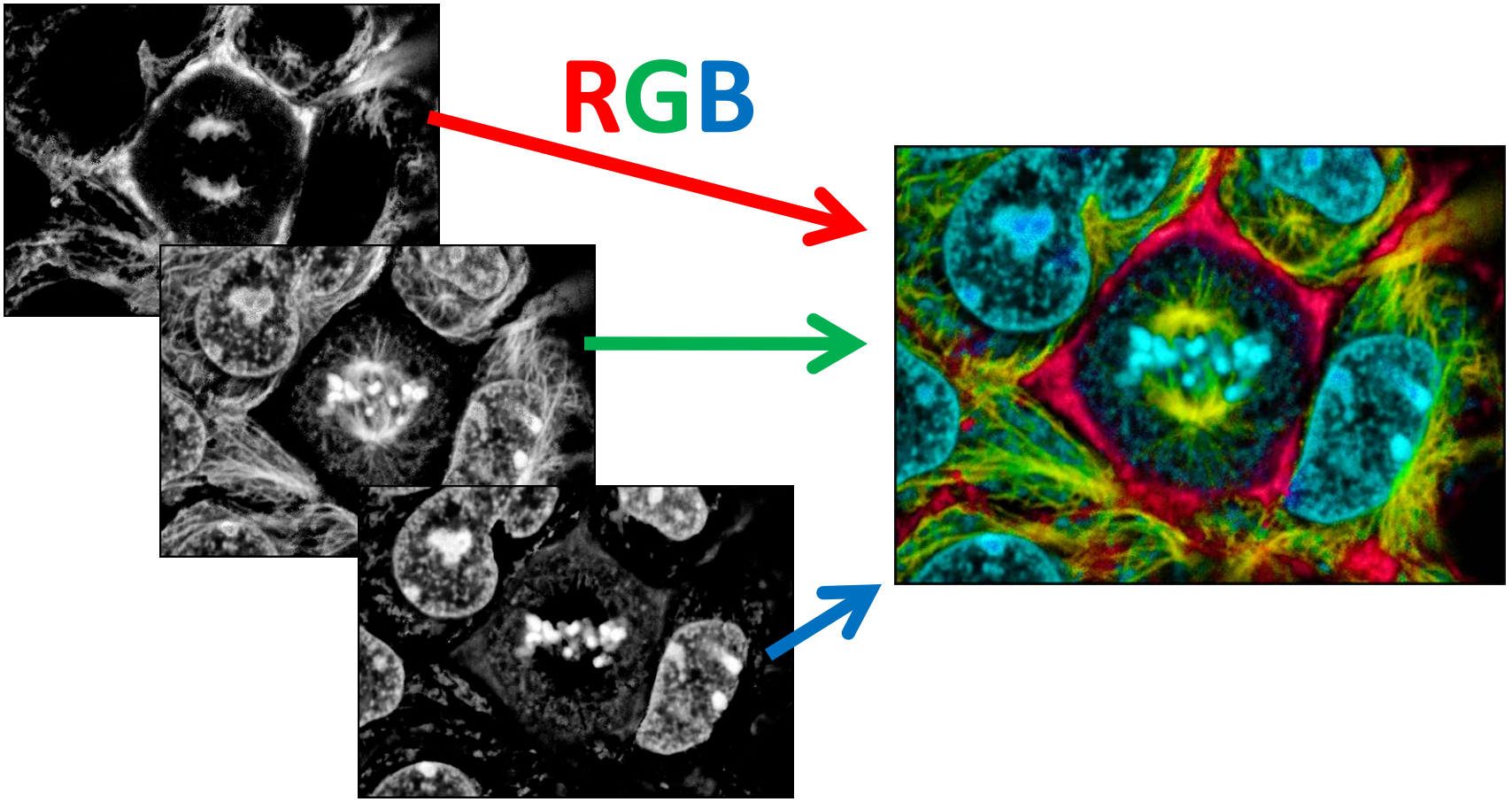
What a color picture is, at least with your camera.

RED

GREEN

BLUE

Color images are (usually) channel mixtures of intensities

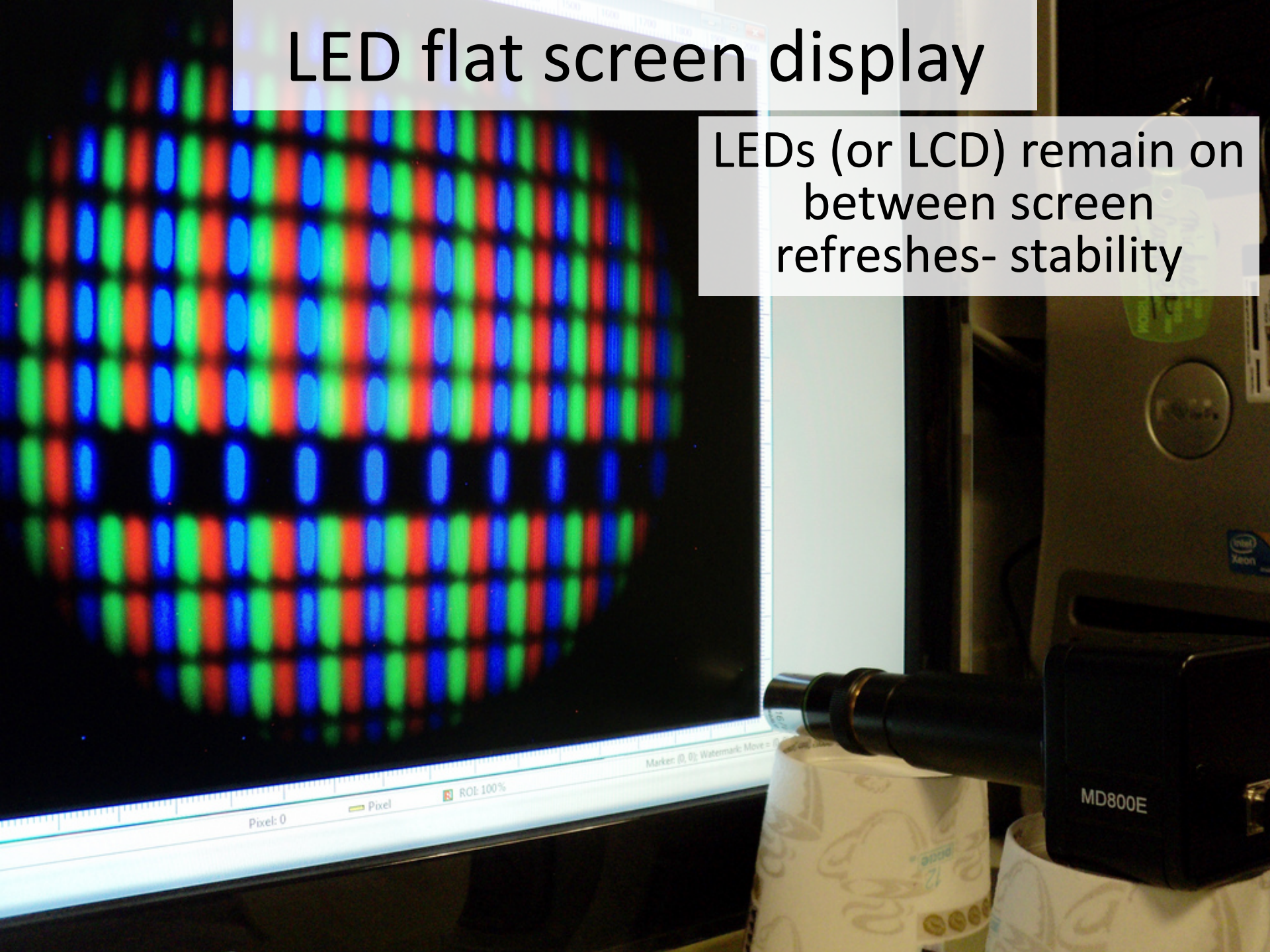


Color TV with high res comb

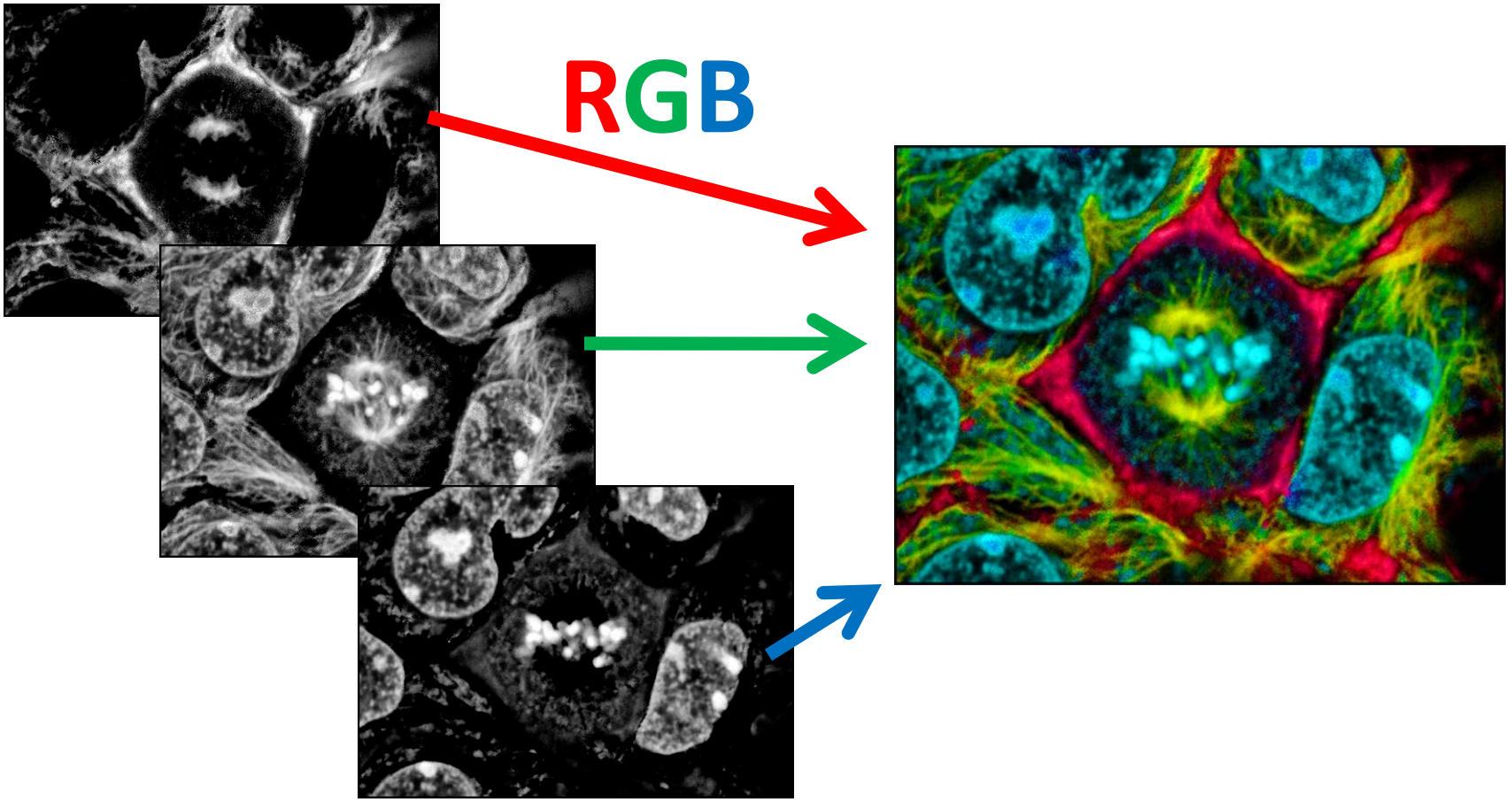
Scanning beams cause flicker.

LED flat screen display

LEDs (or LCD) remain on between screen refreshes- stability

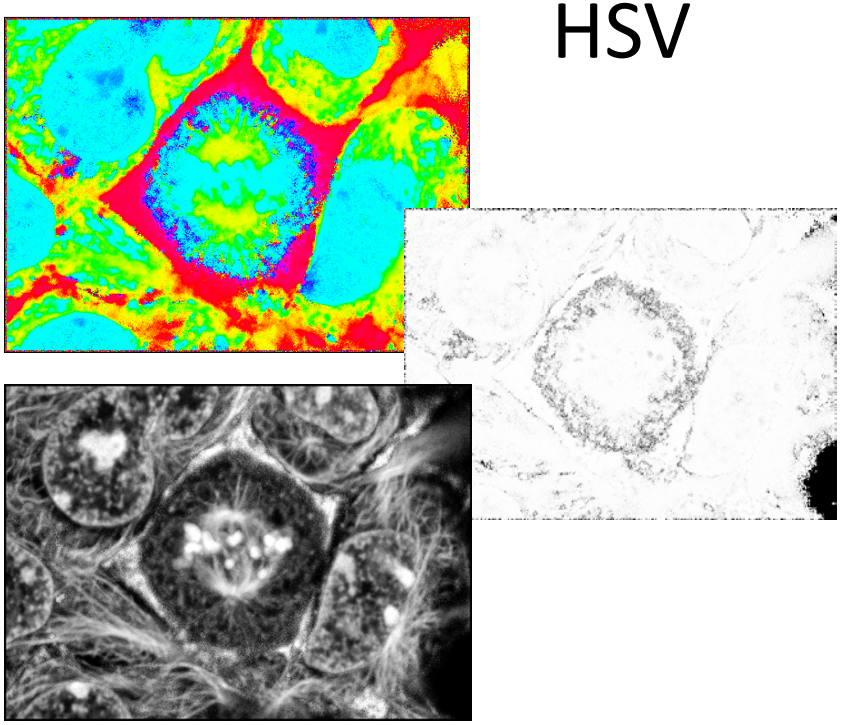


Color images are (usually) channel mixtures of intensities

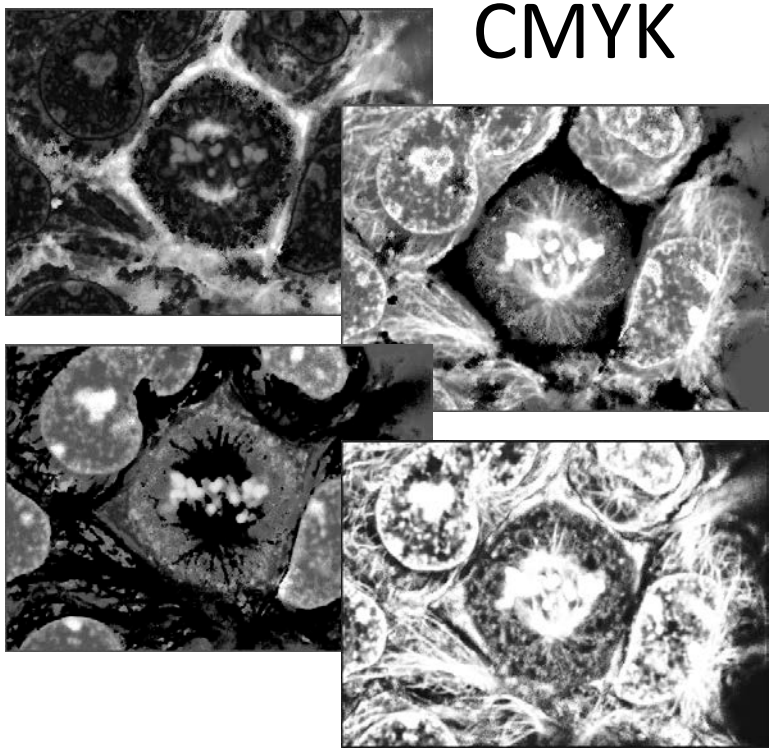


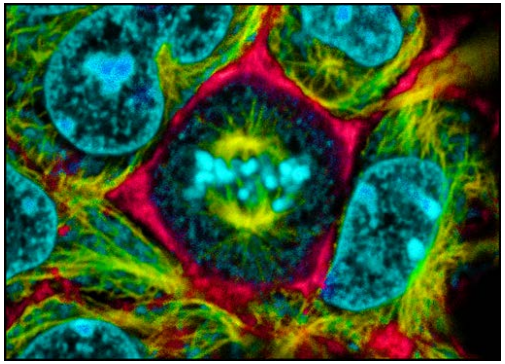
Less Common

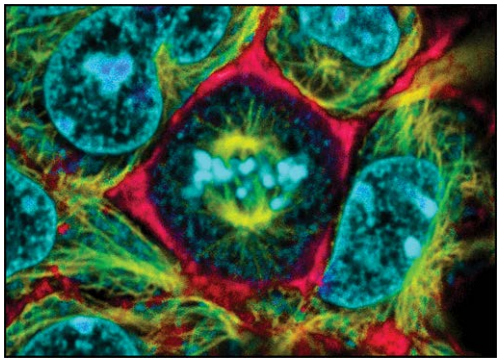
HSV



CMYK







Channels

- LUTs (Look Up Tables)

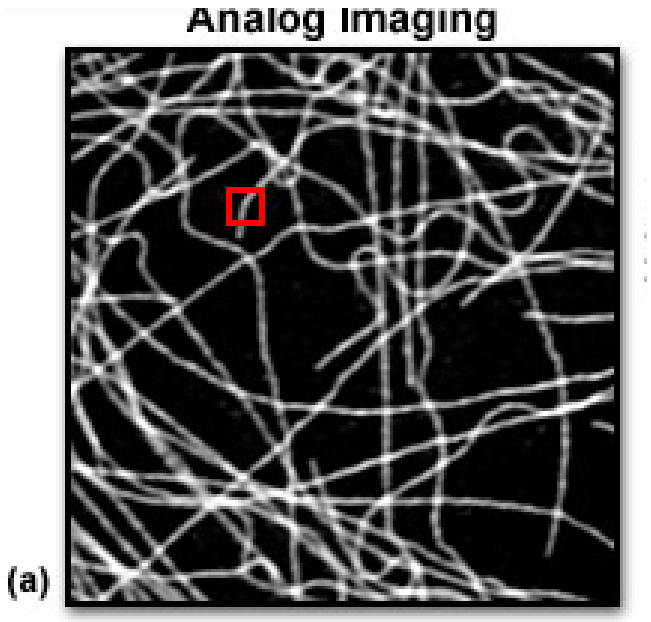
Exercise with color spaces

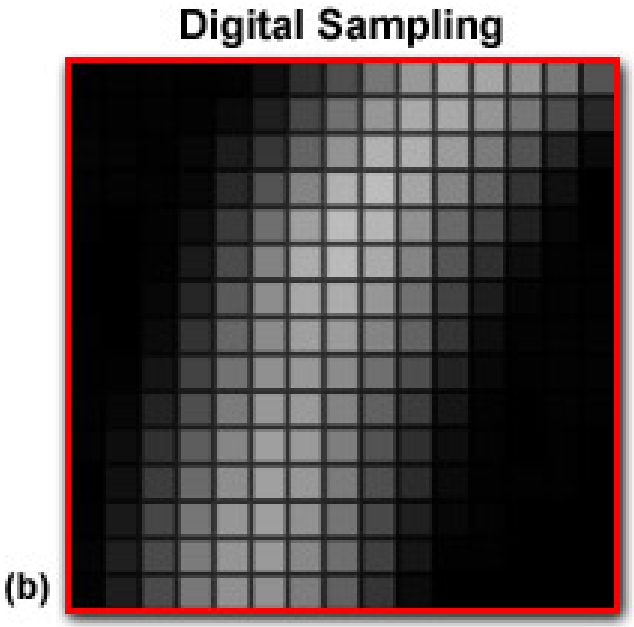
- Import confocal images as multichannel (hyperstack)
- Change colors per channel
- Manipulate and measure in channels
- Convert to RGB
- Make an instant multi-panel figure using Montage command
- Add scale bar – compare in multichannel and RGB modes

Images are 2D matrices of numbers

The matrix itself is just numbers. To know more about these numbers, there must be additional tags which are usually stored in a header.

Images are 2D matrices of numbers





Pixel Quantization

(c)

2	3	3	2	3	16	47	78	118	152	170	165	149	119	83
3	2	3	3	11	31	72	113	150	170	168	148	118	78	41
5	3	2	7	28	51	100	147	177	175	155	123	82	38	10
3	3	3	8	41	83	132	177	188	165	132	98	52	15	0
2	0	3	16	59	111	160	188	181	145	105	72	31	7	0
0	0	3	26	75	131	172	186	170	132	85	47	13	3	2
0	0	7	38	90	141	168	172	150	113	67	28	5	3	2
2	0	10	51	103	139	159	155	132	98	52	13	3	3	3
2	2	20	67	114	144	152	139	111	78	34	8	2	3	3
3	7	34	78	123	152	154	131	96	60	21	3	0	3	2
3	13	49	93	134	159	154	126	83	47	13	3	0	2	0
0	16	60	106	142	160	152	123	78	43	10	2	2	2	0
2	25	70	118	150	159	145	116	72	31	7	3	0	0	0
5	31	74	123	149	154	136	110	64	21	2	2	0	0	0
3	29	74	119	144	145	124	96	52	11	0	0	0	0	0

To know more about these numbers, there must be additional tags which are usually stored in a header.

There may also be addl tags between

123	11	13	8	1	109	51	218	221	147	195	13	45	153	253	88			
					244	204	106	0	102	177	86	135	152	244	215			
					105	98	247	213	79	18	241	208	58	136	91			
					10	84	196	134	194	76	211	191	9	110	79			
					83	164	147	114	213	92	184	28	38	213	209			
					217	88	250	128	118	235	54	164	137	167	26			
					103	38	22	155	146	31	78	220	136	163	174			
					107	130	20	101	38	190	81	22	169	252	51			
					107	176	252	34	93	49	208	189	80	4	157			
					26	87	22	78	43	36	214	249	107	250	17			
					164	127	25	116	60	155	46	134	125	128	30			
					213	39	180	56	121	19	6	60	132	77	85	5	7	11

Exercise: Look at metadata in our confocal images.

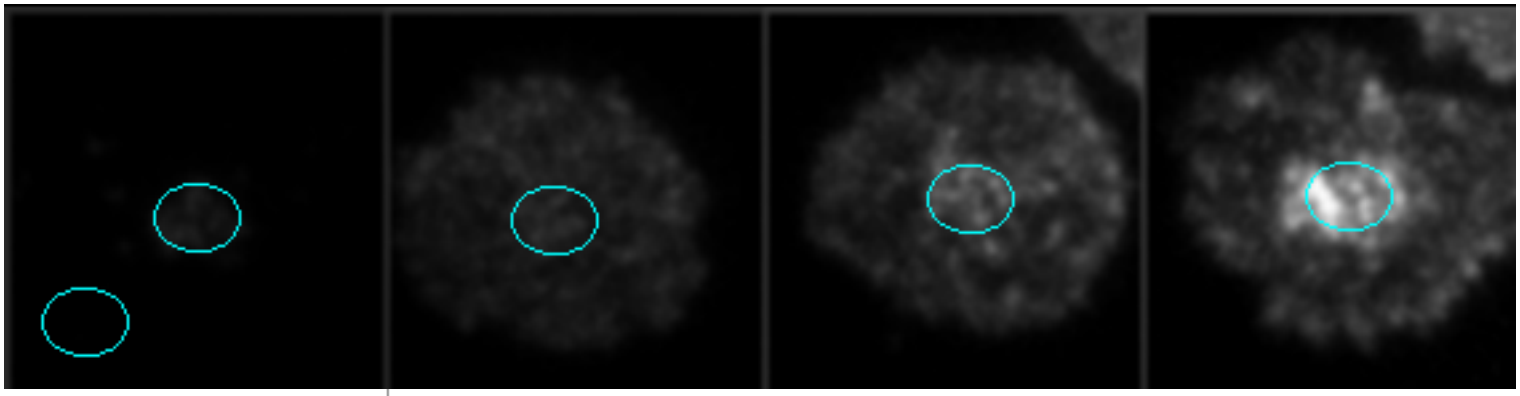
- Using LOCI plugins, import native format files with show metadata and XLM boxes clicked.
- Image > Properties…
- Image > Show Info…

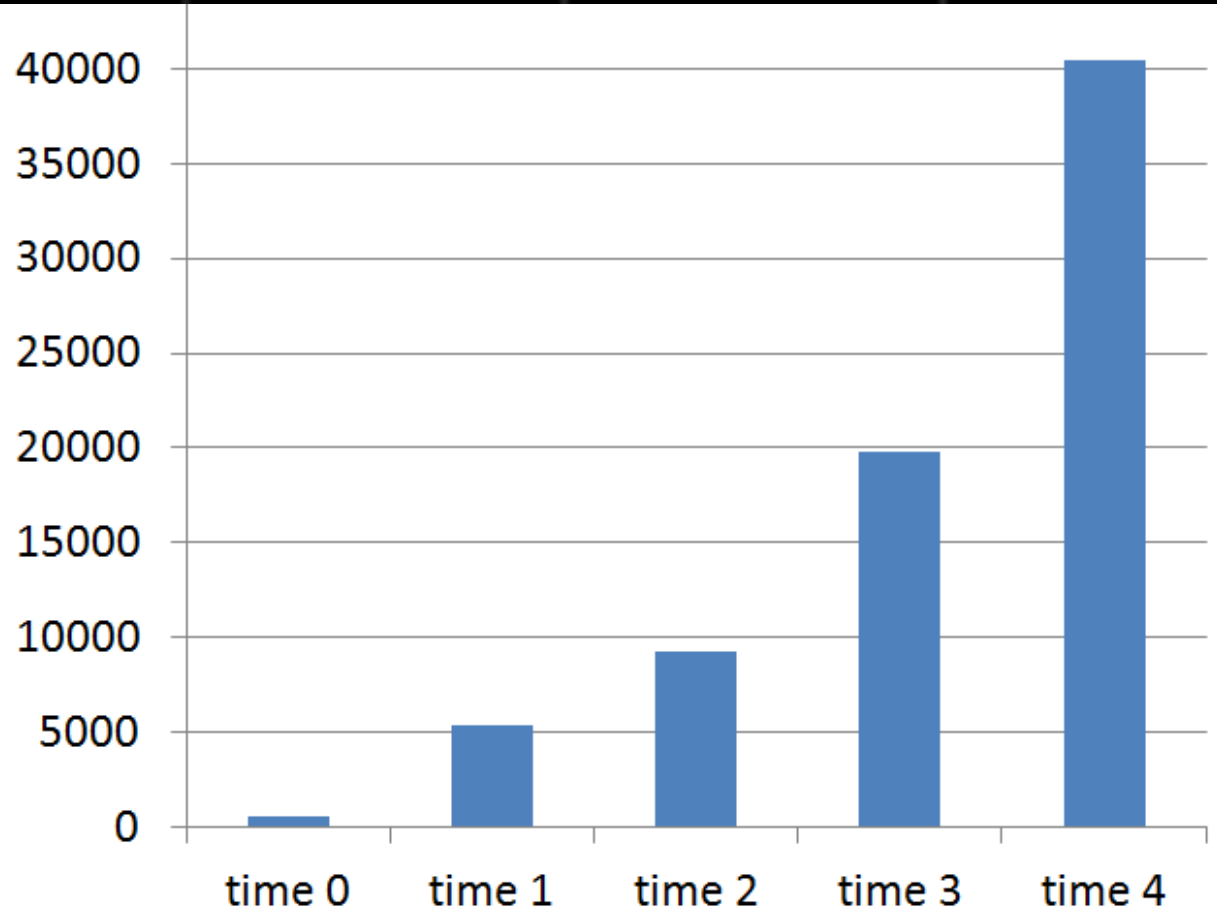
Images are 2D matrices of numbers

We may quantify these numbers.

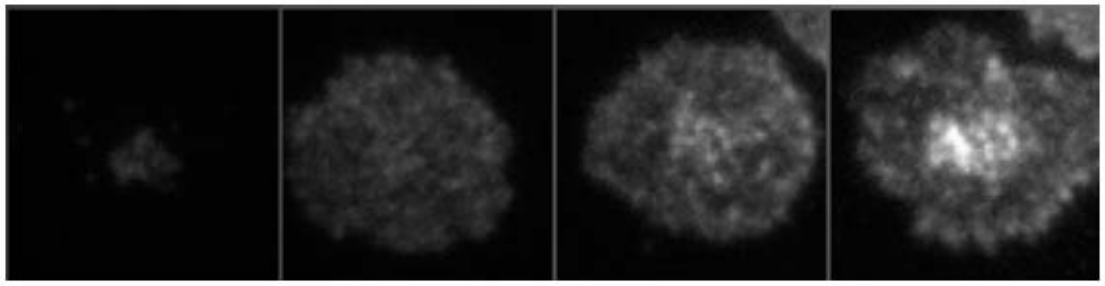
Manipulate the numbers.

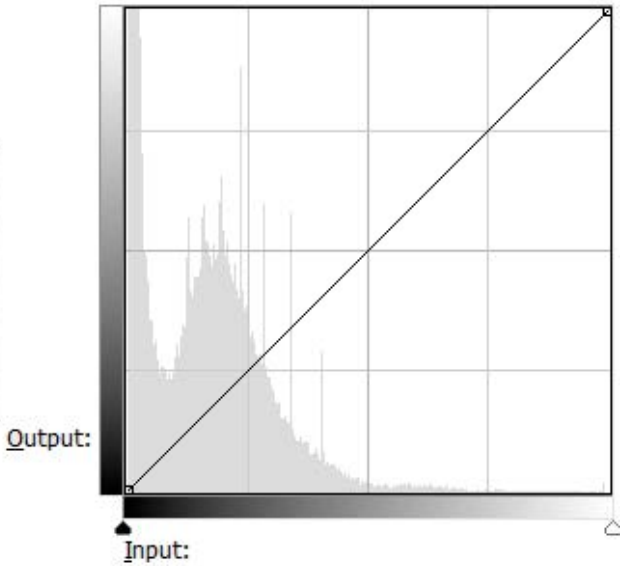
Operations that change the way images look *without* changing the raw data and operations that change the raw data.

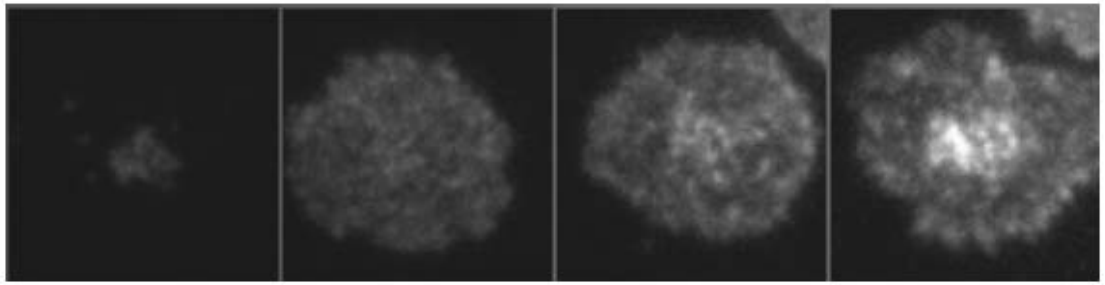


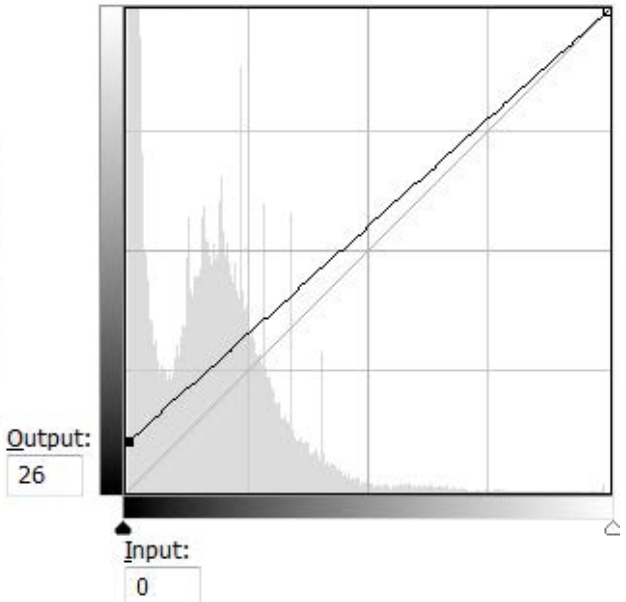

40000
35000
30000
25000
20000
15000
10000
5000
0
time 0
time 1
time 2
time 3
time 4

We have difficulty seeing subtleties in the darks of images







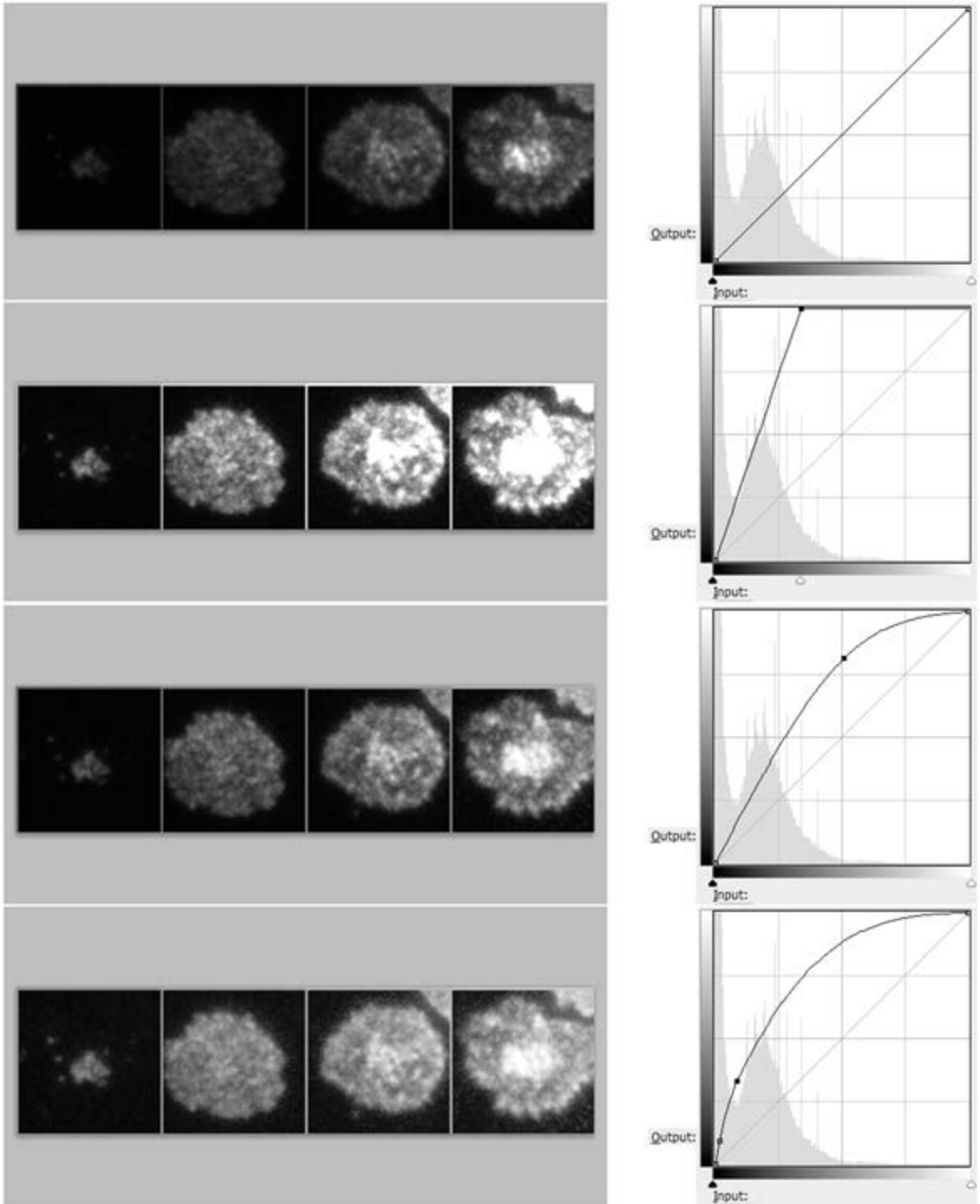


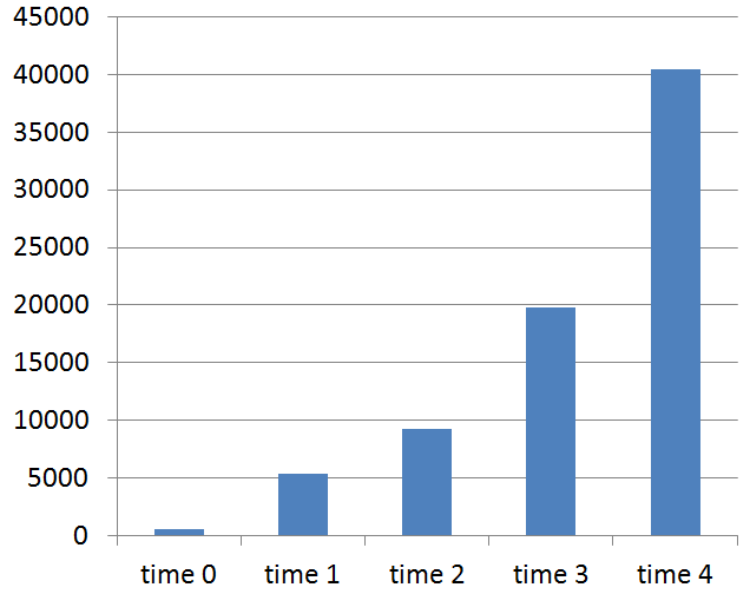
What is gamma?

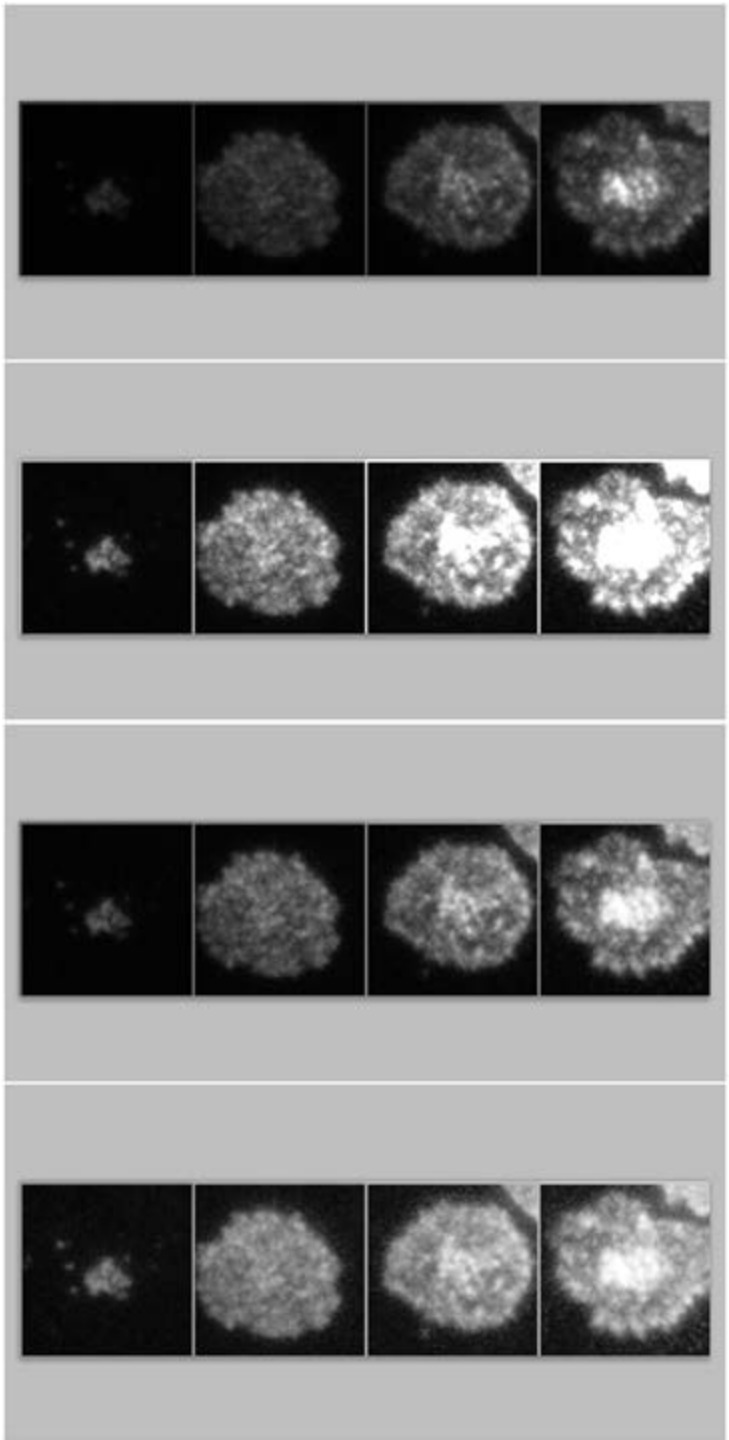
It is a non-linear contrast adjustment.

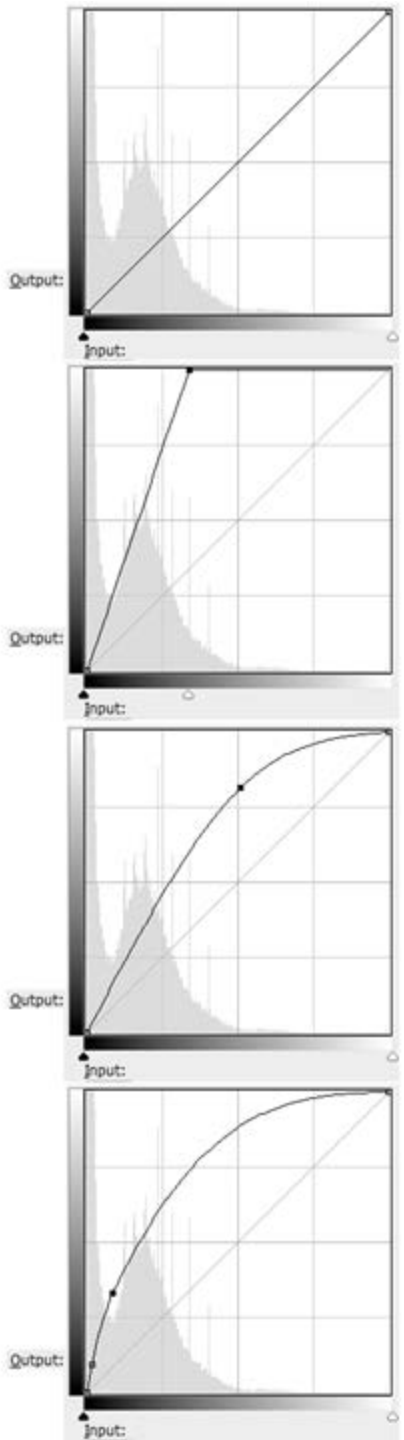
We use it

- when the dynamic range of data is too large to display linearly;
- to selectively boost contrast in a narrow range;
- because we (our eyes and brains) do not discriminate darks and lights linearly.

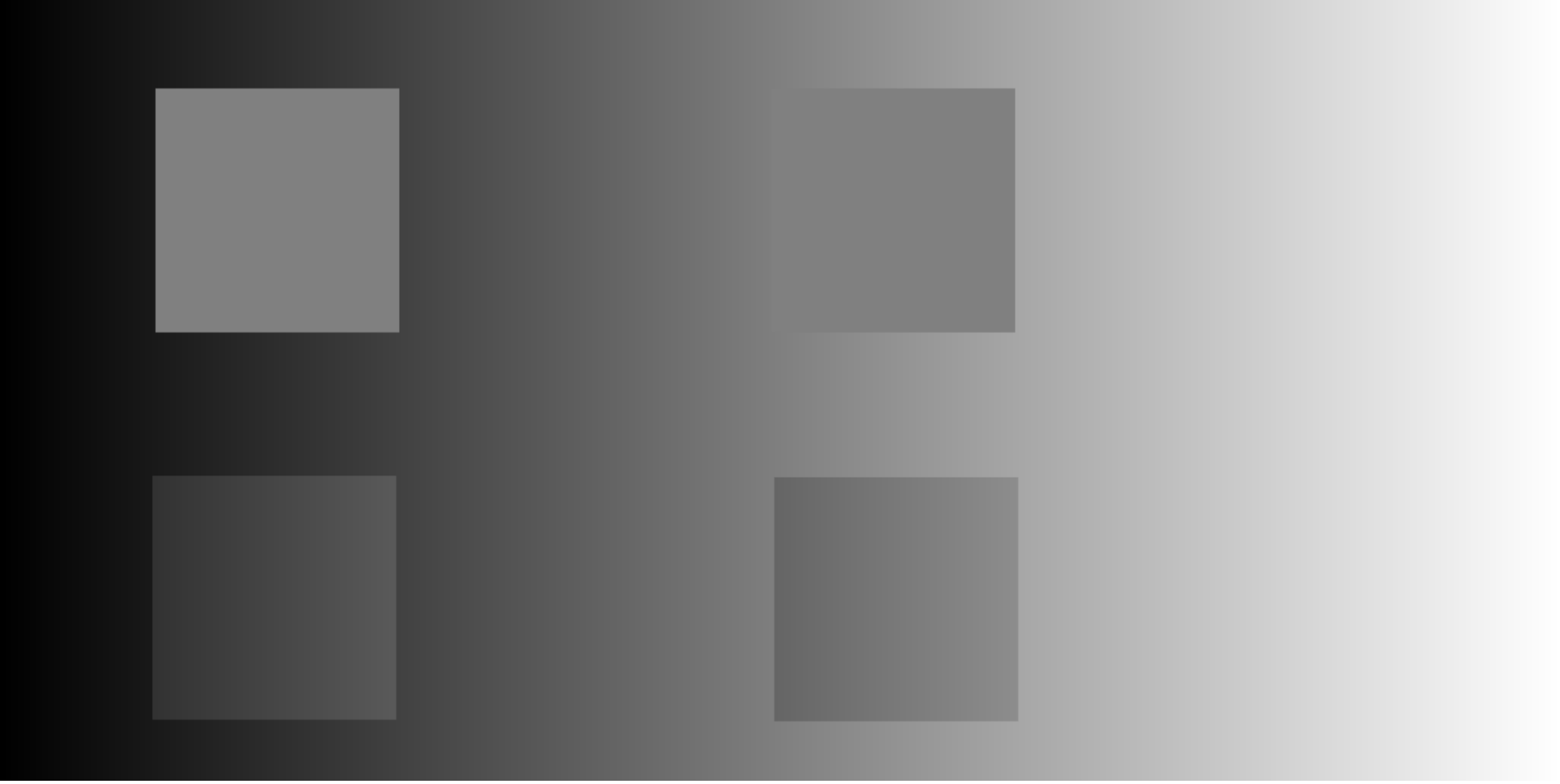

Output:
Input:
Output:
Input:
Output:
Input:
Output:
Input:


45000
40000
35000
30000
25000
20000
15000
10000
5000
0
time 0
time 1
time 2
time 3
time 4

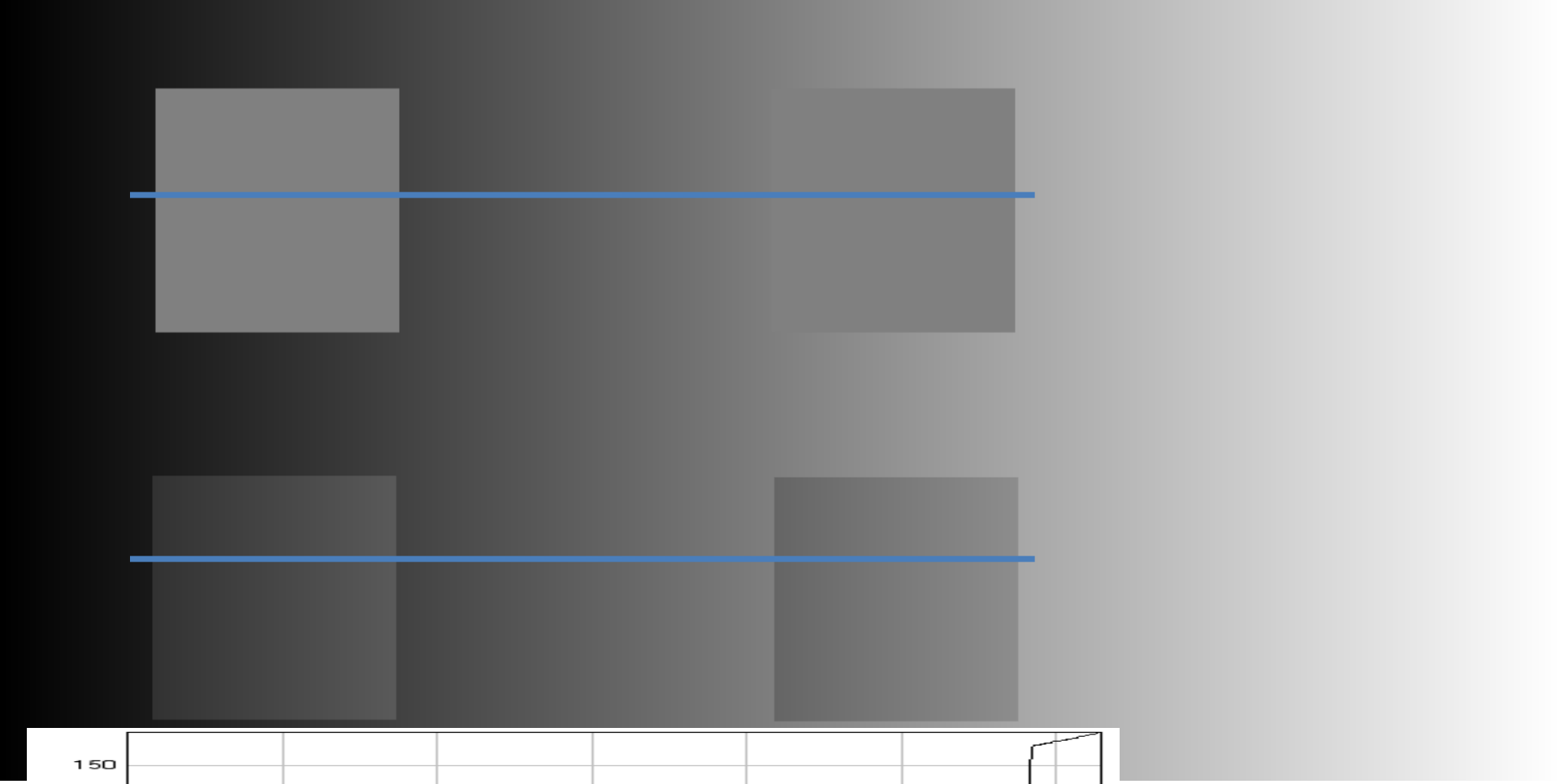


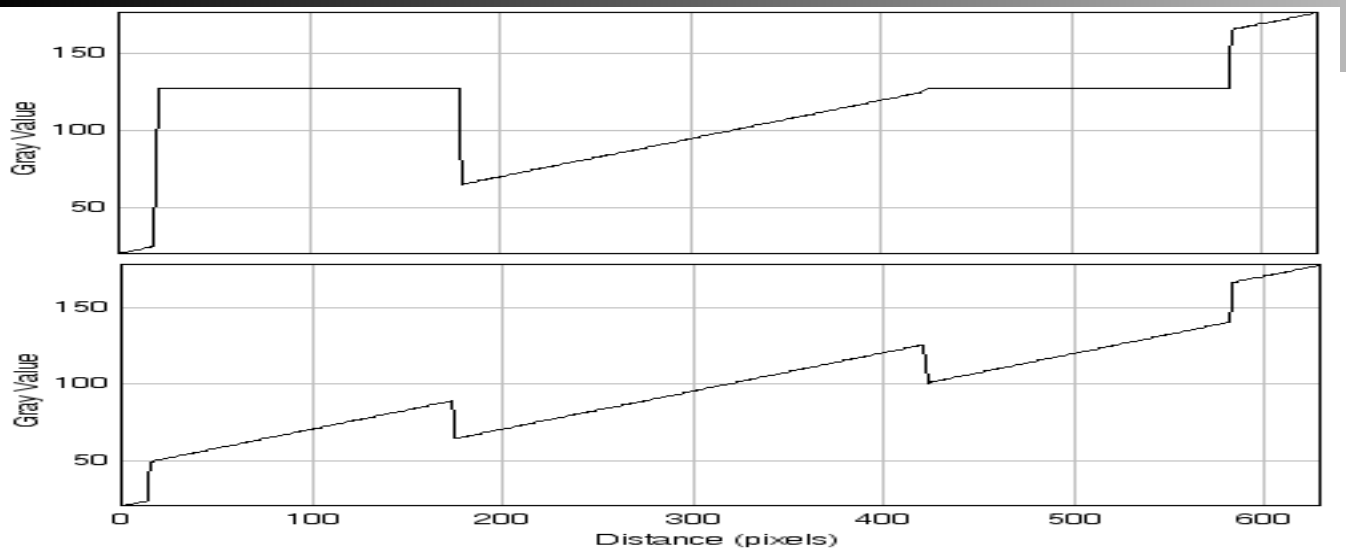

Output:
Input:
Output:
Input:
Output:
Input:
Output:
Input:

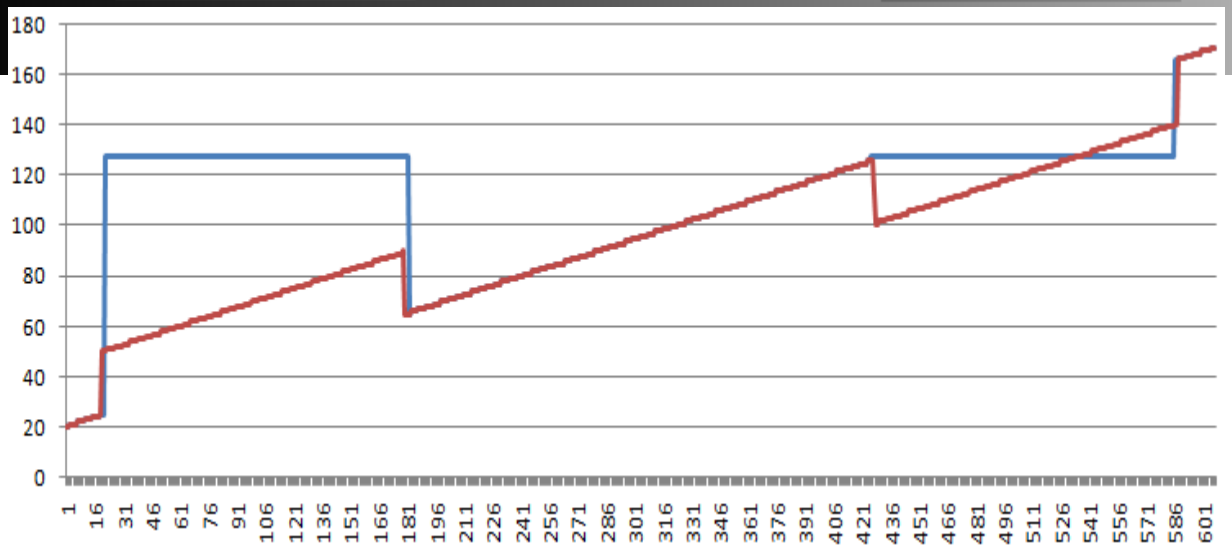
Importance of Computer Image Analysis Because Often We Cannot Trust Our Eyes



Are these squares
1.) the same or different intensities and
2.) are they uniform or shifting (gradient) intensities?




150
100
50
Gray Value
150
100
50
Gray Value
0
100
200
300
400
500
600
Distance (pixels)


180
160
140
120
100
80
60
40
20
0
1
16
31
46
61
76
91
106
121
136
151
166
181
196
211
226
241
256
271
286
301
316
331
346
361
376
391
406
421
436
451
466
481
496
511
526
541
556
571
586
601

Most Quantification of Intensity

Fluorescence intensity usually linear relationship to protein or other chemical.

Absorption intensity usually inverse log relationship and very sensitive to thickness of sample.

Most measurements are of fluorescence combined with area.

Area

Count the number of pixels.

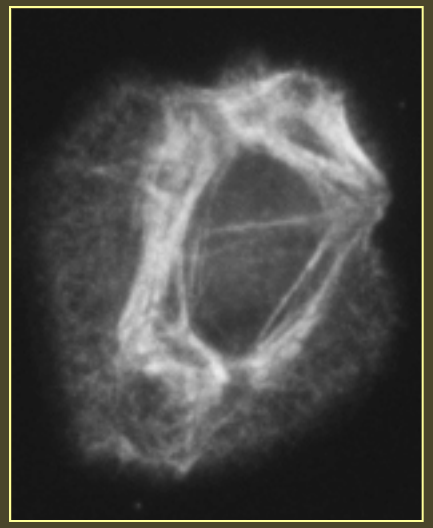
(In old days, cut out from photos and weighed or manually counted with overlaid grid.)

Intensity

Photometer in eyepiece of microscope.

Masked portions of image.

Quantification of mass and concentration in cells by fluorescent microscopy and computer analysis



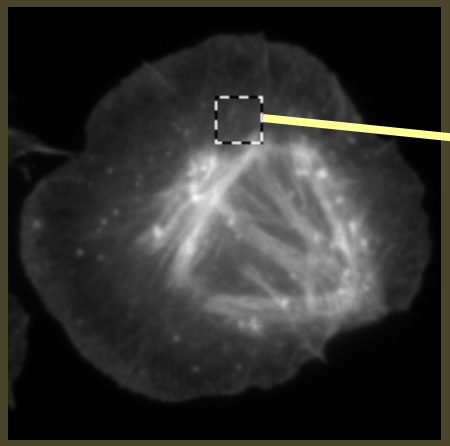
= ???

There are two types of measurements we get from intensity information.

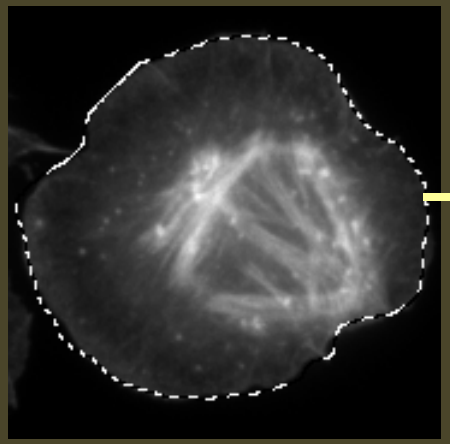
1. Mass (total amount of a species in a given area or volume)
2. Concentration (amount of mass per unit area or volume)

Mass

Mass = integrated intensity = sum of all pixel values in a given area



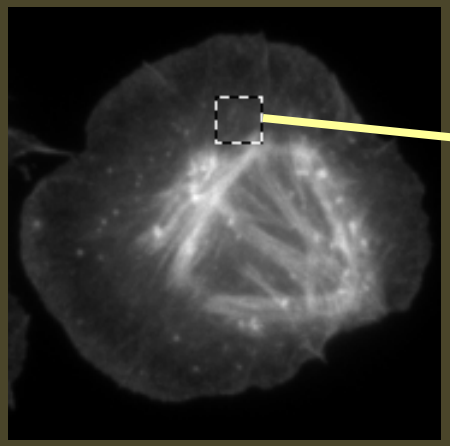
238,088



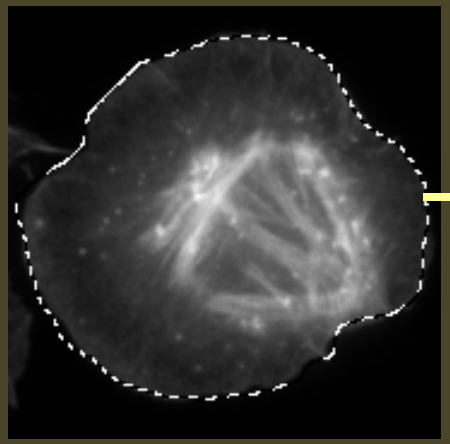
11,852,009

Concentration

Concentration = mass per unit area (# of pixels) = mean mass



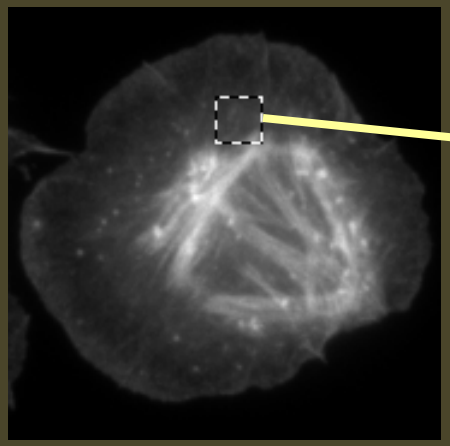
595.22



625.04

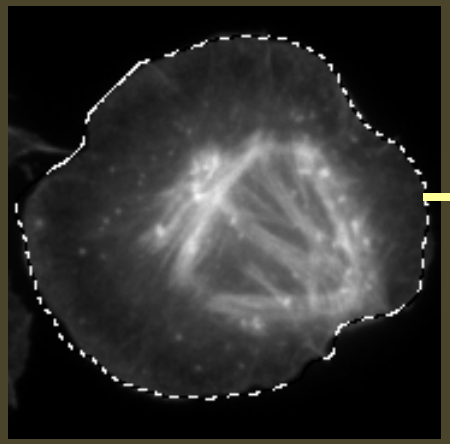
Concentration

Concentration = mass per unit area (# of pixels) = average mass



238,088 / 400 =

595.22



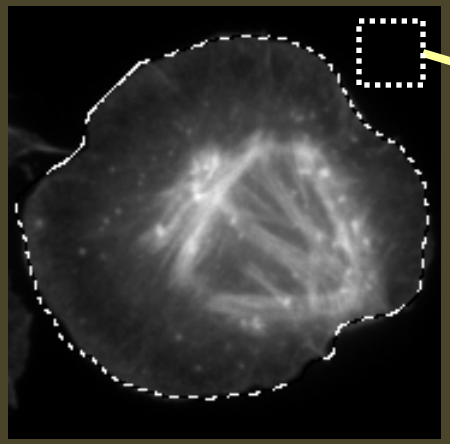
11,852,009 / 18962 =

625.04

Whoops, we skipped a step!

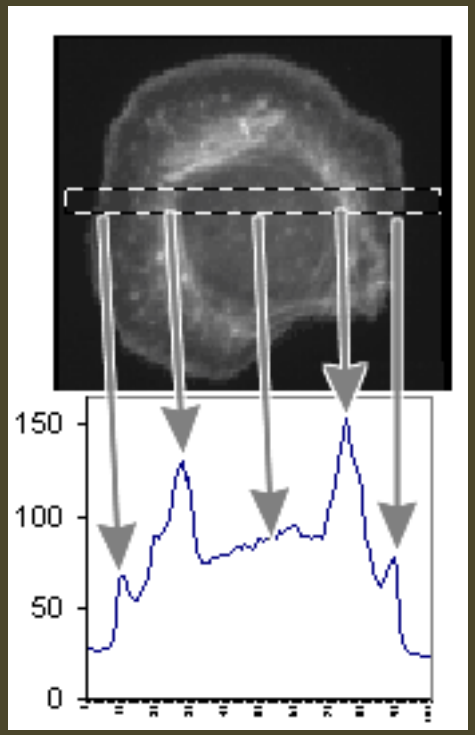
The previous measurements assumed an ideal situation where everything that was non-cell was pure black or zero.

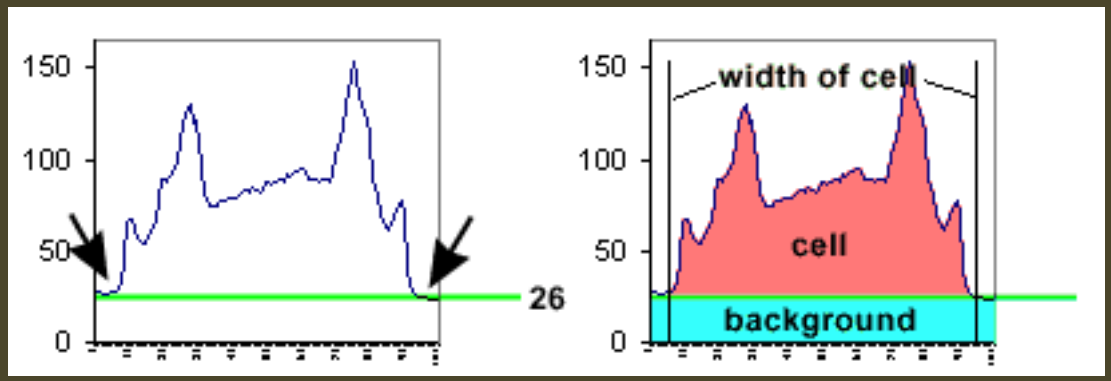
But when we collect images, this isn't true!



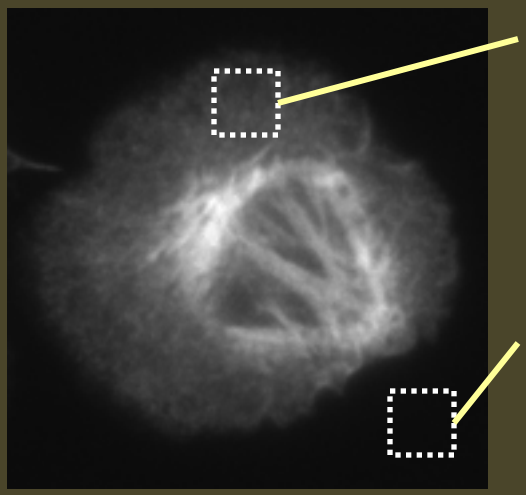
440 +/- 10

Area under the curve





Subtract the background

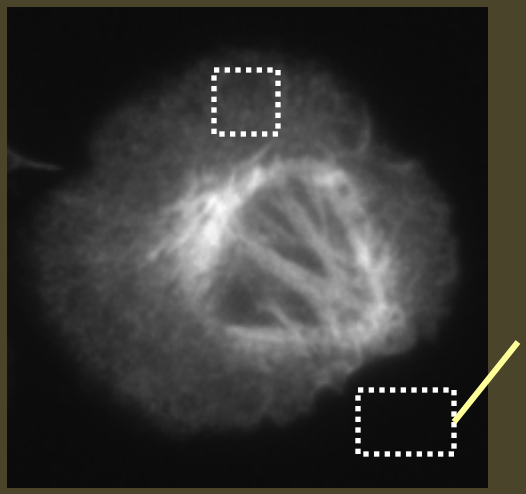


Let's say that this box is 10 X 10 pixels.
Let's say its integrated intensity = 100,000.

Let's say that this box is 10 X 10 pixels.
Let's say its integrated intensity = 44,000.

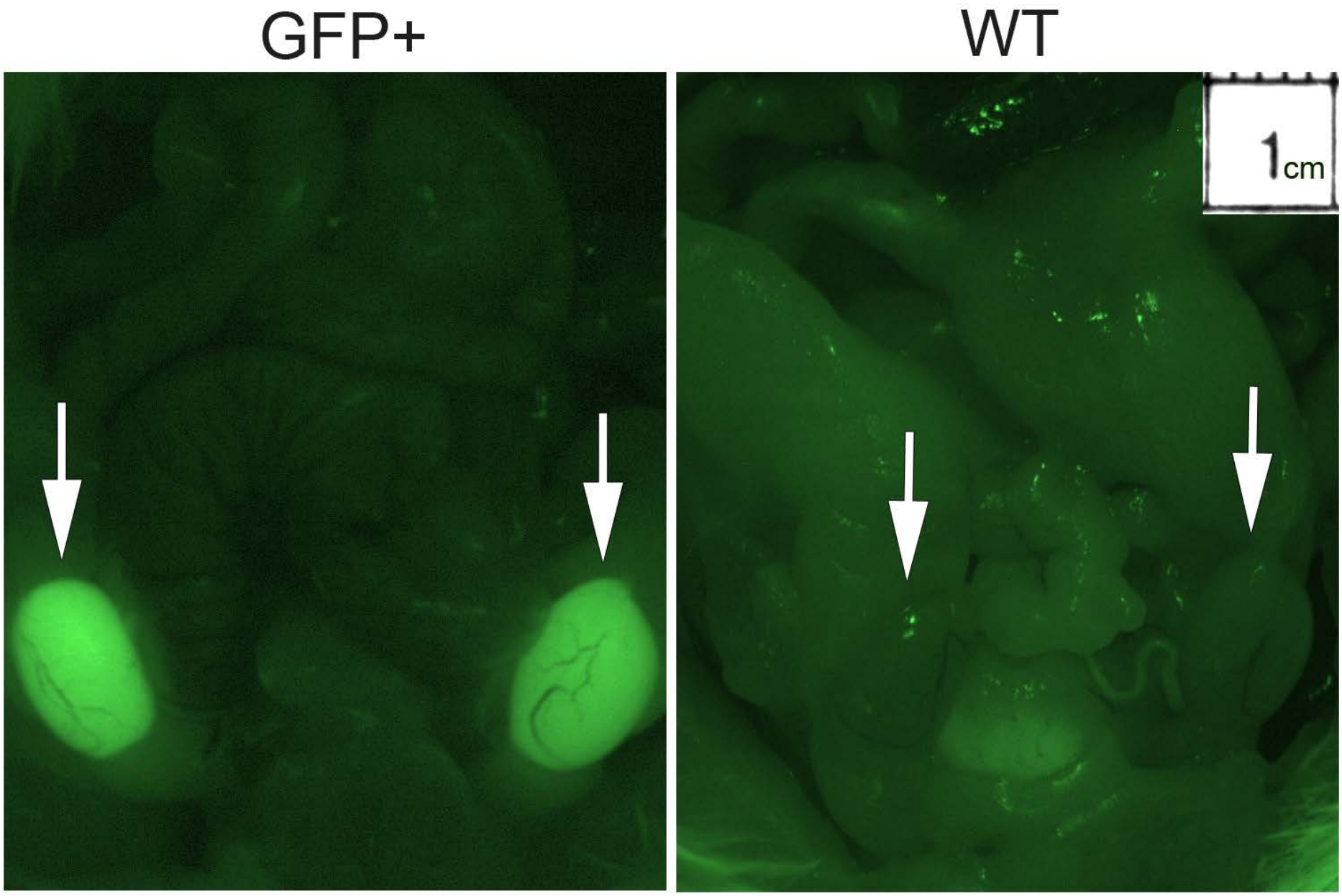
The mass of the box within the cell is really
100,000 – 44,000 = 56,000

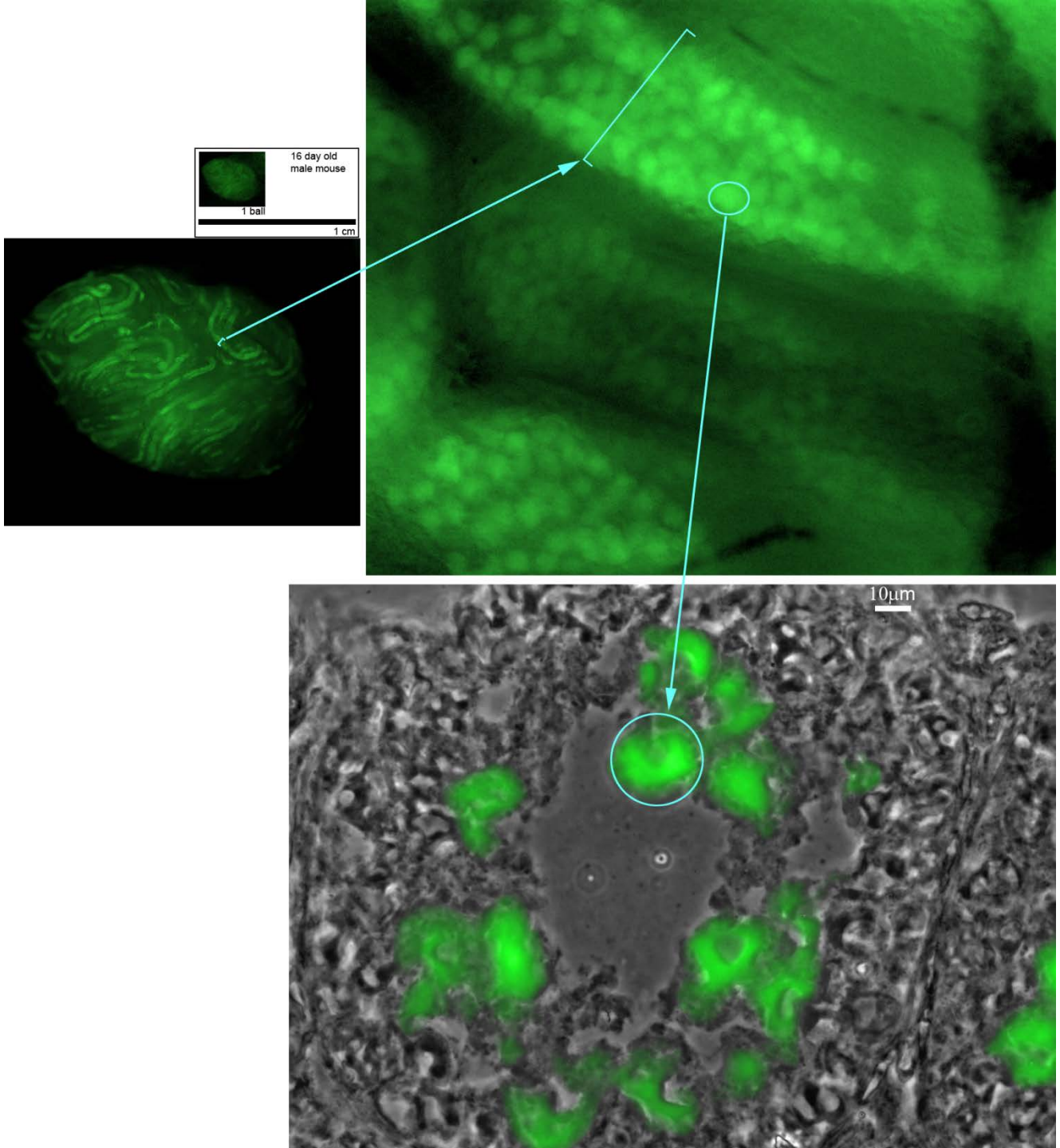
Subtract the background

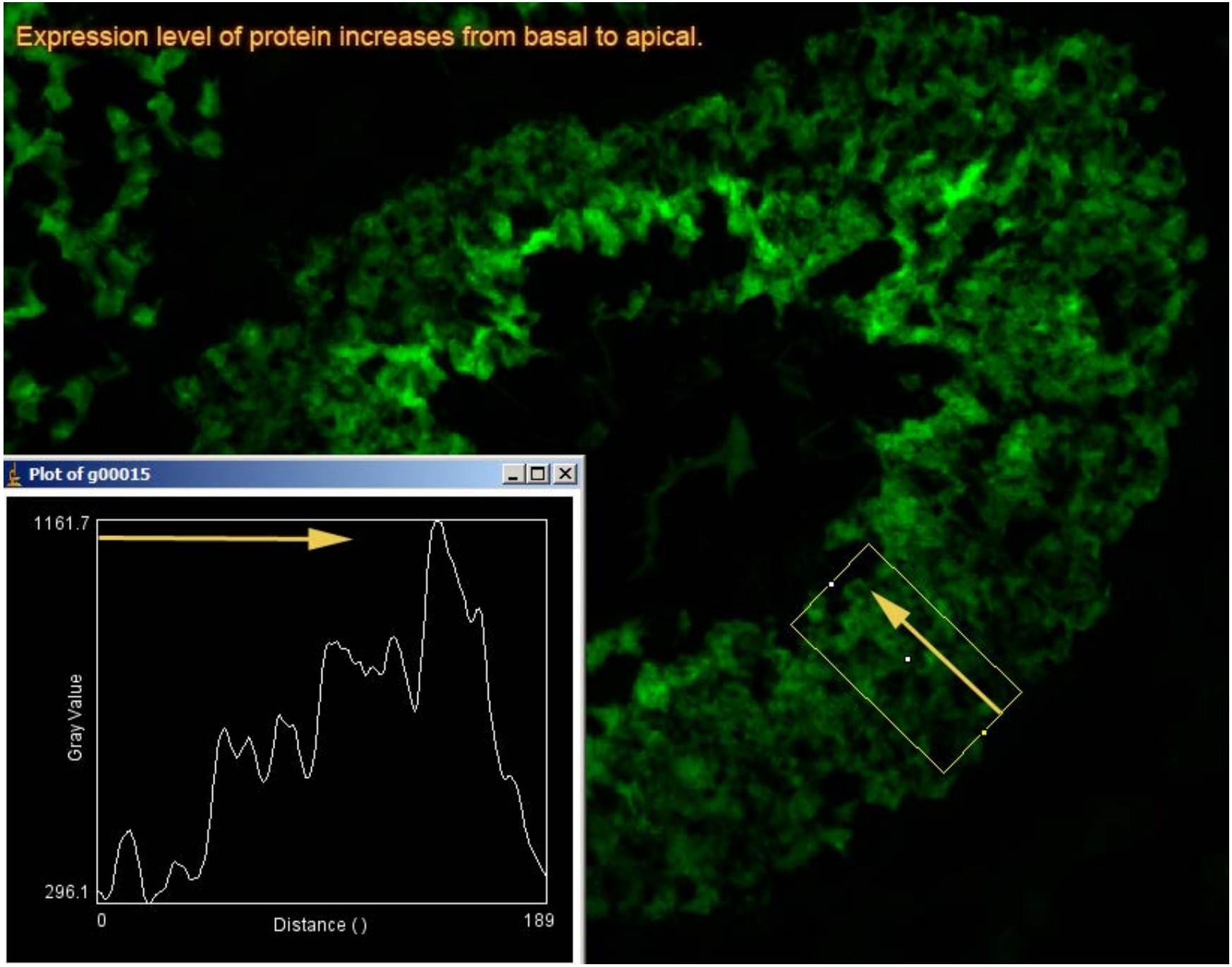


Because the background area may vary in size, it it simpler to always do the math based on the concentration.

The size or shape of this box doesn't matter.
All we care about is that its
Mass / area = 440.


GFP+
WT
1cm

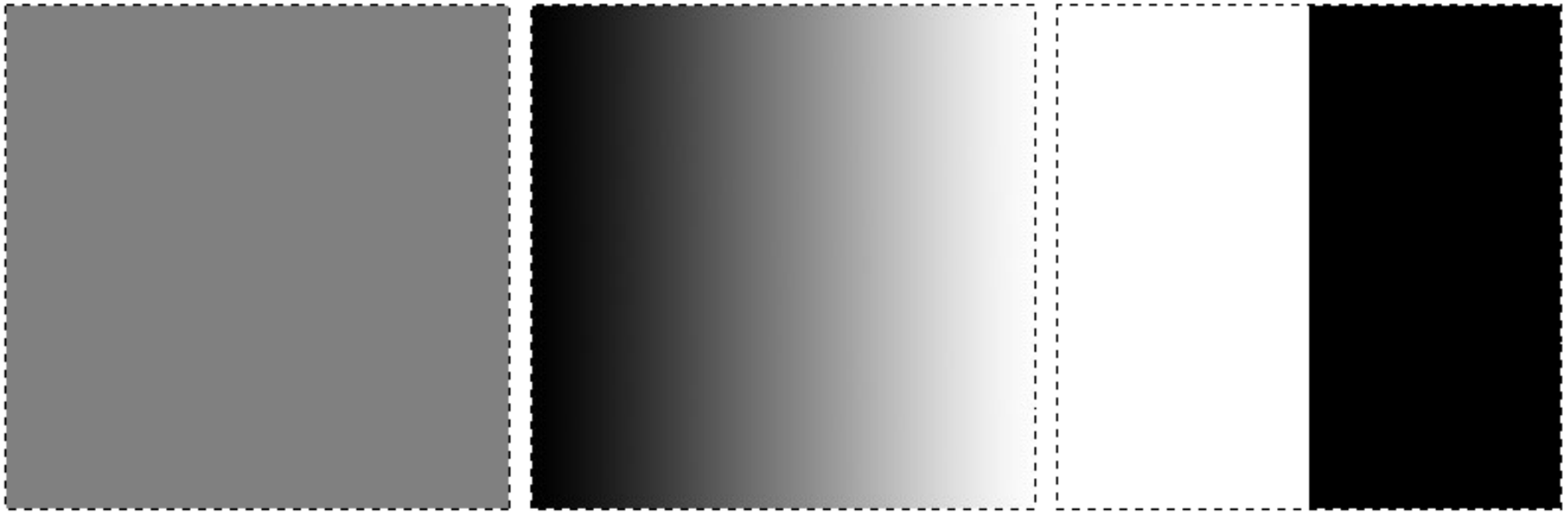

16 day old
male mouse
1 ball
1 cm
10μm


Expression level of protein increases from basal to apical.
Plot of g00015
1161.7
296.1
Gray Value
0
Distance ( )
189

Summary of Basic Intensity Measurements

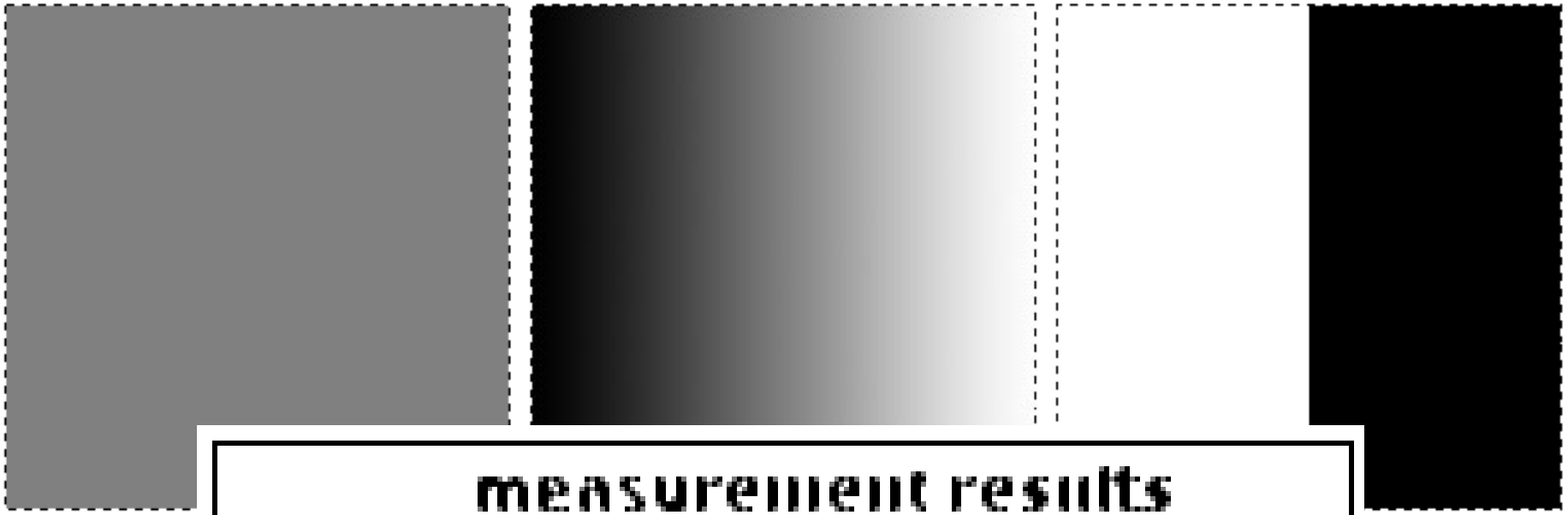
- Background
- Range of intensities
- Maximum value *not saturated*

Distribution of Intensities



Images are 8 bit grayscale (values from 0 to 255).
All three images have mean values of 128.

Distribution of Intensities

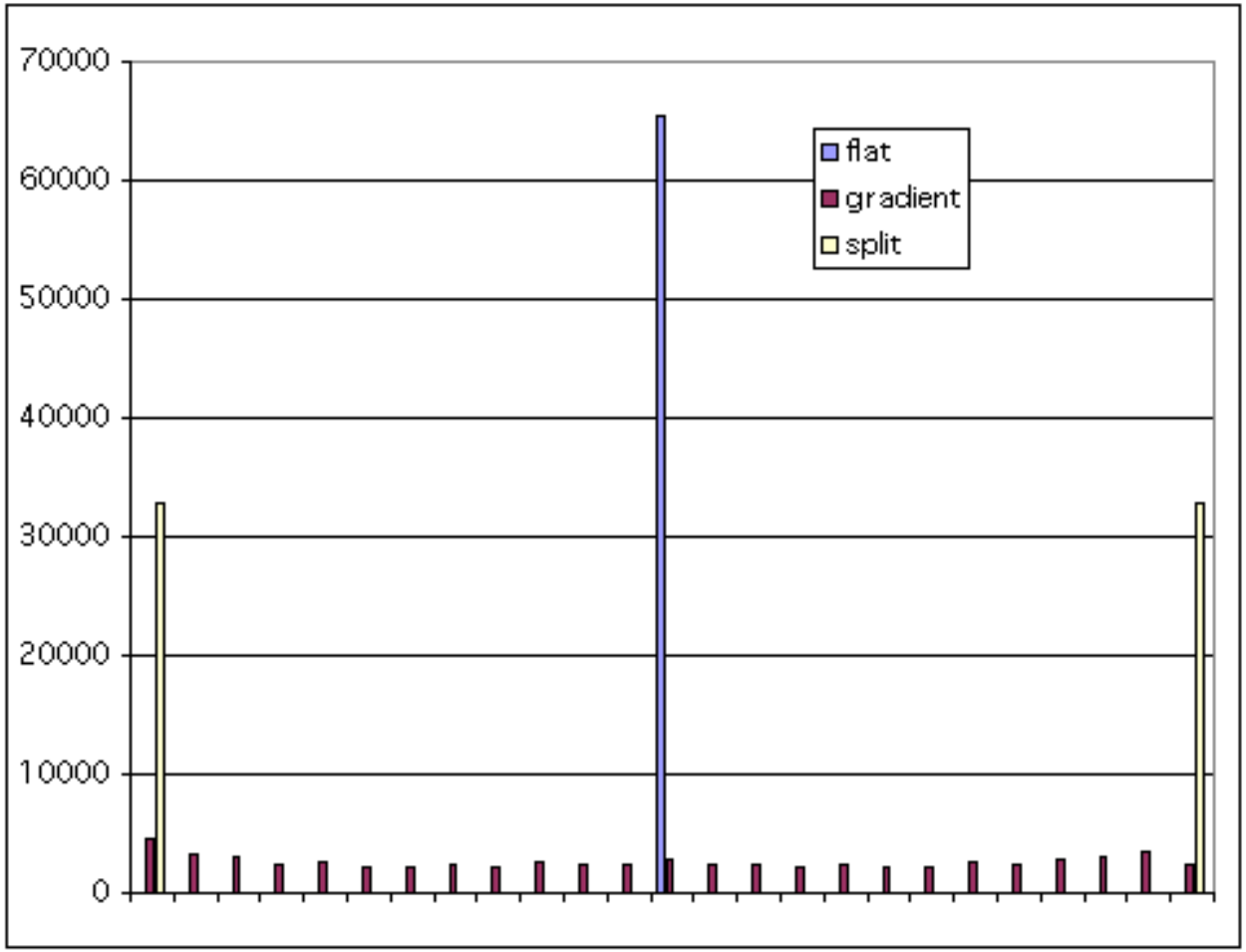


measurement results			
	0 Meas. #	1 Mean	2 Stand.Dev.
0	1.00	128.00	0
1	2.00	127.00	79.18
2	3.00	127.50	127.50

Distribution of Intensities



Bin Value	flat	gradient	split
0	0	4463	32768
10	0	3157	0
21	0	3036	0
31	0	2495	0
42	0	2610	0
52	0	2276	0
63	0	2207	0
73	0	2442	0
83	0	2189	0
94	0	2510	0
104	0	2311	0
115	0	2444	0
125	65536	2748	0
135	0	2394	0
146	0	2489	0
156	0	2218	0
167	0	2439	0
177	0	2203	0
188	0	2265	0
198	0	2543	0
208	0	2443	0
219	0	2861	0
229	0	2958	0
240	0	3480	0
250	0	2355	32768
sum:	6.55E+04	6.55E+04	6.55E+04



Automated Segmentation and Measuring

Noise

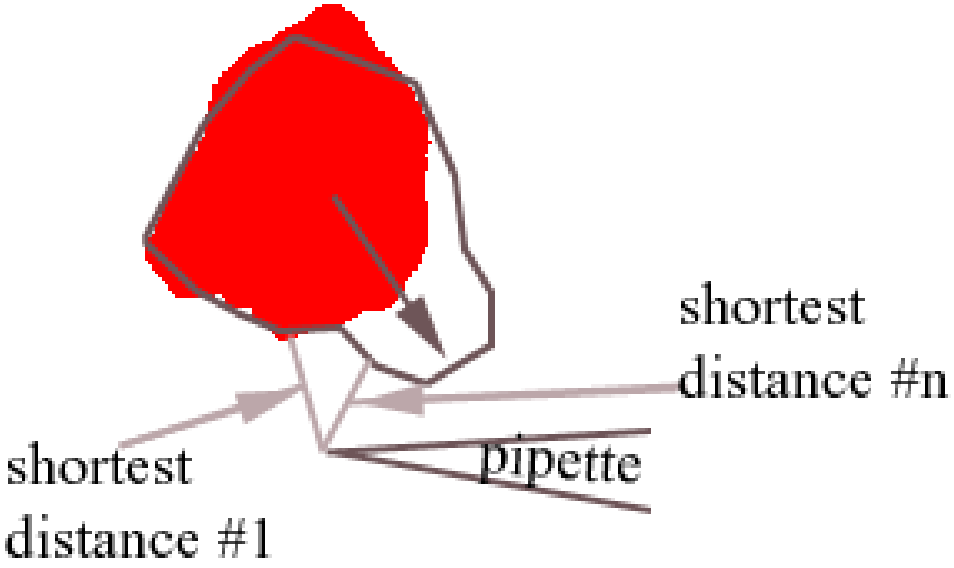
Flatfield correction

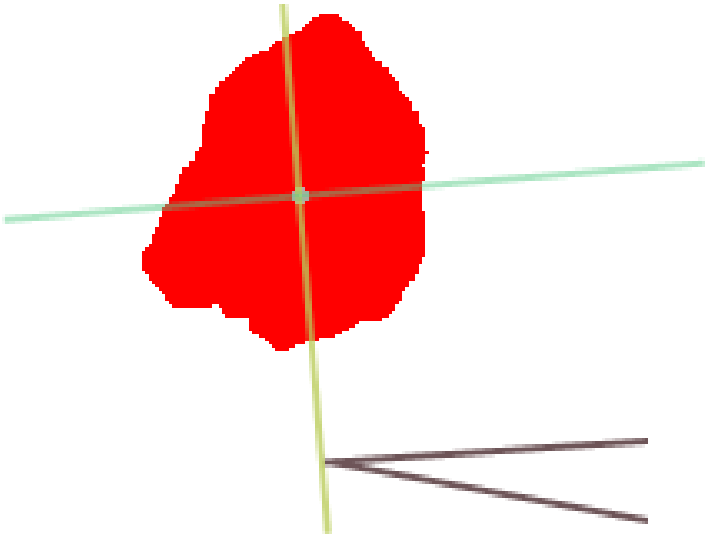
- Adjacent image
- Out of focus image
- Calculated or blurred image
  (does not eliminate high frequency noise, e.g. dirt in lightpath)
- Estimate with high pass filter
  (accentuates high frequency noise)

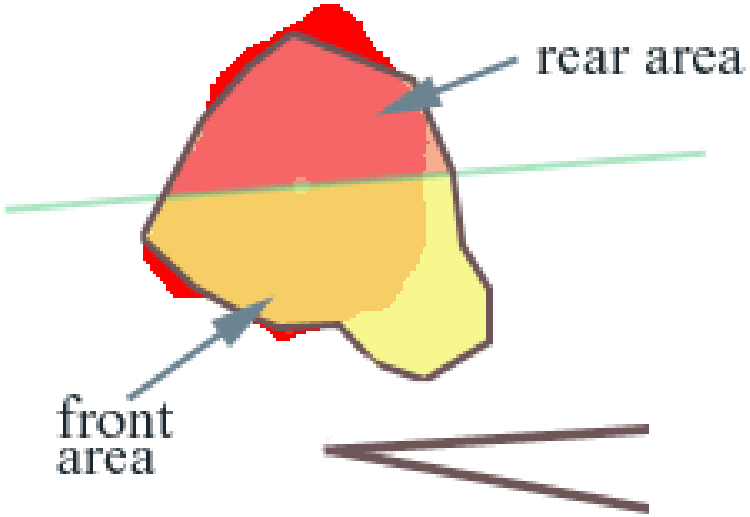
Measuring Change in Shape

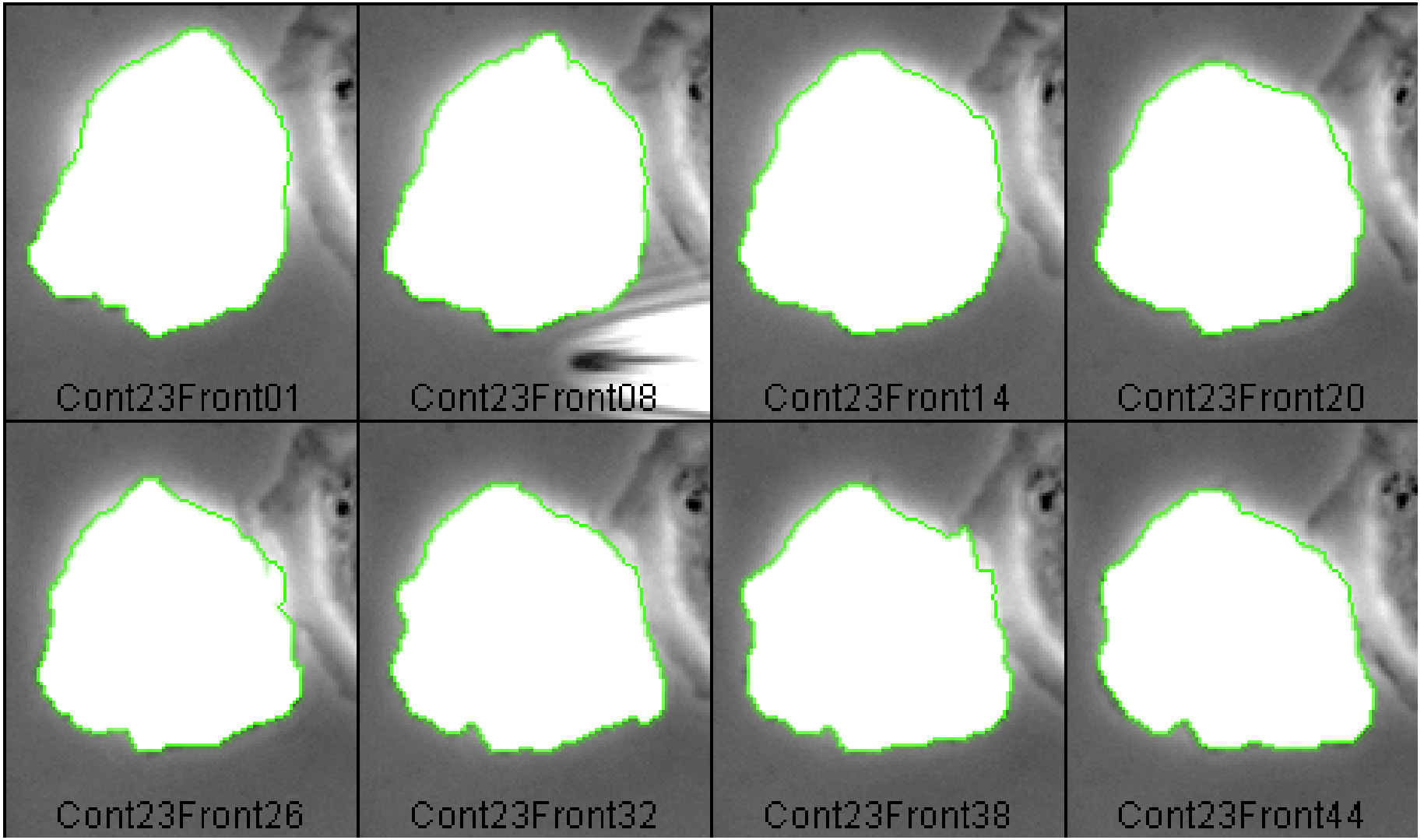
- Movement in a particular direction
- Ratio area to perimeter
- Ratio min and max axes

Chemotaxis

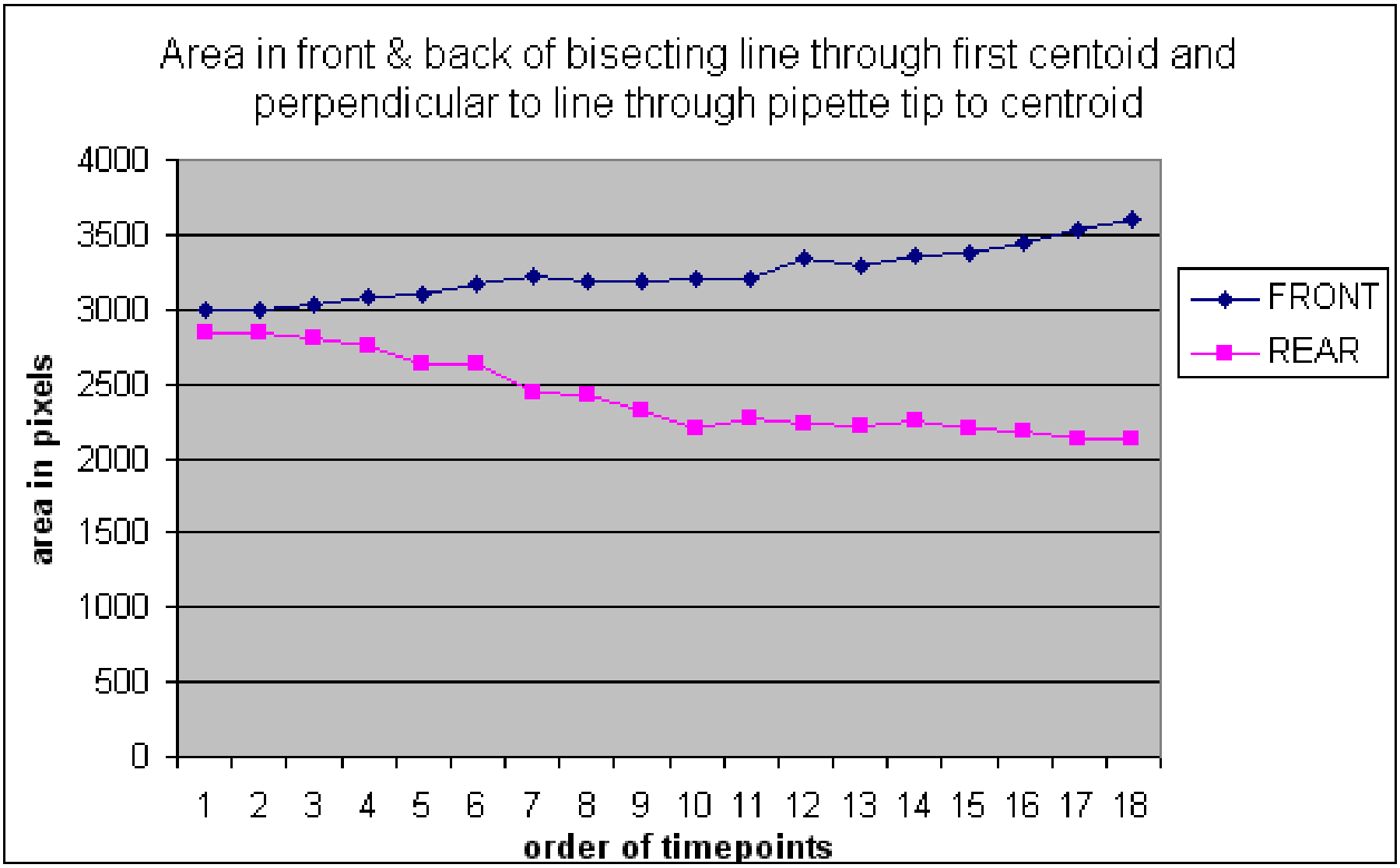

shortest
distance #n
shortest
distance #1
pipette



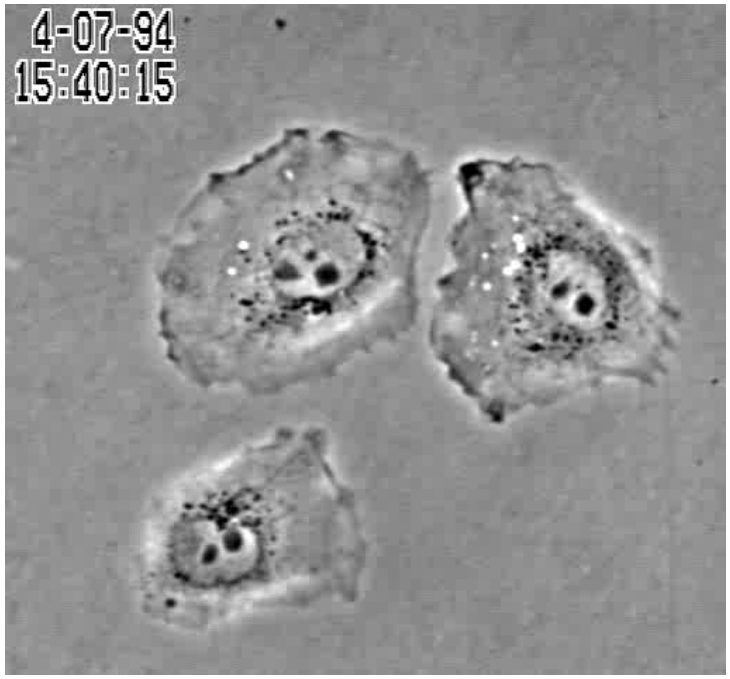

rear area
front
area


Cont23Front01
Cont23Front08
Cont23Front14
Cont23Front20
Cont23Front26
Cont23Front32
Cont23Front38
Cont23Front44

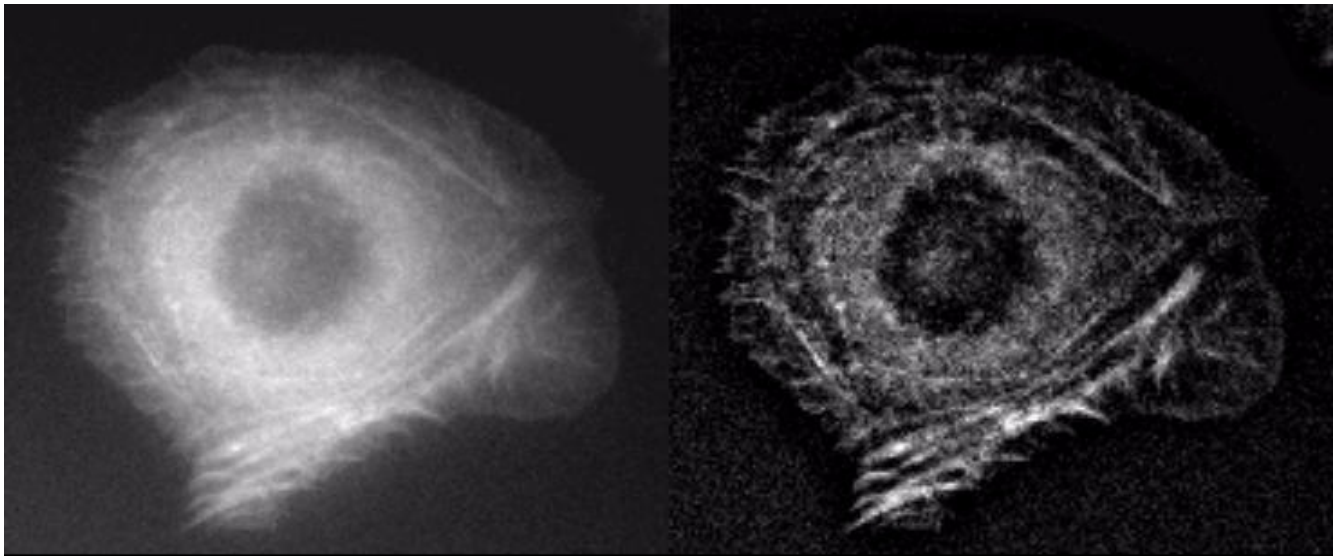
FRONT	Area	X	Y	REAR	Area	X	Y	FRONT/REAR
1	2994	107	111	1	2836	106	71	1.055712271
2	2994	107	111	2	2836	106	71	1.055712271
3	3024	108	111	3	2808	105	72	1.076923077
4	3078	108	111	4	2757	105	72	1.116430903
5	3092	109	111	5	2638	104	74	1.172100076
6	3168	109	112	6	2631	103	74	1.204104903
7	3221	110	112	7	2440	102	75	1.320081967
8	3187	110	112	8	2418	102	75	1.318031431
9	3193	109	112	9	2318	101	76	1.377480587
10	3209	110	112	10	2207	100	76	1.454009968
11	3205	111	112	11	2267	100	76	1.413762682
12	3341	111	112	12	2239	100	76	1.492184011
13	3290	111	112	13	2216	99	77	1.48465704
14	3355	111	113	14	2259	99	77	1.485170429
15	3382	111	113	15	2196	98	77	1.54007286
16	3440	113	113	16	2174	98	78	1.582336707
17	3541	113	113	17	2135	97	78	1.658548009
18	3606	115	113	18	2123	98	78	1.698539802


Area in front & back of bisecting line through first centoid and perpendicular to line through pipette tip to centroid
area in pixels
4000
3500
3000
2500
2000
1500
1000
500
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
order of timepoints
FRONT
REAR

EGF Upshift

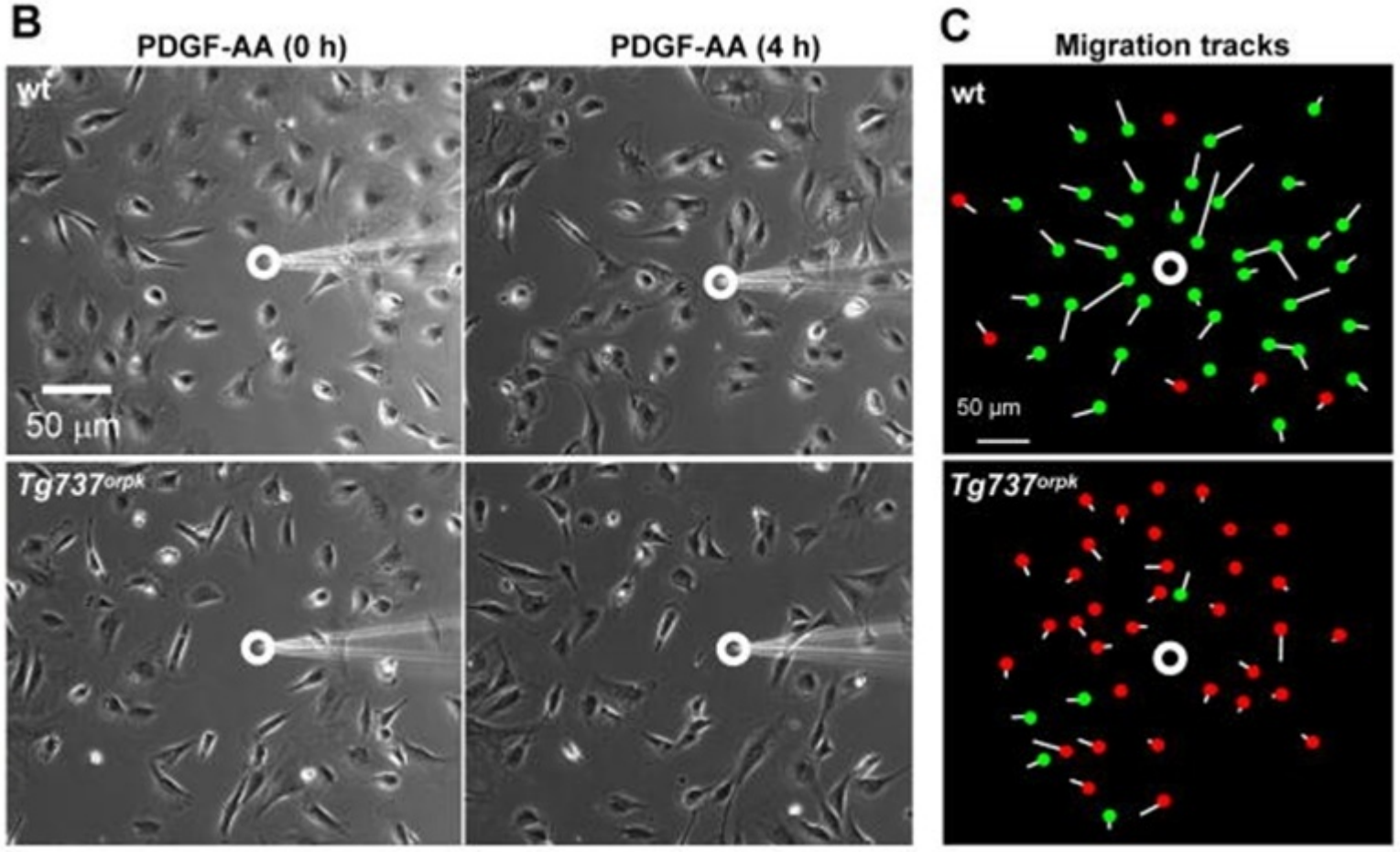


GFP Fusion

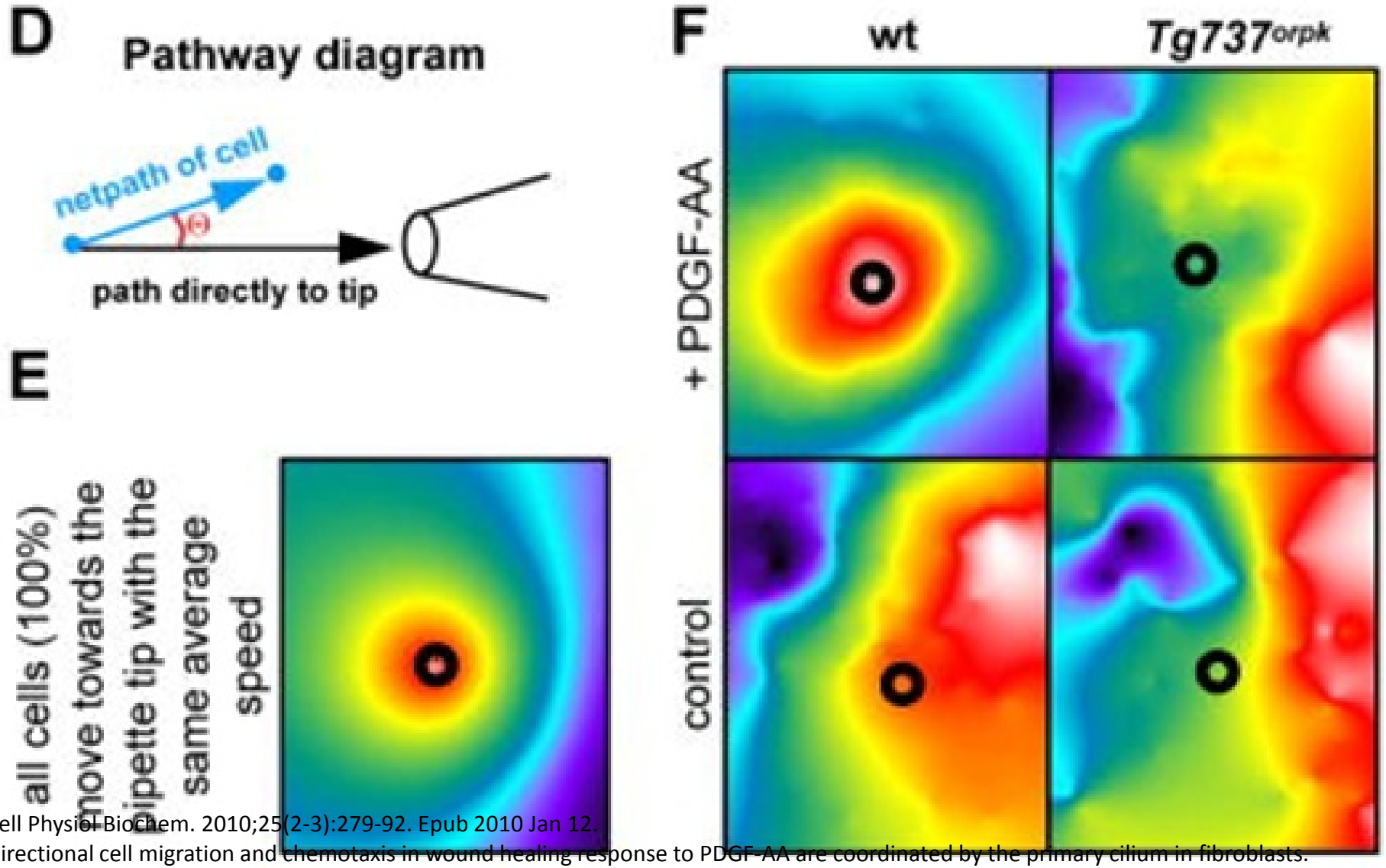


EGF Kinetics graph here

Chemotaxis quantification



Cell Physiol Biochem. 2010;25(2-3):279-92. Epub 2010 Jan 12.
Directional cell migration and chemotaxis in wound healing response to PDGF-AA are coordinated by the primary cilium in fibroblasts.
Schneider L, Cammer M, Lehman J, Nielsen SK, Guerra CF, Veland IR, Stock C, Hoffmann EK, Yoder BK, Schwab A, Satir P, Christensen ST.

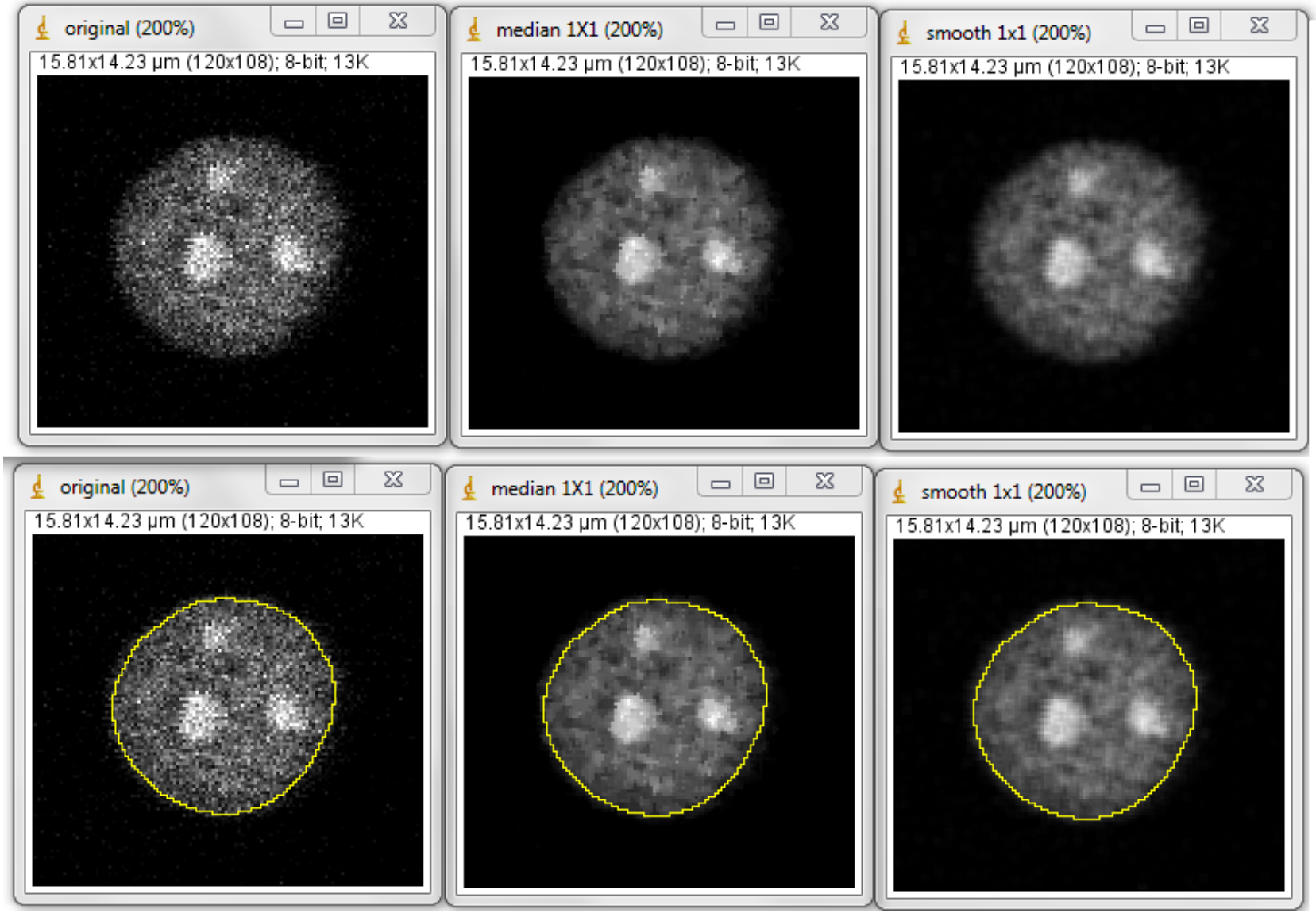


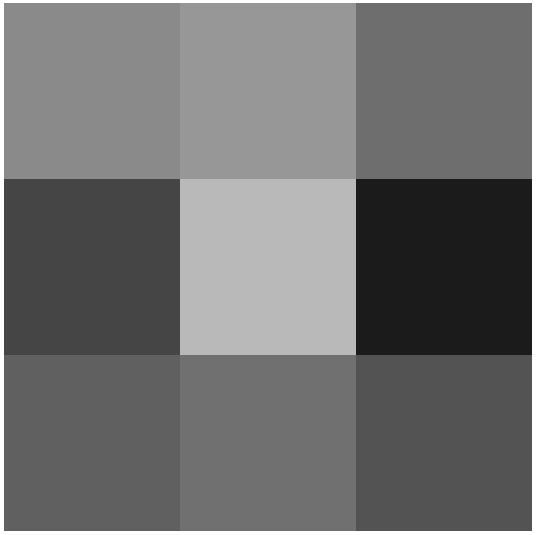
Cell Physiol Biochem. 2010;25(2-3):279-92. Epub 2010 Jan 12.
Directional cell migration and chemotaxis in wound healing response to PDGF-AA are coordinated by the primary cilium in fibroblasts.
Schneider L, Cammer M, Lehman J, Nielsen SK, Guerra CF, Veland IR, Stock C, Hoffmann EK, Yoder BK, Schwab A, Satir P, Christensen ST.

Important take-home messages

- Raw Data Sacrosanct
- Save uncompressed
- Keep a back-up in a separate location
- Do not change contrast
- Do not resize
- Don’t change in other ways
- GIGO: Measurements not useful without well planned experiments with proper controls

Two simple ways to filter noise





138	151	110
69	185	27
96	112	83

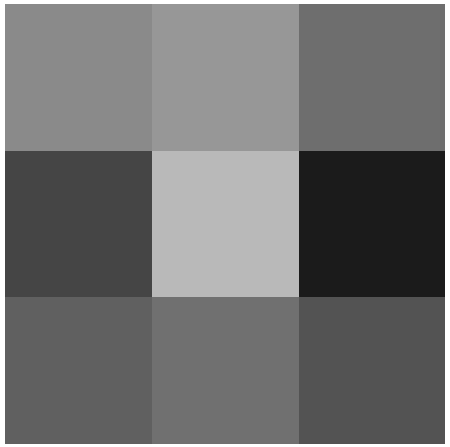
138	151	110
69	185	27
96	112	83

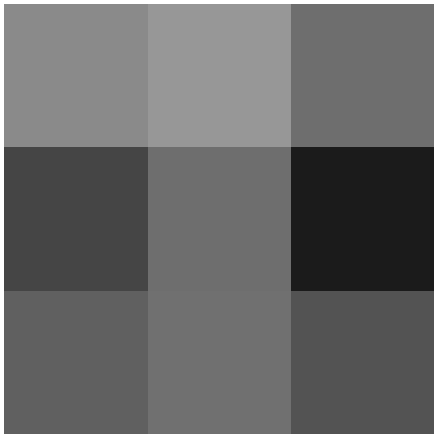
27
69
83
96
110
112
138
151
185

138	151	110
69	185	27
96	112	83

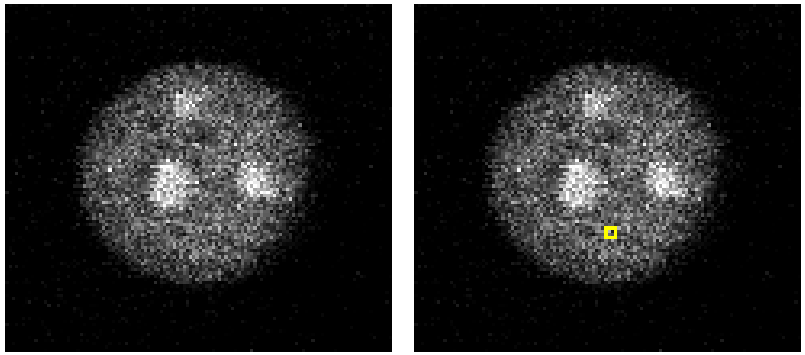
Replace with median value

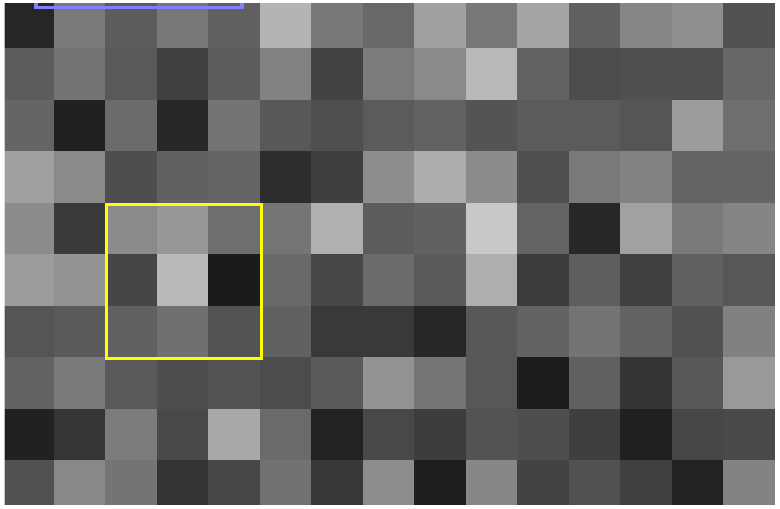
138	151	110
69	110	27
96	112	83



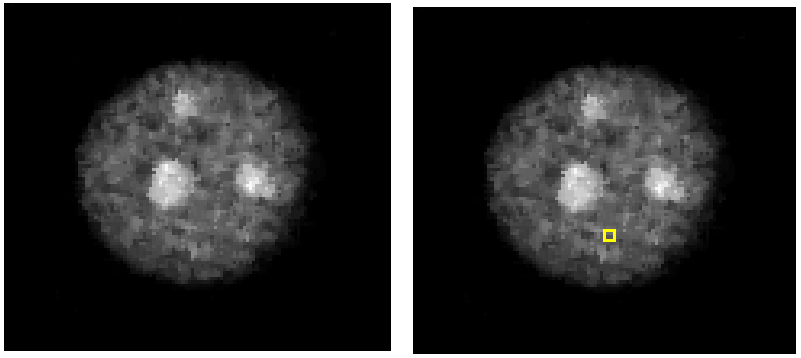


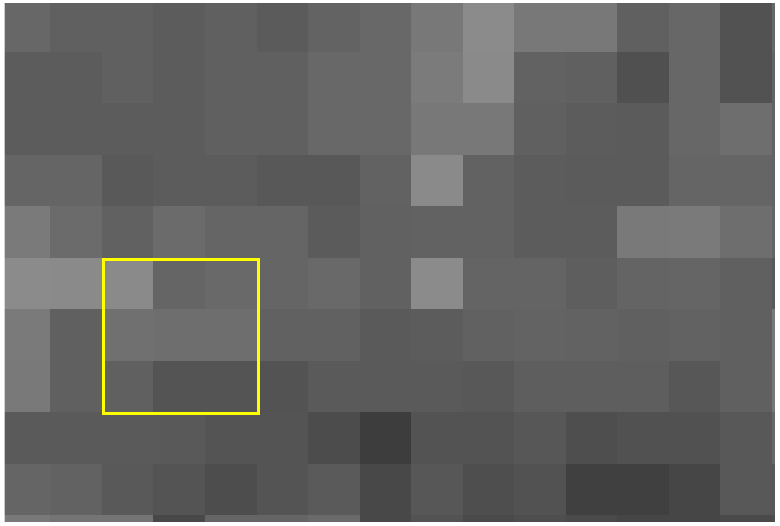
Original



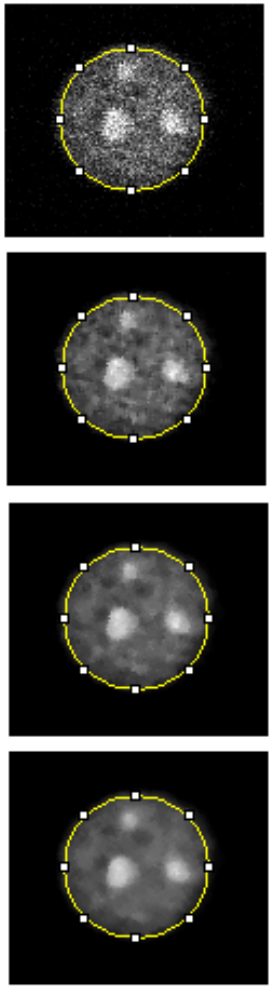


Median 3X3



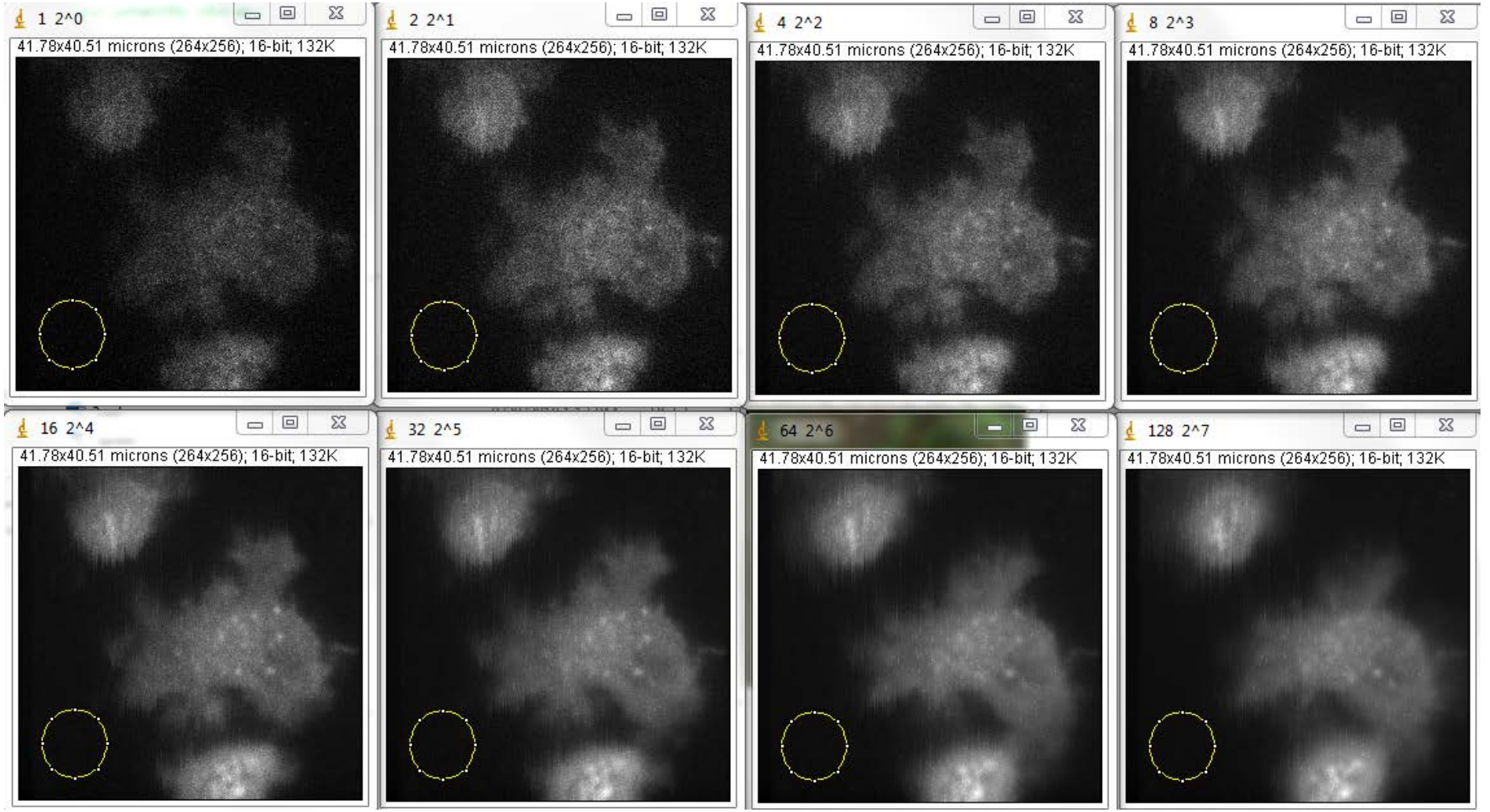


Is this valid for intensity quantifications?

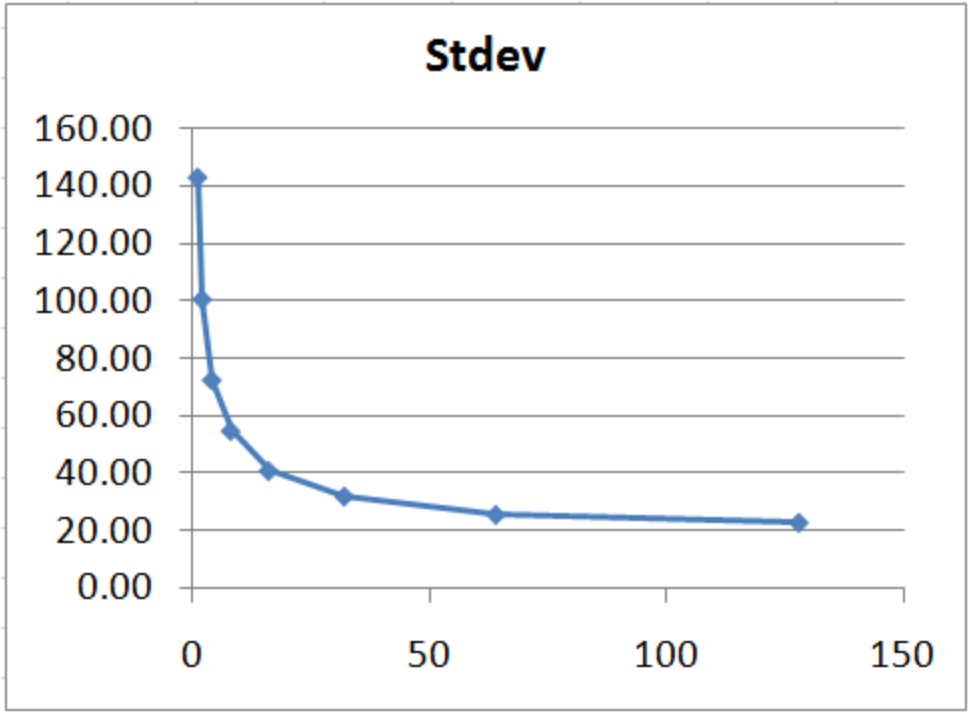


	Label	Area	Mean	StdDev
1	ORIGINAL	60.5	93.5	48.7
2	median 3X3	60.5	90.9	36.3
3	median 5X5	60.5	90.1	33.9
4	median 7X7	60.5	89.3	32.4

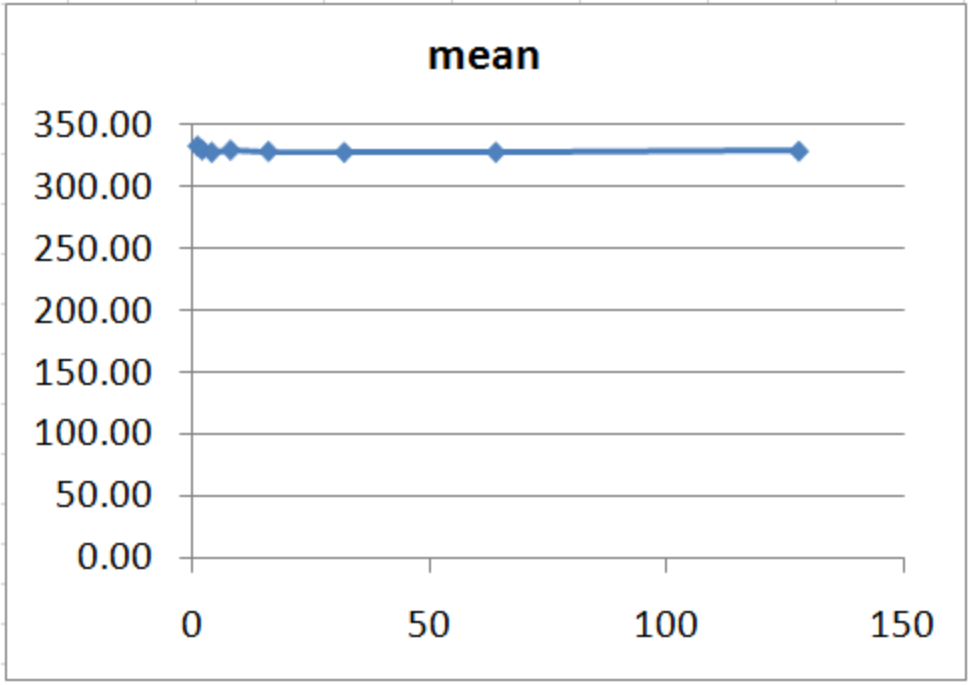
Averaging serial frames



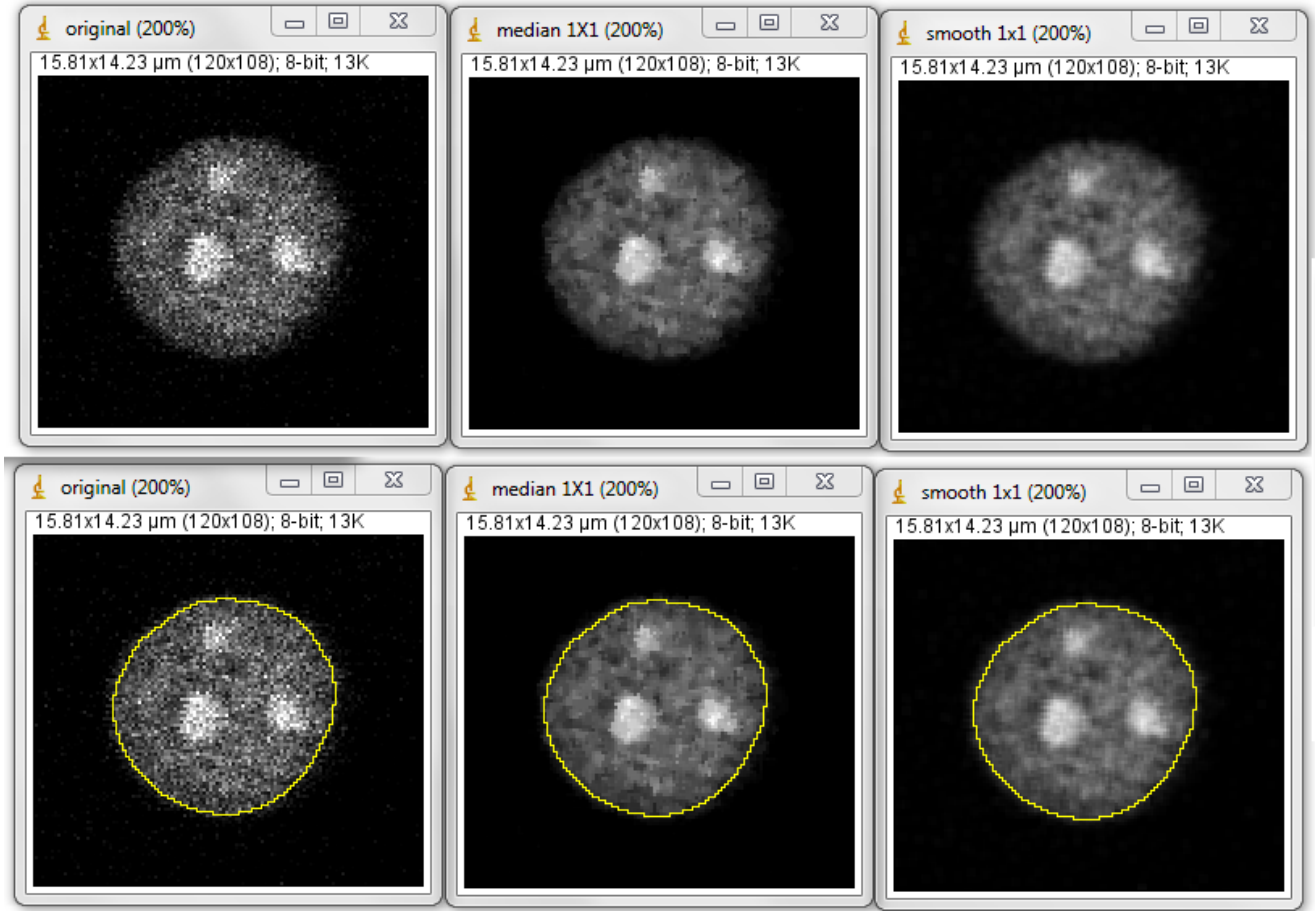
average	Stdev
1	142.95
2	100.55
4	72.40
8	54.77
16	40.82
32	31.98
64	25.56
128	22.79



average	mean
1	333
2	330
4	328
8	330
16	329
32	328
64	328
128	329



Two simple ways to filter noise

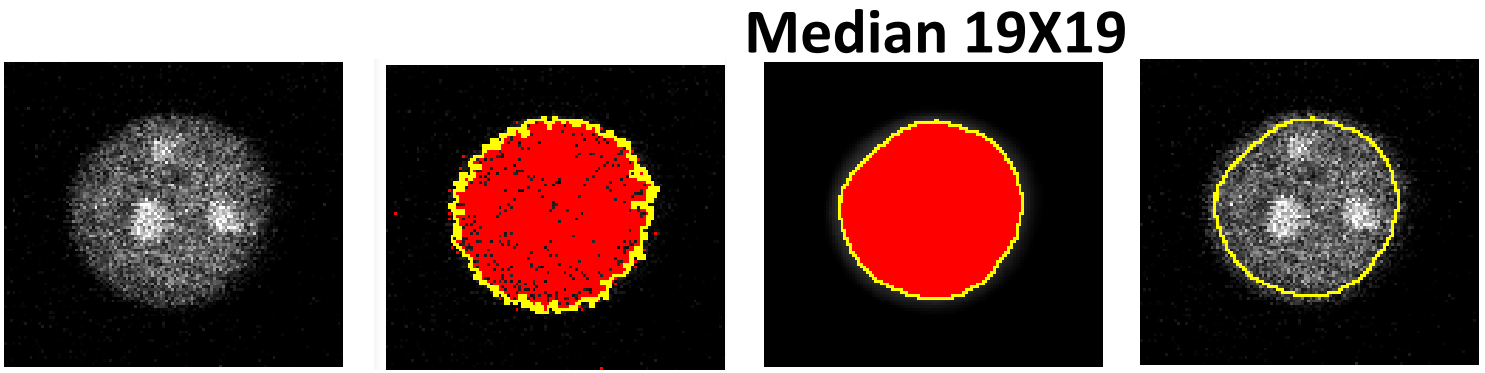


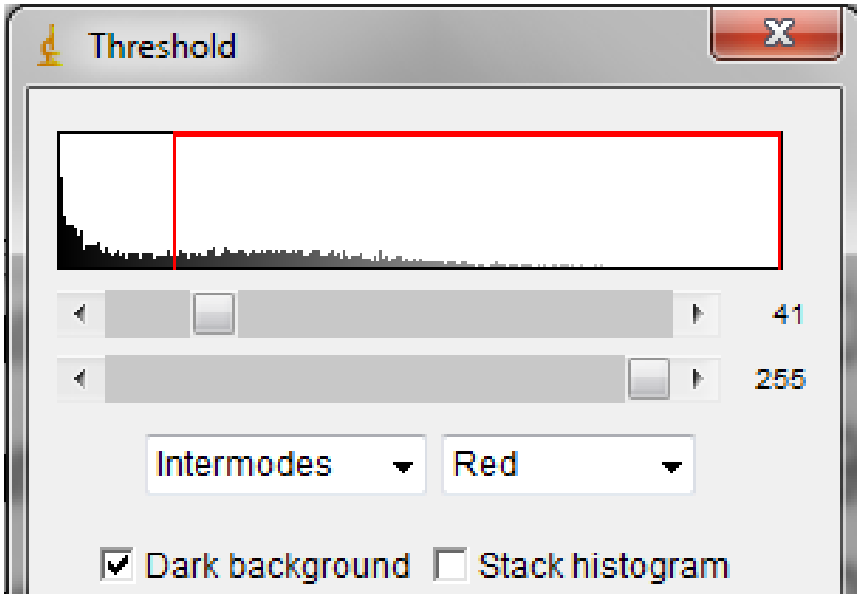
Results

File Edit Font Results

	Label	Area	Mean	StdDev
1	original	60.9	93.6	48.4
2	median 1X1	60.9	91.0	36.0
3	smooth 1x1	60.9	93.3	34.2

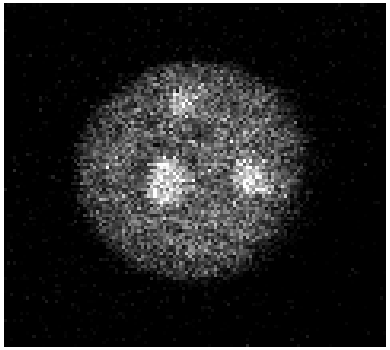
Median filtering good for automated feature detection by intensity

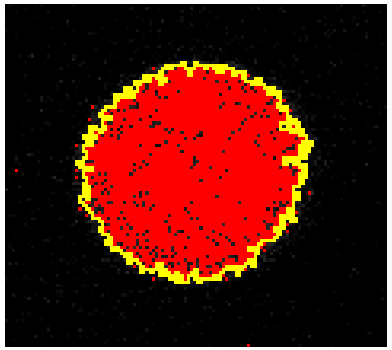


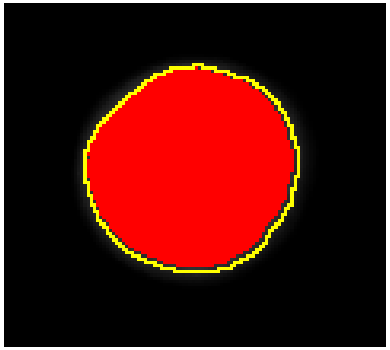


Median good for removing noise for spatial measurements

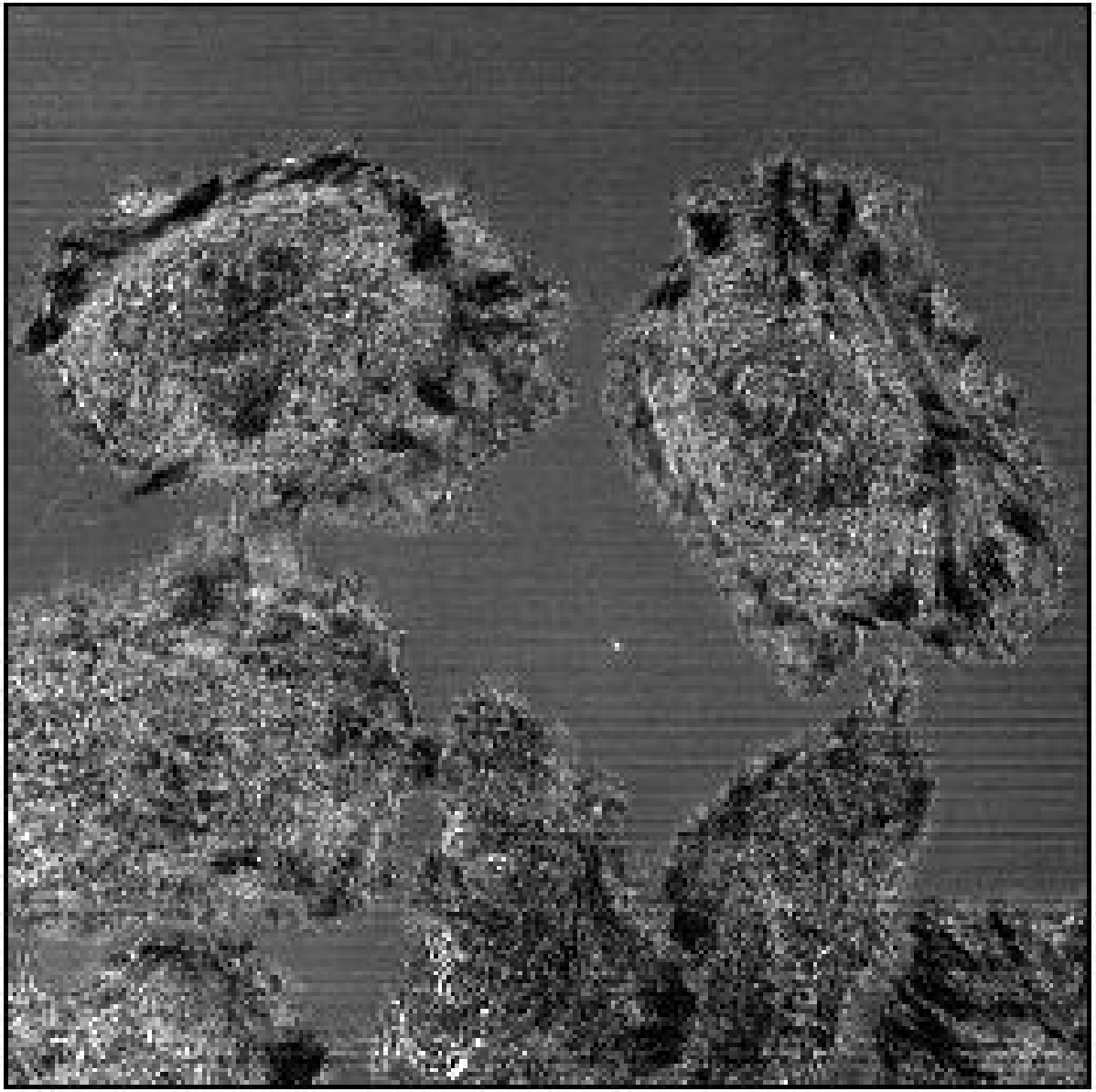
	Label	Area	Perim.	Circ.	Round
1	ORIGINAL	61.6	56.7	0.2	0.9
2	median	57.8	28.4	0.9	0.9

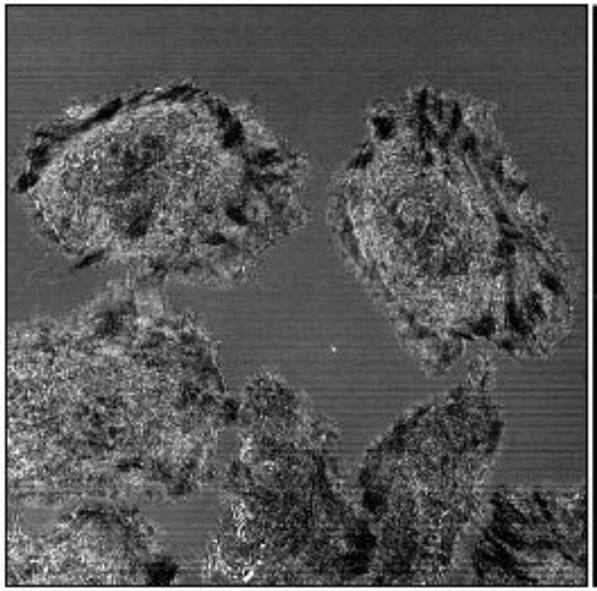




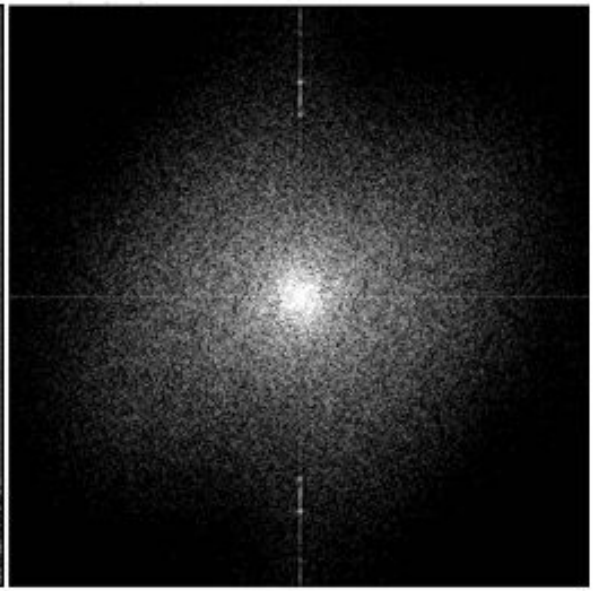


Filtering periodic noise

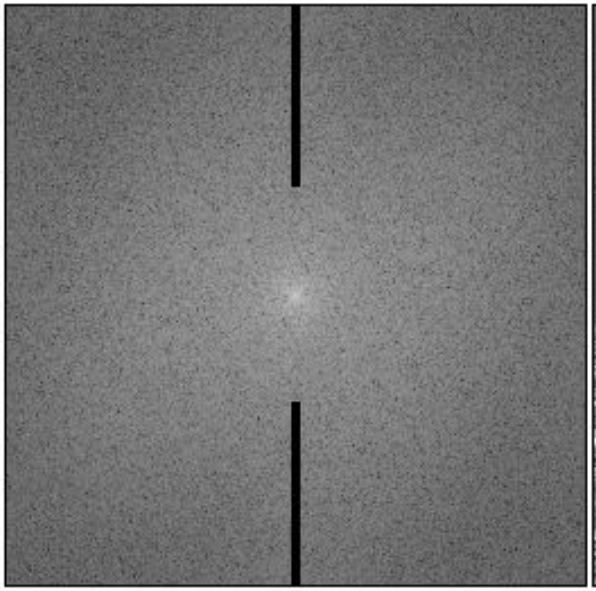




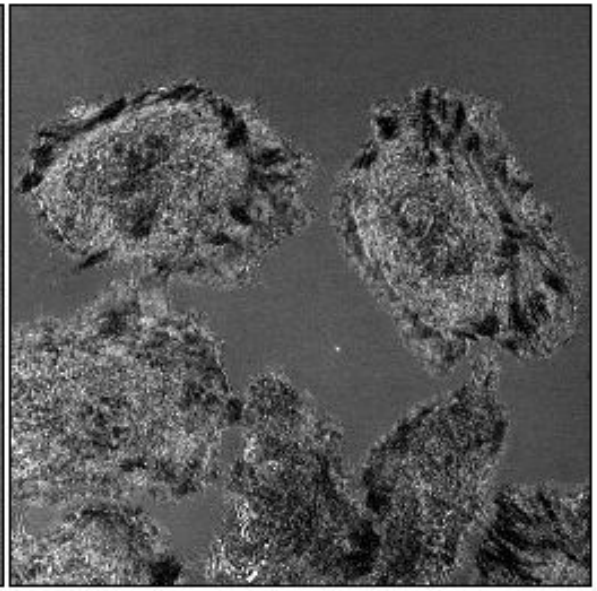
The original image. Reflectance mode of the confocal using the 458 nm line of an Ar laser. Note the horizontal lines.



The power spectrum calculated by ImageJ, contrast enhanced to show the bright spots that represent the X axis fluctuation.

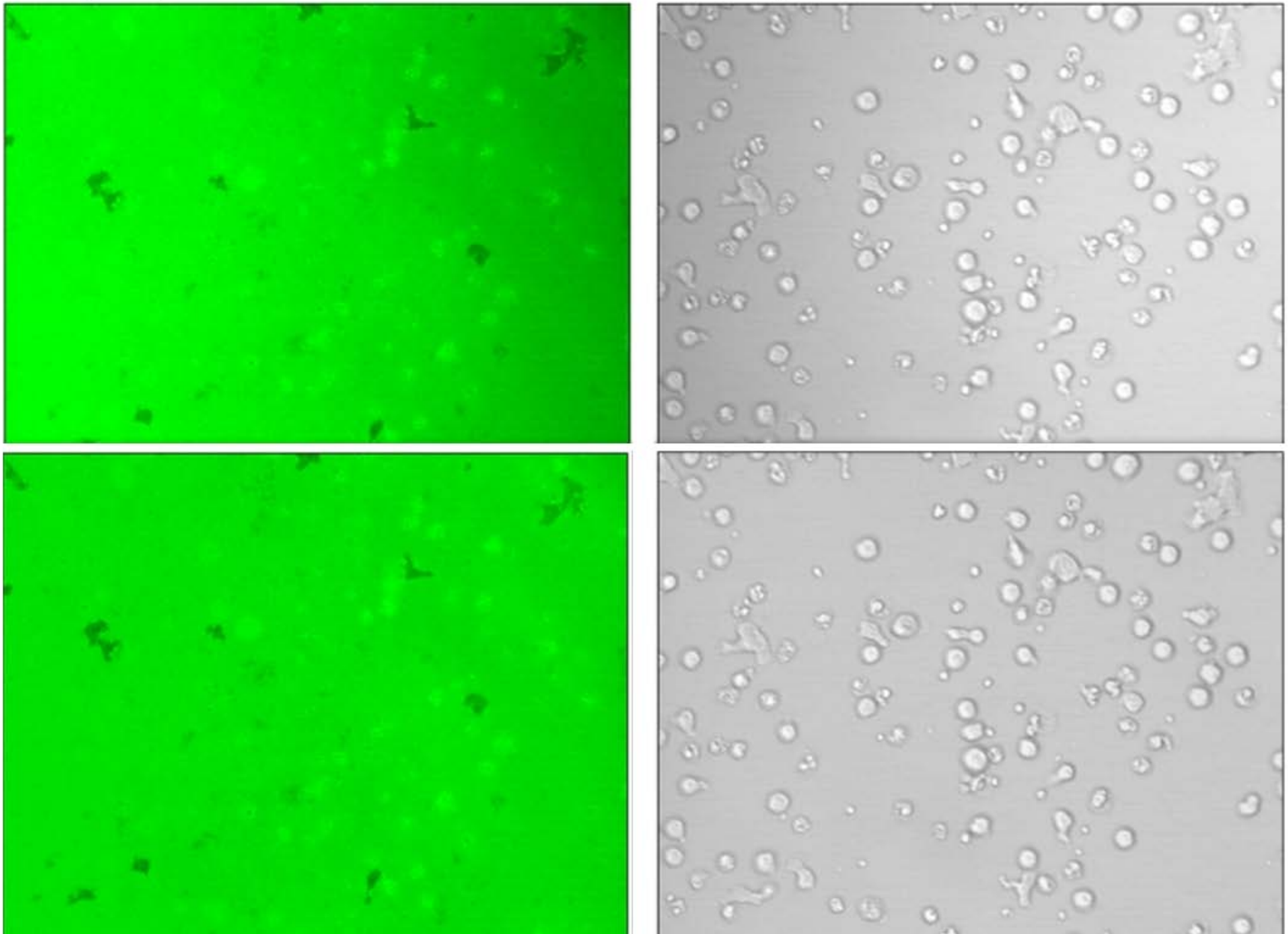


The power spectrum with masks drawn on it.

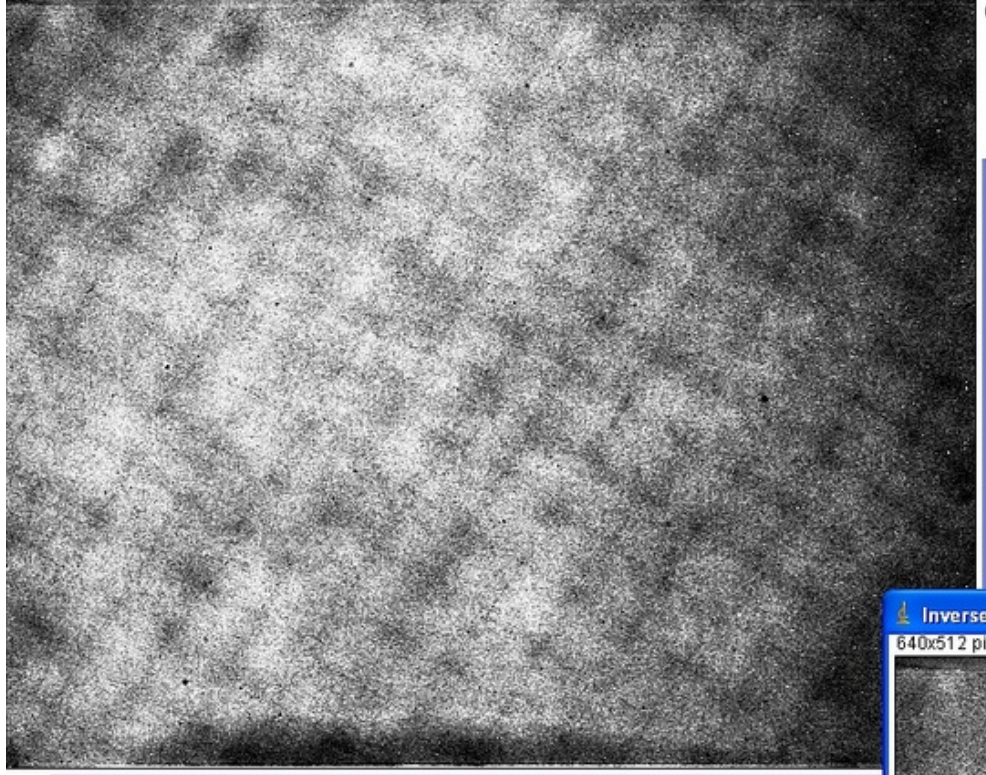


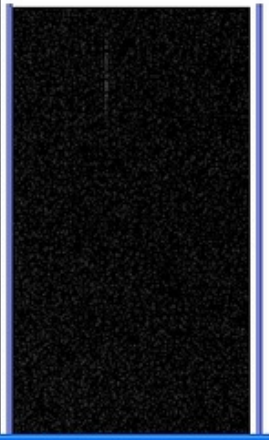
The inverse transform applying the masks.

Here is an example where the raw data have uneven illumination from left to right. The raw data are 512X512 pixels. By applying a high pass filter from 128 to 0 pixels, the low frequency variation is removed; the resulting bottom images are flatter.

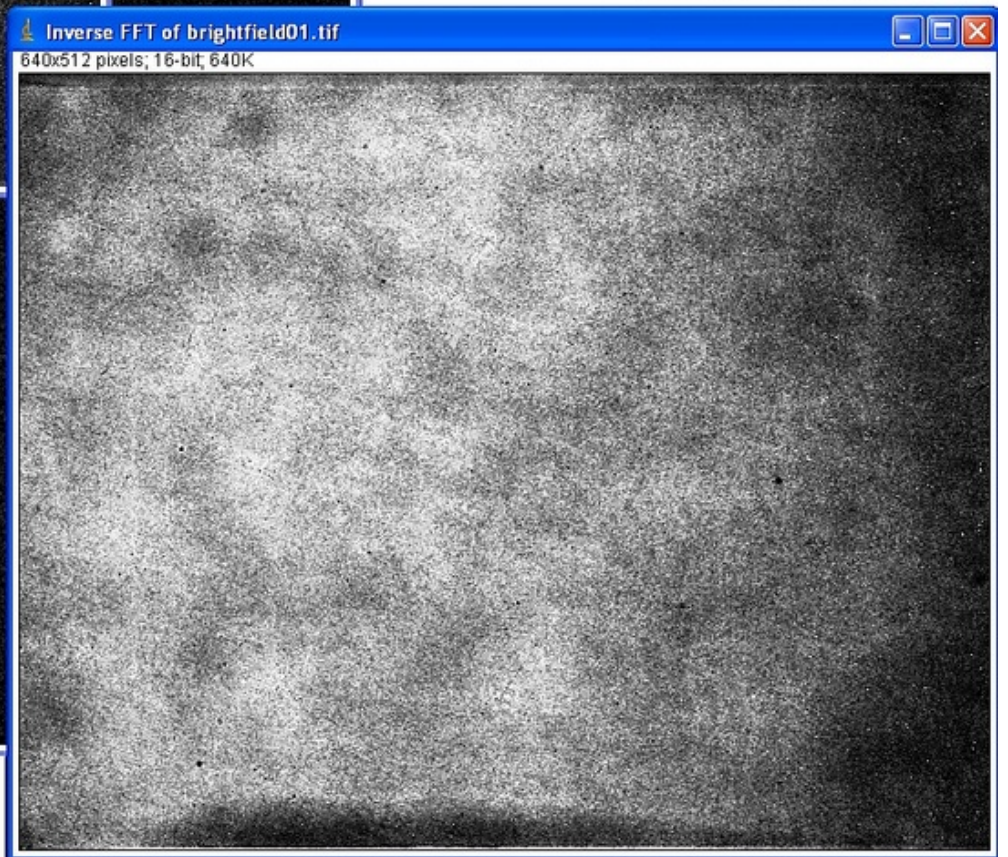


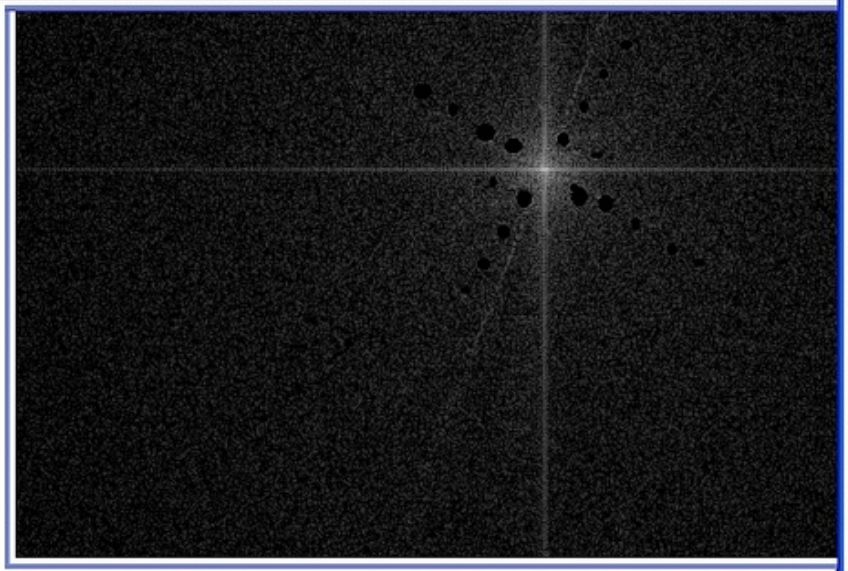
Original spinning disk image





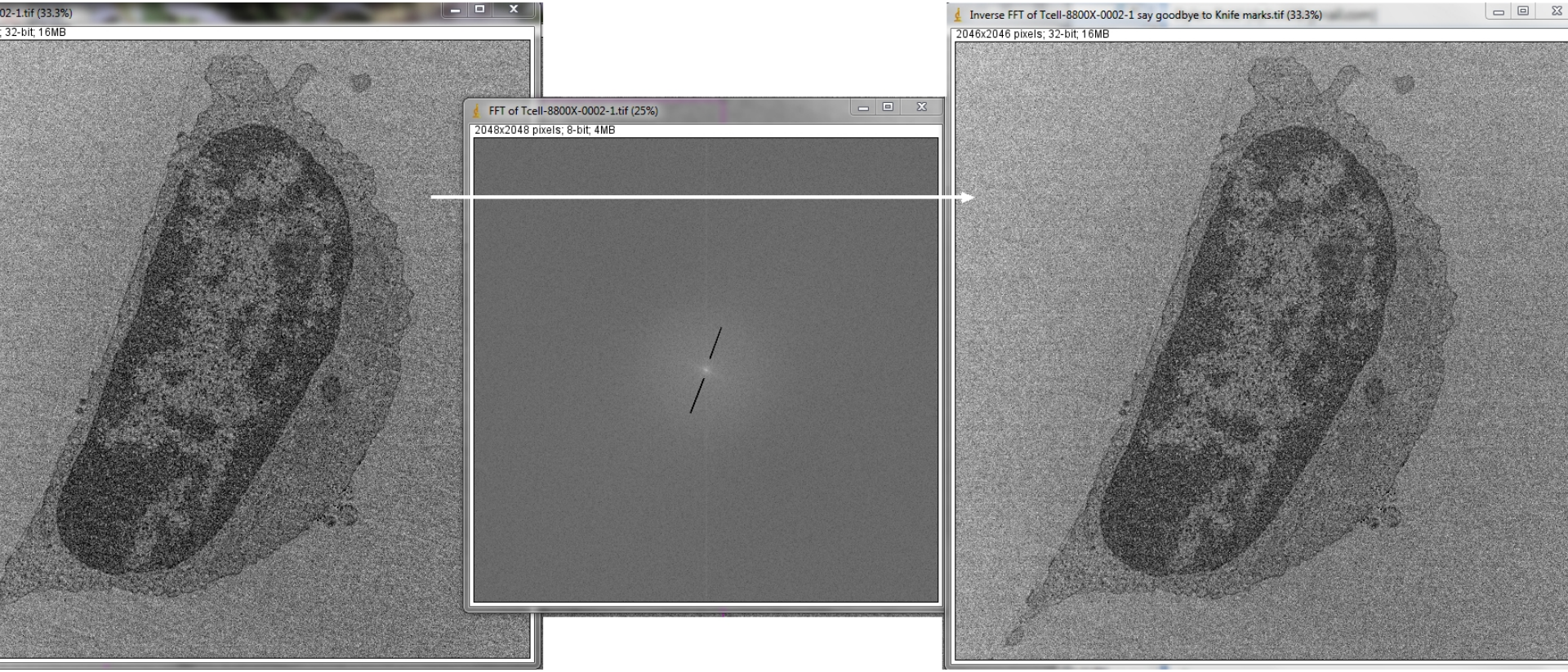
Inverse FFT with mask



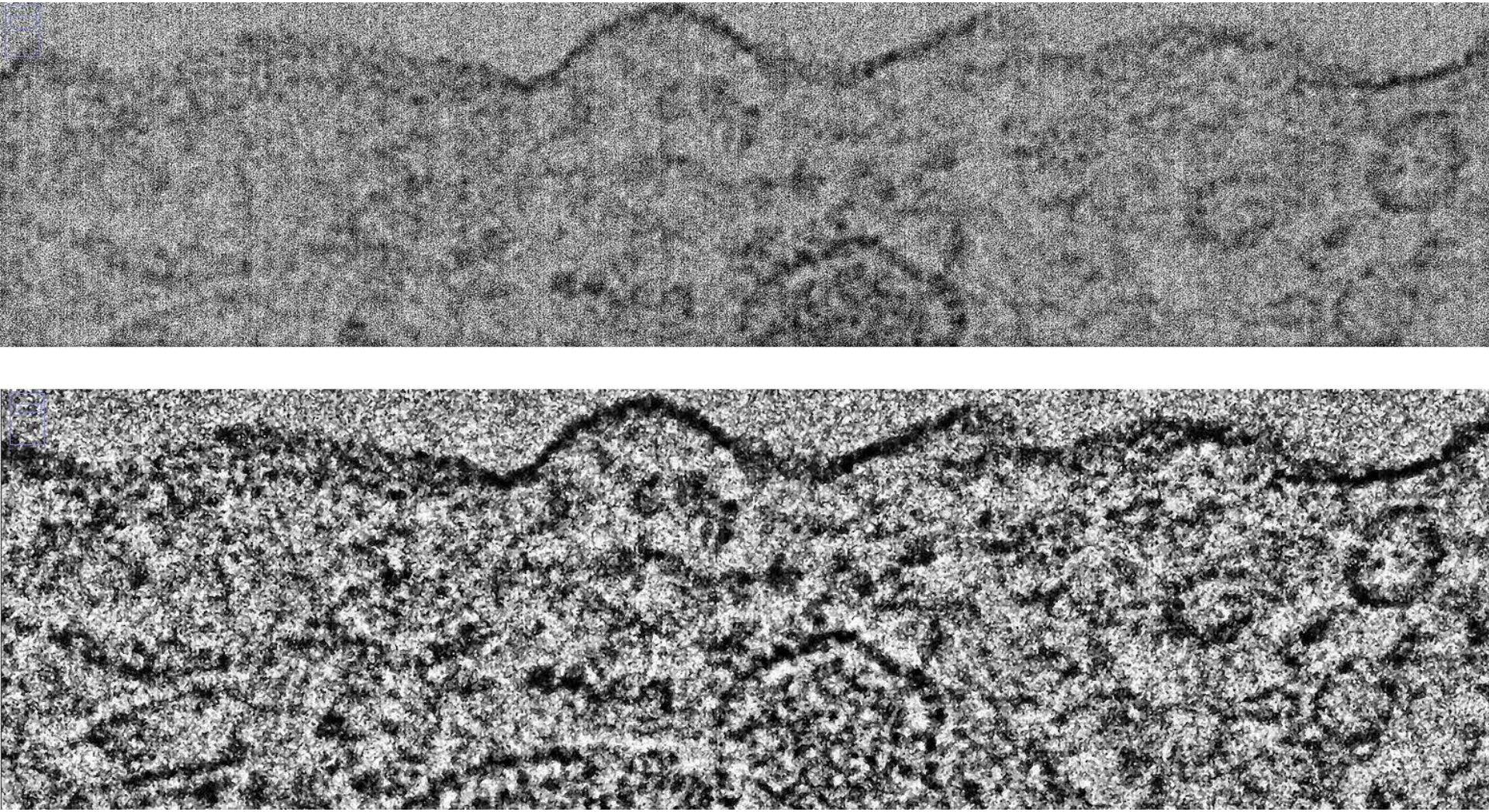


power spectrum with mask drawn in black

Transmission Electron Micrograph Knife Marks Minimized



Transmission Electron Micrograph Enhanced



Tcell-66000X-0009 fftcustom outliers1x1dark median1X1 CLAHE144-245-3.tif

Flatfield Correction

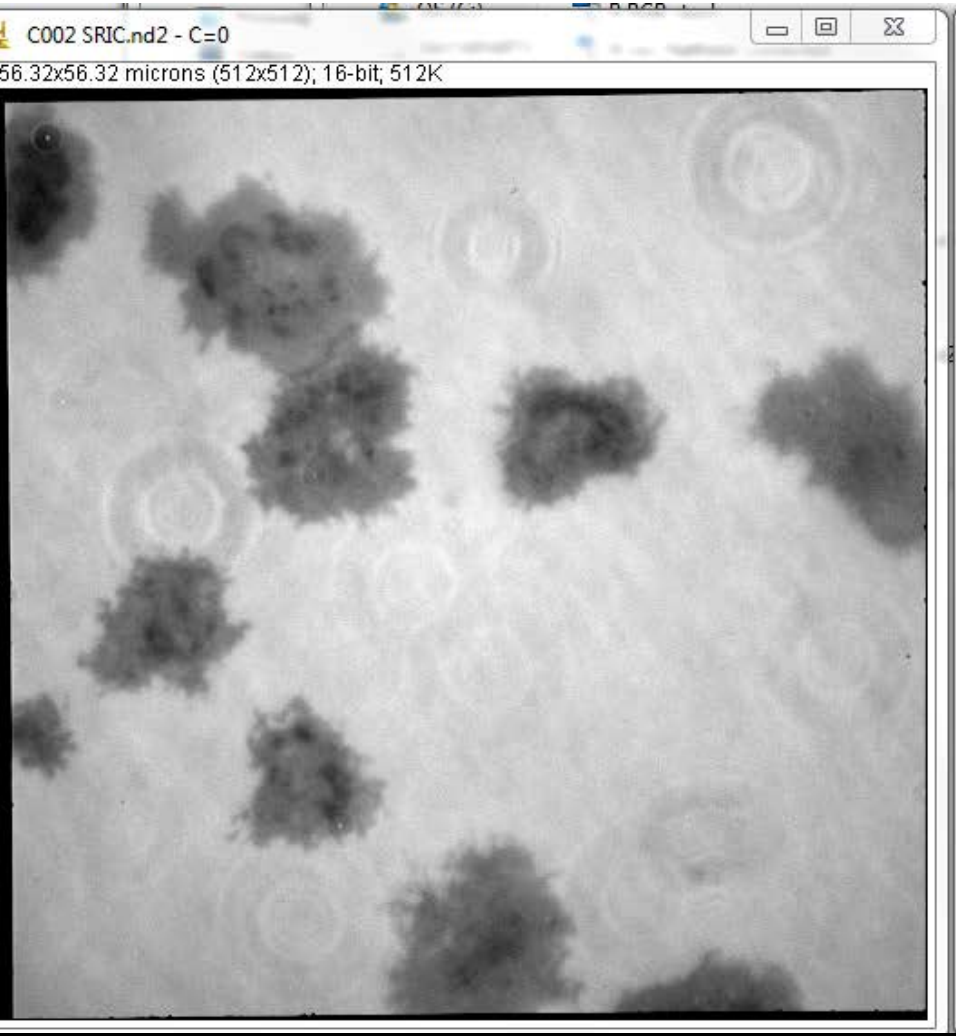
- What is it?
- Why do you want to use it?

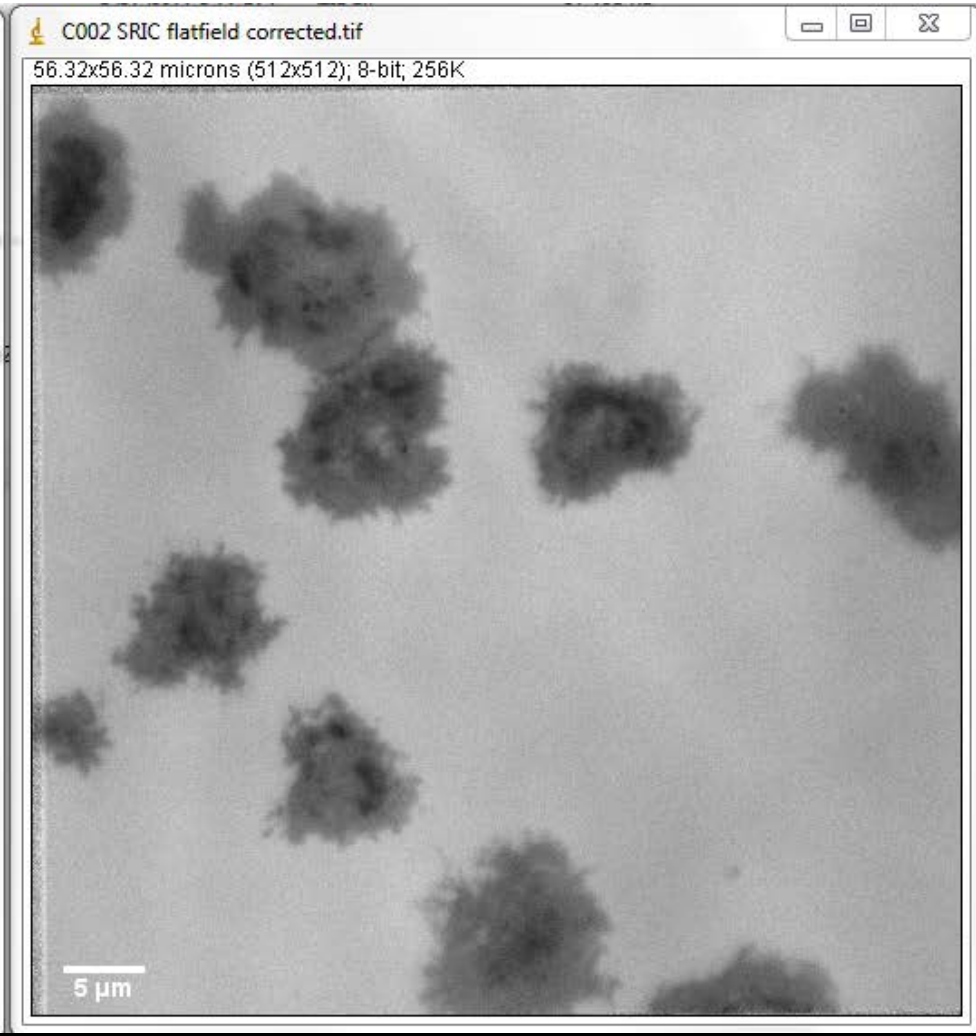
  Remove dirt in optics.
  Mask periodic noise.
  Consistent intensity measurements across field.
  Increase contrast.
- Examples & how to do it

  To remove dirt, divide by high frequency low noise image.
  To remove broad uneven lighting, divide by low frequency image.

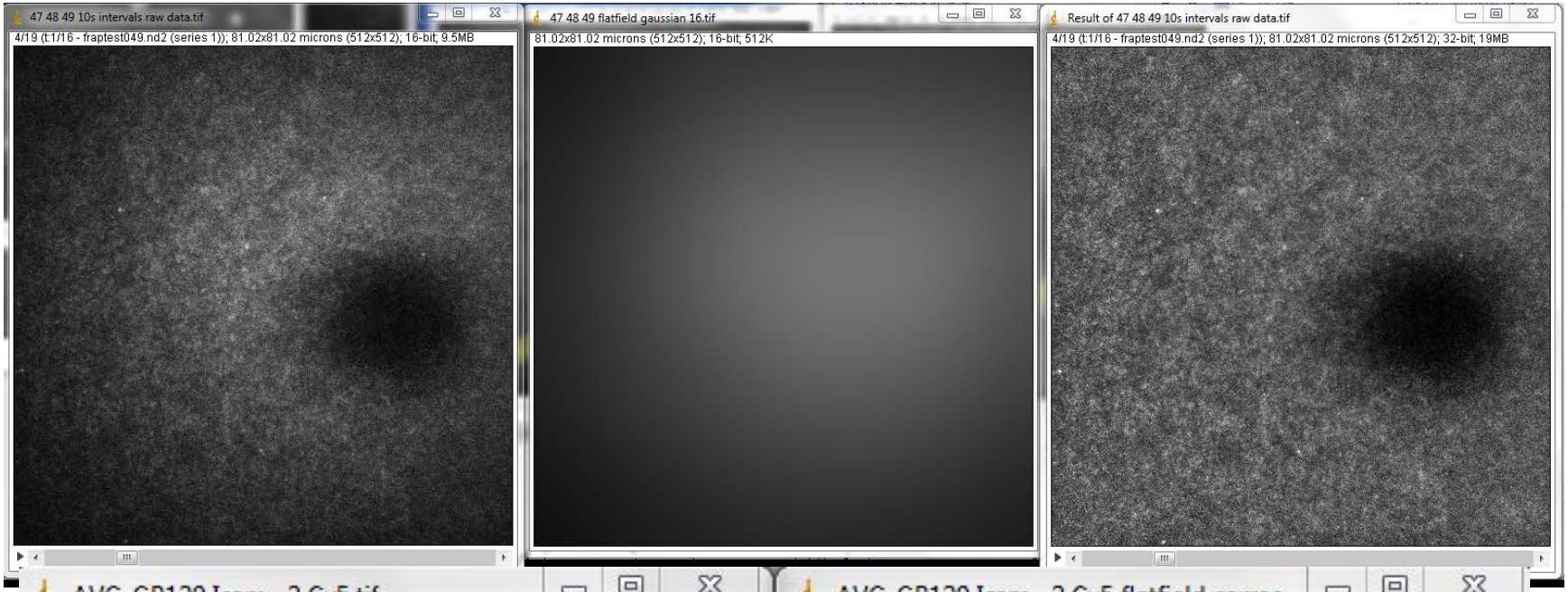
To remove fine detail, divide by low noise image.

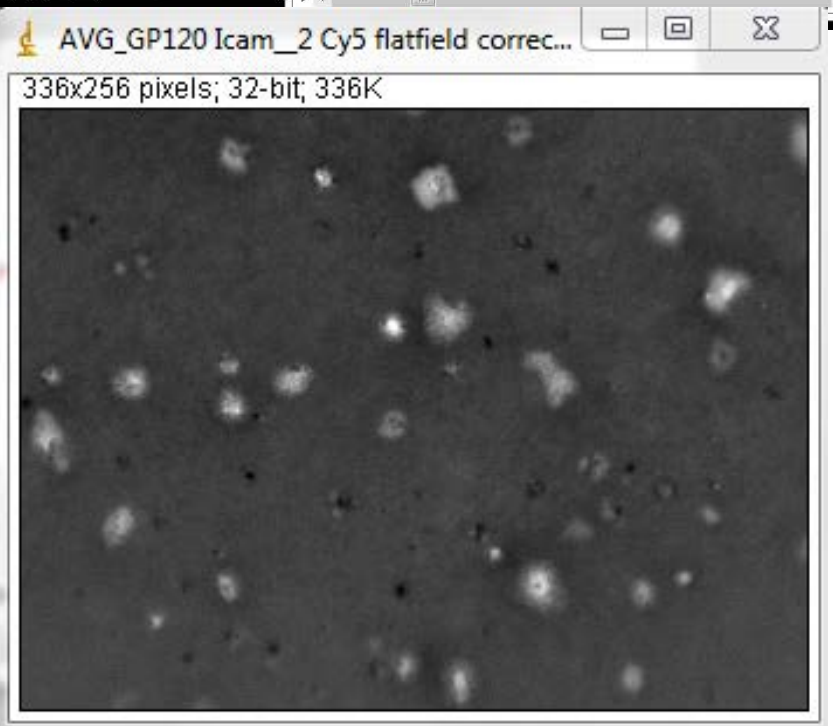
Example using real collected background image:





Examples using synthetic background image; divide by Gaussian blurred or median filtered image:



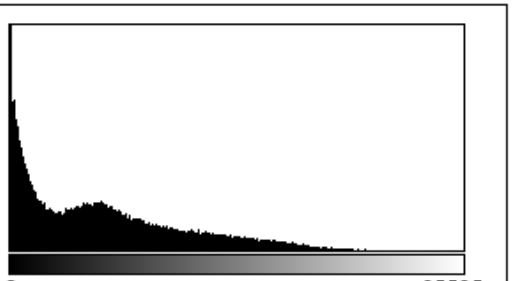


If measuring all the protein in a cell based on intensity, are confocal and high N.A. preferable? Explain.

An important but often overlooked concern is to avoid using programs that alter the image without the user being aware of it. For example, carefully correcting the colors in an image using Photoshop and then placing it in Powerpoint for presentation will cause changes even on the same computer screen (as well discarding pixels and reducing resolution if copy-and-paste is used for the transfer). In addition the image may appear different on another computer monitor, or when using a projector. Printing an image will also alter colors [.] … Placing images into a figure using Illustrator makes it all too easy to adjust the size or position of an image that will result in interpolation and alteration of pixel values (rotation is especially bad in this regard). Placing images into a web page for viewing can cause all of these problems at once. And using any lossy compression method such as jpeg will discard potentially important information that cannot be recovered.

From http://www.drjohnruss.com/downloads/ethics.pdf

Histogram of C1-TCRplus_8_SIR_ALX



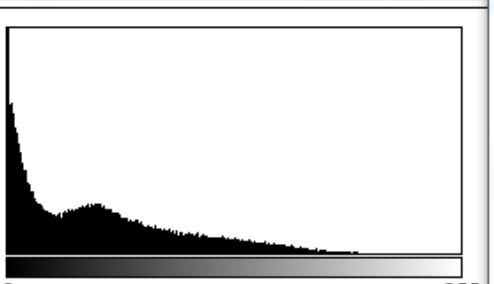
Count: 107124
Mean: 11161.573
StdDev: 12187.719
Bins: 256
Min: 0
Max: 65535
Mode: 0 (24355)
Bin Width: 255.996

List Copy Log Live

value=27135.586
count=335

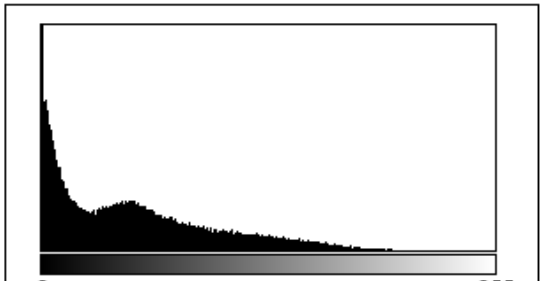
C1-TCRplus_
10/81 (c:1/2; z:

Histogram of Word



Count: 104229
Mean: 44.793
StdDev: 47.716
Min: 0
Max: 255
Mode: 0 (20680)

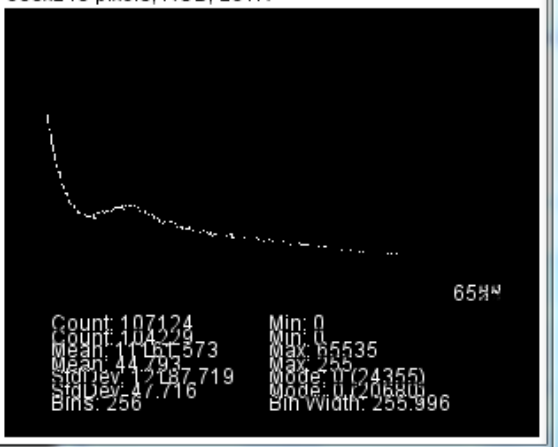
Histogram of PPT



Count: 104229
Mean: 44.793
StdDev: 47.716
Min: 0
Max: 255
Mode: 0 (20680)

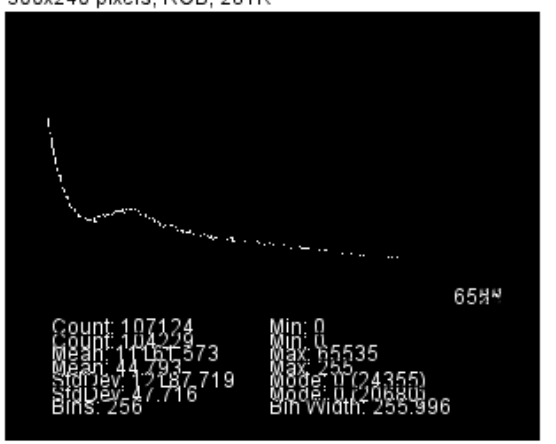
Result of Histogram of C1-TCRpl...

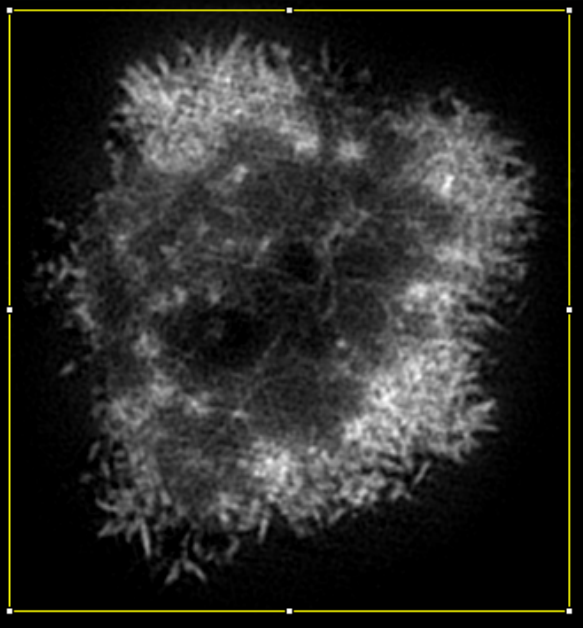
300x240 pixels; RGB; 281K



Result of Histogram of C1-TCRpl...

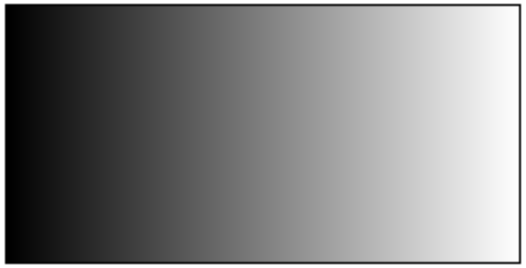
300x240 pixels; RGB; 281K



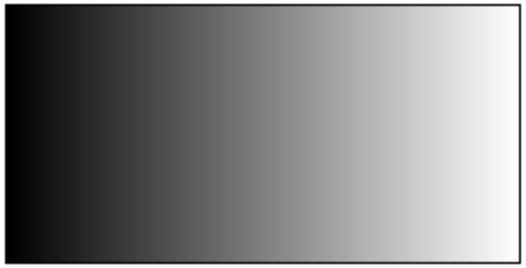


Every Video Card and Screen Have Their Own Biases

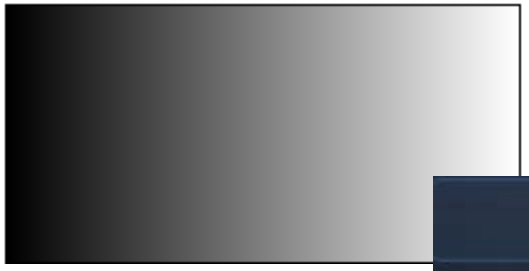
original



Original screen snapshot gamma 1.0

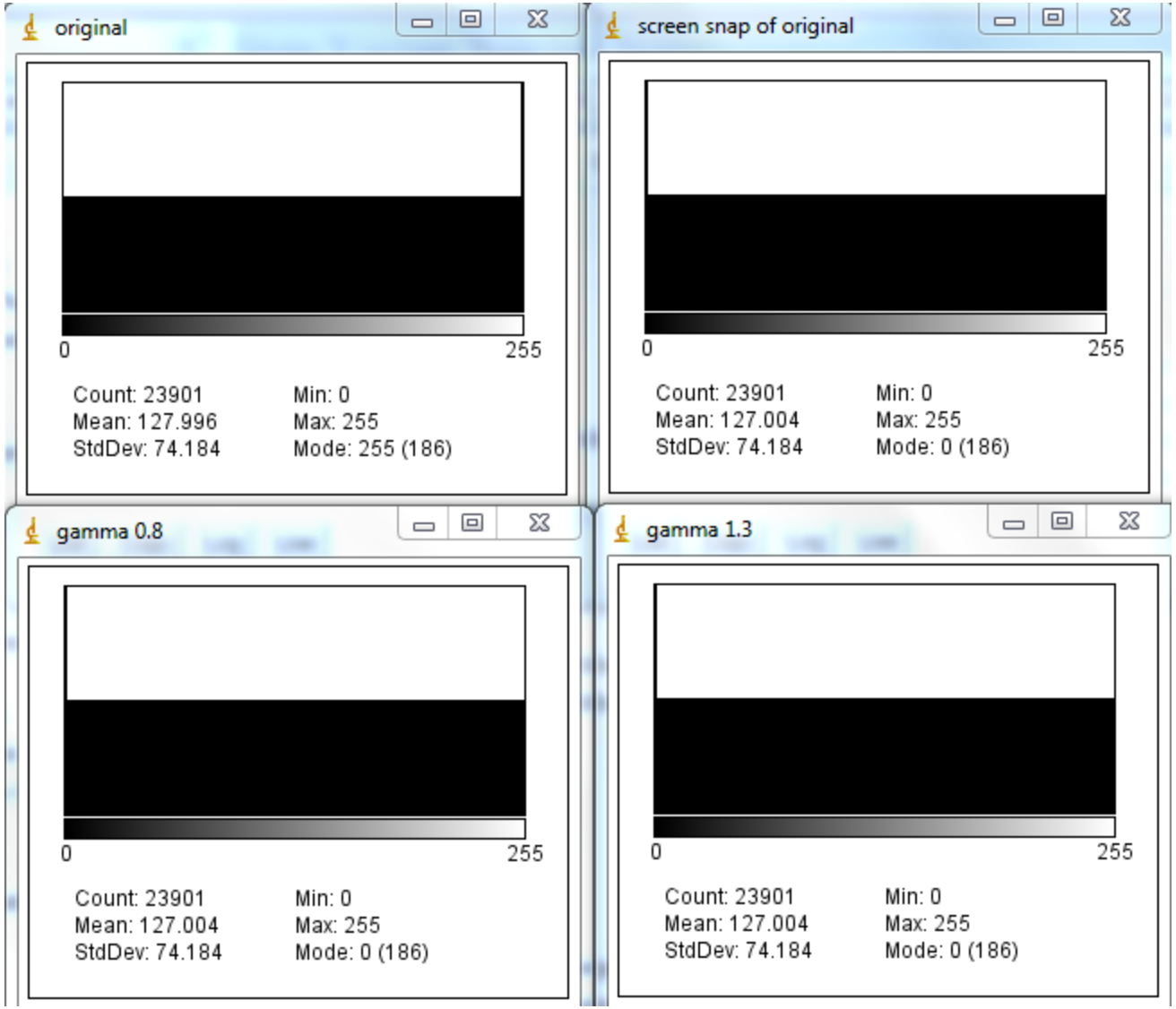


Gamma 0.8

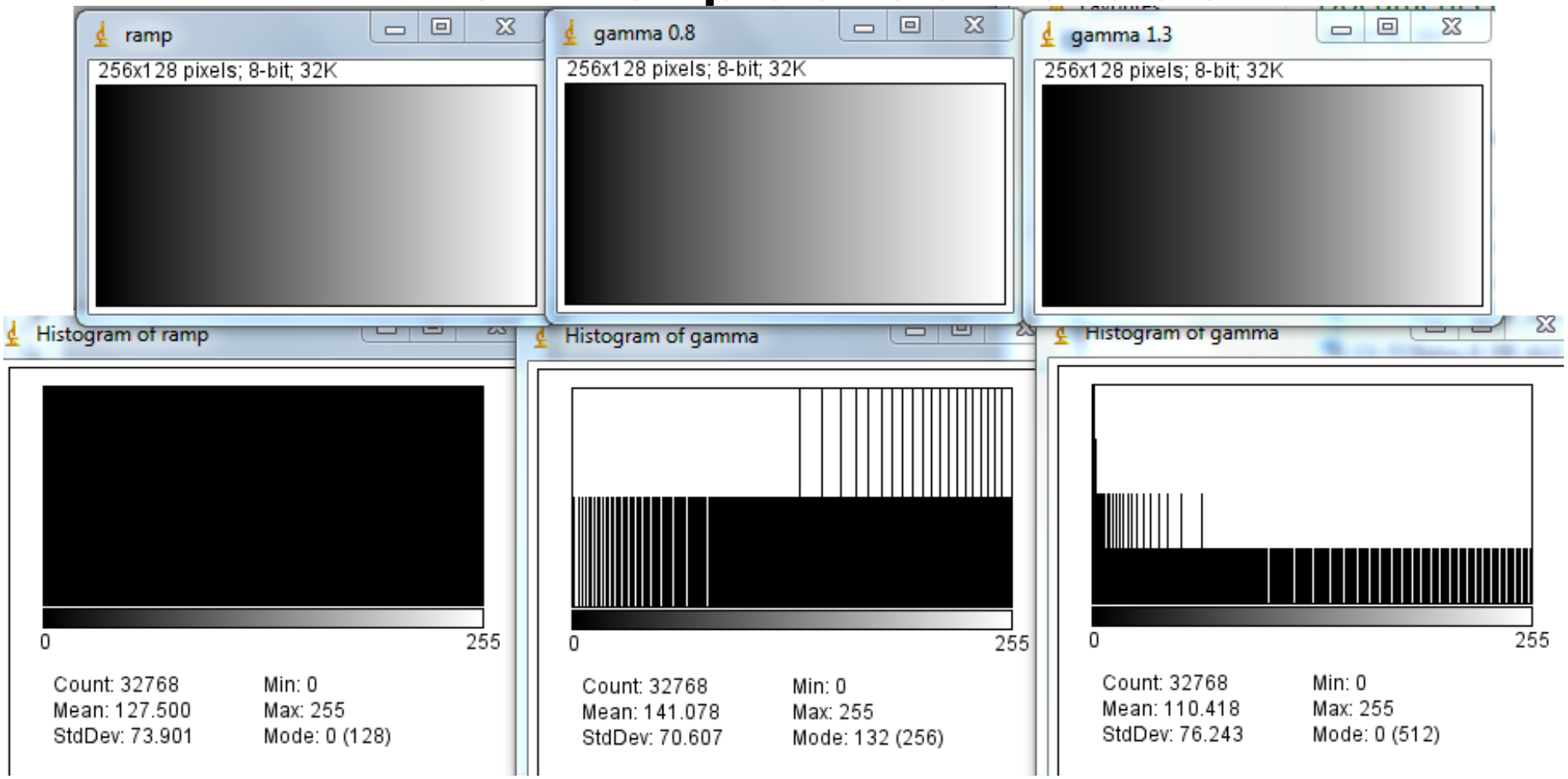


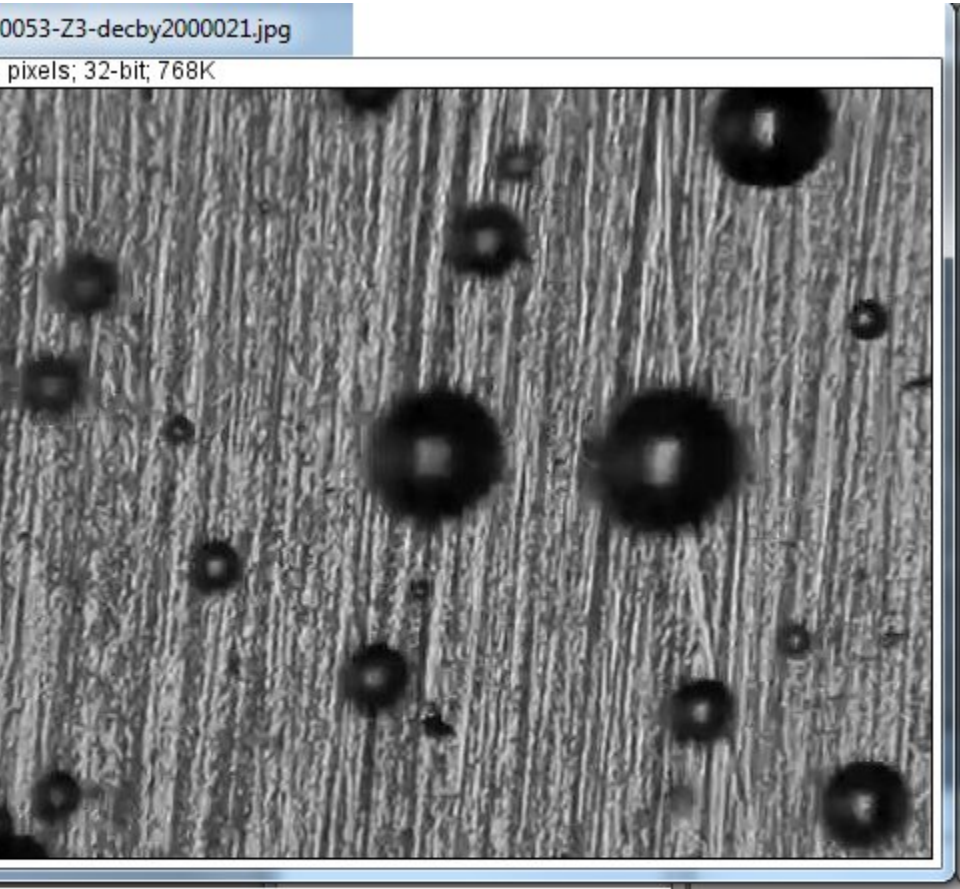
Gamma 1.3

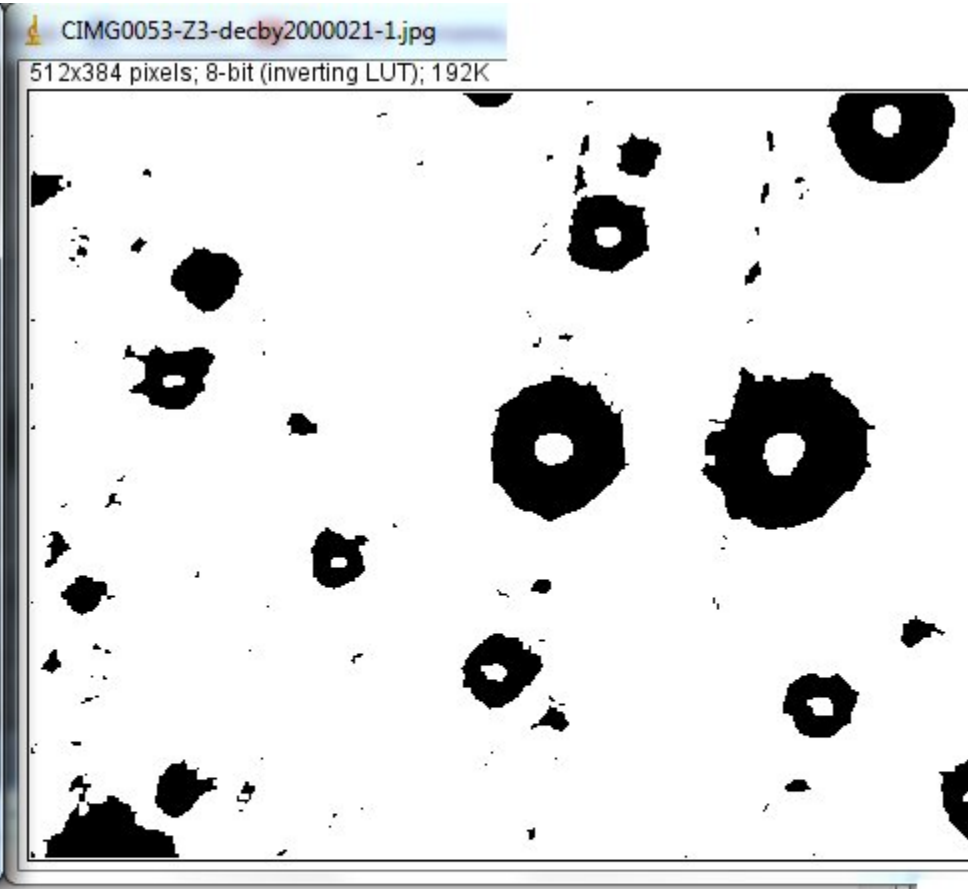
Screen snaps actually record raw data, not displayed LUT



What I expected to see





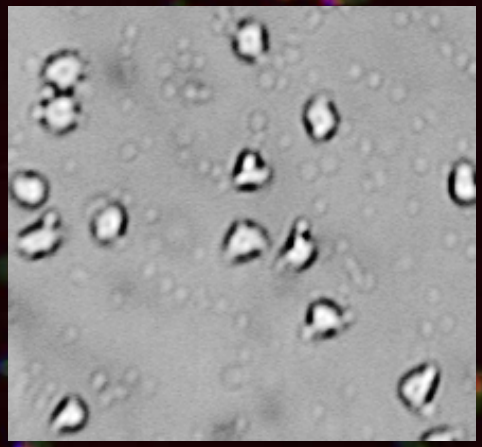



```
selectWindow("CIMG0053-Z3-decby2000021.jpg");
run("Duplicate...", "title=CIMG0053-Z3-decby2000021-1.jpg");
run("32-bit");
run("Median...", "radius=6");
run("South");
//run("Threshold...");
setAutoThreshold("Default");
setAutoThreshold("Default");
run("Convert to Mask");
run("Erode");
```

A few examples of ethical choices.

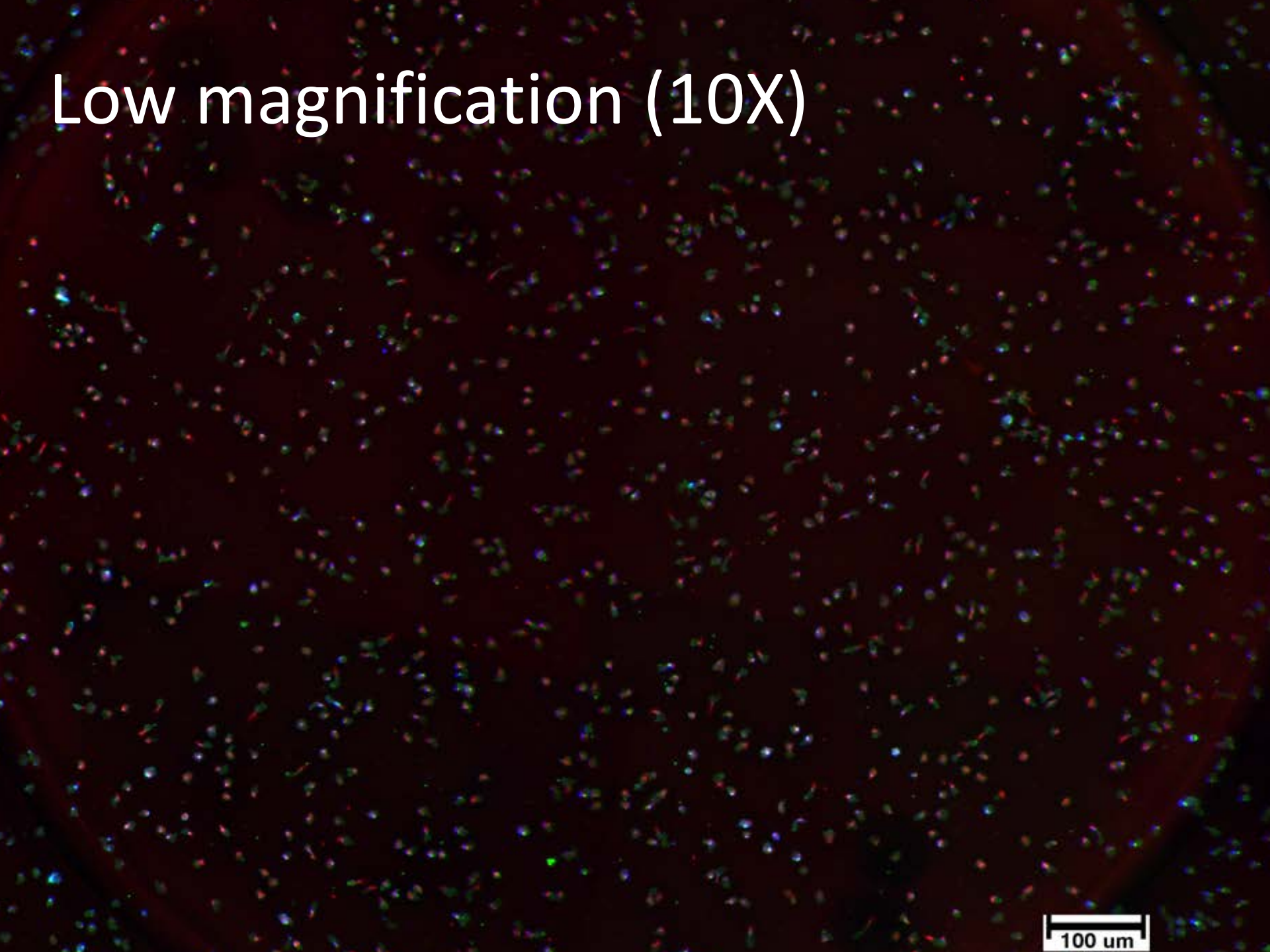
How to choose fields of cells fairly for quantification

Low magnification (10X)

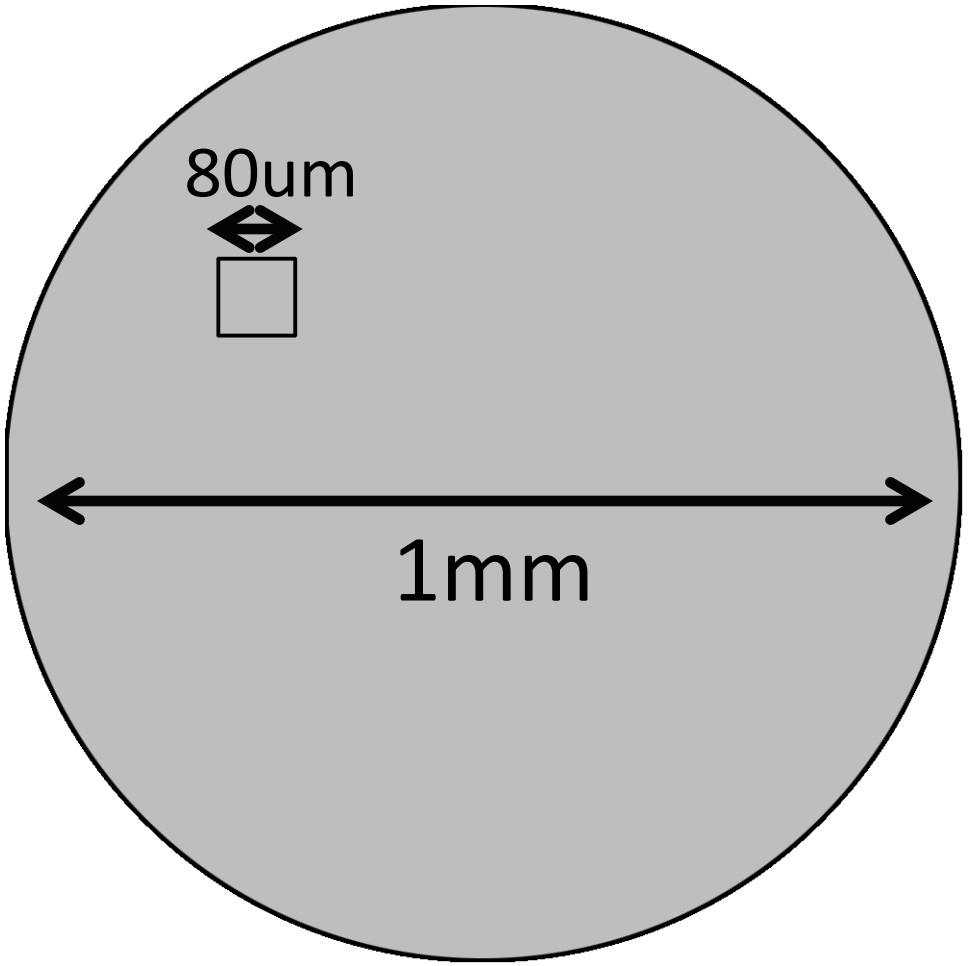


100 um

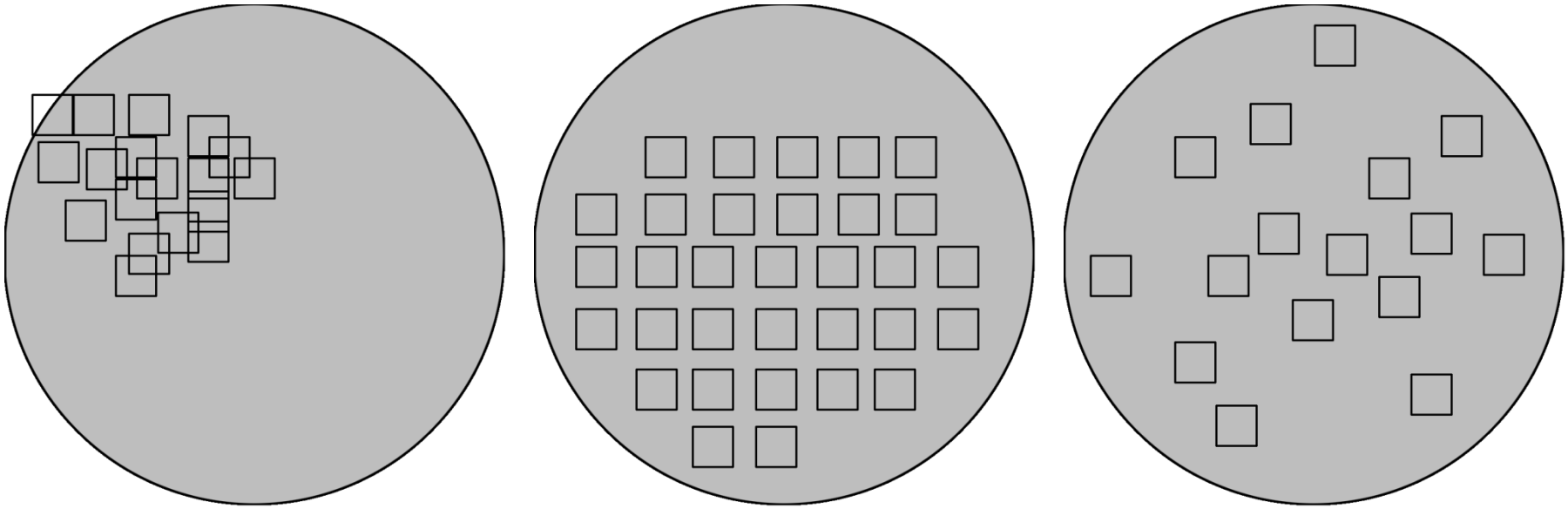
Low magnification (10X)



Imaging “representative” or random cells on a bilayer



Distribution of fields imaged

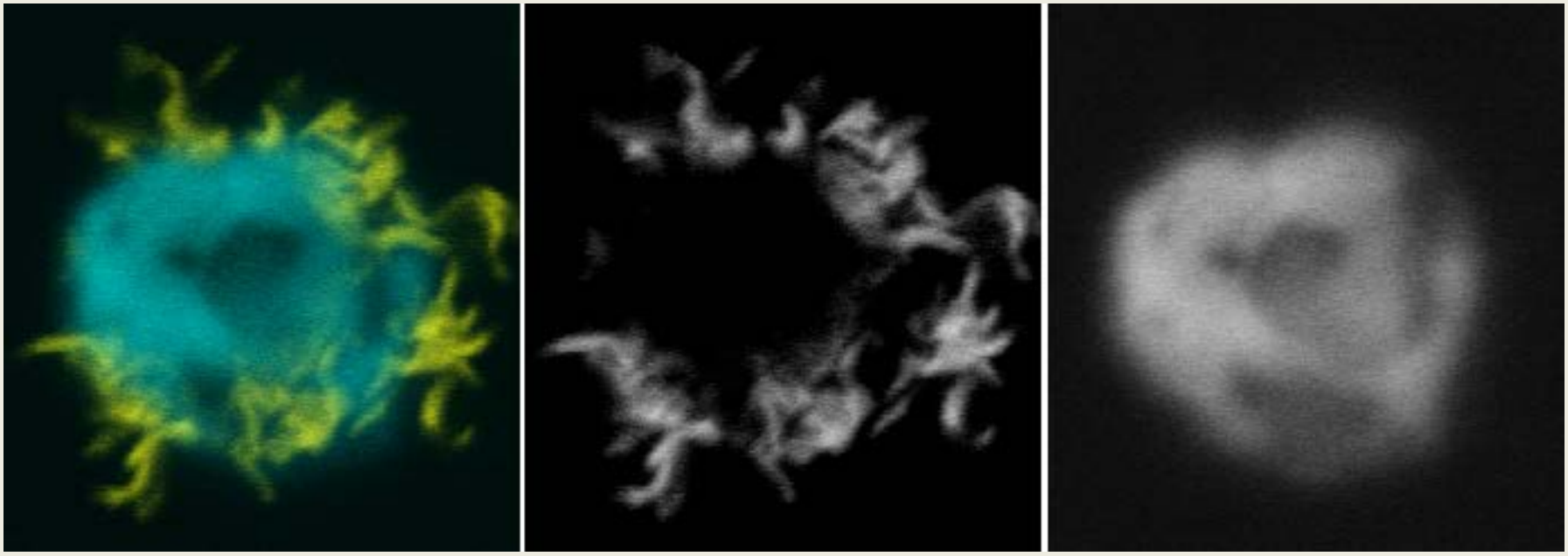


NO **YES** **YES**

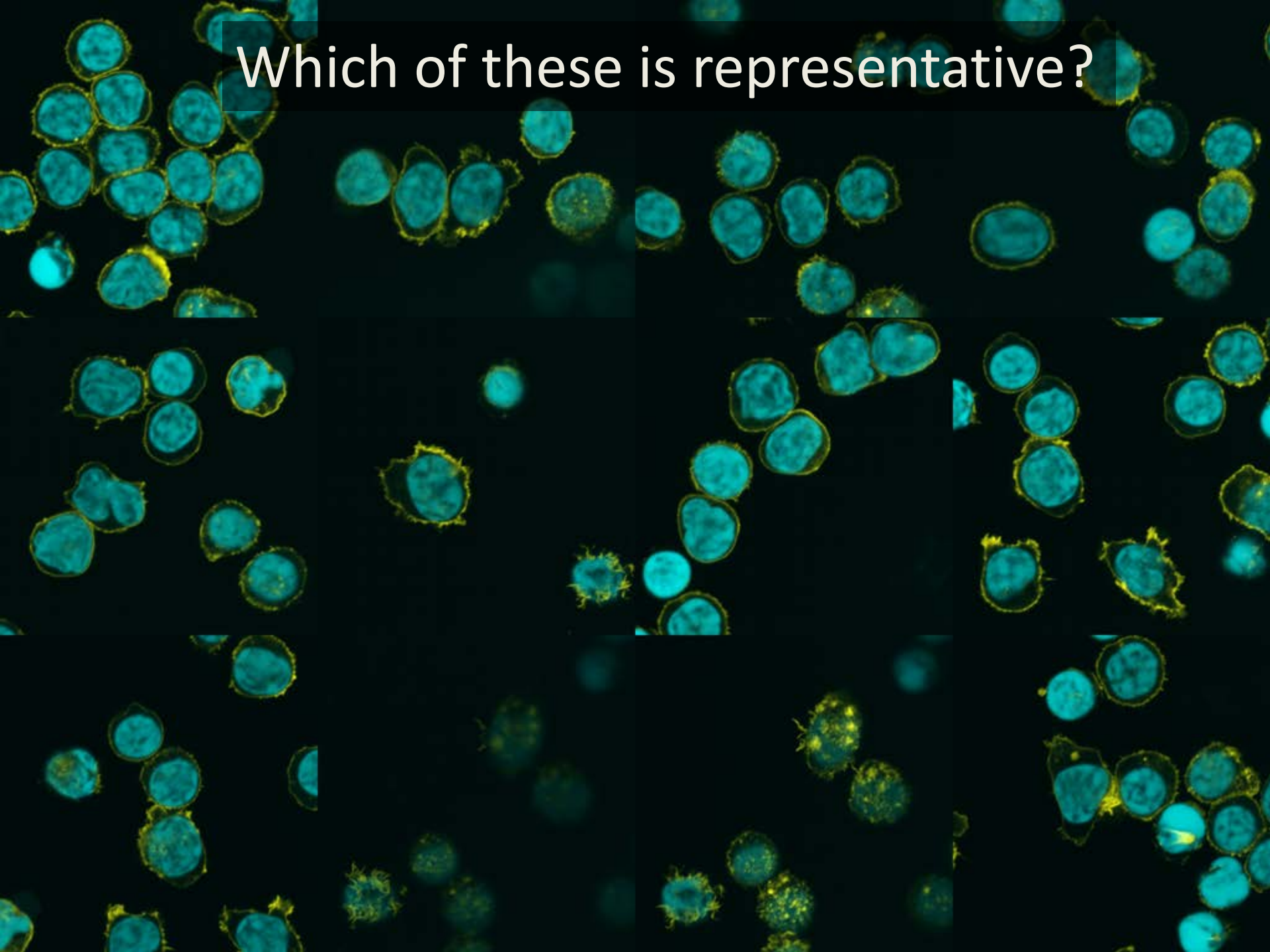
Showing the Typical Cell

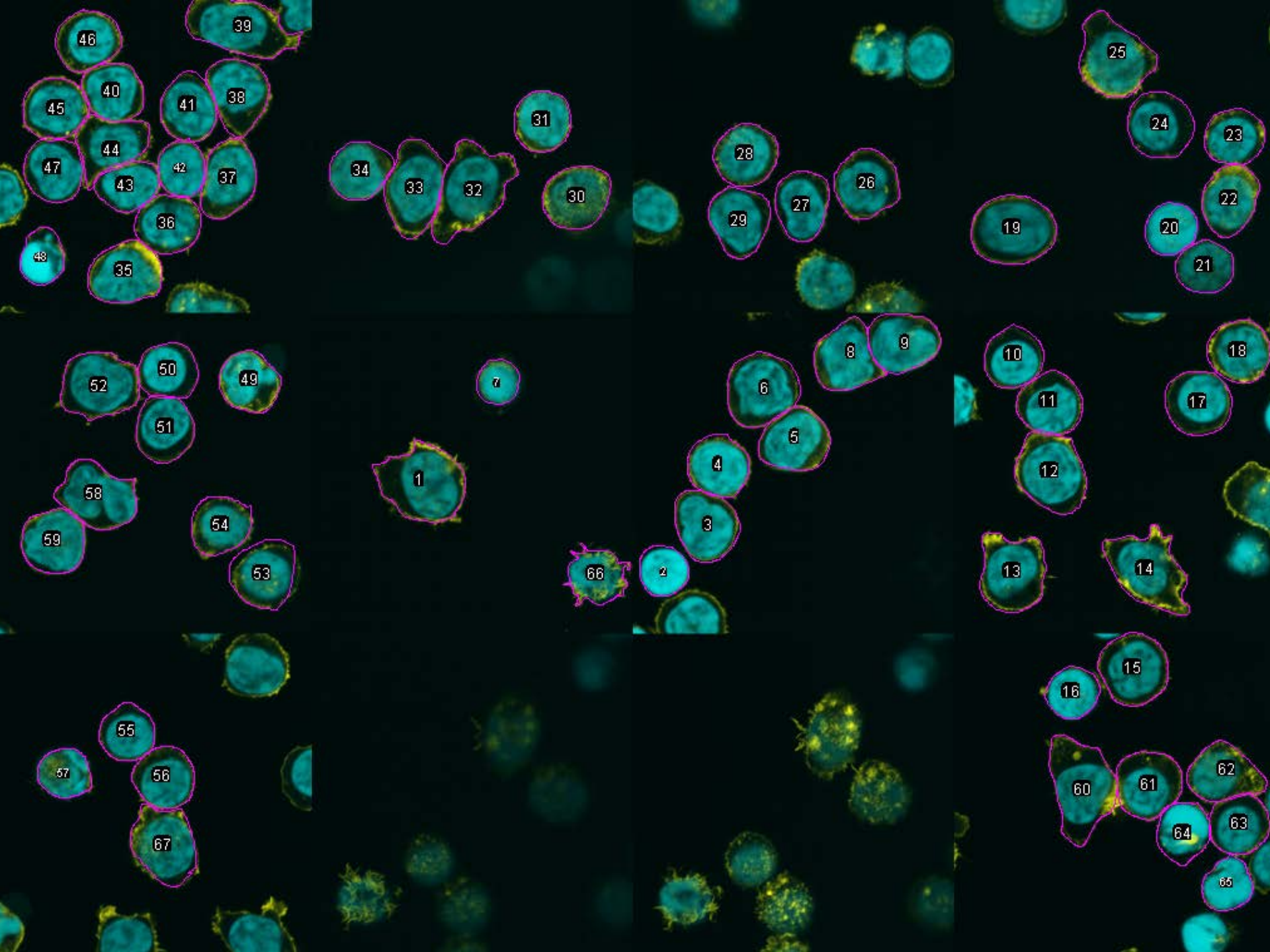
You have hundreds of pictures of cells but for the figure in your paper you are going to show one. How do you choose it?

Fig. 1 Showing this cell supports hypothesis that cells are small & have a lot of ruffles after treatment.



Which of these is representative?

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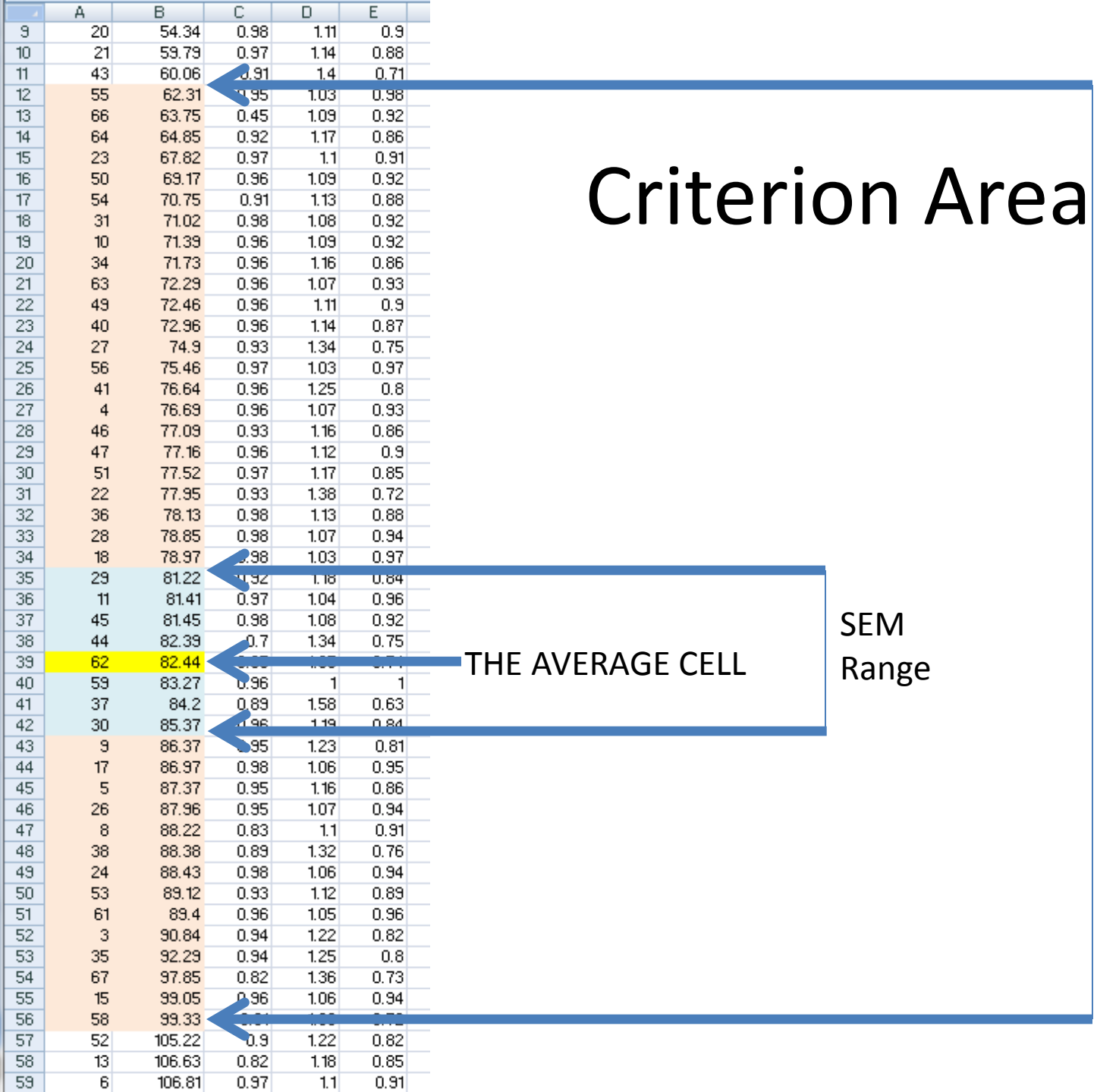
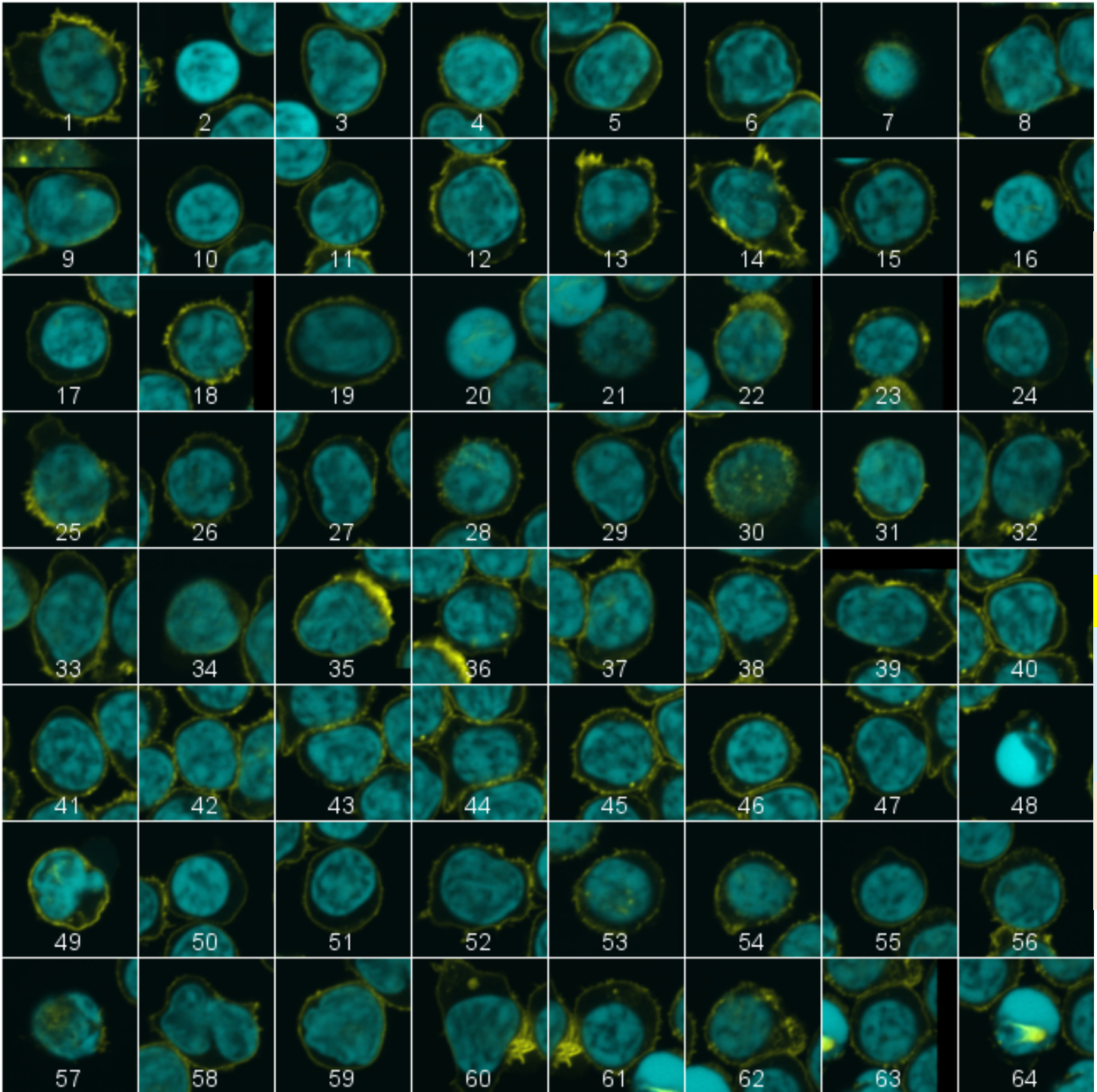
	A	B	C	D	E	F	G	H	I
1		Area (um^2)	Circ.	AR	Round			Average	
2	1	129.04	0.83	1.21	0.83		Area um^2	**82.52**	
3	2	49.97	0.97	1.06	0.94		stdev	20.50	
4	3	90.84	0.94	1.22	0.82		n=	67	
5	4	76.69	0.96	1.07	0.93		SEM	2.50	
6	5	87.37	0.95	1.16	0.86				
7	6	106.81	0.97	1.1	0.91				
8	7	38.17	0.98	1.08	0.92				
9	8	88.22	0.83	1.1	0.91				
10	9	86.37	0.95	1.23	0.81				
11	10	71.39	0.96	1.09	0.92				
12	11	81.41	0.97	1.04	0.96				
13	12	110.95	0.91	1.34	0.75				
14	13	106.63	0.82	1.18	0.85				
15	14	116.23	0.63	1.56	0.64				
16	15	99.05	0.96	1.06	0.94				
17	16	53.68	0.97	1.08	0.93				
18	17	86.97	0.98	1.06	0.95				
19	18	78.97	0.98	1.03	0.97				
20	19	118.18	0.97	1.24	0.81				
21	20	54.34	0.98	1.11	0.9				
22	21	59.79	0.97	1.14	0.88				
23	22	77.95	0.93	1.38	0.72				
24	23	67.82	0.97	1.1	0.91				
25	24	88.43	0.98	1.06	0.94				
26	25	113.08	0.85	1.24	0.81				
27	26	87.96	0.95	1.07	0.94				
28	27	74.9	0.93	1.34	0.75				
29	28	78.85	0.98	1.07	0.94				

Criterion Area

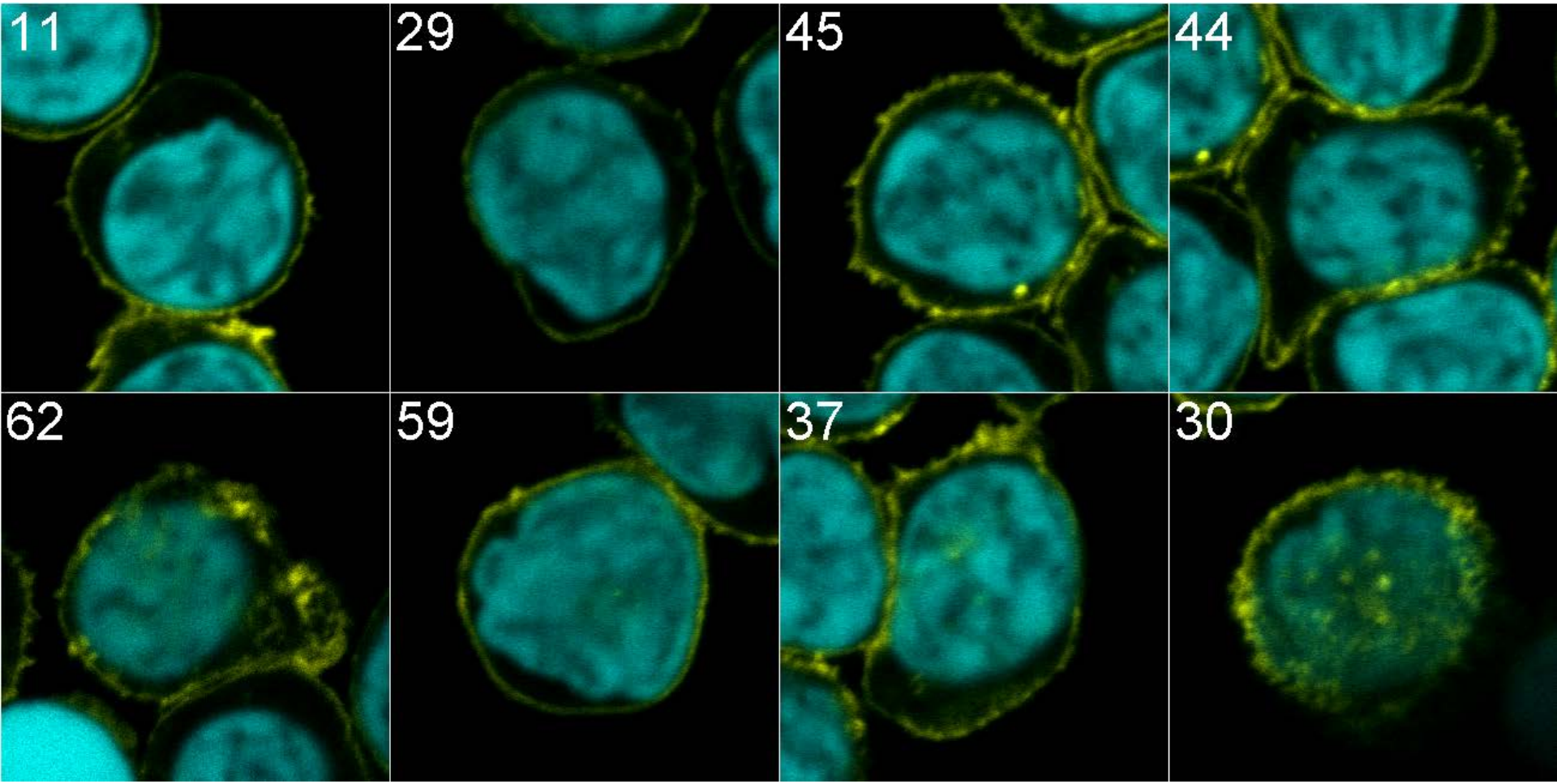


	A	B	C	D	E
9	20	54.34	0.98	1.11	0.9
10	21	59.79	0.97	1.14	0.88
11	43	60.06	0.91	1.4	0.71
12	55	62.31	0.95	1.03	0.98
13	66	63.75	0.45	1.09	0.92
14	64	64.85	0.92	1.17	0.86
15	23	67.82	0.97	1.1	0.91
16	50	69.17	0.96	1.09	0.92
17	54	70.75	0.91	1.13	0.88
18	31	71.02	0.98	1.08	0.92
19	10	71.39	0.96	1.09	0.92
20	34	71.73	0.96	1.16	0.86
21	63	72.29	0.96	1.07	0.93
22	49	72.46	0.96	1.11	0.9
23	40	72.96	0.96	1.14	0.87
24	27	74.9	0.93	1.34	0.75
25	56	75.46	0.97	1.03	0.97
26	41	76.64	0.96	1.25	0.8
27	4	76.69	0.96	1.07	0.93
28	46	77.09	0.93	1.16	0.86
29	47	77.16	0.96	1.12	0.9
30	51	77.52	0.97	1.17	0.85
31	22	77.95	0.93	1.38	0.72
32	36	78.13	0.98	1.13	0.88
33	28	78.85	0.98	1.07	0.94
34	18	78.97	0.98	1.03	0.97
35	29	81.22	0.92	1.18	0.84
36	11	81.41	0.97	1.04	0.96
37	45	81.45	0.98	1.08	0.92
38	44	82.39	0.7	1.34	0.75
39	62	82.44	0.96	1	1
40	59	83.27	0.96	1	1
41	37	84.2	0.89	1.58	0.63
42	30	85.37	0.96	1.19	0.84
43	9	86.37	0.95	1.23	0.81
44	17	86.97	0.98	1.06	0.95
45	5	87.37	0.95	1.16	0.86
46	26	87.96	0.95	1.07	0.94
47	8	88.22	0.83	1.1	0.91
48	38	88.38	0.89	1.32	0.76
49	24	88.43	0.98	1.06	0.94
50	53	89.12	0.93	1.12	0.89
51	61	89.4	0.96	1.05	0.96
52	3	90.84	0.94	1.22	0.82
53	35	92.29	0.94	1.25	0.8
54	67	97.85	0.82	1.36	0.73
55	15	99.05	0.96	1.06	0.94
56	58	99.33	0.91	1.09	0.92
57	52	105.22	0.9	1.22	0.82
58	13	106.63	0.82	1.18	0.85
59	6	106.81	0.97	1.1	0.91



36	78.13
28	78.85
18	78.97
29	81.22
11	81.41
45	81.45
44	82.39
62	82.44
59	83.27
37	84.2
30	85.37
9	86.37
17	86.97

Cells that are within SEM of AVG area



- **ImageJ Interest Group [IMAGEJ@LIST.NIH.GOV]** on behalf of **Rasband, Wayne (NIH/NIMH) [E] [rasbandw@MAIL.NIH.GOV]**
- **Subject:** Count negative pixels in a stack
- **To:** IMAGEJ@LIST.NIH.GOV
-
- Saturday, August 17, 2013 12:00 AM
- On Aug 16, 2013, at 10:48 AM, sreeyuth wrote:

> Hi,
>
> I have around 440 images in my stack. From each image, I want to get the
> total number of pixels having negative values. So in the end I expect a list
> which has just 2 columns, Slice Number and No. of pixels having negative
> values in that slice.

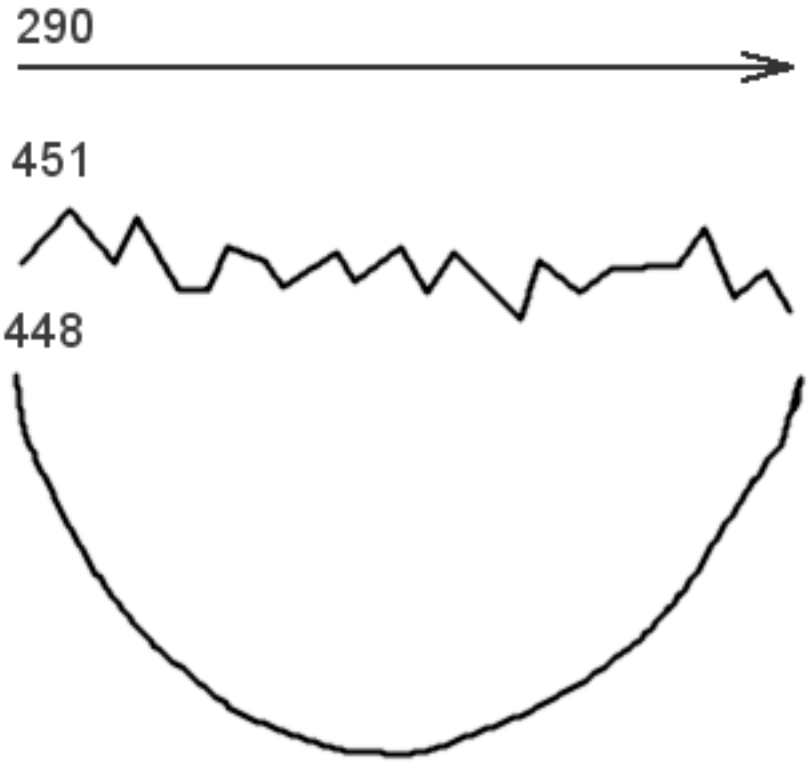
Here is a macro that does this:

```
saveSettings;
run("Clear Results");
run("Set Scale...", "distance=0 known=0");
setThreshold(-99999, -0.00001);
run("Set Measurements...", "area limit");
for (n=1; n<=nSlices; n++) {
  setSlice(n);
  run("Measure");
}
restoreSettings;
```

-wayne

Why to ask the ImageJ listserv for help

Netpath/totalpath may not tell characteristics of movement



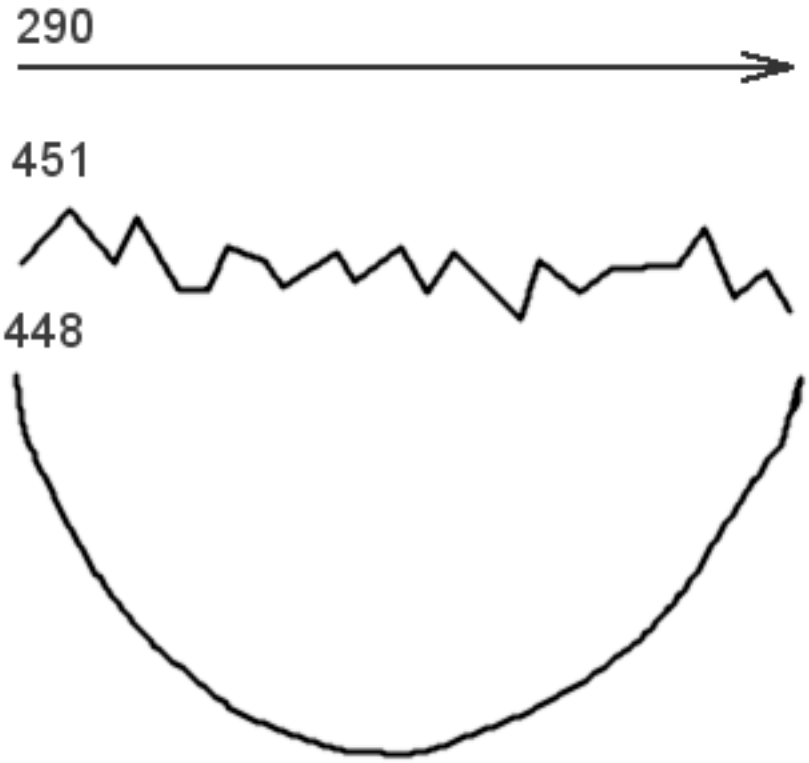
netpath = 290 totalpath = 290

netpath = 290 totalpath = 450

netpath = 290 totalpath = 450

Ratio 0.64

Netpath/totalpath may not tell characteristics of movement



netpath = 290 totalpath = 290

netpath = 290 totalpath = 450

netpath = 290 totalpath = 450

Ratio 0.64

Also, irregular velocity vs. constant speed…

The Size Of The Ruler Problem

Message: The spatial and temporal resolution changes the answer. Choose magnification, time intervals, and duration of experiment appropriate for your questions. Be careful comparing to others' results.

For example:

To see ruffling, high mag, high speed, short duration.

To see synapse formation and release, low mag, long intervals, long duration.