

Quantitative Digital Image Analysis

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Outline

- Overview of digital image analysis
- Application to single cells using microscopy
- Examples using fixed cells and tissues
 - ♦ human histopathology, model organisms, ex vivo cultures of isolated cells
 - ♦ multiplexing (CyclF/MxIF)
- Live-cell imaging of cellular dynamics: Ca^{2+} flux, kinase activity
- Live demo of FIJI



Quantifying Features From Images

- *How many types of objects?*
- *How many objects of each type?*
- *What are the distinguishing features of each type?*
- *What information do you think a computer should use to count and classify each object? (segmentation)*
- *Do the identified boundaries define the object size? (2D vs 3D)*



What if you obtained new data?

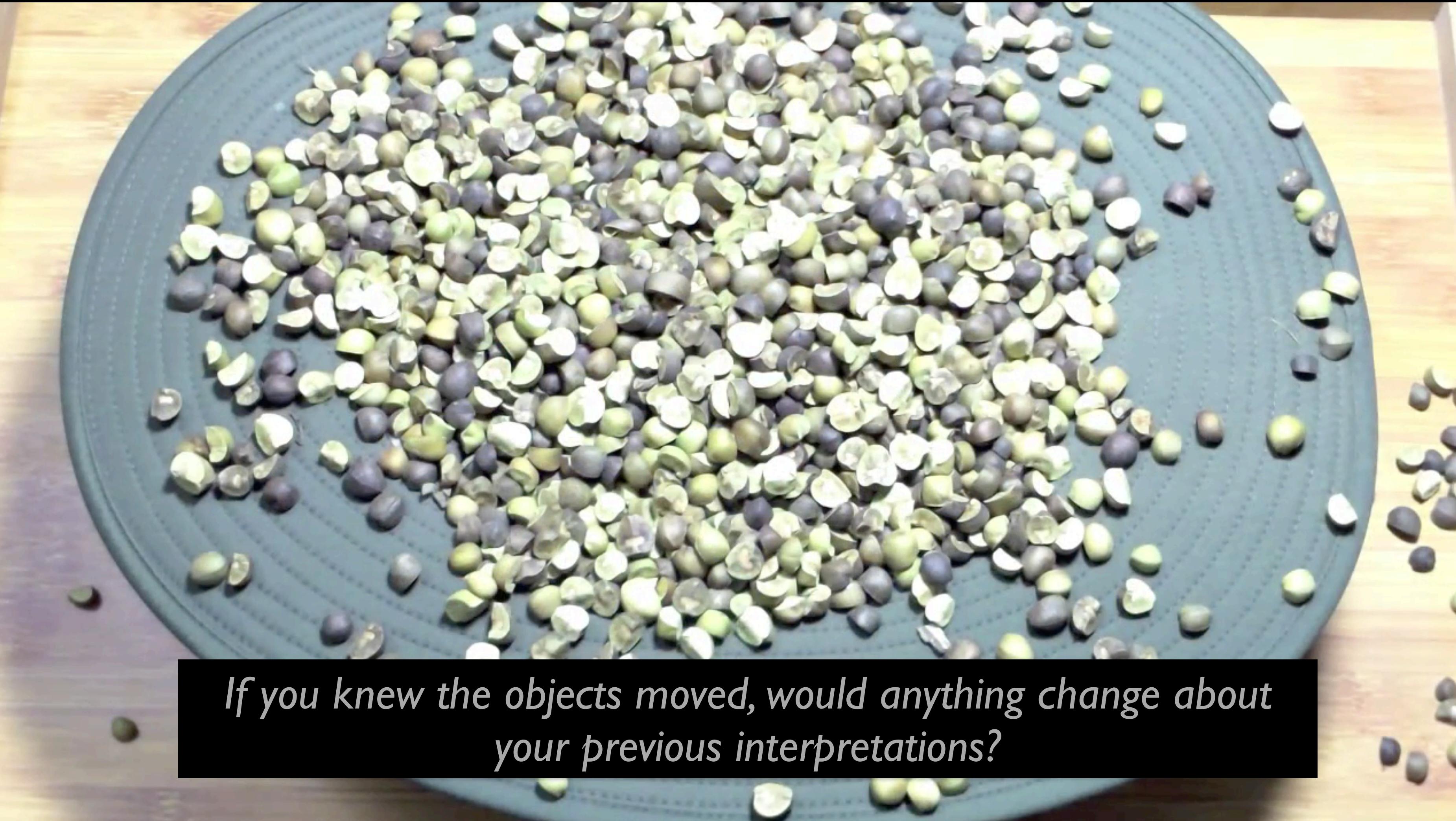
*Would each new individual fit
into one of the previously
defined types?*

*Would you modify your
classification scheme?*

*What differences may be due to
how data were acquired?
(technical vs experimental
variation)*



What about changes in time?



If you knew the objects moved, would anything change about your previous interpretations?

Microscopy and Digital Imaging

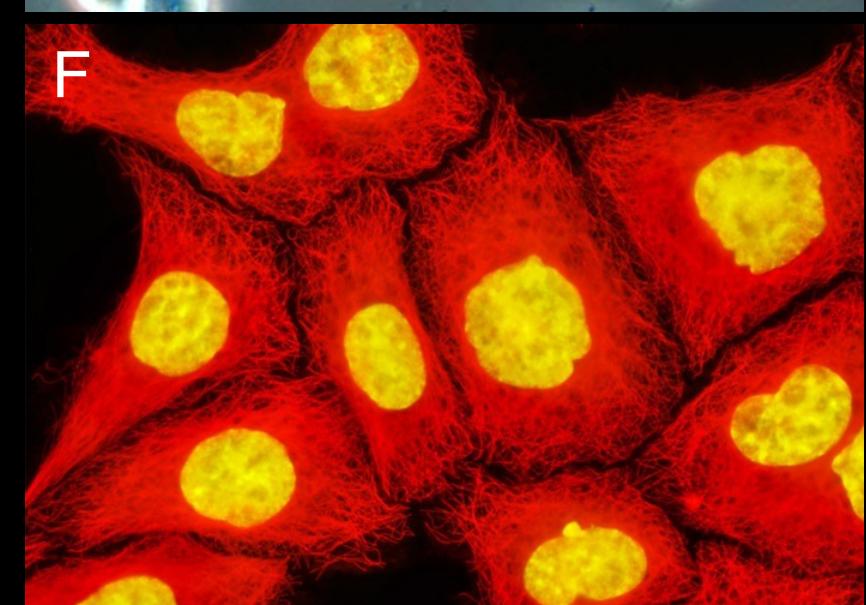
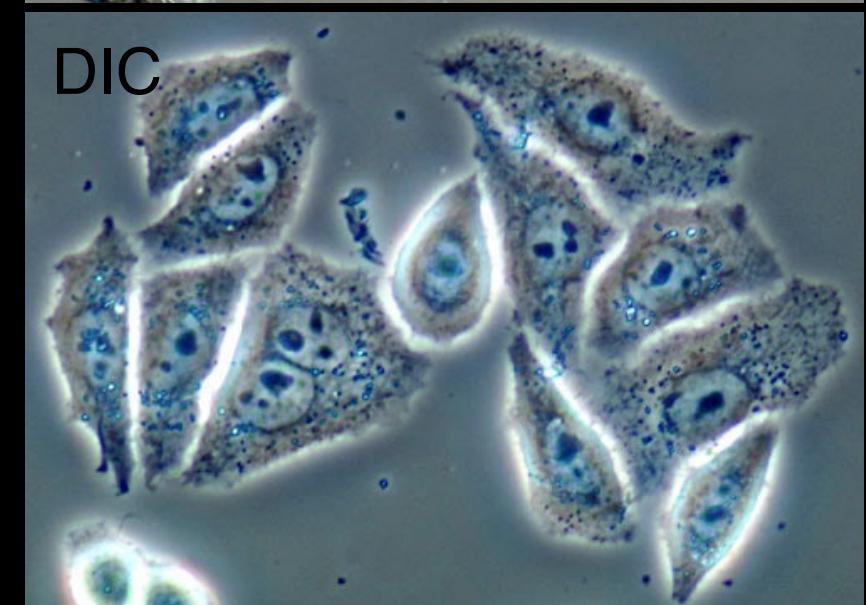
Direct visualization of cells using lenses (many modalities)

Advantages

- Spatial information can be maintained (intact tissues)
- Many optical properties of cells can be measured
- Biochemical properties of cells can be queried with light-reactive reagents (e.g., chromophores, fluorophores, dye-conjugated antibodies, etc.)
- Can be coupled to moving stage or optics and digital imaging to increase throughput
- Repeated analysis of same cells is possible (e.g. dynamic studies of live cells)

Disadvantages

- Information obscured by out-of-focus light
- Cells must be in the same focal plane (aided by confocal z-scanning or LLSM)
- Must optimize tradeoffs in terms of acquisition speed, spatial resolution, phototoxicity, excitation/emission wavelengths of light, and more.

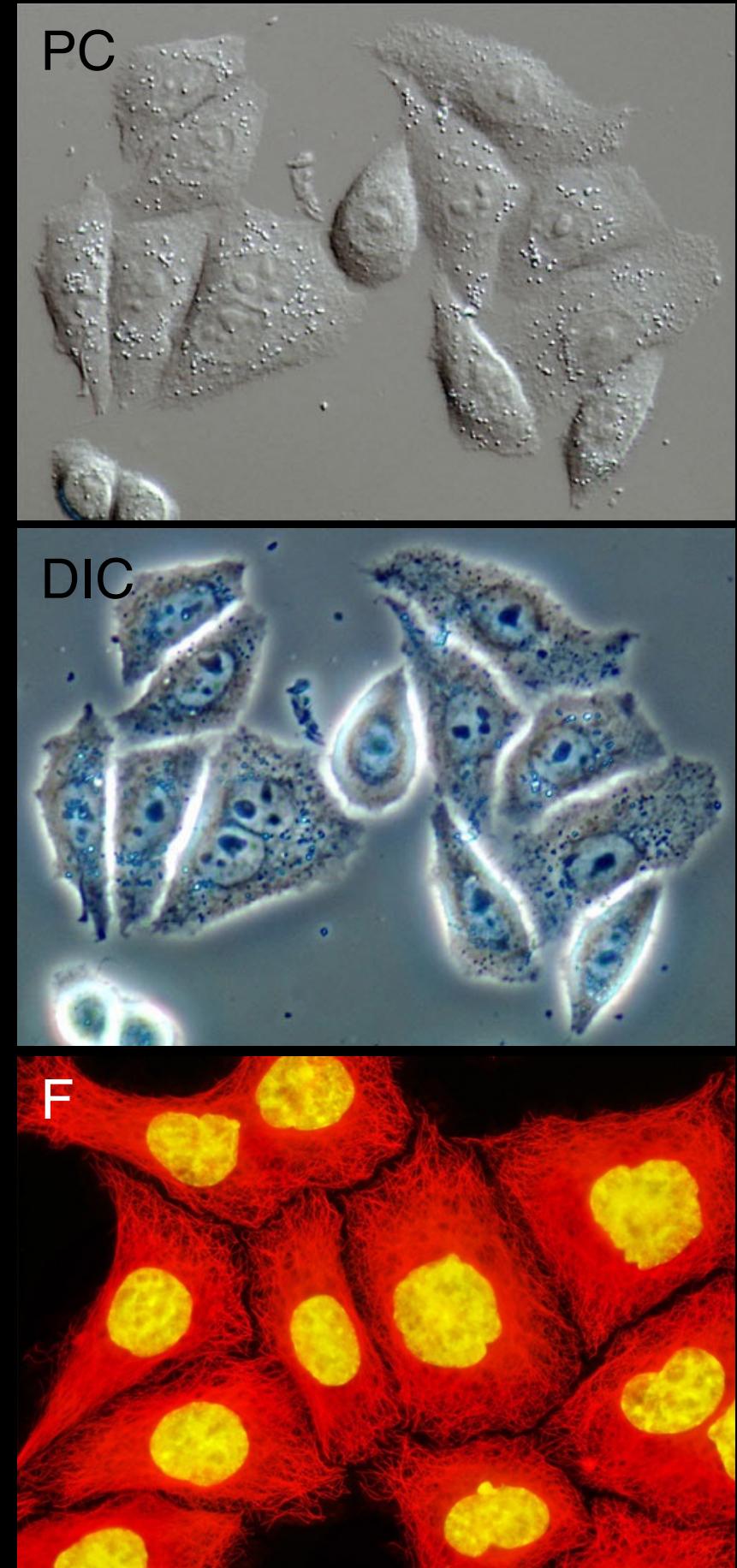


HeLa cells

Images from Nikon's Microscopy U.
www.microscopyu.com

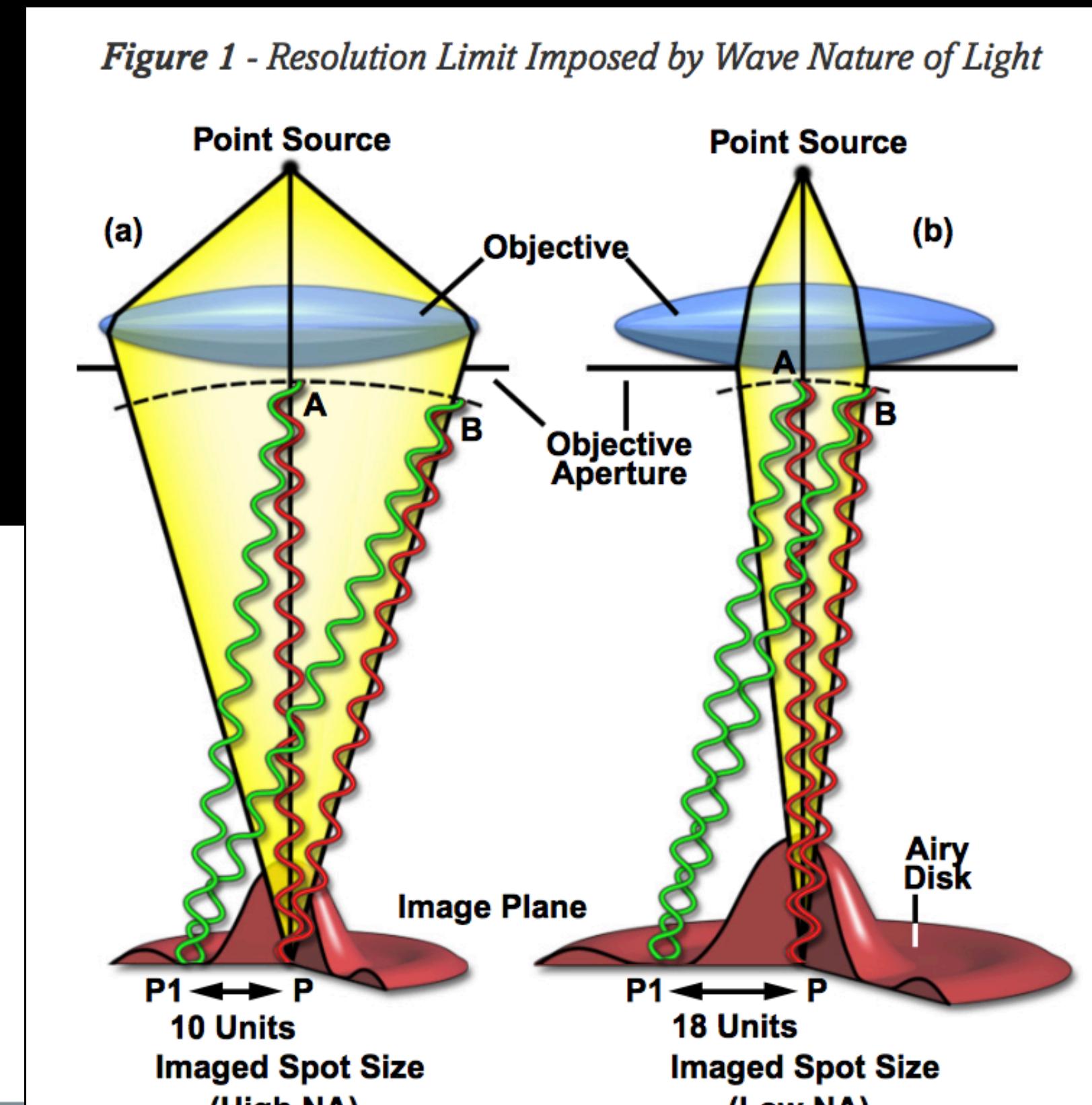
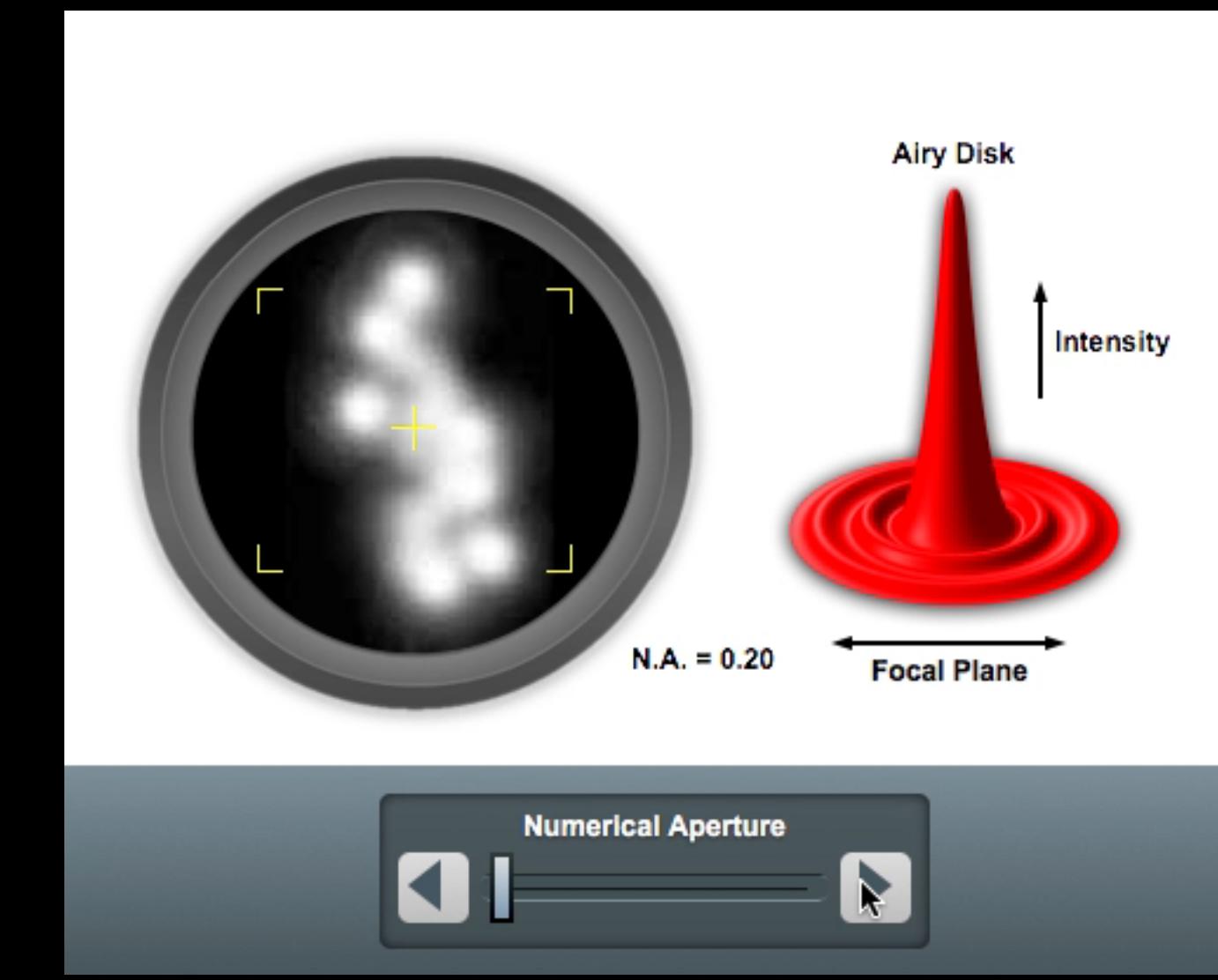
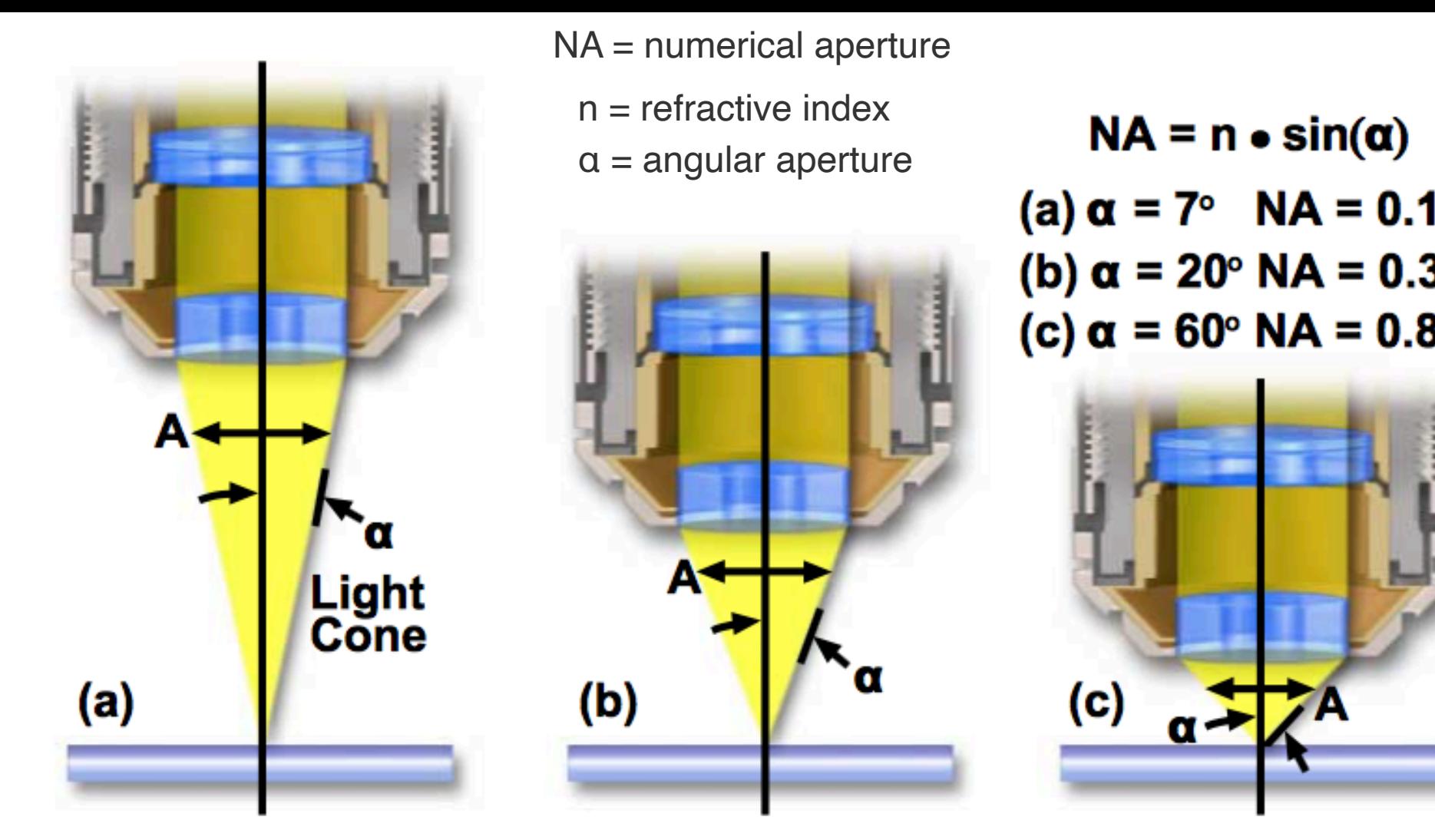
Microscopy and Digital Imaging

- Images from typical transmitted light microscopy have relatively low contrast
- Contrast can be enhanced with phase or differential interference
- But best signal-to-noise is obtained from fluorescence conferred by dyes or fluorescent proteins (e.g. GFP)



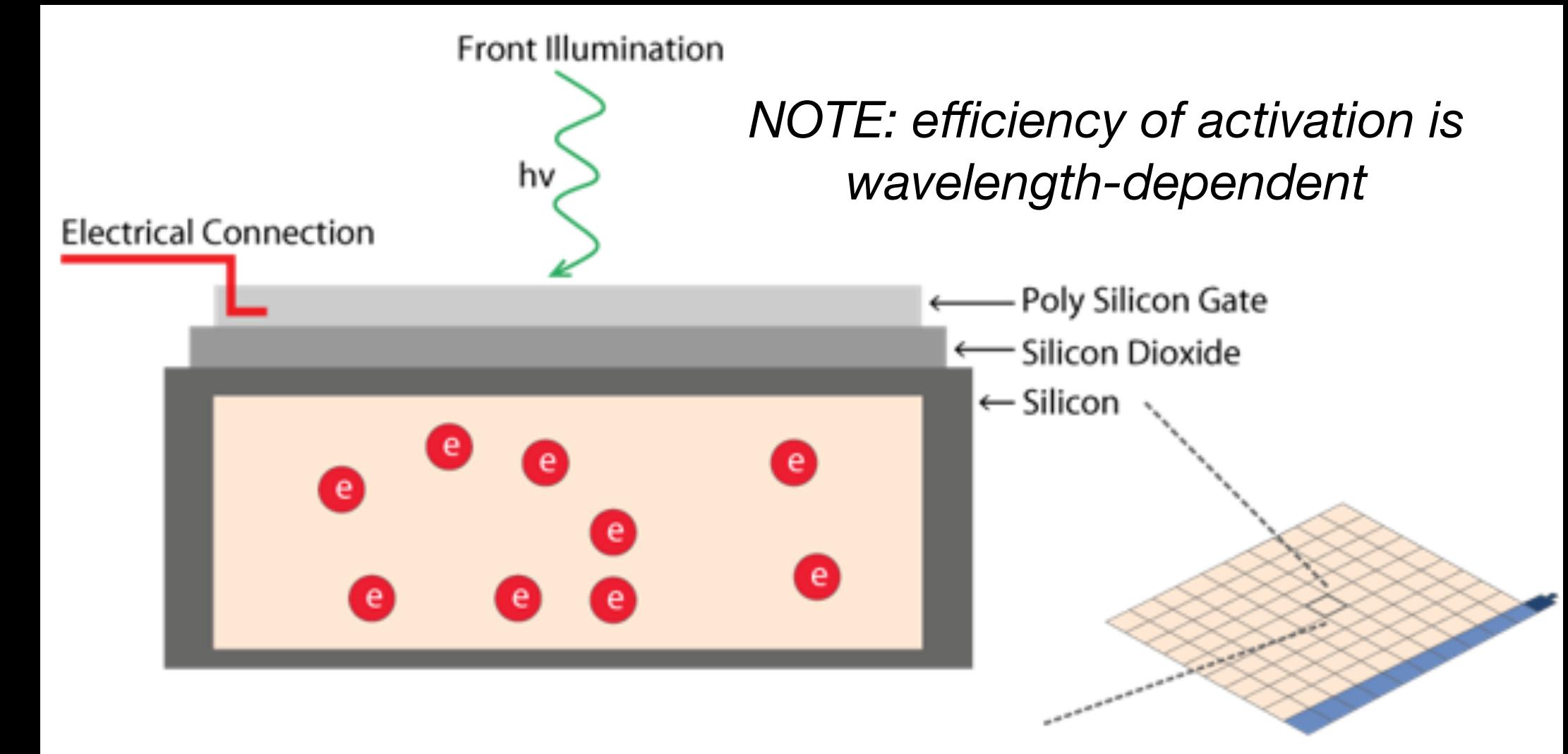
Microscopy Imaging

- Light is passed through lenses to magnify and focus it onto the sensor array
- Spatial resolution is based on several factors, including magnification, numerical aperture, sensor density and array size.



Digital Imaging

- Digital cameras collect light energy on arrays of sensors arranged in a grid (pixels)
- Light activates electrons on each sensor, which are collected and converted into a digital value based on pixel (bit) depth of the sensor (range of values)
- The numerical value of each pixel represents the intensity of the optical image averaged over the sampling interval (space and time)
- Saturation is achieved when a sensor is filled to capacity (maximum depth); information from any additional light is lost
- The resulting array of numeric values is a digital image

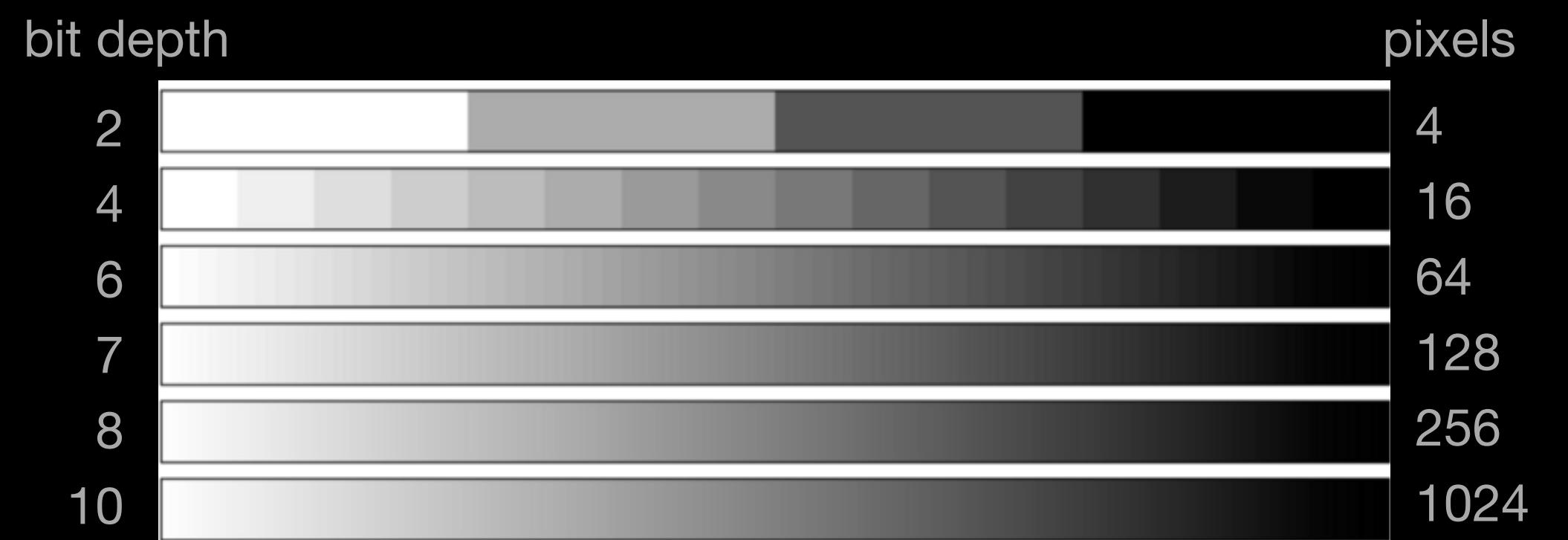


NOTE: efficiency of activation is wavelength-dependent

Common sensor array types

CCD
CMOS

https://en.wikipedia.org/wiki/Charge-coupled_device
https://en.wikipedia.org/wiki/Active-pixel_sensor
complementary metal-oxide-semiconductor



Anatomy of a Digital Image

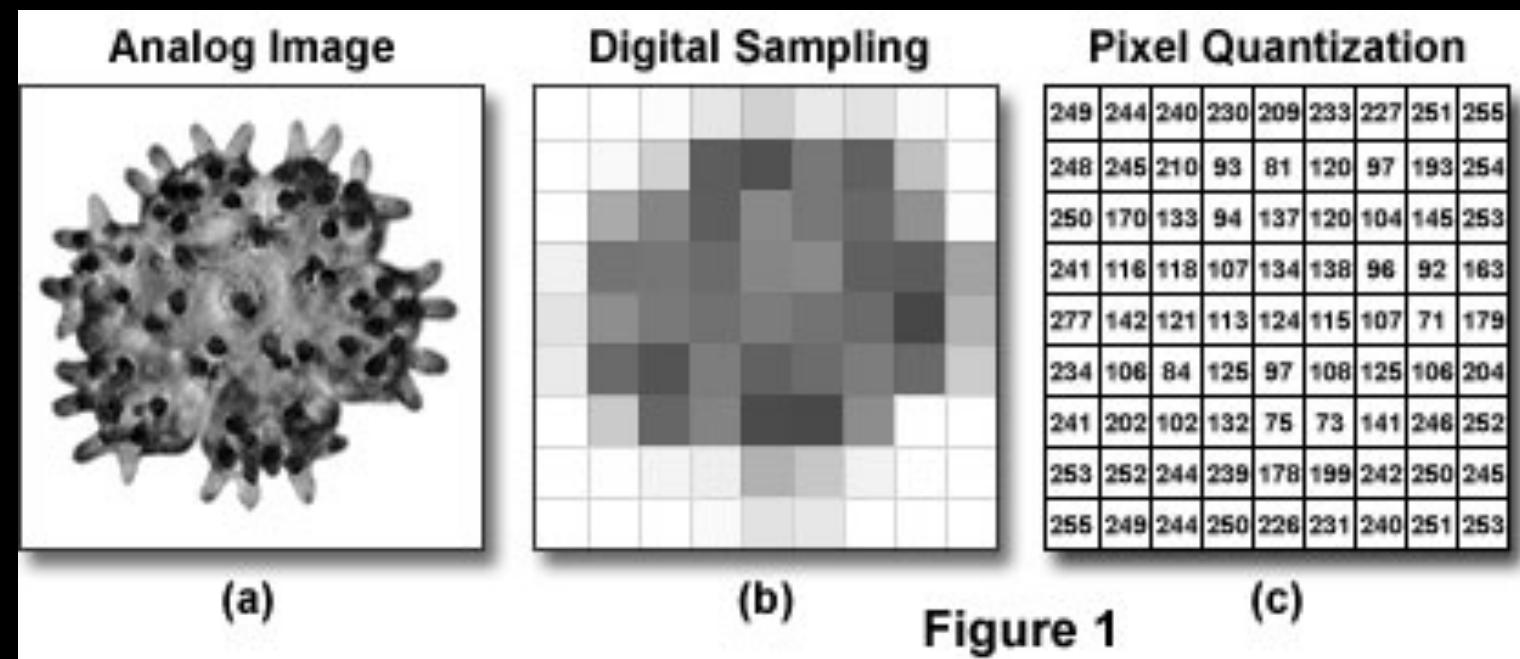
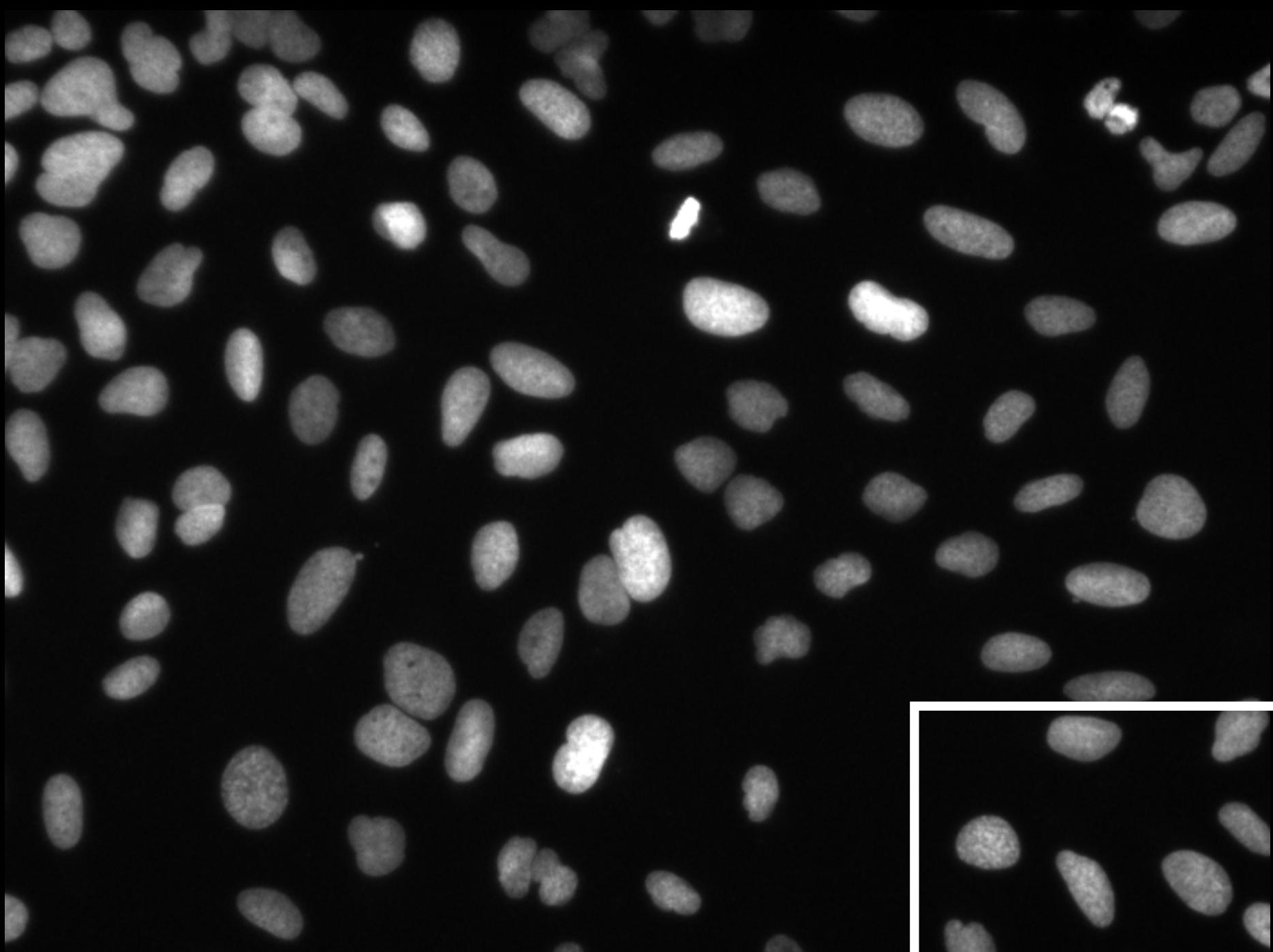


Figure 1

- Essentially a matrix of intensity values (pixels) captured by the sensor array
- Many different file formats; common standard is Tagged Image File Format (TIFF)
- Multiple channels (e.g. color), multiple pages (e.g. time points or z-sections), and form of compression (default is none) can all be included within a single file



Anatomy of a Digital Image

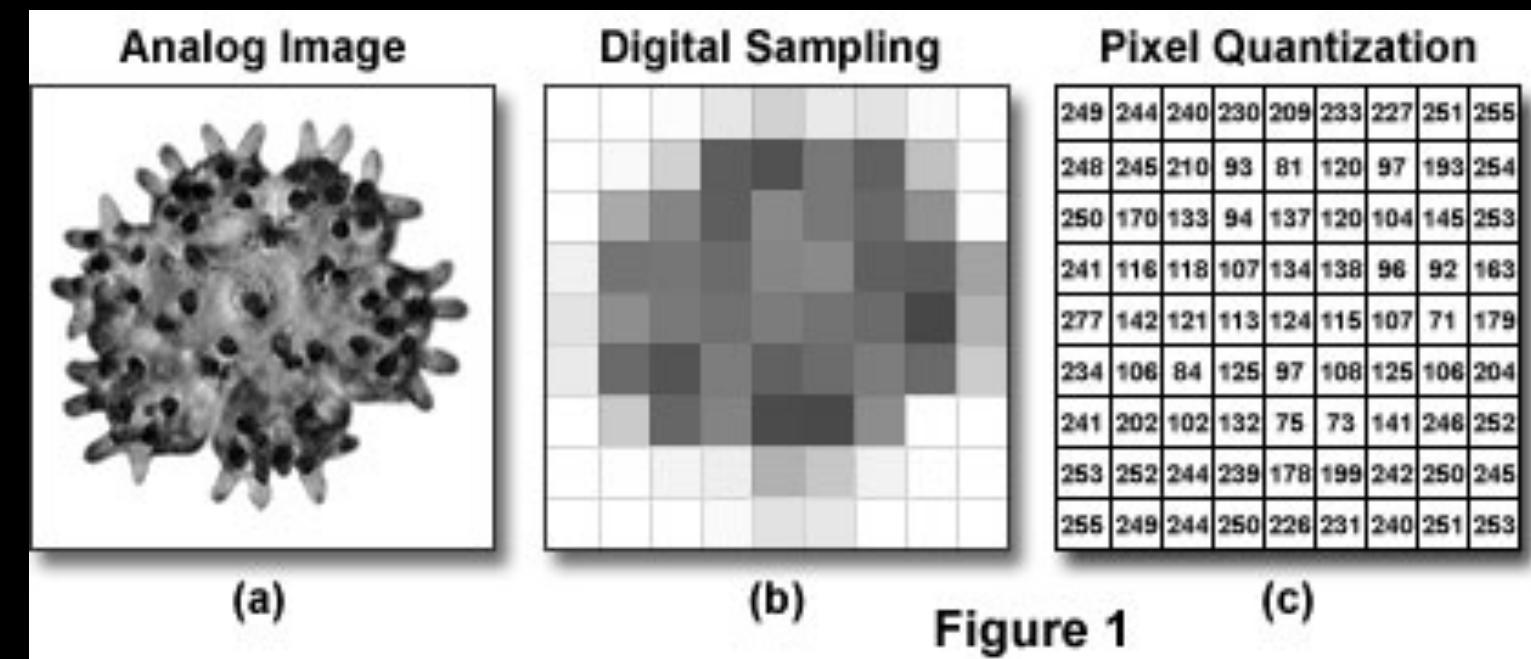
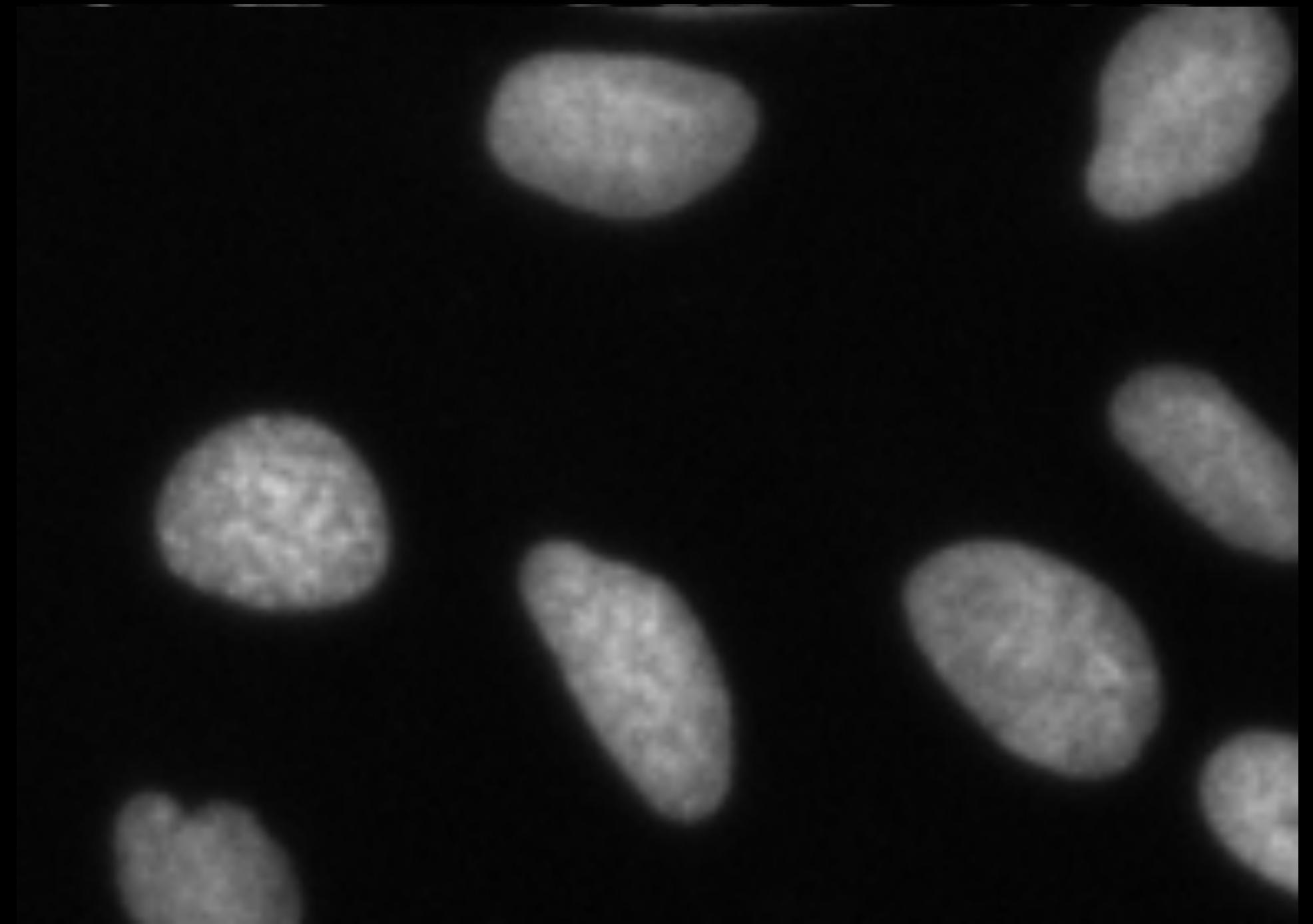


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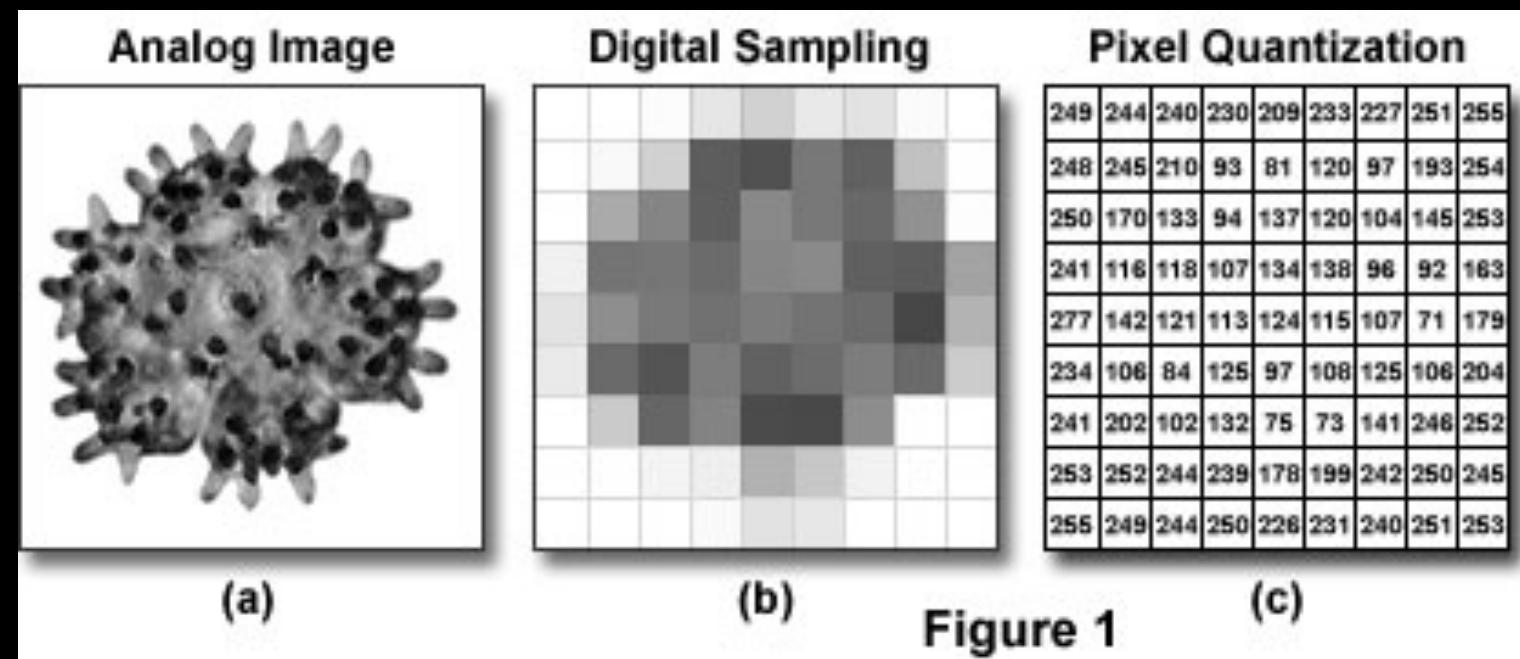
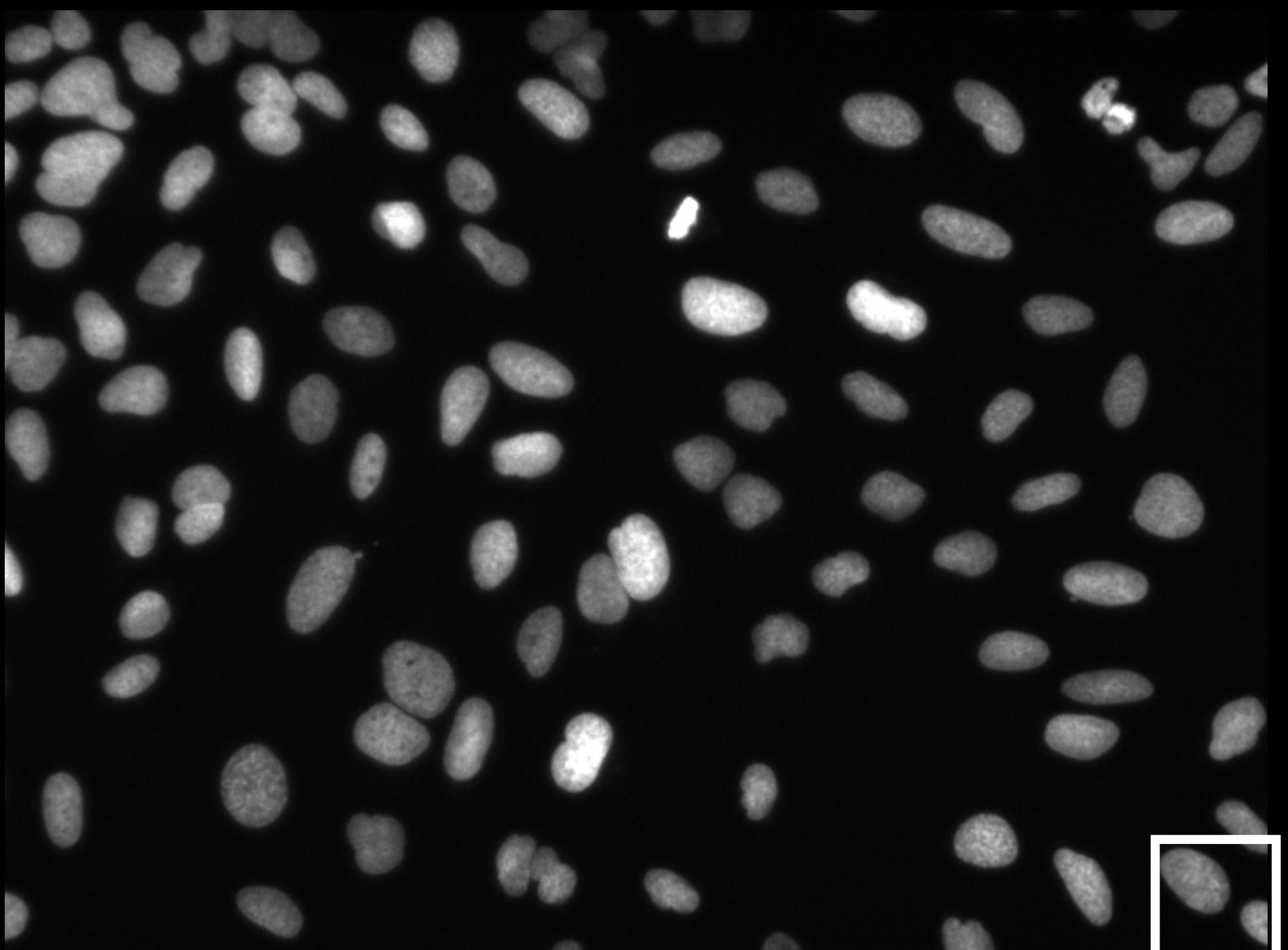
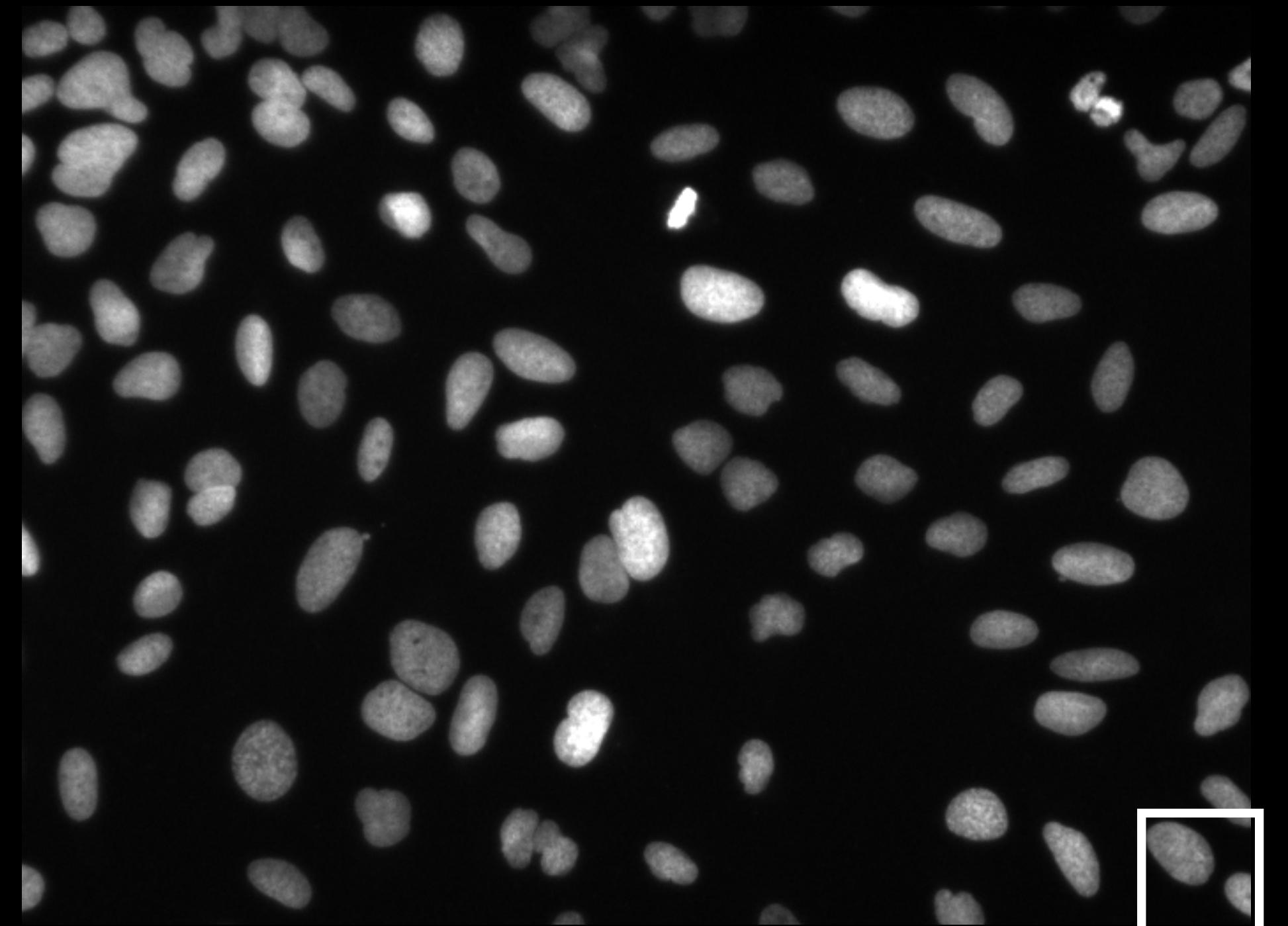
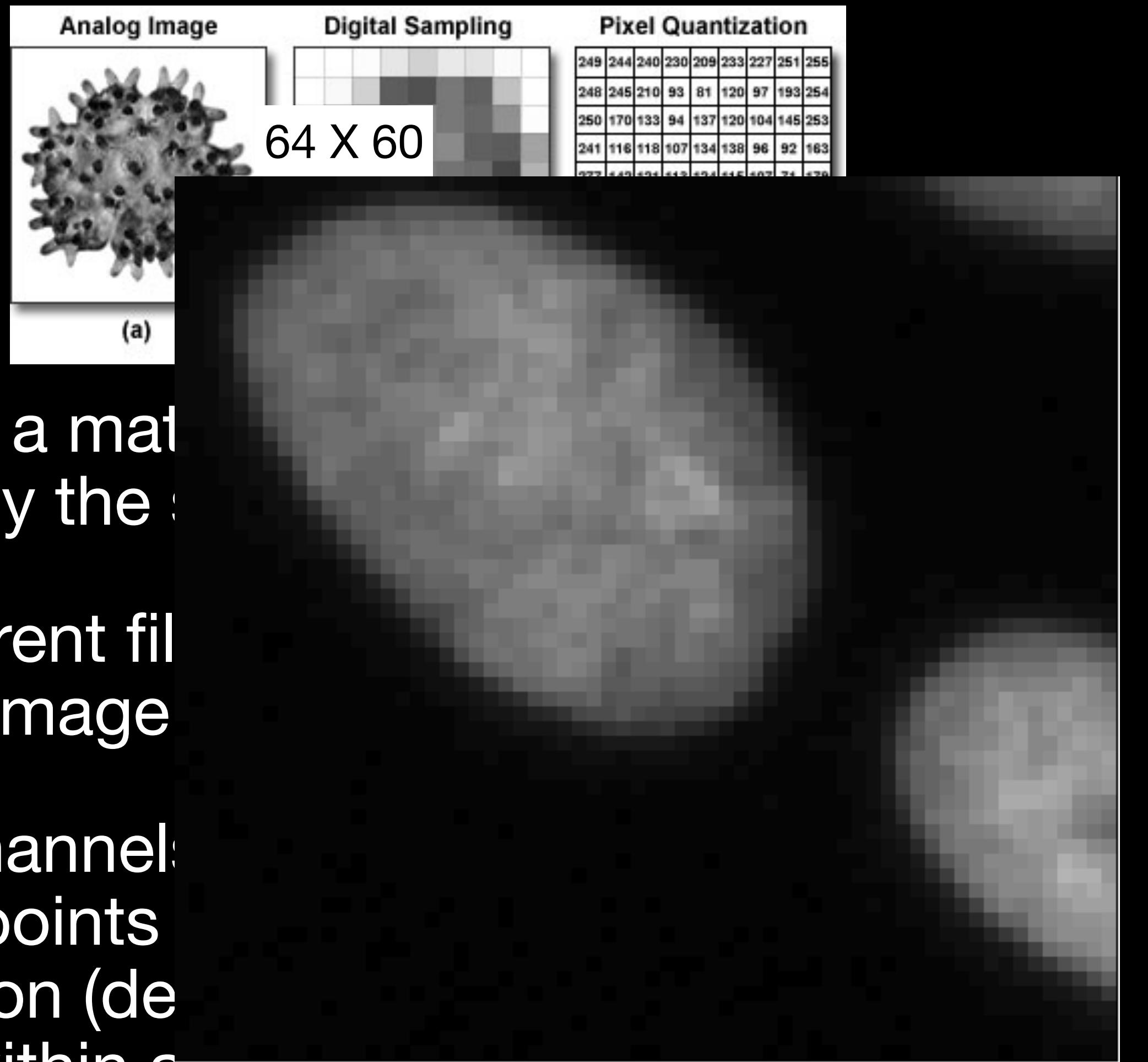


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Anatomy of a Digital Image



- Essentially a matrix of pixels captured by the sensor
- Many different file formats exist. One is Tagged Image File Format (TIFF)
- Multiple channels can be combined (e.g. time points) and compressed (decompressed) and included within a single file.

Image Processing

- Steps required to extract relevant information
- Often requires multiple steps that may include: flat-field or background correction, contrast enhancement, bit depth scaling, filtering, binarization/thresholding, many more
- Segmentation: the process of identifying relevant objects and determining their boundaries within an image

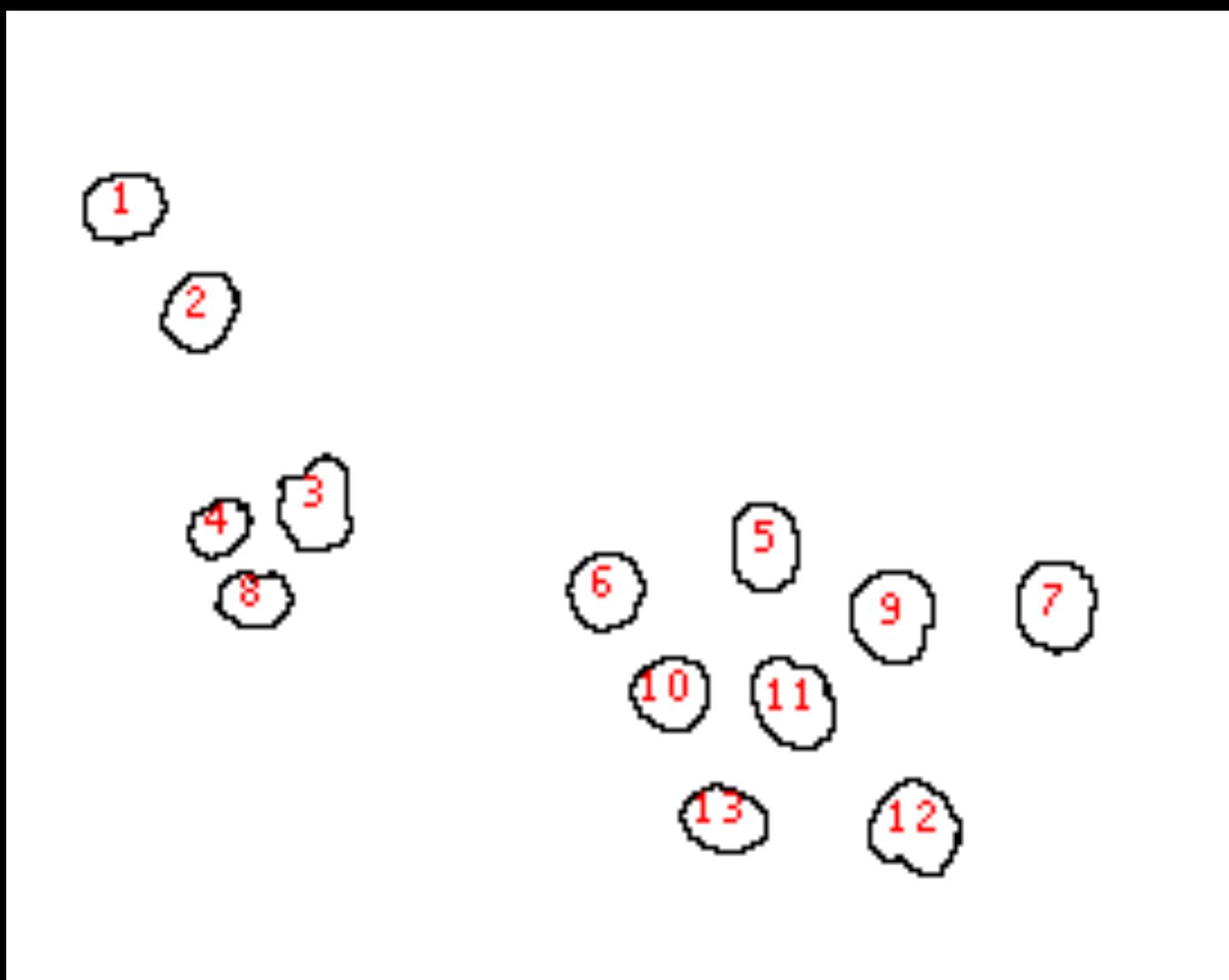
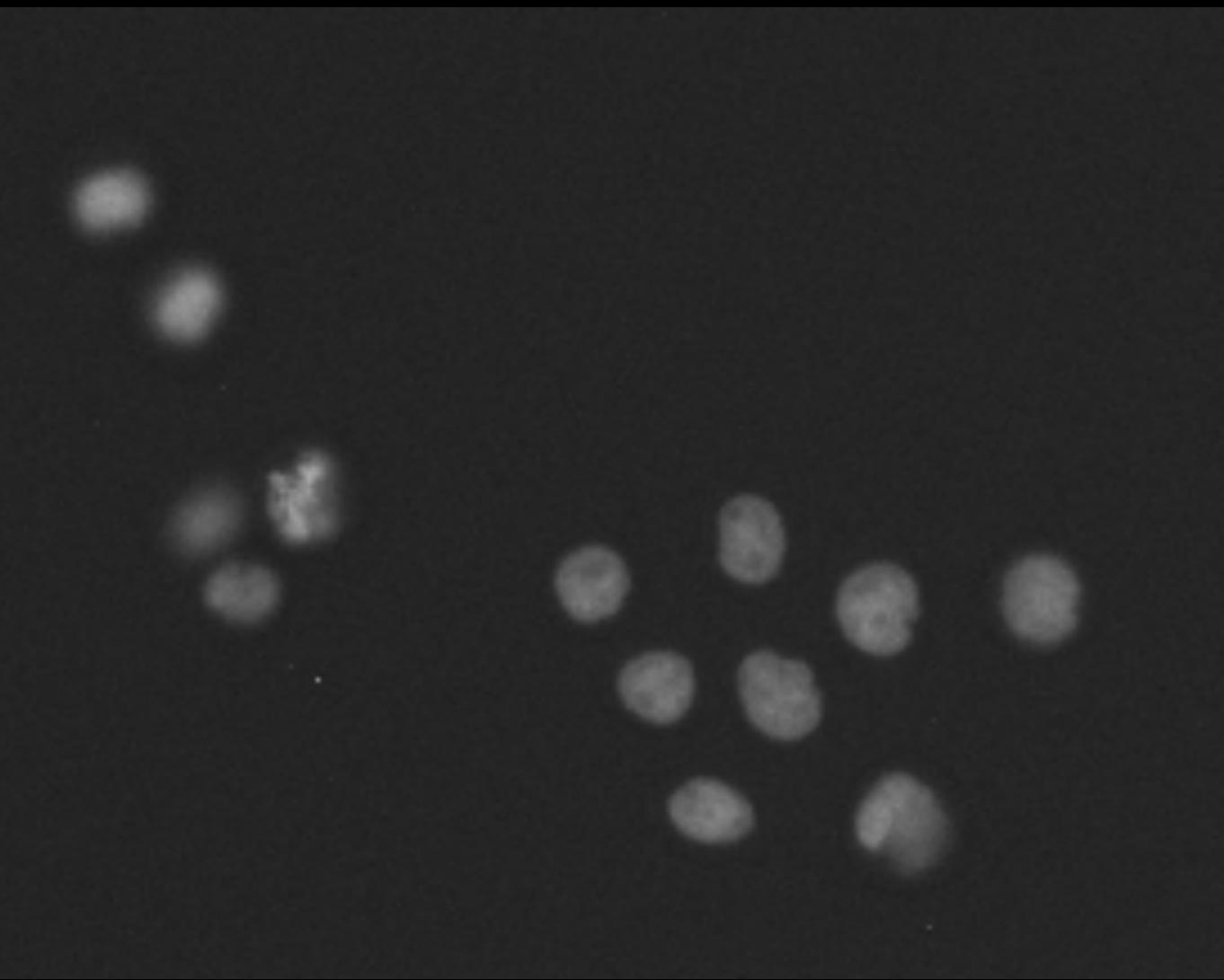
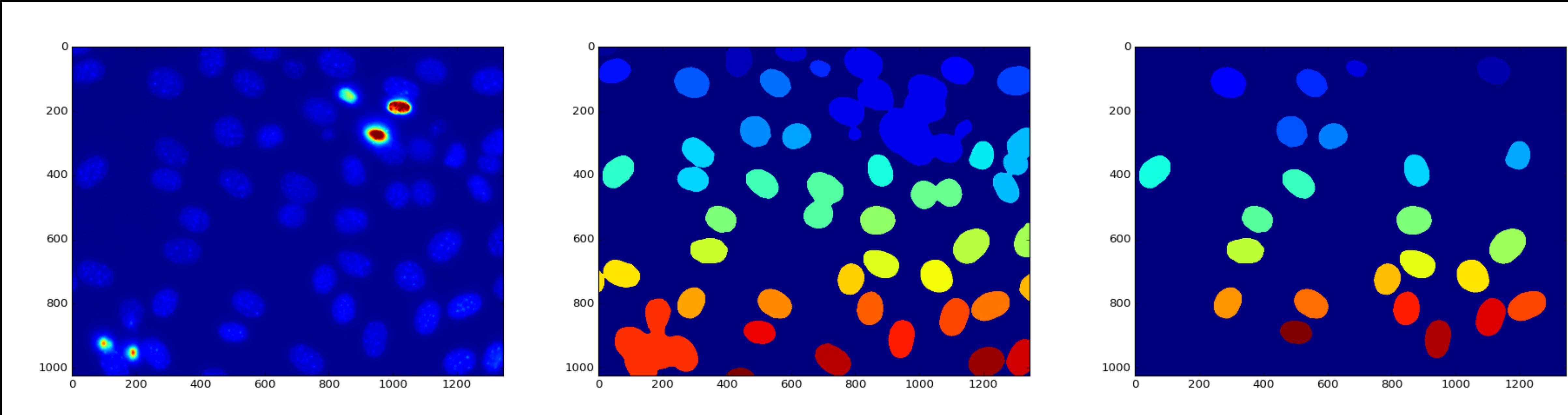


Image Segmentation

- The process of identifying relevant objects and determining their boundaries within an image
- Many different approaches; often requires complex algorithms



(Some) applications of digital image analysis

- Digital histopathology (H&E, immunohistochemical staining)
- Cyclic/multiplexed immunofluorescence of large tissue sections
- Calcium signaling in live cells
- Live-cell reporters of kinase activity
- Change in cell number over time in response to drug treatment

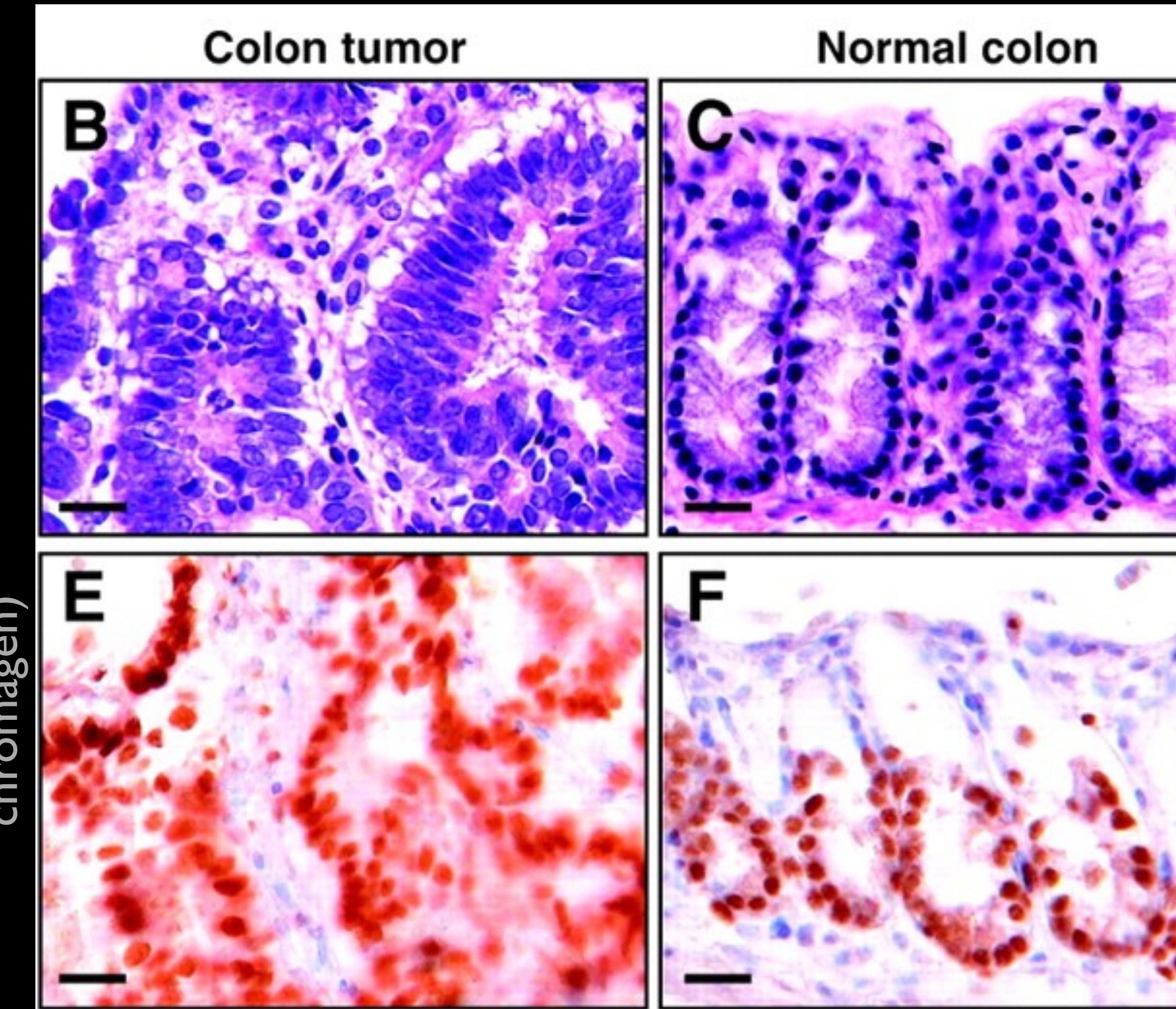
Microscopy Images of Human Tissues

Cancer Histopathology

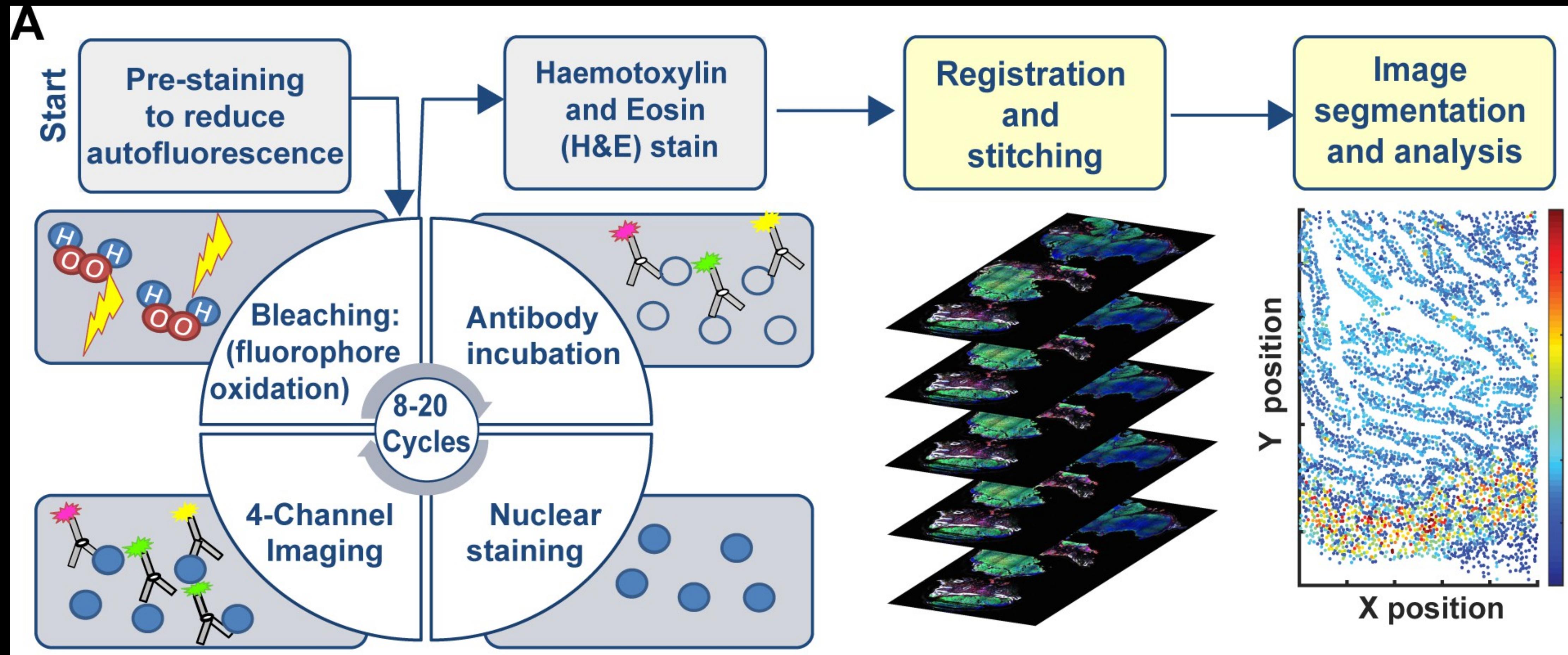
*What differences
are important/
relevant?*

*How could you
quantify the
differences
between the two
samples?*

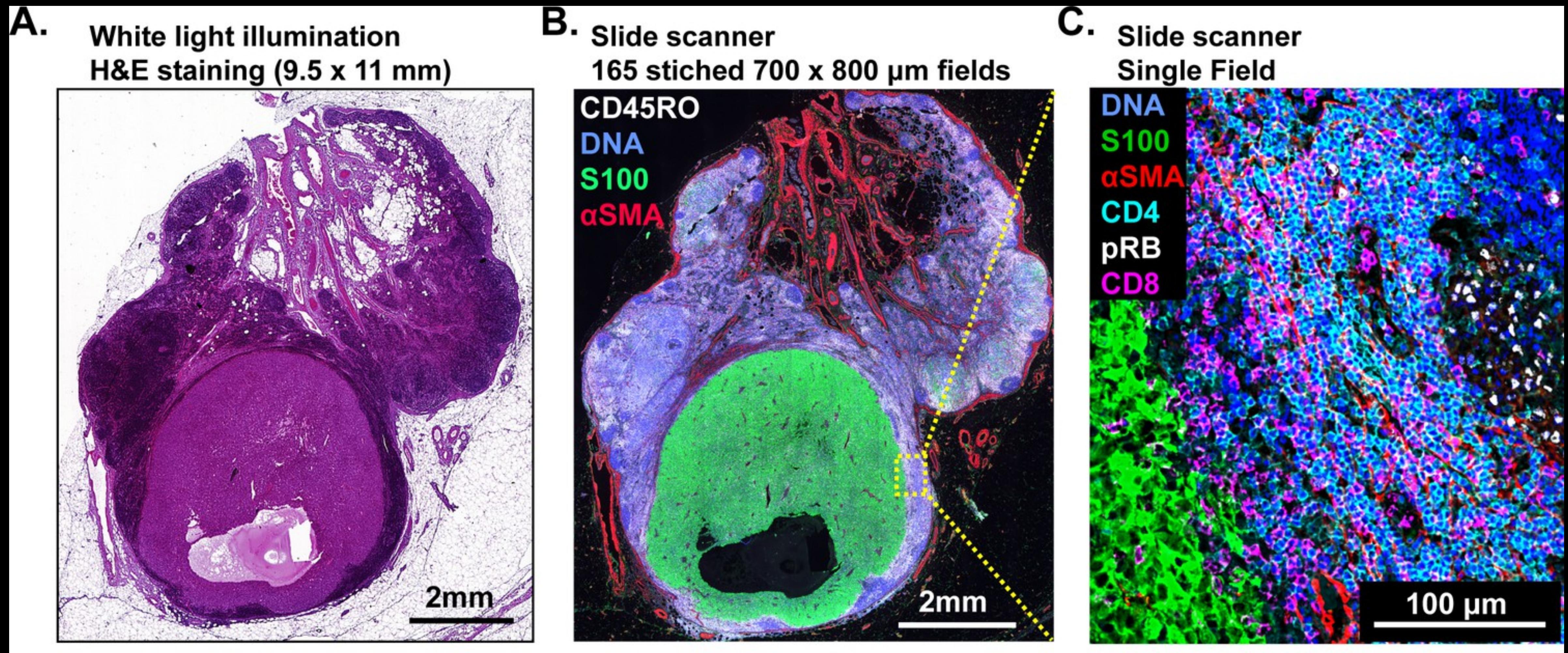
H&E
MCM4 (HRP/
chromagen)



Cyclic Immunofluorescence



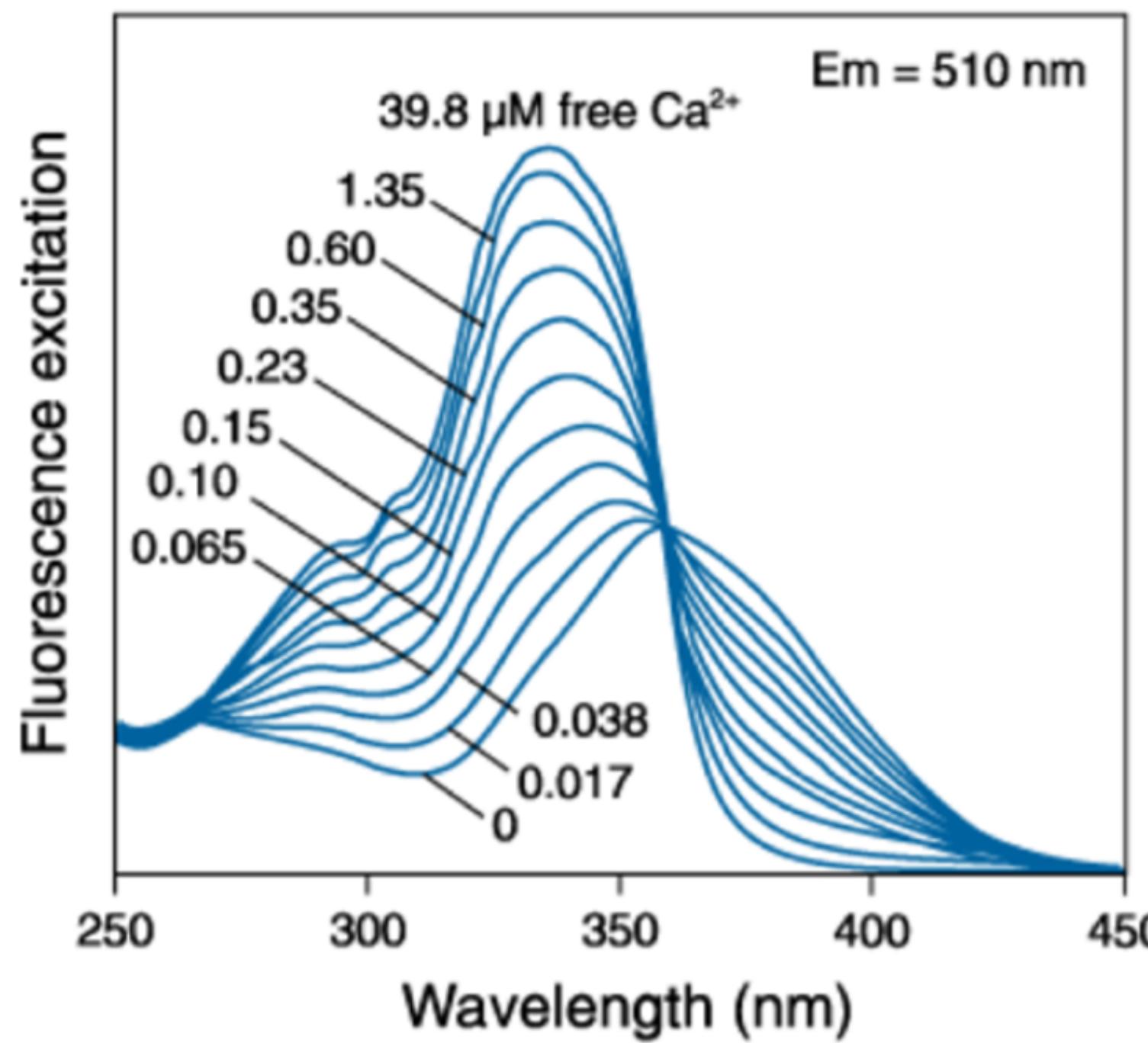
Cyclic Immunofluorescence



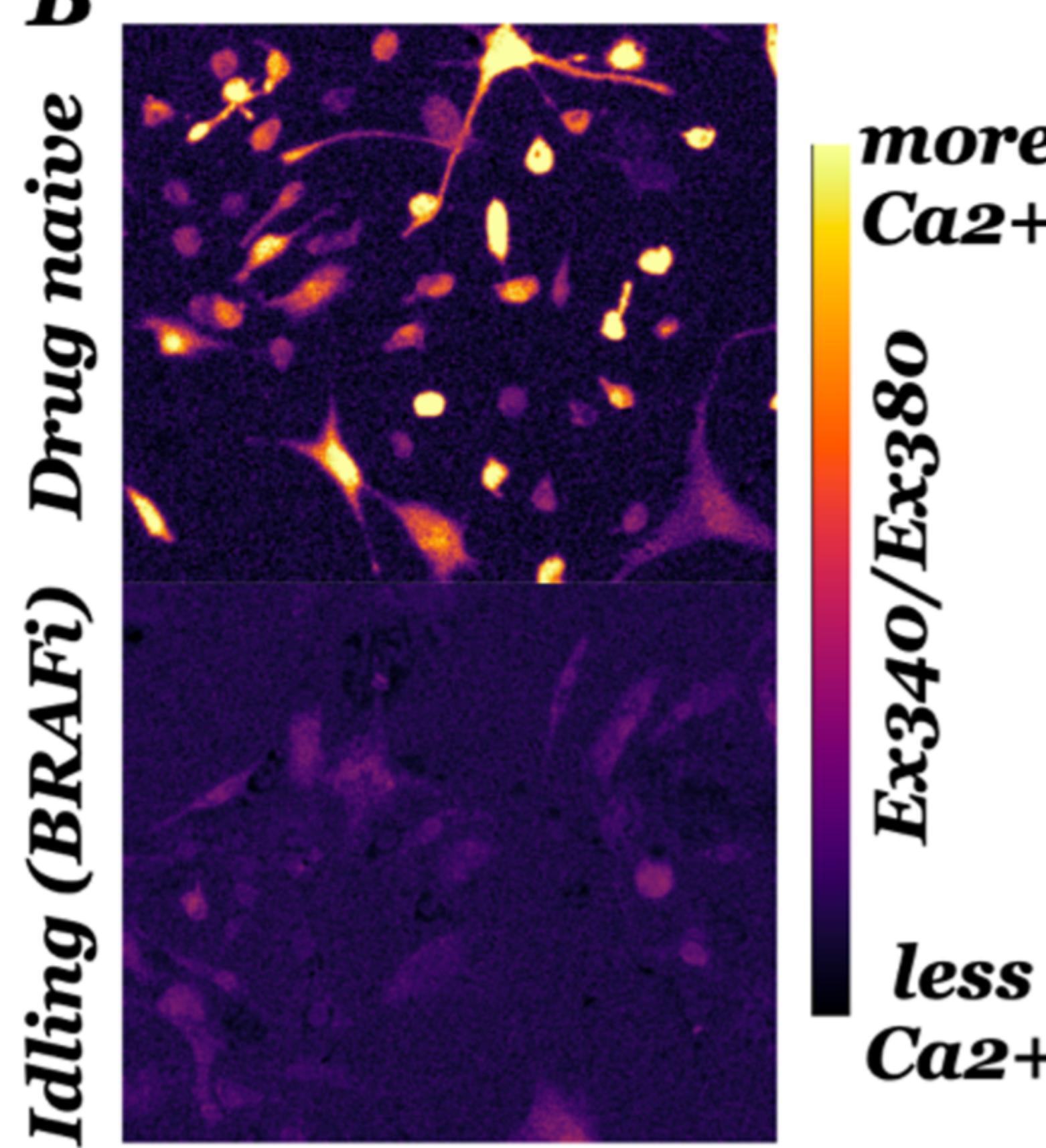
Calcium signaling in BRAF-mutant melanoma after treatment with BRAFi

A

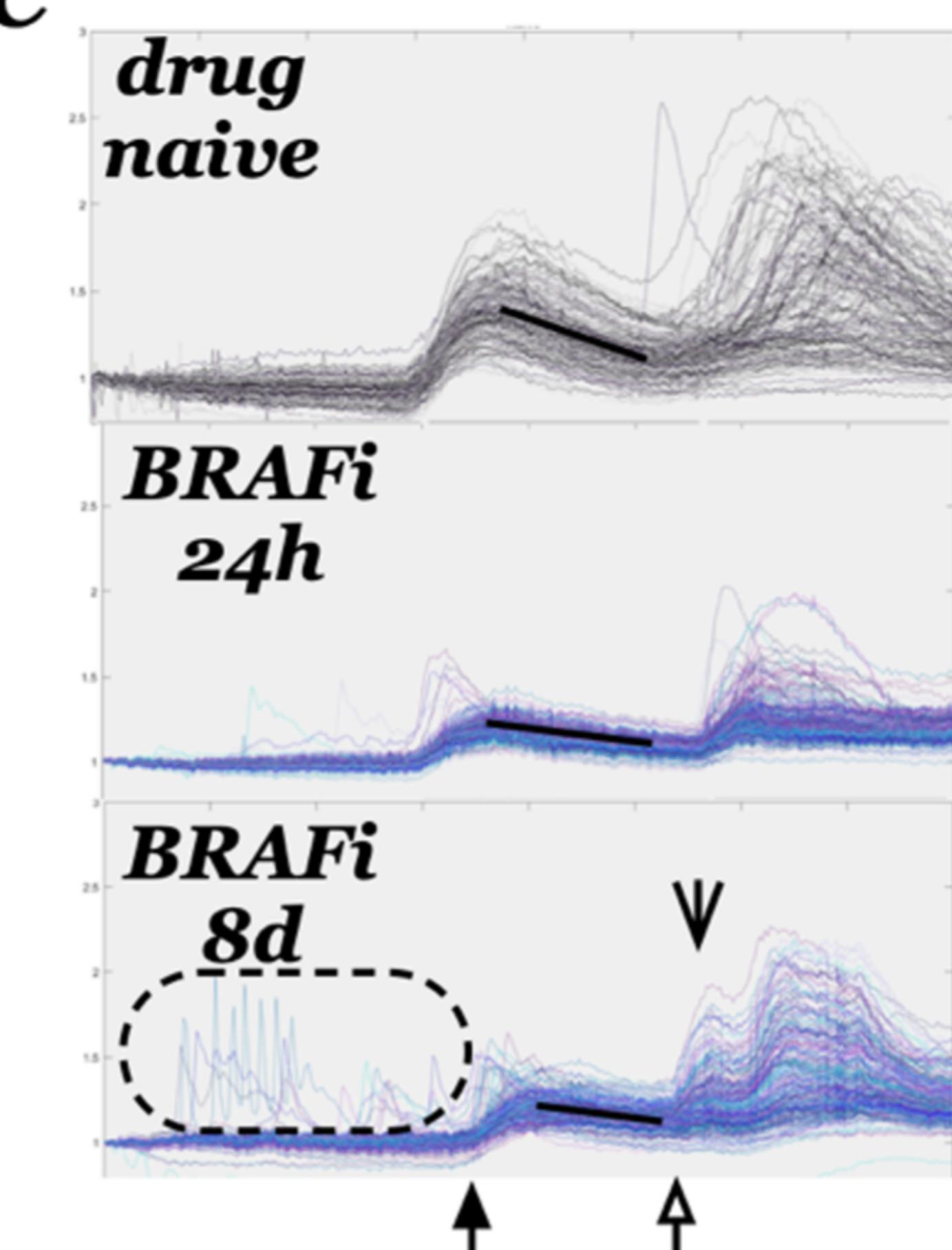
Ca²⁺-dependence of fura-2 fluorescence



B

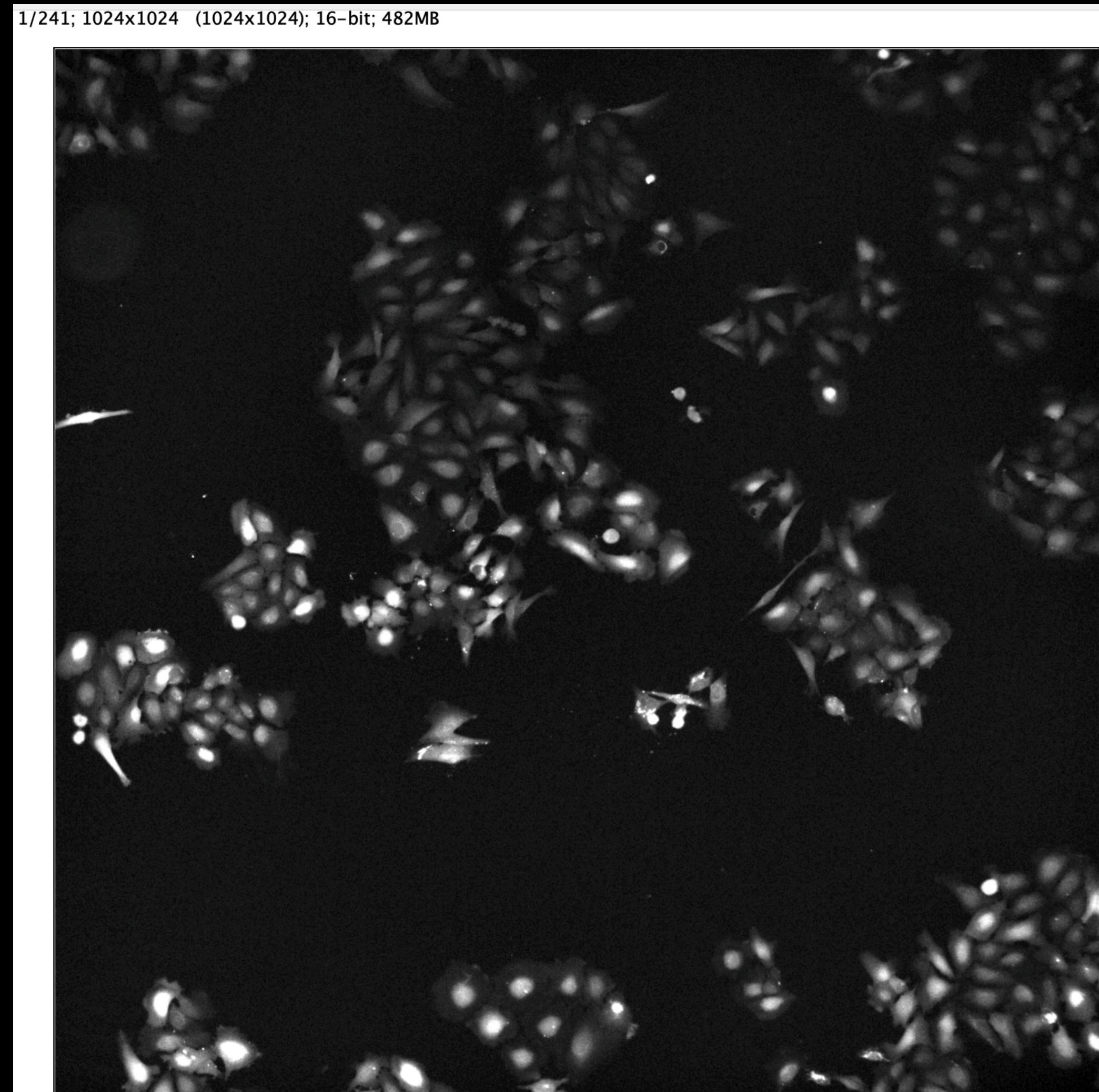


C



Calbryte-520 (calcium-binding fluorophore)

A375 melanoma cells treated with BRAFi; 5 sec/frame



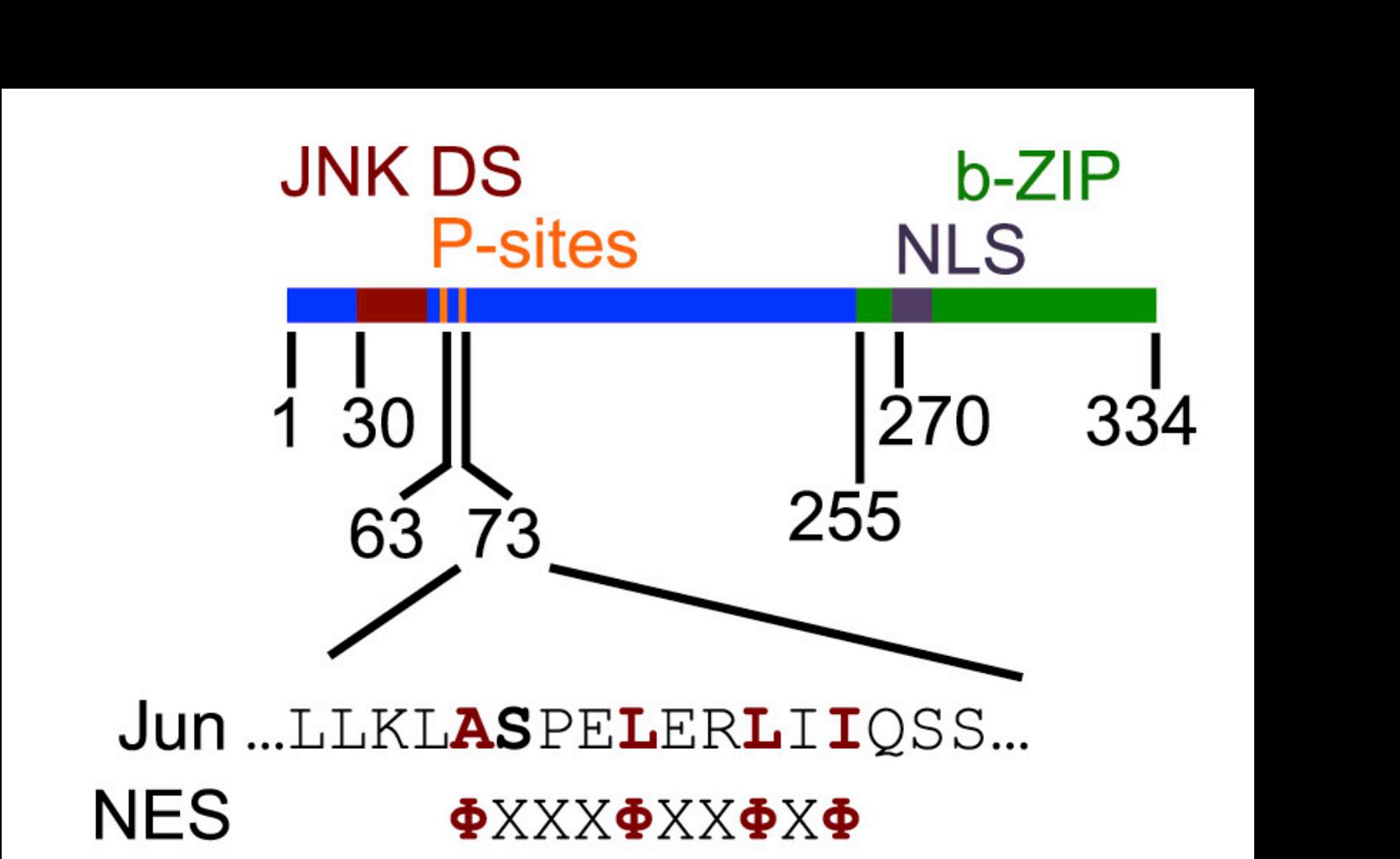
Quantifying Kinase Activity in Live Cells

Cell CellPress

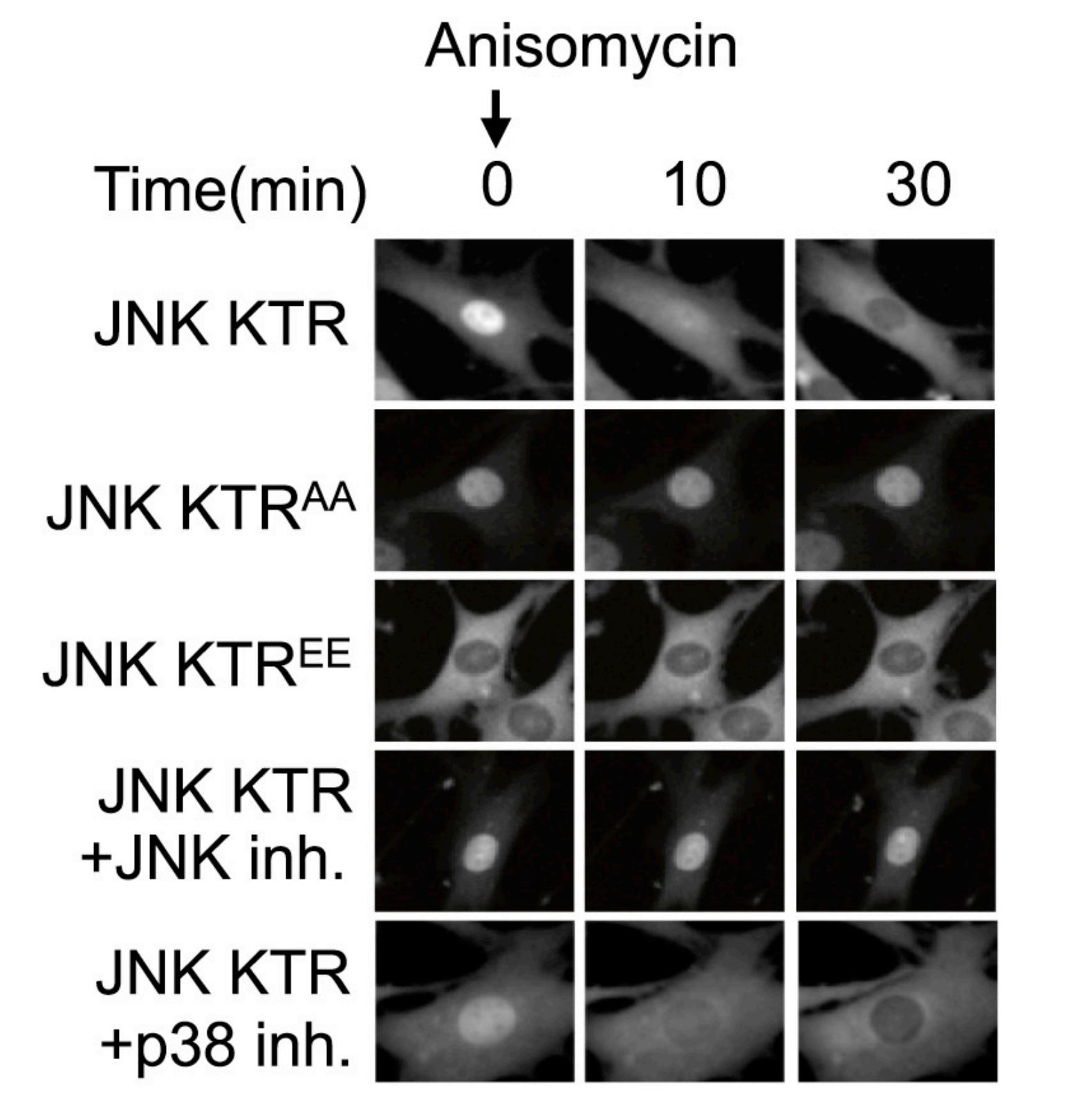
Volume 157, Issue 7, 19 June 2014, Pages 1724-1734

Resource
High-Sensitivity Measurements of Multiple Kinase Activities in Live Single Cells

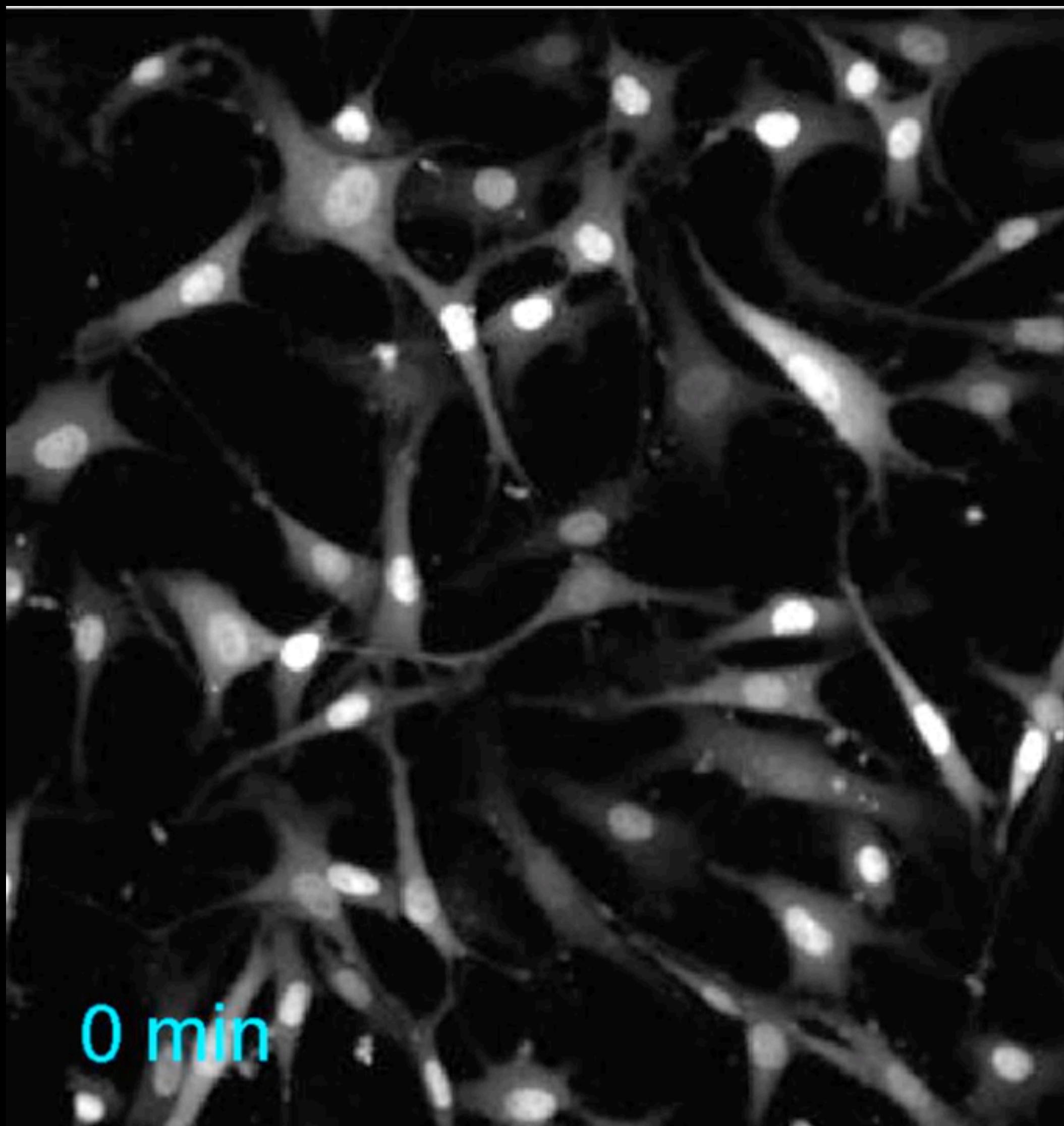
Sergi Regot ¹  , Jacob J. Hughey ¹, Bryce T. Bajar ¹, Silvia Carrasco ¹, Markus W. Covert ¹



The diagram illustrates the structure of the Jun protein. It features a central blue horizontal bar representing the protein backbone. Key regions are highlighted: 'JNK DS P-sites' in red at the N-terminus (residues 1-30), a 'b-ZIP' domain in green at the C-terminus (residues 255-334), and an 'NLS' (Nuclear Localization Signal) in purple (residues 270-290). Below the backbone, two sets of vertical lines indicate phosphorylation sites: one set from residue 1 to 73, and another set from 255 to 334. A sequence of the protein's amino acids is shown below, with a red 'AS' motif highlighted. At the bottom, a red 'NES' (Nuclear Export Signal) sequence is shown with the motif 'ΦXXXΦXXΦXΦ'. Numerical labels 1, 30, 63, 73, 255, 270, and 334 are placed along the backbone to mark specific residues.



Quantifying Kinase Activity in Live Cells



NIH3T3 cells with JNK-KTR
+ 50 ng/ml anisomycin @ 15 min
+ JNKi @ 85 min

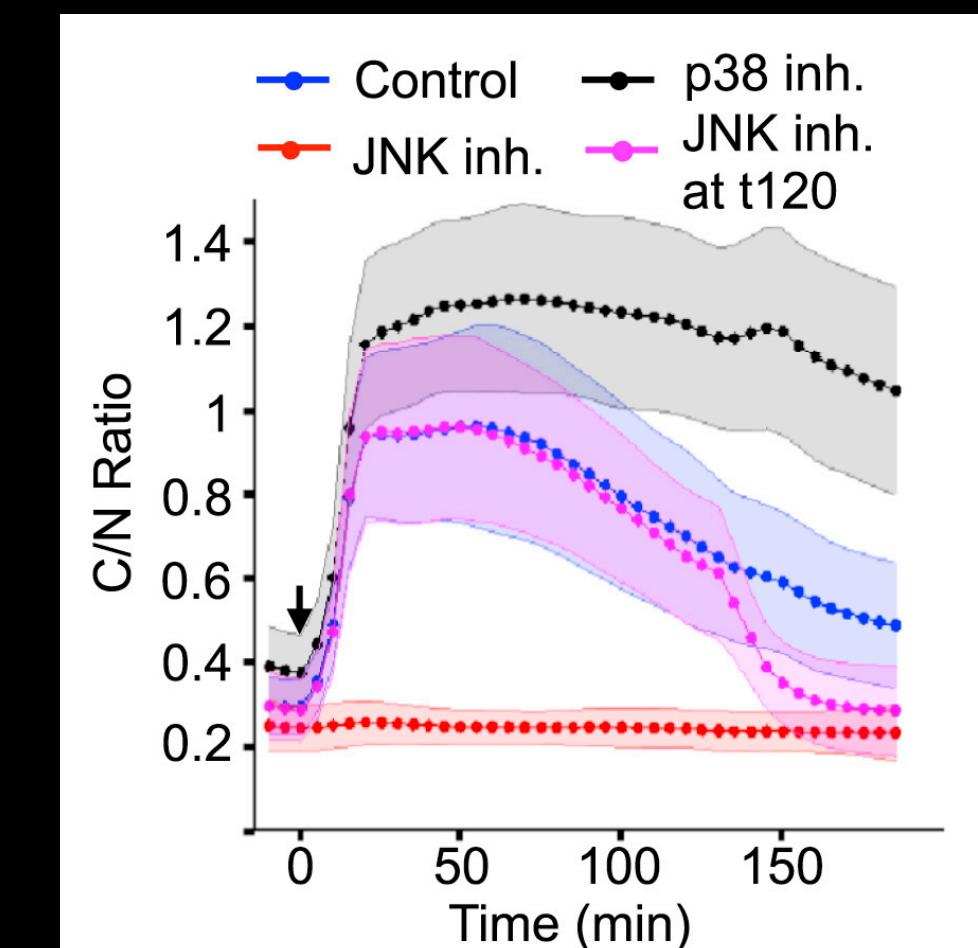
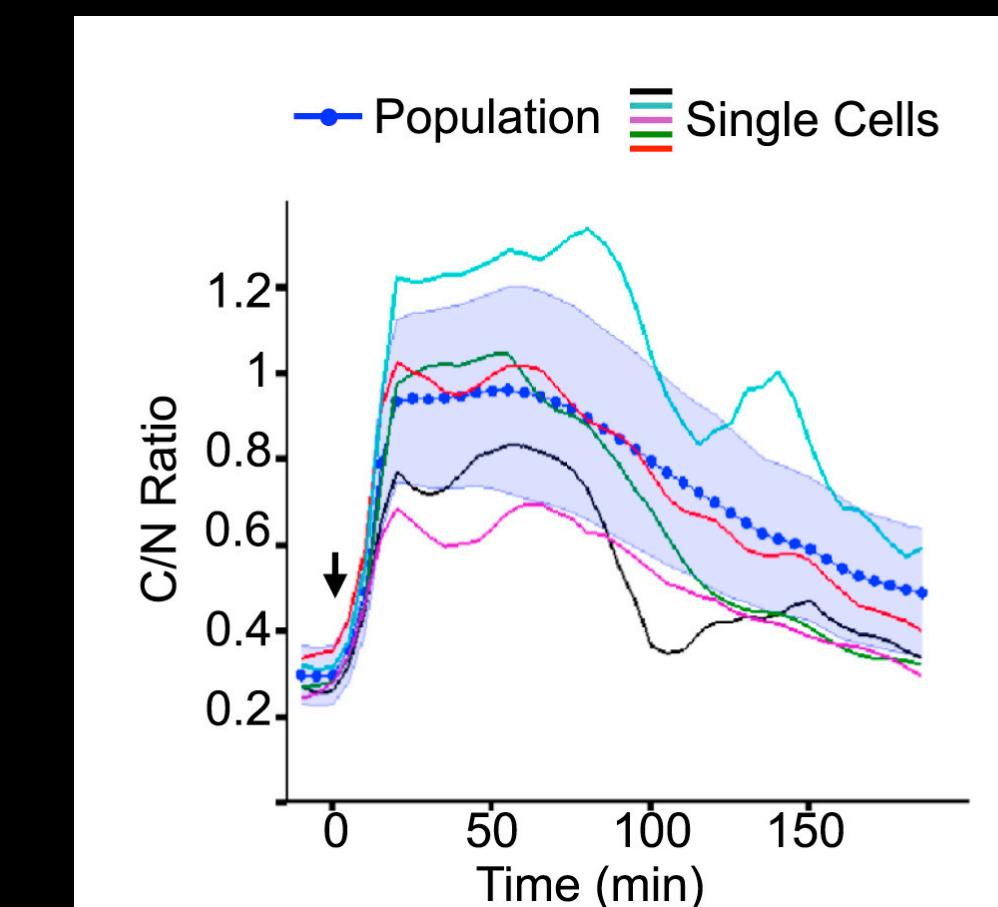


Image Analysis Software (examples)

- ImageJ/FIJI



<https://imagej.net/>

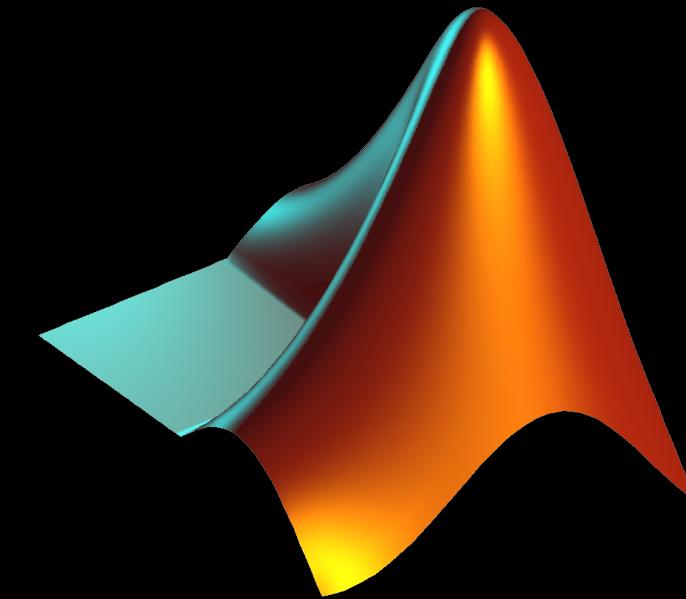
- CellProfiler



<https://www.cellprofiler.org/>

- Matlab

* *Vanderbilt students/faculty
can obtain a free license*



<https://mathworks.com>

Live demo of FIJI

Image manipulation and analysis



Example images: PC, DIC, 2-channel fluorescence, time series

- Getting image information
- Brightness & contrast: histogram (adjusting visualization only)
- Color: monochrome, RGB, channels, composite, pseudocolor, splitting/merging channels
- Segmentation/count objects: subtract background; threshold; convert to mask; Gaussian blur; watershed; analyze particles