# Classic Segmentation with Python

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BoBiAC
Boston Bioimage Analysis Course | 2025
CITE
Core for Imaging Technology & Education
/
HARVARD MEDICAL SCHOOL

# Slides & feedback from Team CITE & IAC!

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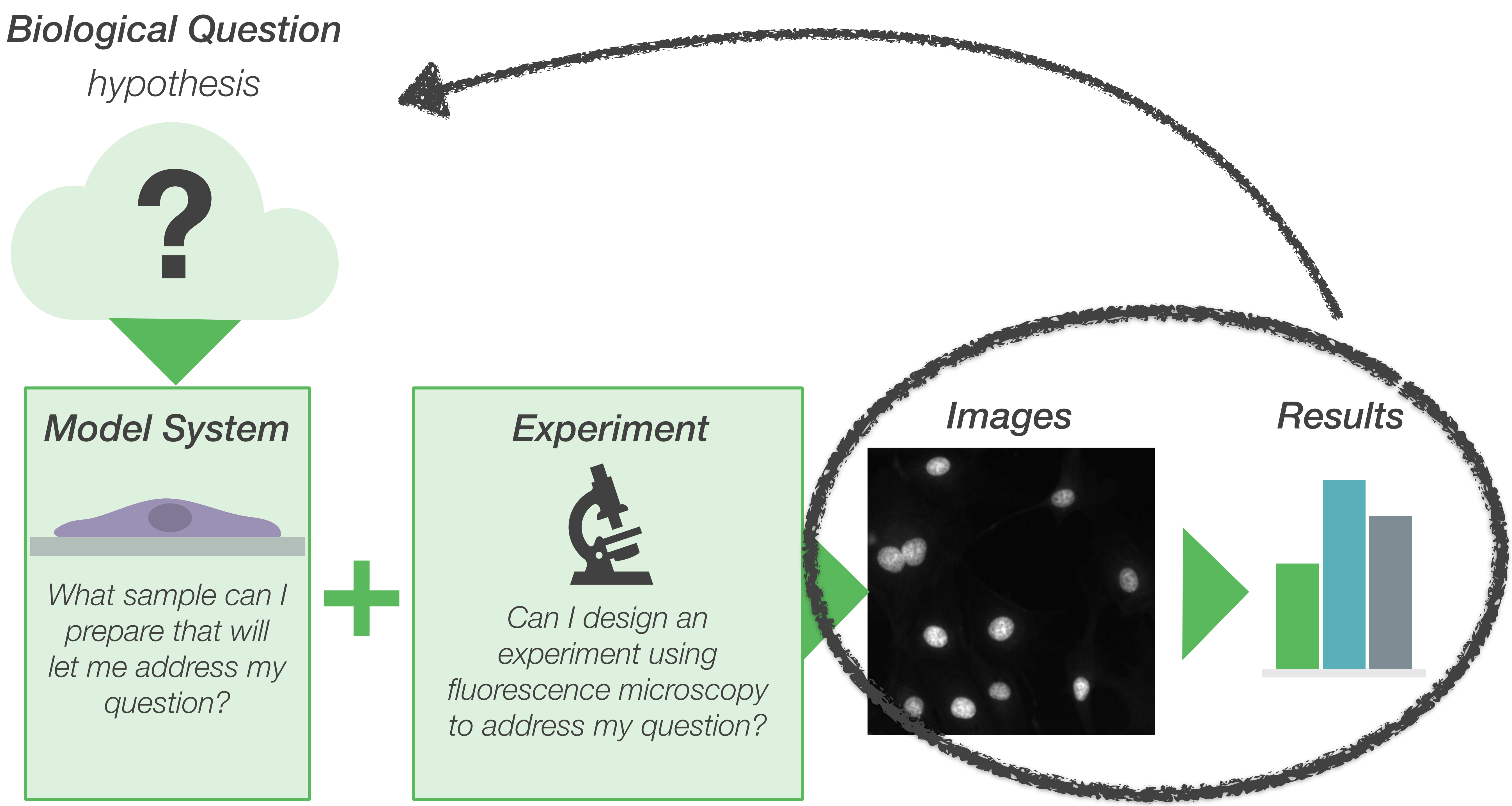
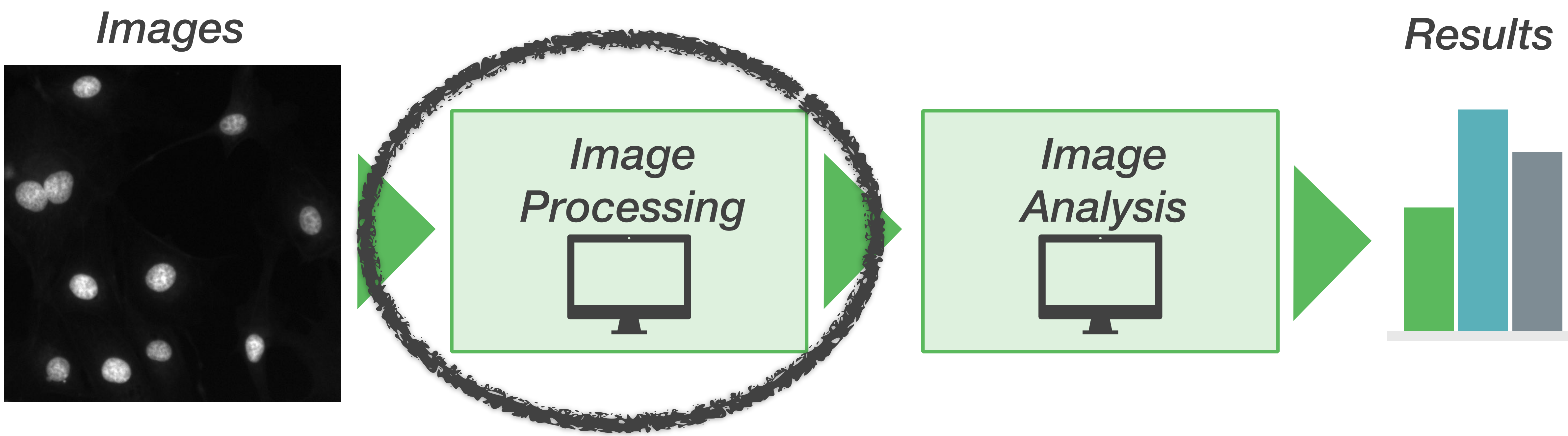

Biological Question
hypothesis
?
Model System
What sample can I prepare that will let me address my question?
+
Experiment
Can I design an experiment using fluorescence microscopy to address my question?
Images
Results



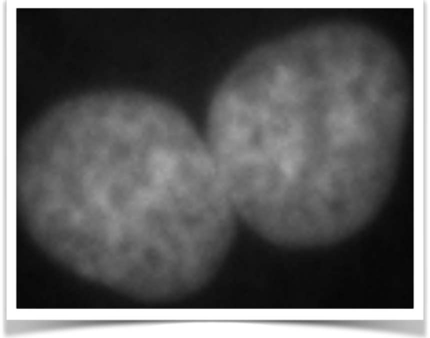

## *Today's Focus:*

**Analysis Goal:** Make measurements on objects in images

**Processing Goal:** *Select* individual objects in images

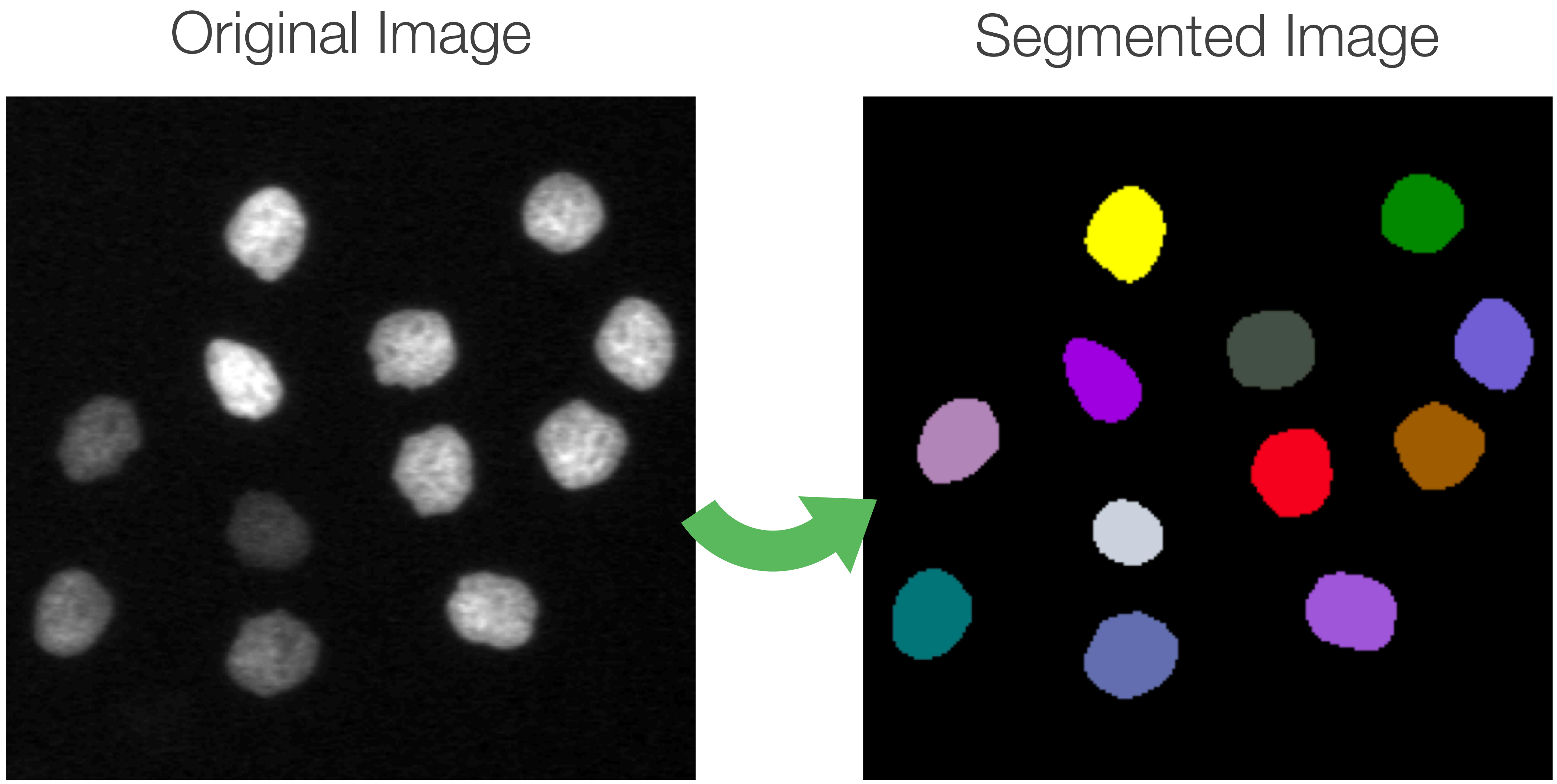
# Example Dataset

| | |
|---|---|
| ***WHAT*** | Fluorescence images of mammalian cell monolayer |
| ***LABEL*** | DAPI: a fluorophore that binds to minor grooves of dsDNA |
| ***DATASET*** | Widefield fluorescence images |



DAPI is a nuclear marker!

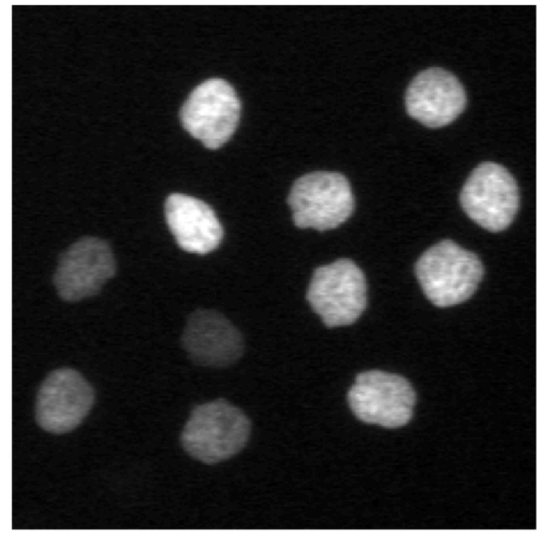
# **Analysis Goal:** Make a measurement on images of nuclei




To measure nuclei properties, we need to ***select*** each individual nucleus. This process is called ***image segmentation***.

# **2 types of segmentation:** Semantic & Instance

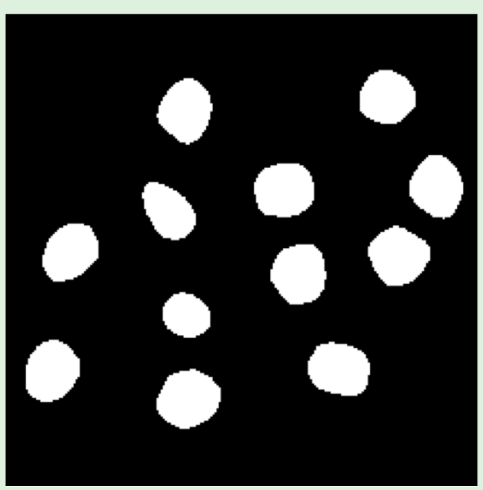
**Raw Image**



## Semantic Segmentation

All objects treated as the same category

**Example:**



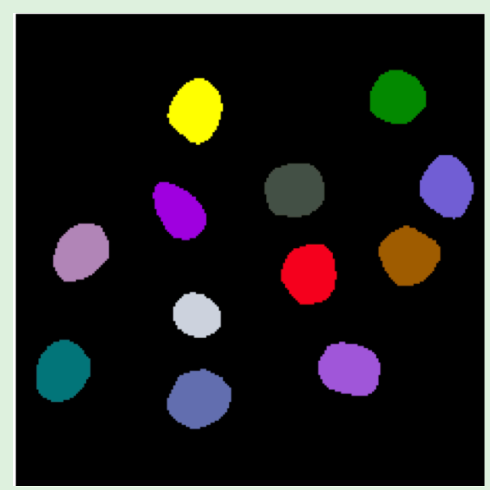
**Categories:**

Nucleus

## Instance Segmentation

Each object is distinguished as separate and has a unique label

**Example:**



**Categories:**

Nucleus 1

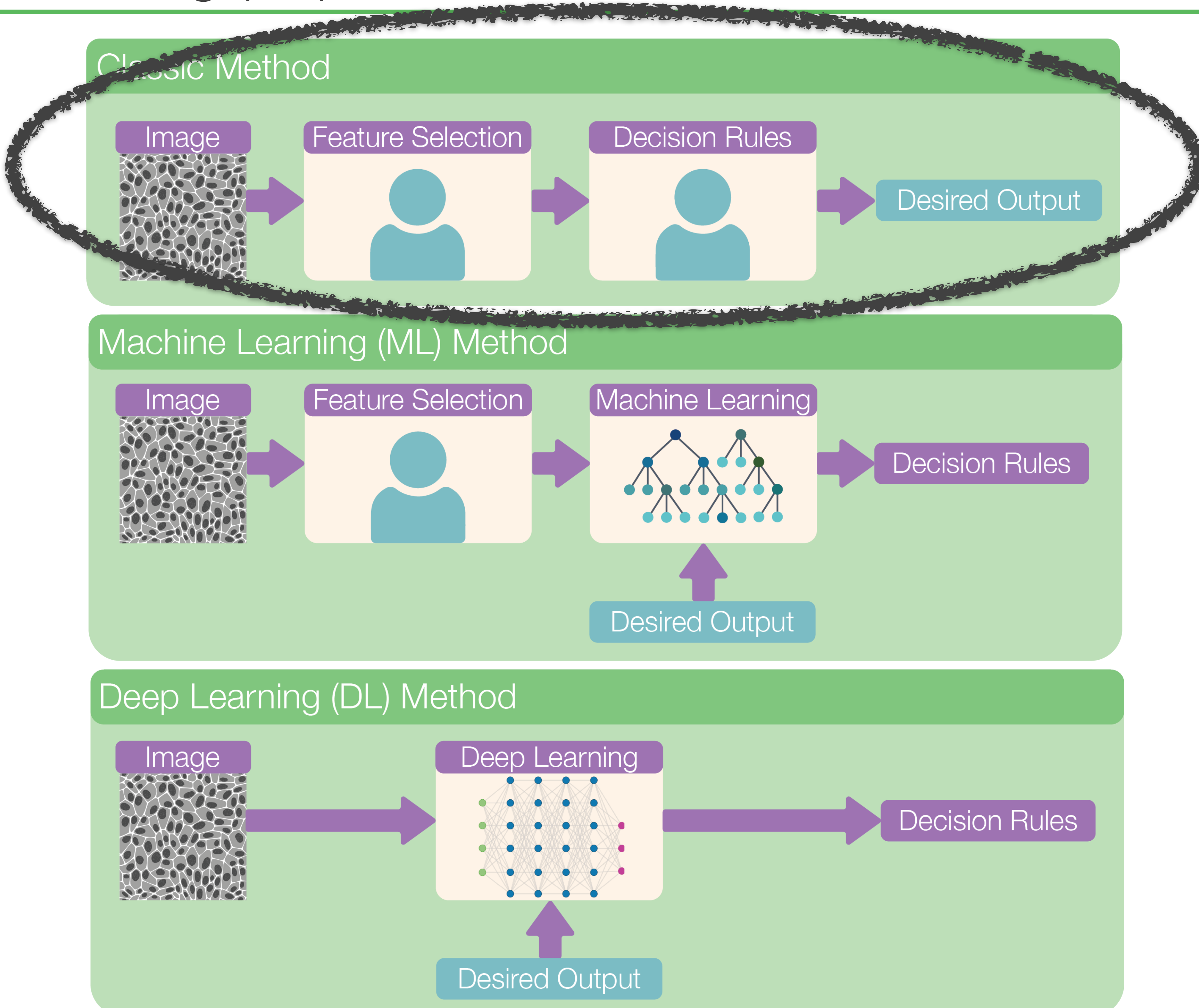
Nucleus 2

Nucleus 3

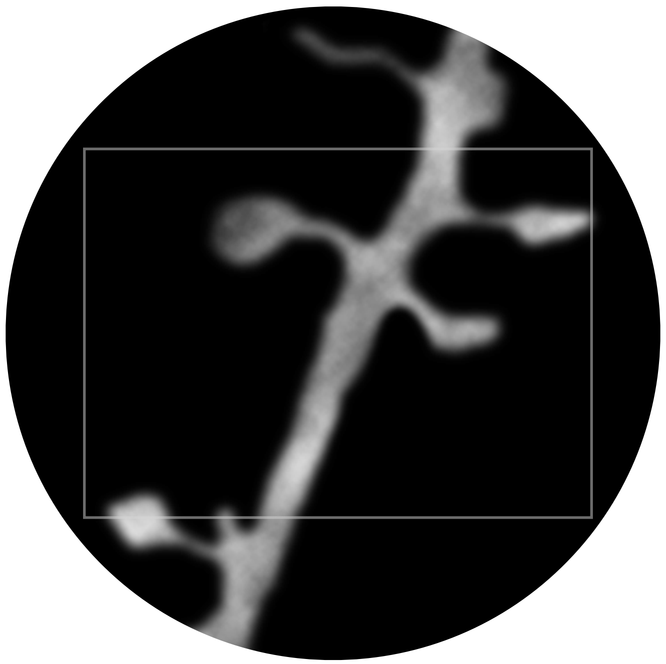
…

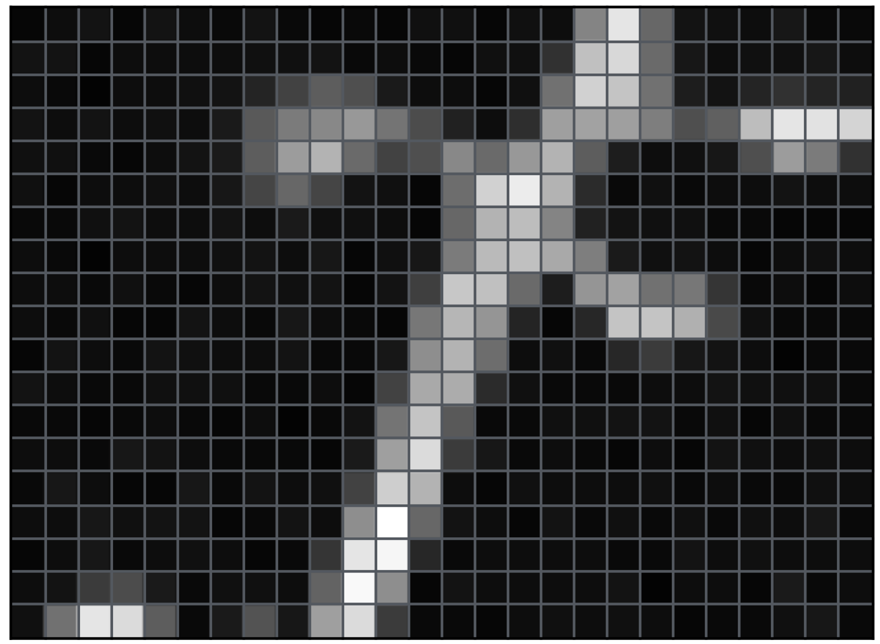
We can achieve either segmentation type with different ***pipelines***.

# **3 pipelines:** Classic, Machine Learning (ML), & Deep Learning (DL)



# The **classic pipeline** takes advantage of an image's spatial organization of intensity values





| | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 6 | 13 | 19 | 6 | 19 | 13 | 9 | 19 | 9 | 6 | 9 | 6 | 16 | 16 | 6 | 16 | 13 | 132 | 229 | 103 | 19 | 16 | 13 | 23 | 9 | 9 |
| 19 | 19 | 6 | 13 | 13 | 13 | 13 | 16 | 16 | 19 | 9 | 13 | 9 | 6 | 16 | 16 | 49 | 192 | 216 | 106 | 23 | 13 | 16 | 16 | 23 | 13 |
| 13 | 9 | 4 | 13 | 13 | 16 | 19 | 36 | 66 | 93 | 79 | 26 | 13 | 13 | 6 | 16 | 113 | 209 | 196 | 113 | 29 | 19 | 36 | 49 | 36 | 33 |
| 19 | 13 | 19 | 13 | 16 | 13 | 26 | 89 | 123 | 136 | 152 | 116 | 76 | 33 | 13 | 46 | 159 | 162 | 159 | 126 | 79 | 96 | 189 | 229 | 226 | 212 |
| 16 | 16 | 9 | 6 | 13 | 19 | 26 | 93 | 156 | 179 | 106 | 66 | 79 | 136 | 106 | 152 | 179 | 93 | 29 | 13 | 16 | 23 | 79 | 156 | 123 | 49 |
| 16 | 6 | 13 | 13 | 16 | 13 | 23 | 69 | 103 | 69 | 19 | 16 | 6 | 109 | 209 | 236 | 179 | 43 | 9 | 16 | 9 | 13 | 13 | 19 | 13 | 13 |
| 9 | 9 | 16 | 19 | 13 | 13 | 19 | 13 | 26 | 16 | 16 | 13 | 6 | 103 | 179 | 189 | 132 | 33 | 19 | 16 | 16 | 9 | 9 | 6 | 6 | 6 |
| 13 | 9 | 4 | 13 | 13 | 13 | 16 | 19 | 13 | 23 | 6 | 16 | 23 | 123 | 186 | 192 | 169 | 126 | 26 | 16 | 19 | 13 | 6 | 13 | 16 | 13 |
| 13 | 13 | 9 | 16 | 9 | 6 | 13 | 19 | 16 | 19 | 6 | 19 | 63 | 199 | 192 | 106 | 29 | 149 | 162 | 113 | 119 | 53 | 9 | 13 | 6 | 13 |
| 13 | 9 | 16 | 6 | 6 | 19 | 13 | 9 | 23 | 13 | 9 | 6 | 119 | 182 | 149 | 36 | 6 | 39 | 196 | 196 | 176 | 73 | 16 | 9 | 9 | 9 |
| 6 | 19 | 13 | 9 | 19 | 16 | 13 | 13 | 19 | 9 | 9 | 23 | 142 | 179 | 109 | 13 | 16 | 9 | 39 | 59 | 23 | 19 | 13 | 4 | 9 | 9 |
| 19 | 13 | 9 | 9 | 16 | 16 | 16 | 9 | 9 | 13 | 6 | 66 | 169 | 172 | 43 | 16 | 9 | 9 | 9 | 13 | 13 | 19 | 16 | 16 | 16 | 9 |
| 9 | 9 | 6 | 9 | 13 | 9 | 6 | 13 | 4 | 9 | 19 | 116 | 196 | 89 | 9 | 9 | 16 | 16 | 19 | 19 | 9 | 16 | 6 | 16 | 9 | 9 |
| 13 | 13 | 9 | 23 | 19 | 13 | 9 | 9 | 9 | 6 | 26 | 159 | 219 | 59 | 23 | 9 | 13 | 9 | 6 | 13 | 6 | 19 | 16 | 13 | 16 | 13 |
| 9 | 23 | 13 | 6 | 6 | 23 | 9 | 19 | 13 | 16 | 66 | 206 | 179 | 13 | 6 | 16 | 13 | 13 | 13 | 16 | 9 | 13 | 9 | 9 | 16 | 13 |
| 13 | 13 | 23 | 16 | 19 | 19 | 6 | 9 | 19 | 13 | 142 | 255 | 103 | 19 | 13 | 6 | 19 | 9 | 16 | 9 | 16 | 9 | 16 | 13 | 23 | 9 |
| 6 | 13 | 23 | 9 | 13 | 16 | 13 | 6 | 9 | 53 | 229 | 246 | 39 | 9 | 13 | 13 | 13 | 13 | 9 | 9 | 19 | 13 | 16 | 13 | 13 | 13 |
| 13 | 19 | 59 | 76 | 26 | 9 | 16 | 16 | 13 | 99 | 249 | 142 | 6 | 19 | 13 | 13 | 13 | 13 | 19 | 4 | 13 | 13 | 6 | 26 | 9 | 13 |
| 16 | 113 | 229 | 219 | 93 | 9 | 26 | 83 | 23 | 159 | 219 | 59 | 9 | 9 | 6 | 13 | 16 | 13 | 16 | 13 | 6 | 9 | 9 | 16 | 23 | 9 |

**photons**
optical image

**intensity values**
digital image

**intensity values ≠ photons!!**

# classic segmentation with Python

# classic segmentation with Python

- thresholding
- filtering
- labeling a binary mask
- refining a binary mask

# classic segmentation with Python

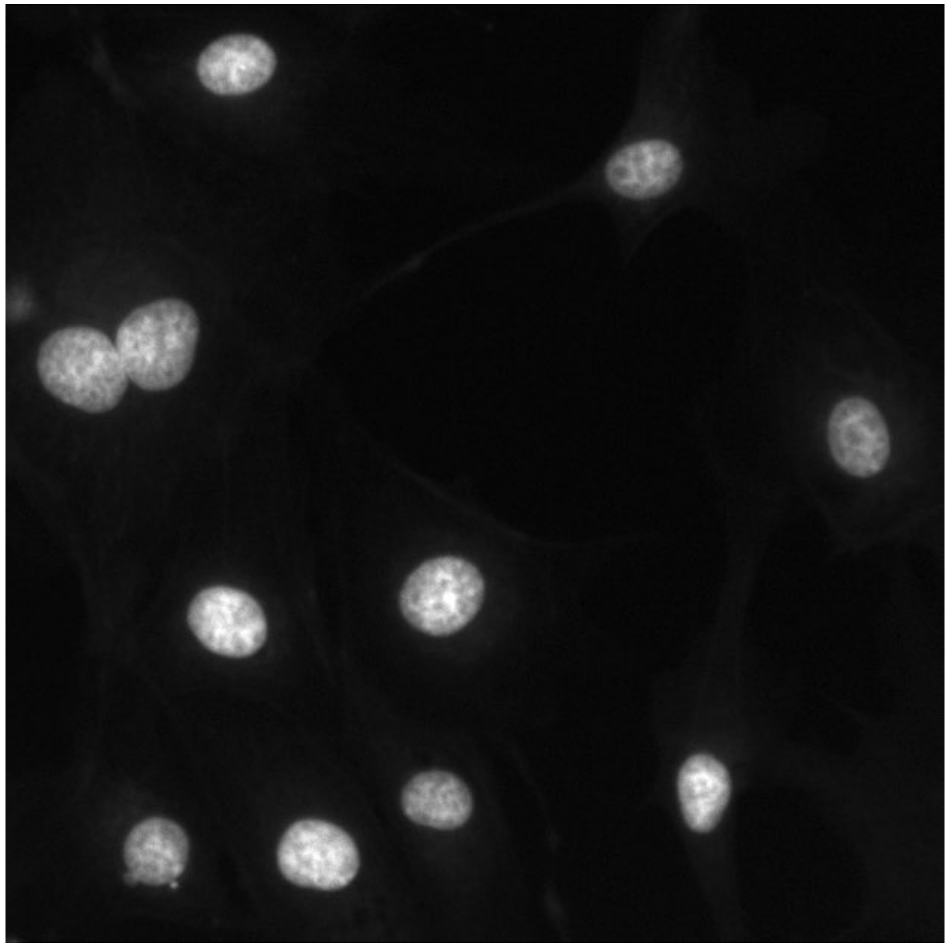
thresholding

filtering

labeling a binary mask

refining a binary mask

Original Image



Nuclei regions have higher intensity values than non-nuclei regions in the image

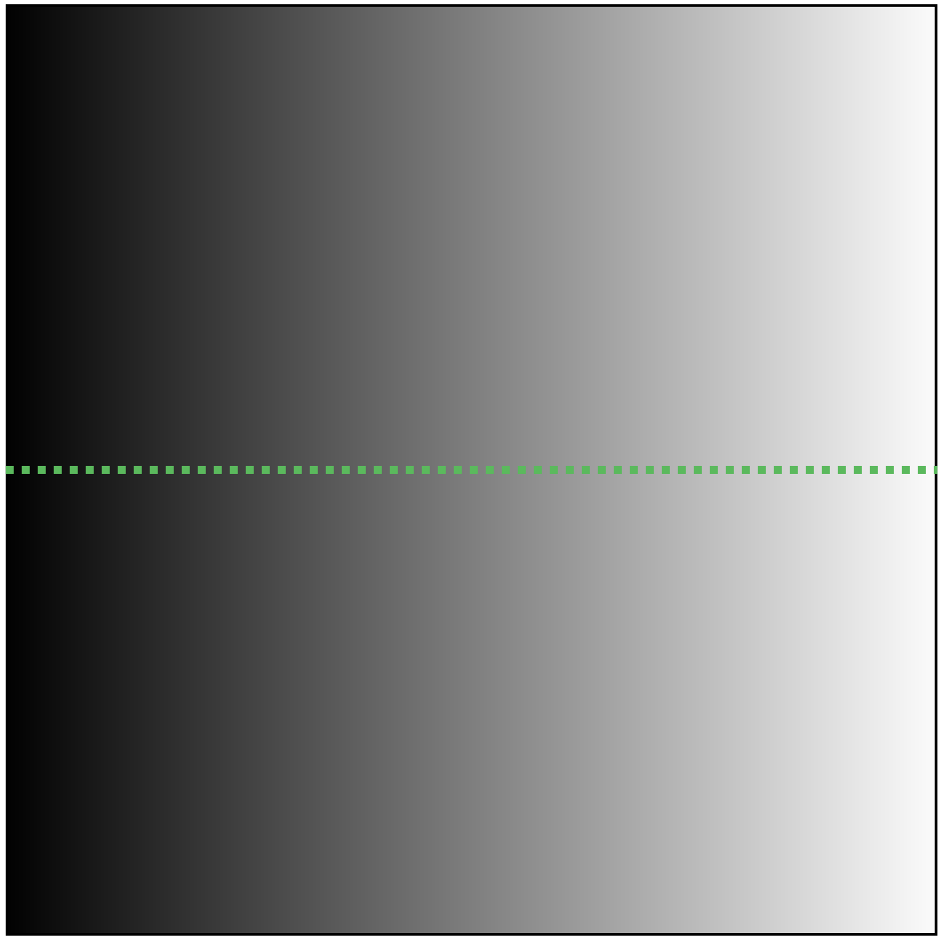
thresholding

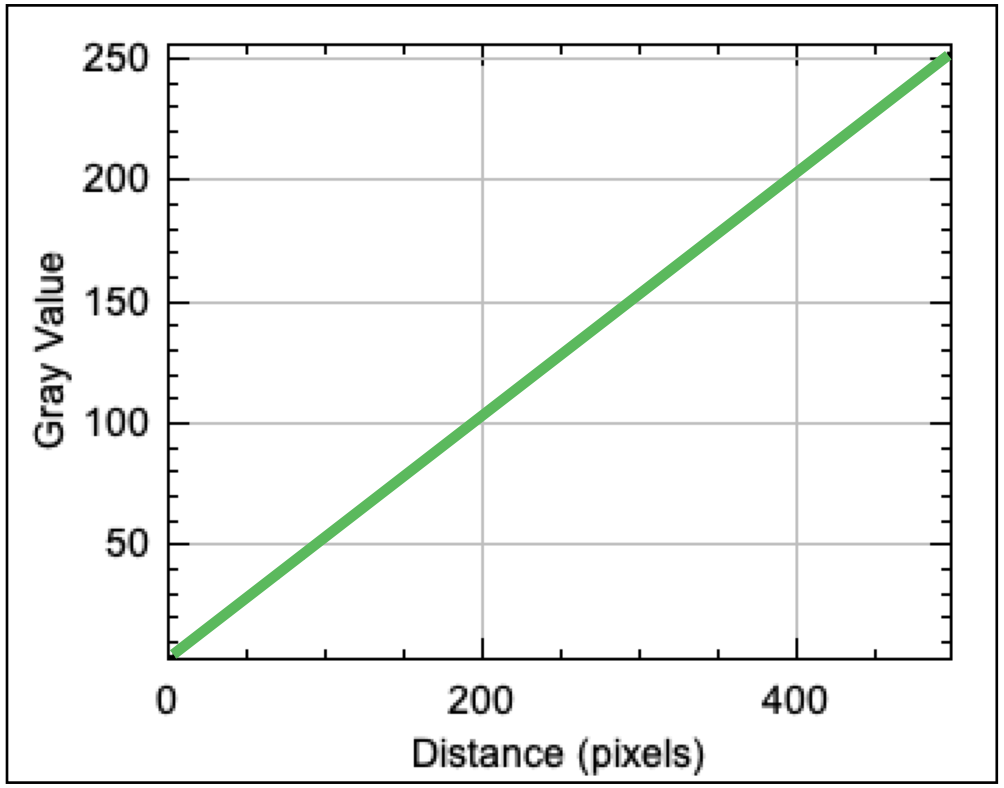
***select*** a ***range*** of ***digital values*** in the image

# thresholding

***select*** a ***range*** of ***digital values*** in the image

**8 bit image (0 - 255)***



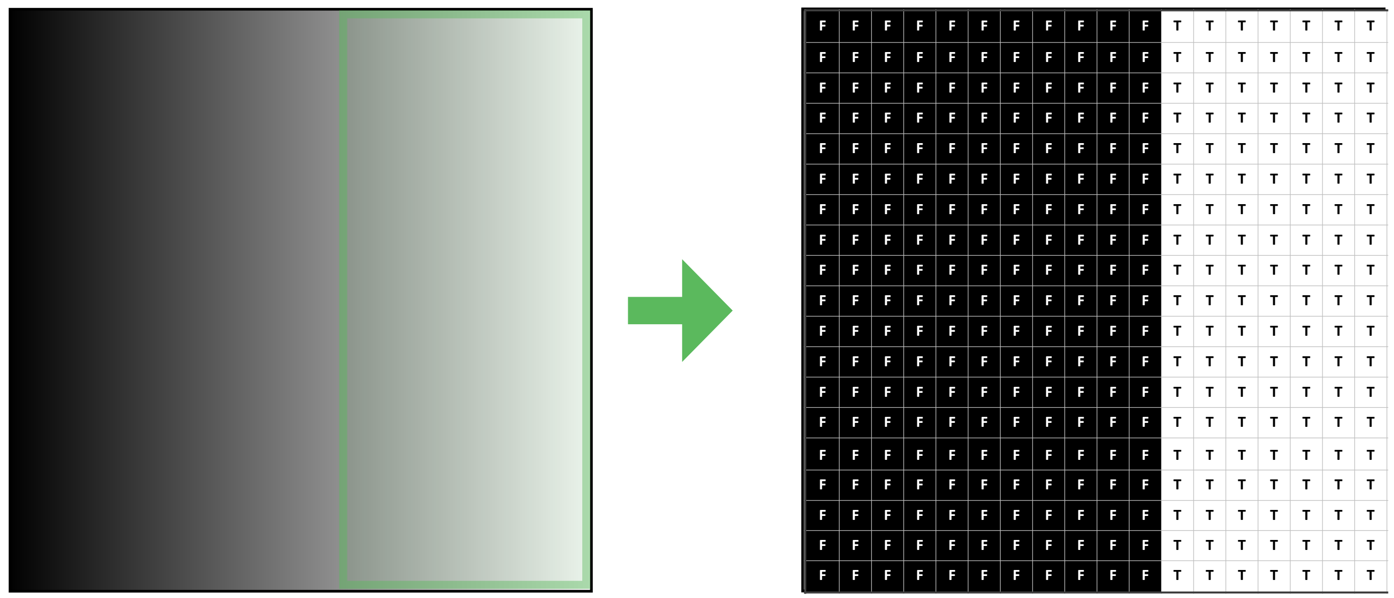



*8 bit image = **each pixel** can have **$2^8$ grey values** = 256 grey values = **range 0-255**

# The result of the thresholding process is a ***binary mask***

A **binary mask** is an image that has only 2 pixel values

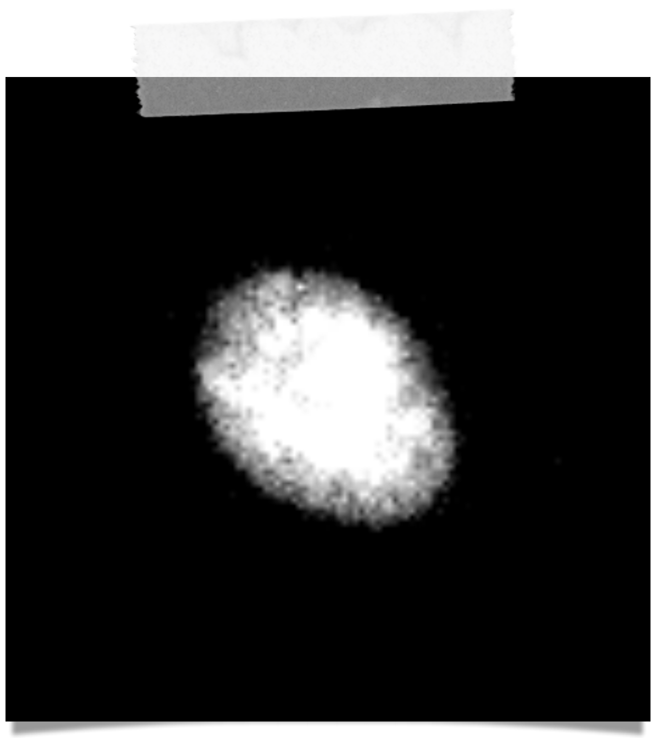
8 bit image (0 - 255) binary_mask



**False:** “discarded” pixels
**True:** selected pixels

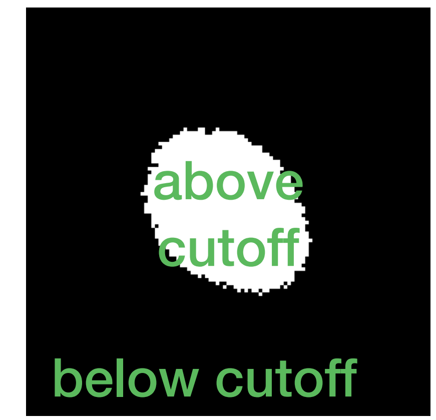
**FIJI’s binary masks have 0 and 255 values.**

# 2 ways to set a threshold



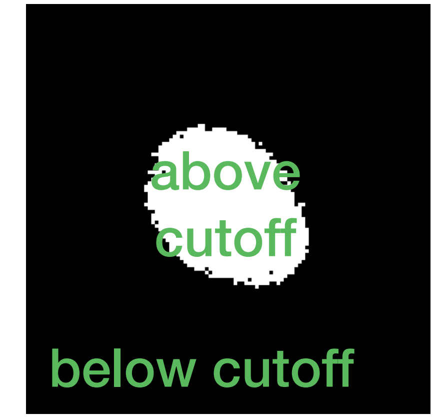
Thresholding by **manually** setting a min intensity count

Image Histogram


Thresholding by **automatically** setting a min intensity count using a thresholding algorithm

Image Histogram


**Use thresholding algorithms so that thresholding will be reproducible across many images.**

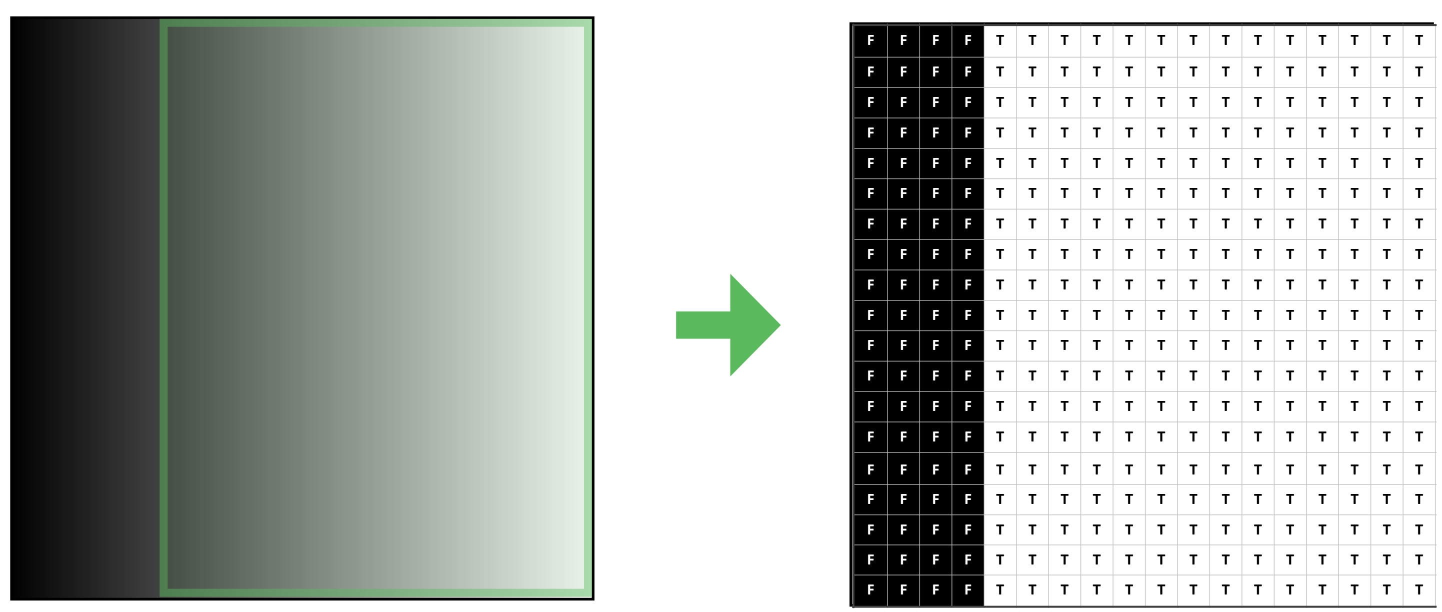
# Thresholding in Python

Thresholding by **manually** setting a min intensity count

[1]:
```python
threshold = 50 # intensity value cutoff
binary_mask = raw_image > threshold
```

8 bit image (0 - 255)

binary_mask



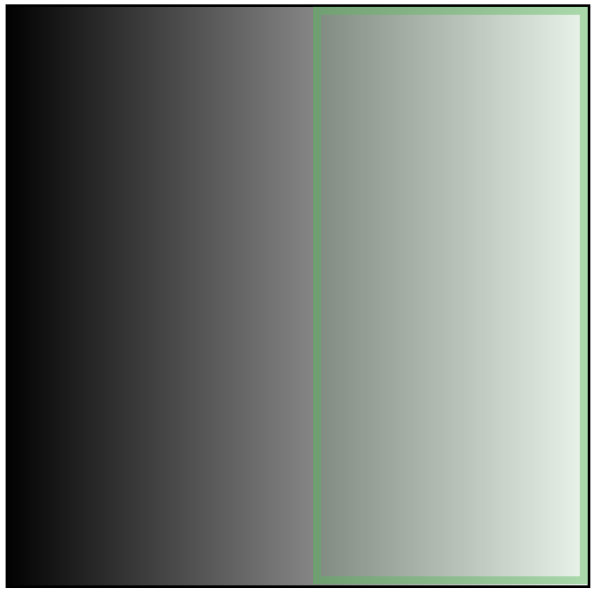
**False:** “discarded” pixels
**True:** selected pixels

# Thresholding in Python

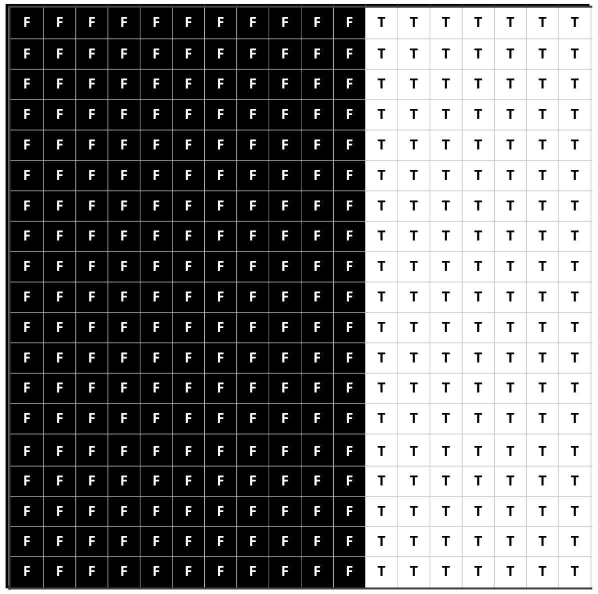
Thresholding by **manually** setting a
min intensity count

[1]:
```
threshold = 100 # intensity value cutoff
binary_mask = raw_image > threshold
```

8 bit image (0 - 255)



binary_mask

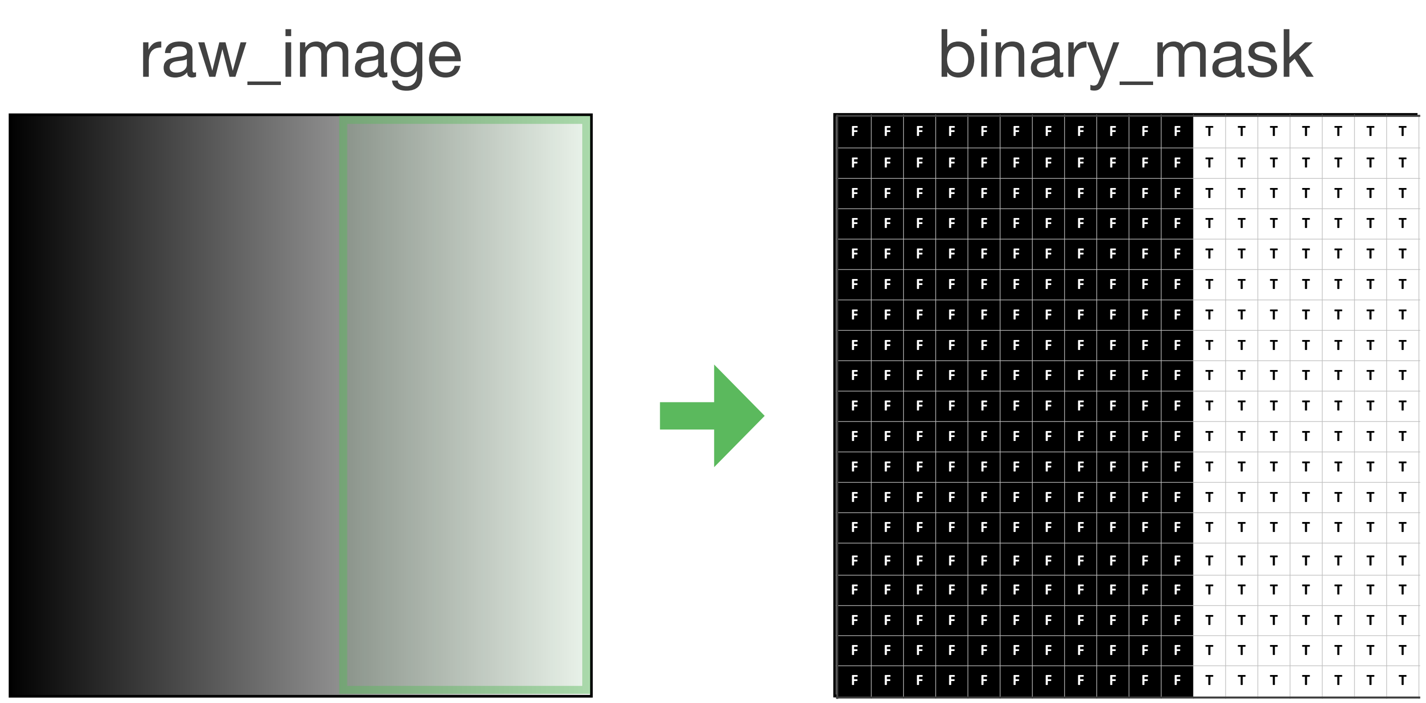


**False:** "discarded" pixels
**True:** selected pixels

# Thresholding in Python

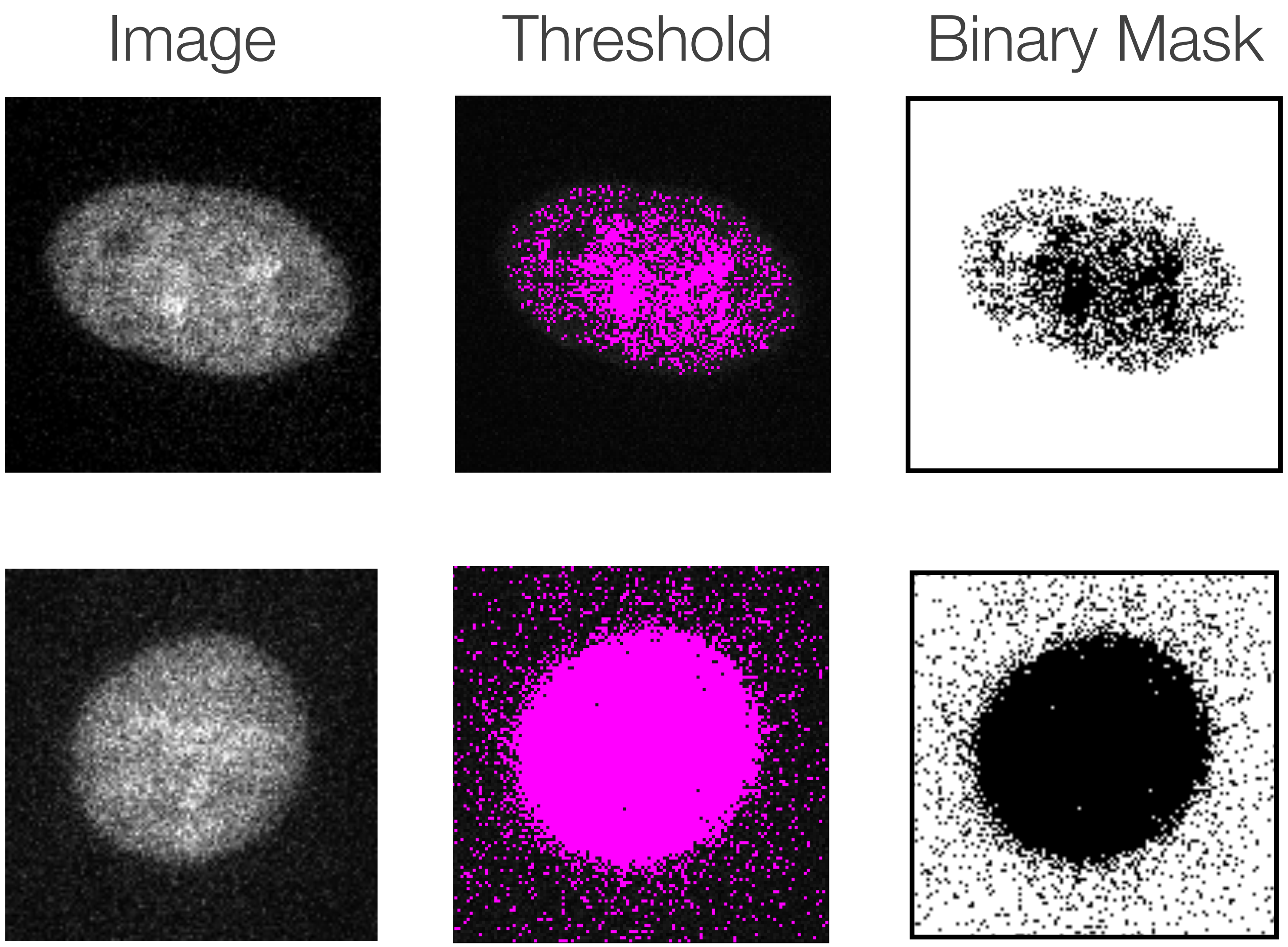
Thresholding by **automatically** setting a min intensity count using a thresholding algorithm

[1]:
```python
# use Otsu thresholding algorithm
from skimage.filters import threshold_otsu
binary_mask = raw_image >
              threshold_otsu(raw_image)
```




**False:** “discarded” pixels
**True:** selected pixels

# Usually if you apply thresholding to the original image, you won't precisely select all or only pixels of interest




## Many factors can contribute to variance in pixel values:

fluorescence label (e.g. DAPI)

background (uneven illumination, out of focus light, aberration)

detector offset

noise (detector read noise, poisson noise, etc.)

# classic segmentation with Python

- thresholding
- ~~filtering~~
- labeling a binary mask
- refining a binary mask

# classic segmentation with Python

filtering

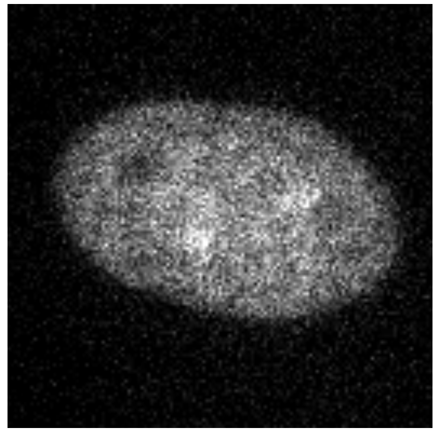
thresholding

~~filtering~~

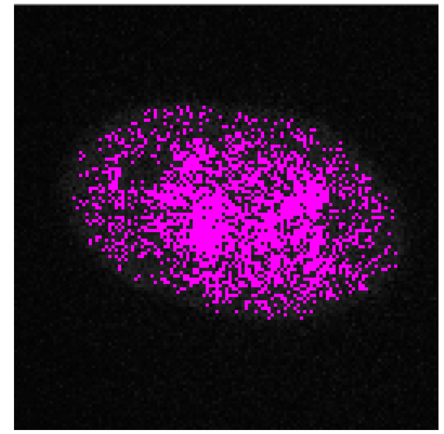
labeling a binary mask

refining a binary mask

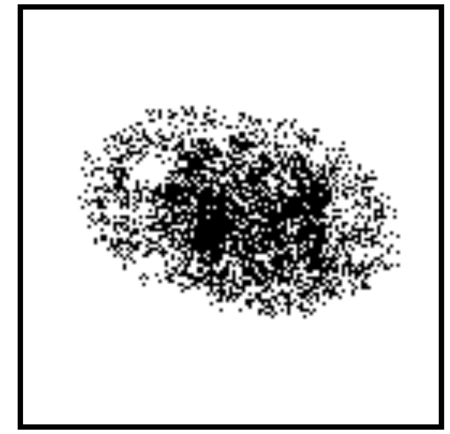
Image



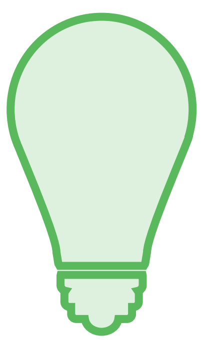
Threshold



Binary Mask

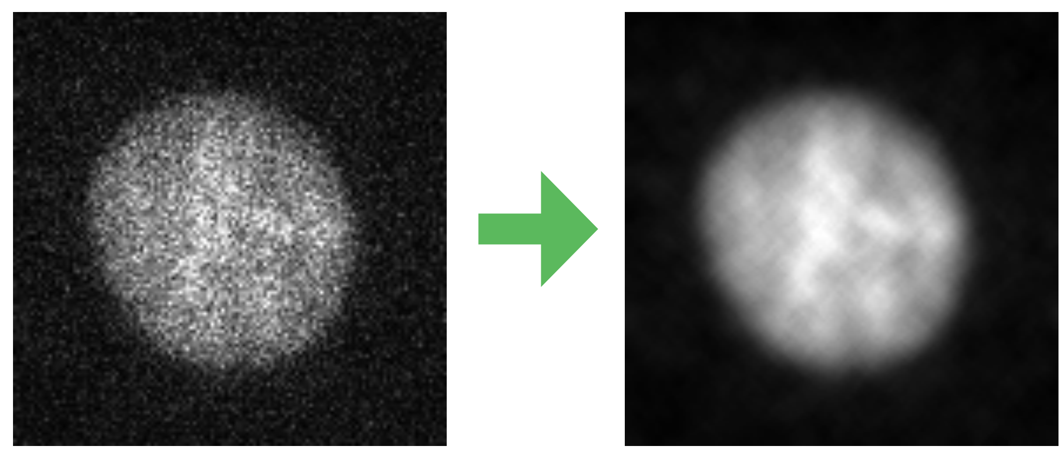


What if we apply an image filter before thresholding?



Image

Filtered Image

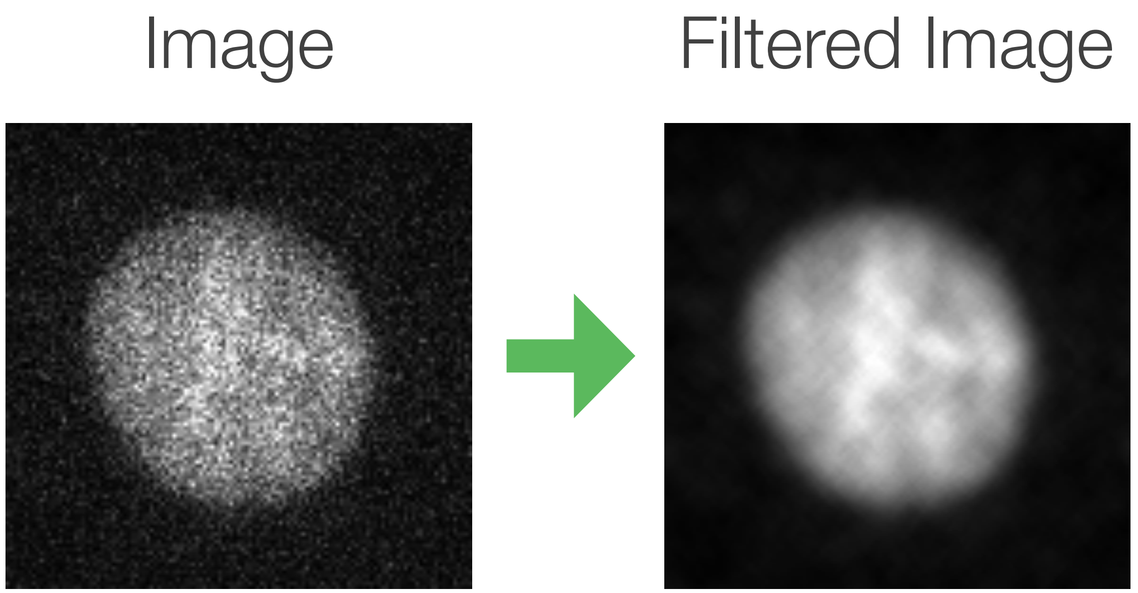


filtering

***Change*** image pixel values using a ***mathematical operation*** to smooth and reduce noise from images.

***Change*** image pixel values using a ***mathematical operation*** to smooth and reduce noise from images.

we are mathematically changing this image's pixel values when we apply a filter.



# How most filters work mathematically

***Convolve*** an image with a ***3x3 kernel***

**Image**

| | | |
|---|---|---|
| $px_1$ | $px_2$ | $px_3$ |
| $px_4$ | $px_5$ | $px_6$ |
| $px_7$ | $px_8$ | $px_9$ |

$px_n$ = pixel value

$\otimes$

**3x3 kernel**

| | | |
|---|---|---|
| $k_1$ | $k_2$ | $k_3$ |
| $k_4$ | $k_5$ | $k_6$ |
| $k_7$ | $k_8$ | $k_9$ |

$k_n$ = value

$$=$$

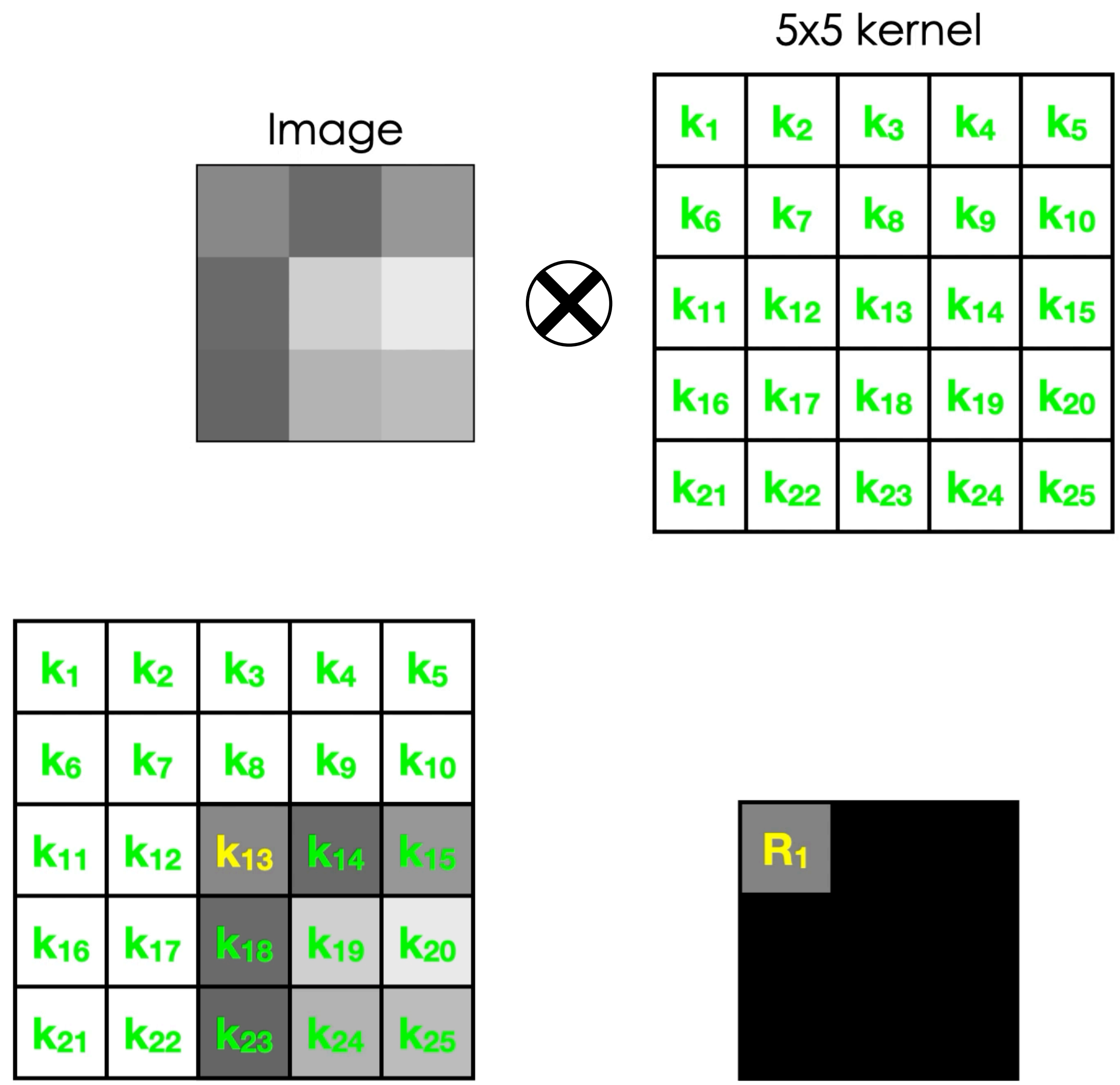
$$
\begin{aligned}
& px_1 * (k_1/k_{sum}) = r_1 \\
+ & px_2 * (k_2/k_{sum}) = r_2 \\
+ & px_3 * (k_3/k_{sum}) = r_3 \\
+ & px_4 * (k_4/k_{sum}) = r_4 \\
+ & px_5 * (k_5/k_{sum}) = r_5 \\
+ & px_6 * (k_6/k_{sum}) = r_6 \\
+ & px_7 * (k_7/k_{sum}) = r_7 \\
+ & px_8 * (k_8/k_{sum}) = r_8 \\
+ & px_9 * (k_9/k_{sum}) = r_9 \\
\hline
& \qquad\qquad\qquad\quad R
\end{aligned}
$$

$k_{sum}$ = sum of $k_n$ ($k_1 + k_2 + k_3 + \ldots$)

**Filtered Image**
(Only central pixel)

?

# ***Convolve*** an image with a ***5x5 kernel***




When you apply a filter, you can specify the kernel size you are convolving with.

# examples of filters good at reducing noise

mean filter

Gaussian blur filter

median filter

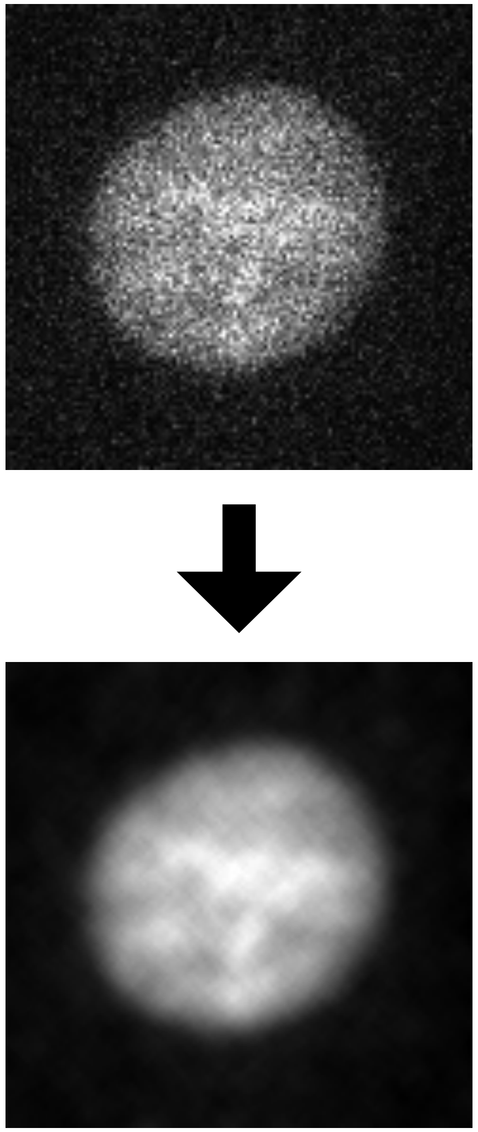
# mean filter

sum values in a list and then divide by total number of values

Footprint refers to the kernel size.

**3x3 kernel**

| | | |
|---|---|---|
| 1 | 1 | 1 |
| 1 | 1 | 1 |
| 1 | 1 | 1 |



**Larger Footprint = Bigger Kernel = Higher Blur**

# examples of filters good at reducing noise

- mean filter
- Gaussian blur filter
- median filter

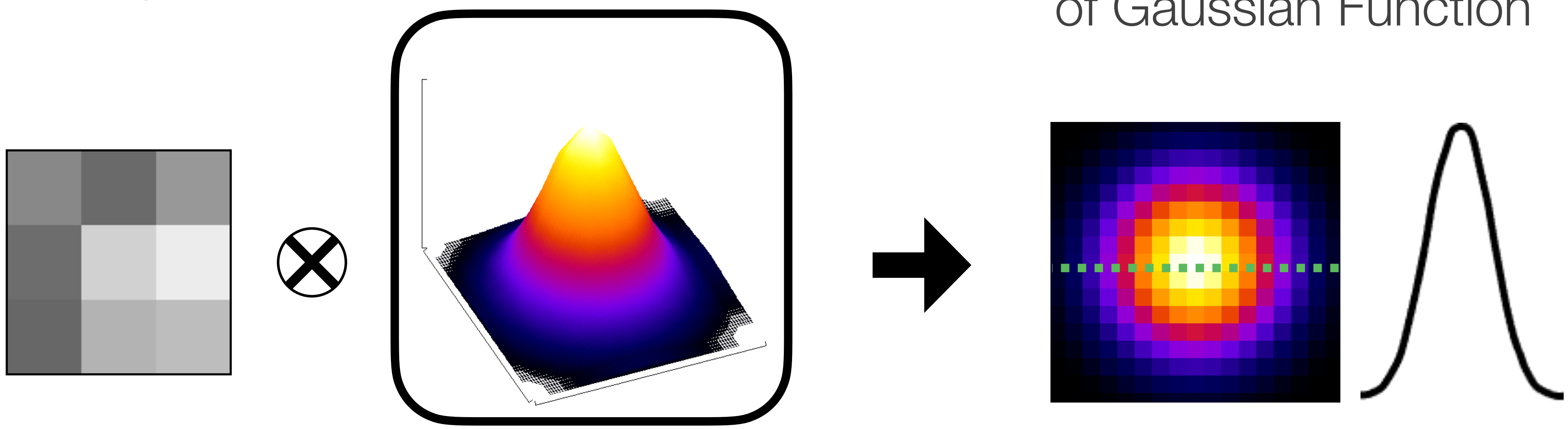
## Gaussian blur filter

multiply each value by Gaussian profile weighting, then divide by total number of values

Image

Gaussian Function

2D top view
of Gaussian Function



Sigma refers to the kernel size.

**Sigma 1 = 5x5 kernel**

**273**

| | | | | |
|---|---|---|---|---|
| 1 | 4 | 7 | 4 | 1 |
| 4 | 16 | 26 | 16 | 4 |
| 7 | 26 | 41 | 26 | 7 |
| 4 | 16 | 26 | 16 | 4 |
| 1 | 4 | 7 | 4 | 1 |

# How is a ***mean filter*** different from a ***Gaussian blur filter***?

mean filter

***VS.***

Gaussian blur filter

In a **Mean Filter**, the **kernel values** are all the **same**

**average**: all pixels are given the same weight.

**25**

| | | | | |
|---|---|---|---|---|
| 1 | 1 | 1 | 1 | 1 |
| 1 | 1 | 1 | 1 | 1 |
| 1 | 1 | 1 | 1 | 1 |
| 1 | 1 | 1 | 1 | 1 |
| 1 | 1 | 1 | 1 | 1 |

**5x5 kernel**

In a **Gaussian Blur Filter**, the **kernel values** follow a **Gaussian profile**

**weighted average**: pixels nearest the center of the kernel are given more weight than those far away from the center.

**273**

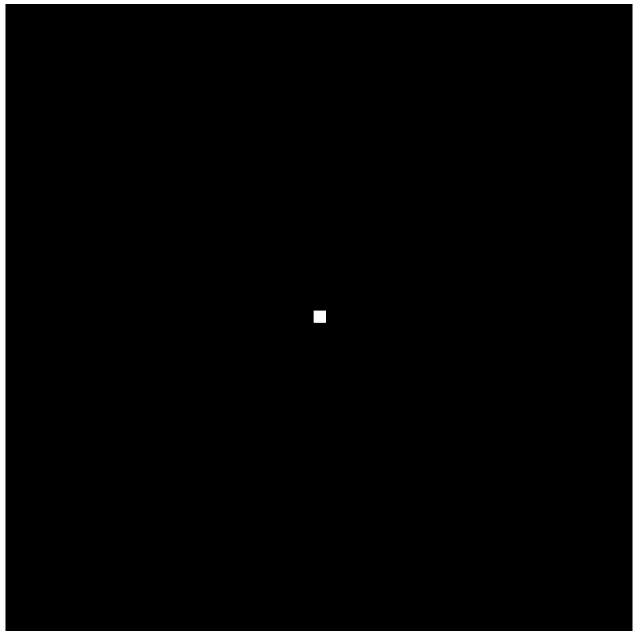
| | | | | |
|---|---|---|---|---|
| 1 | 4 | 7 | 4 | 1 |
| 4 | 16 | 26 | 16 | 4 |
| 7 | 26 | 41 | 26 | 7 |
| 4 | 16 | 26 | 16 | 4 |
| 1 | 4 | 7 | 4 | 1 |

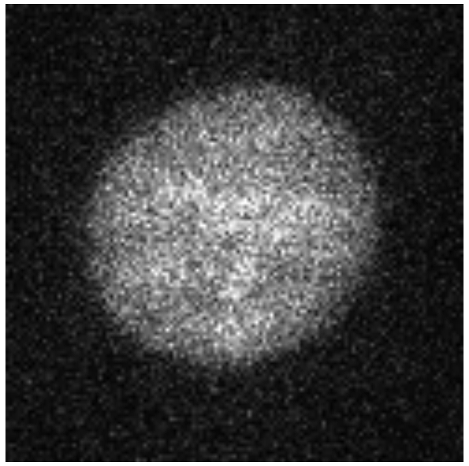
**sigma = 1** **5x5 kernel**

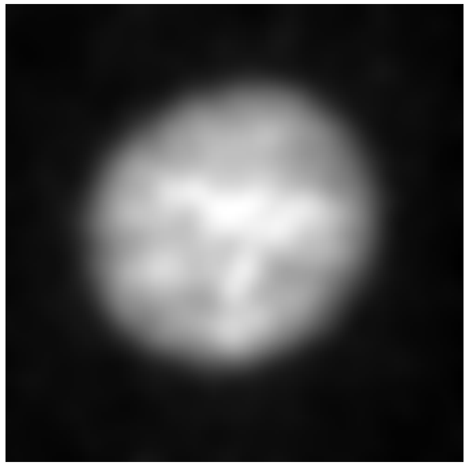
gaussian blur filter

multiply each value by gaussian profile weighting, then divide by total number of values

Image







**Higher Sigma = Bigger Kernel = Higher Blur**

examples of filters good at reducing noise

- ✓ mean filter
- ✓ Gaussian blur filter
- median filter

# median filter

take the middle number in a sorted list of numbers

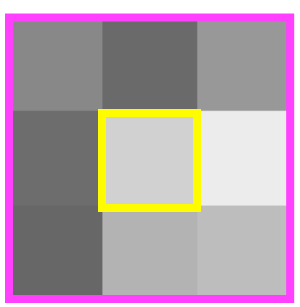
Footprint refers to the kernel size.

**3x3 kernel**

| | | |
|---|---|---|
| 1 | 1 | 1 |
| 1 | 1 | 1 |
| 1 | 1 | 1 |

**Default = 3x3 kernel**

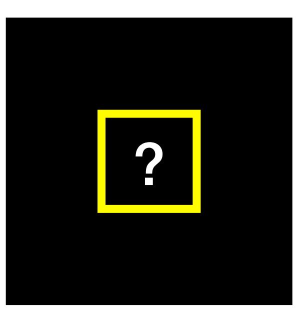
Apply a median filter with 3x3 kernel.
***What is the value of the central pixel in the filtered image?***



| | | |
|---|---|---|
| **136** | **106** | **152** |
| **109** | **209** | **236** |
| **103** | **179** | **189** |

**Look at all of the numbers in this kernel size and find the middle value**

[103, 106, 109, 136, **152**, 179, 189, 209, 236]

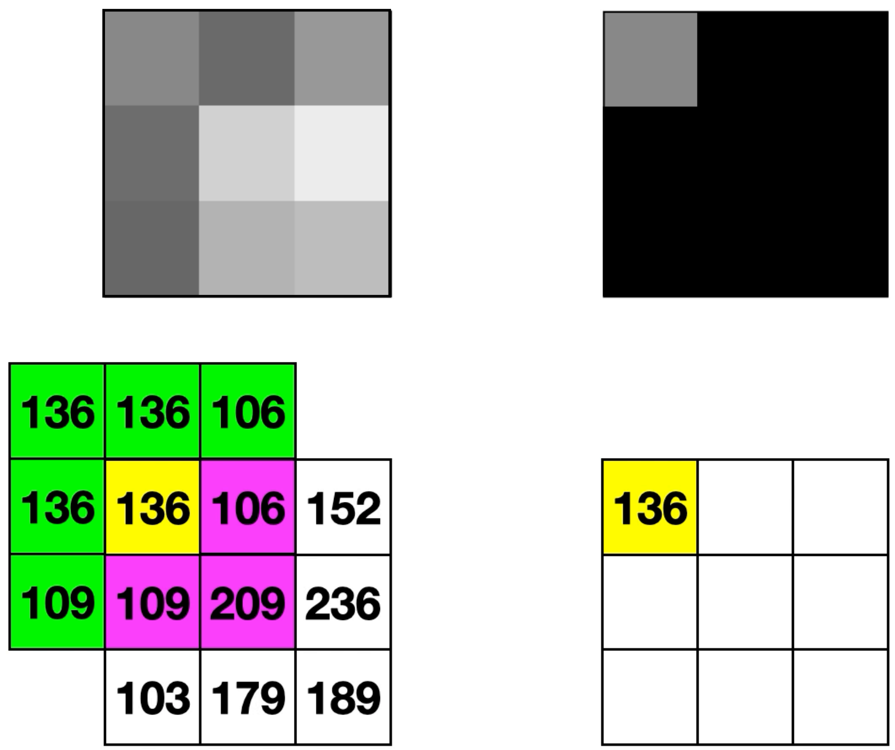



| | | |
|---|---|---|
| | | |
| | **?** | |
| | | |

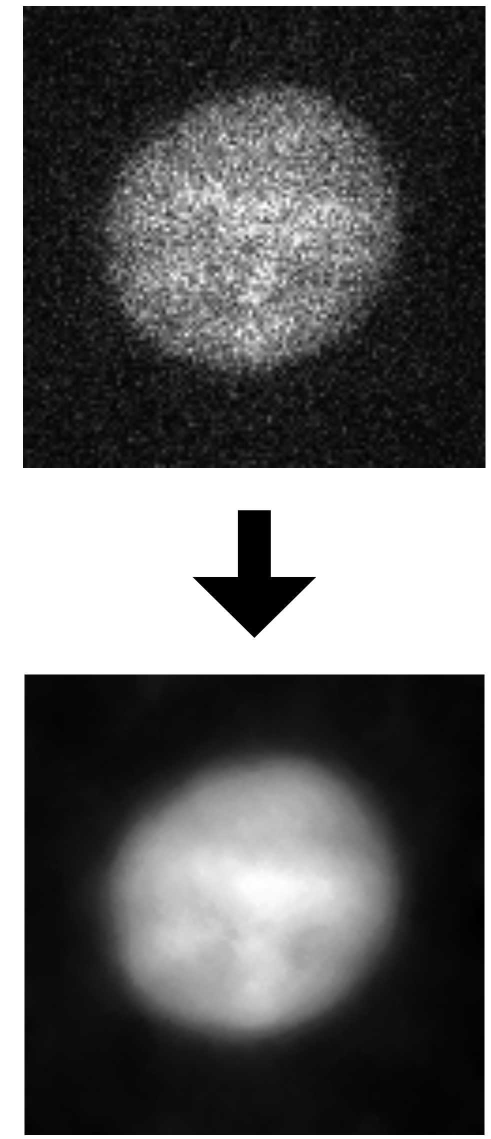
# median filter

take the middle number in a sorted list of numbers

3x3 kernel



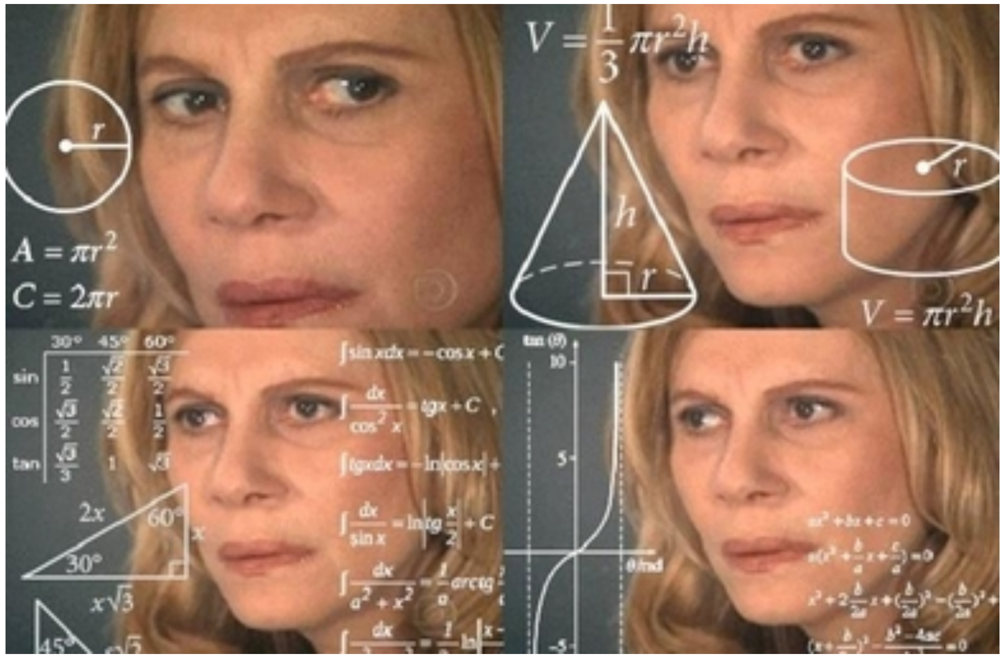

[106, 106, 109, 109, 136, 136, 136, 136, 209]



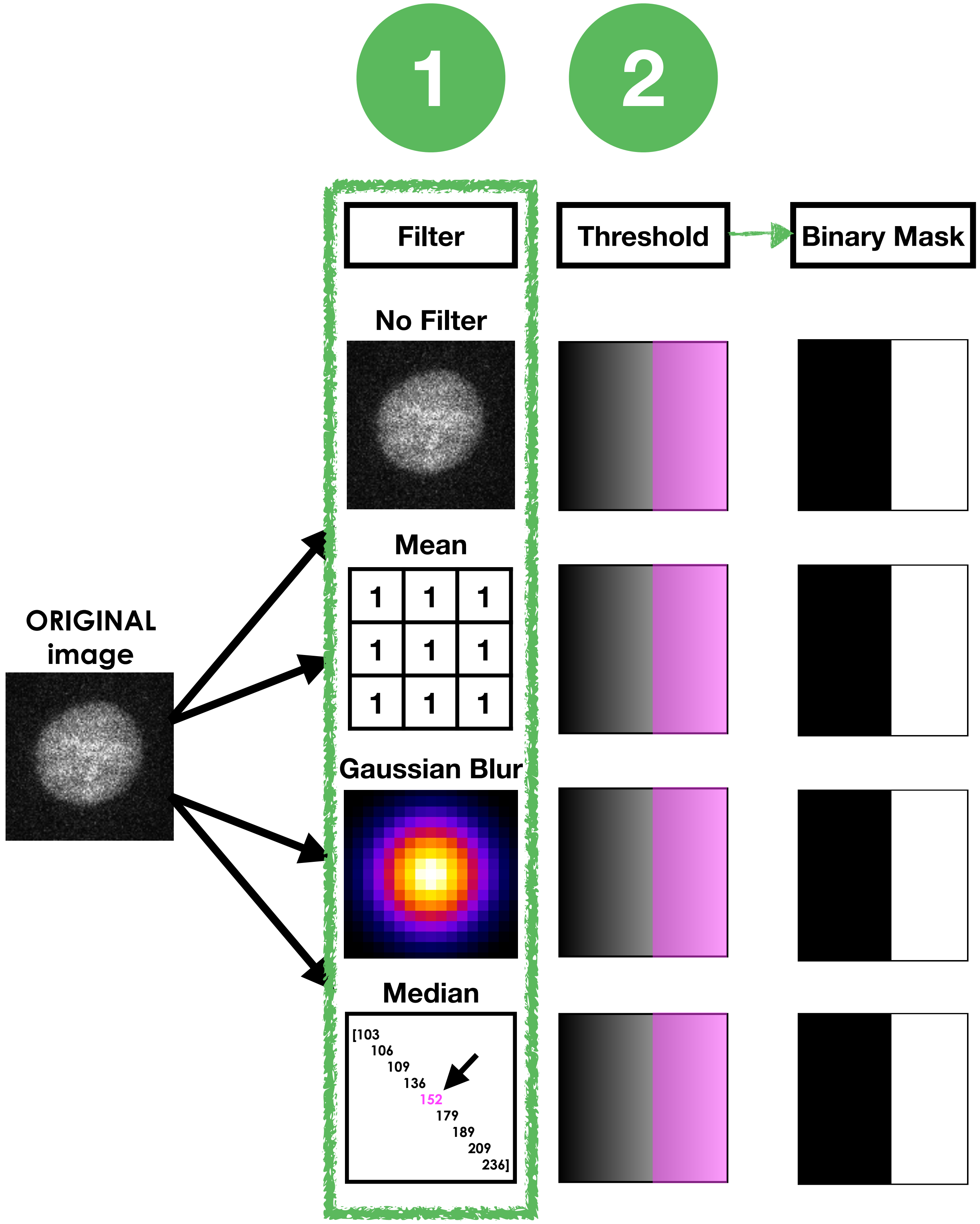
**Bigger Kernel = Higher Blur**
**Median filters don’t use convolution!**

# examples of filters good at reducing noise

- mean filter
- Gaussian blur filter
- median filter




Thinking about filter math can take some time to get used to.




# Which filter should you choose?

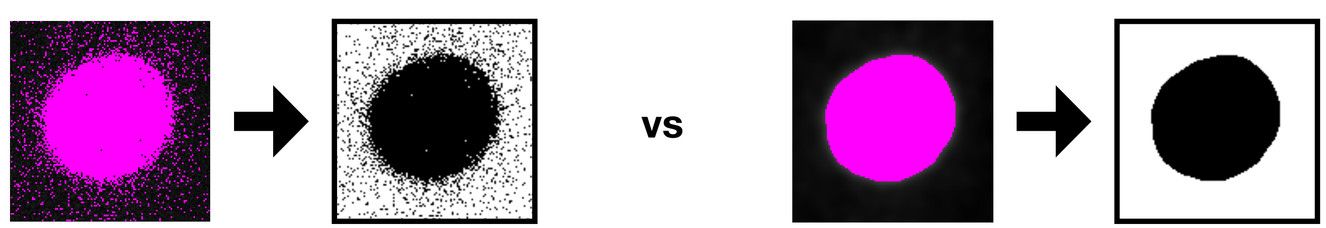
# Choose the filter parameters that give you the best binary mask result

test different filters

- mean filter
- gaussian blur filter
- median filter

…and different kernel sizes

choose the combination that gives you the best binary mask result



# Filtering in Python

Gaussian blur filter

[1]:

```python
# use Gaussian blur filter
from skimage.filters import gaussian
filtered_image = gaussian(raw_image)
```

thresholding algorithm

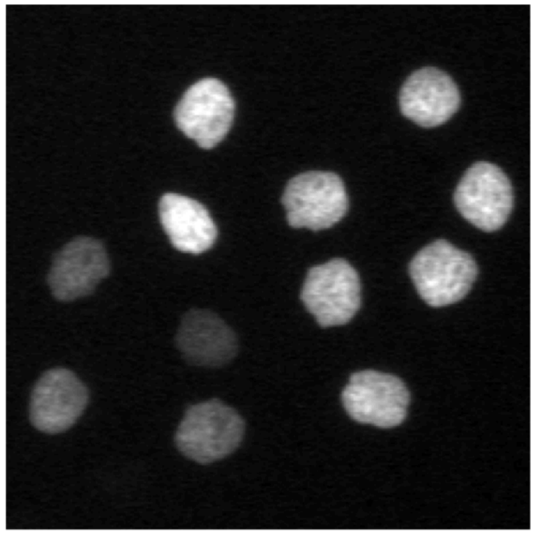
[ ]:

```python
# use Otsu thresholding algorithm
binary_mask = filtered_image >
              threshold_otsu(filtered_image)
```

Now what do we do with the binary mask?

# 2 types of segmentation: Semantic & Instance

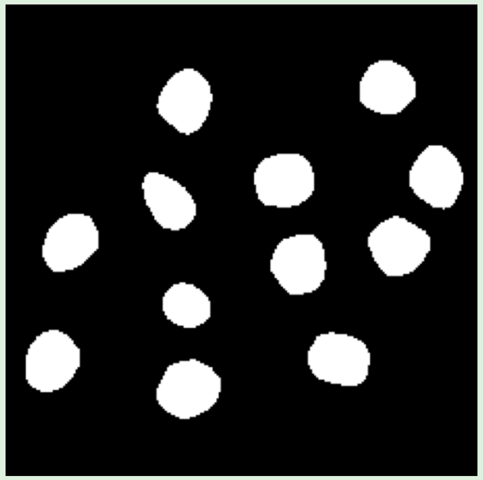
**Raw Image**



## Semantic Segmentation

All objects treated as the same category

**Example:**



**Categories:**

Nuclei

## Instance Segmentation

Each object is distinguished as separate and has a unique label

**Example:**

**Categories:**

Nucleus 1

Nucleus 2

Nucleus 3

…

We need to do another step to accomplish this.

classic segmentation with Python

filtering

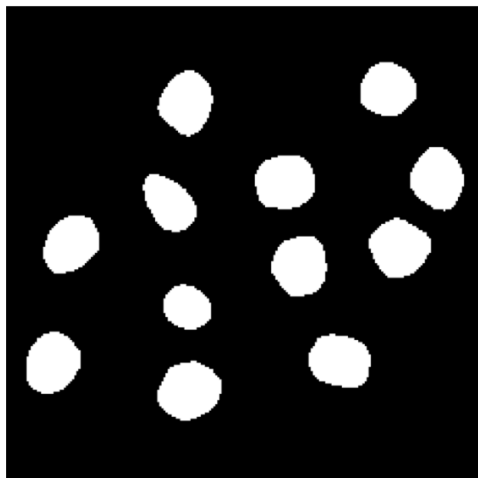
thresholding

~~filtering~~

labeling a binary mask

refining a binary mask

# Now that we have a binary mask, we need a way to ***distinguish individual objects of interest*** in the mask



**Categories:**

Nucleus

**Categories:**

Nucleus 1

Nucleus 2

Nucleus 3

…

# Labeling a mask in Python

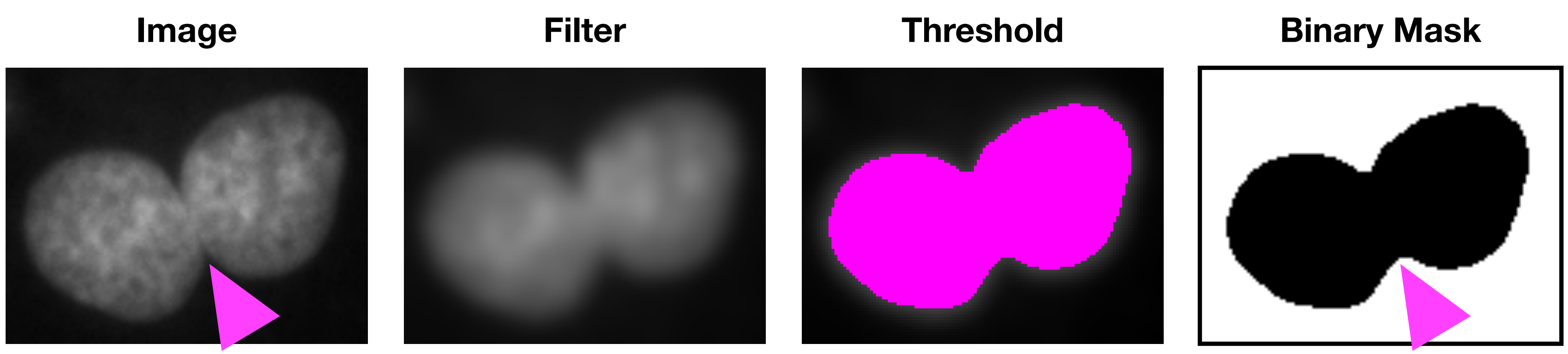
labeling a mask

```
[ ]: from skimage.measure import label
     labeled_image = label(binary_mask)
```

**Lab:**

Classic Segmentation Notebook, Steps 0-4

# What if the binary mask isn’t perfect?




2 or more nuclei in the binary mask image are touching each other, resulting in them being considered as a single object.

# classic segmentation with Python

- filtering
- thresholding
- ~~filtering~~
- labeling a binary mask
- refining a binary mask

# Sometimes binary masks need to be ***refined***

**mask refinement:** additional processing steps applied to a binary mask to more accurately match the image foreground.

## common problems:

**miscellaneous schmutz**

**holes inside objects**

**touching objects**

**Morphological operations:** a family of algorithms that are helpful for modifying object shapes

**Watershed Algorithm:** useful for separating touching objects.

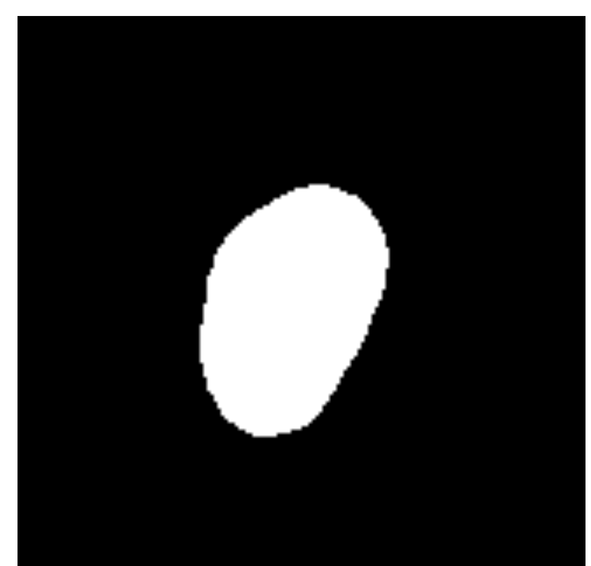
miscellaneous schmutz

**Morphological operations:** a family of algorithms that are helpful for modifying object shapes

opening

~ removes small objects from foreground

```
[ ]: from skimage.morphology import remove_small_objects
     binary_mask_sized = remove_small_objects(
                                   binary_mask,minsize=10)
```



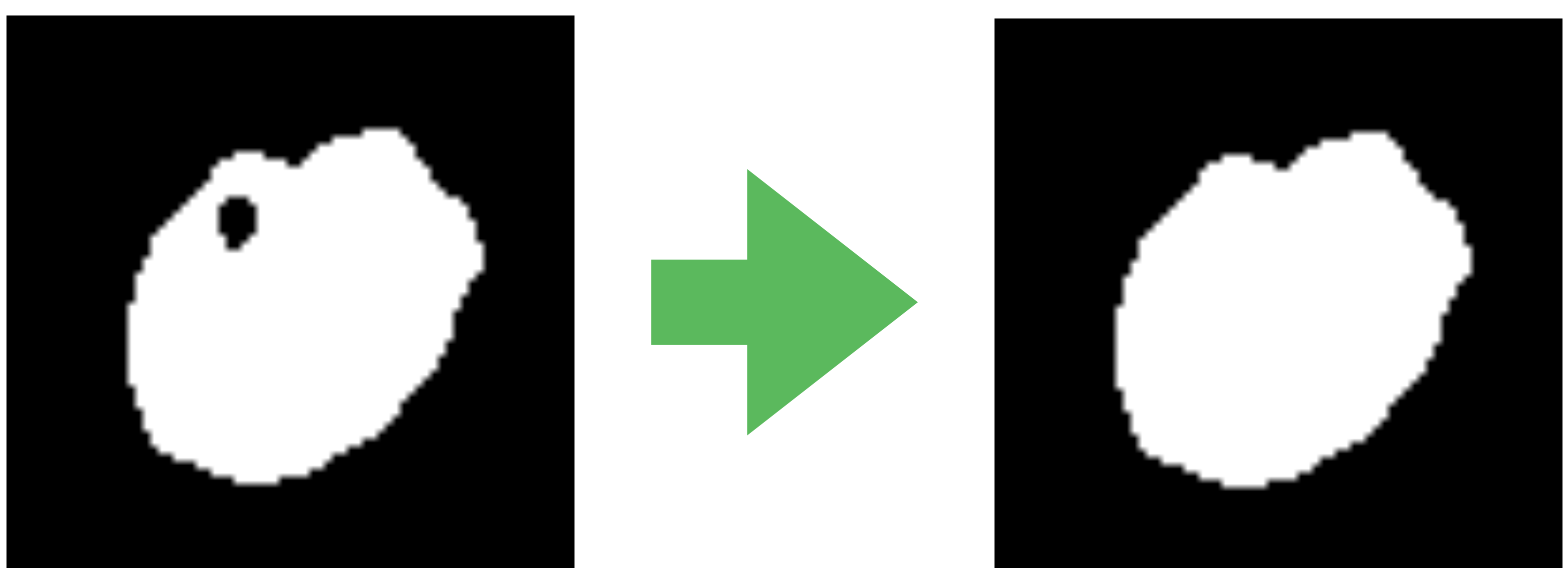
holes inside objects

**Morphological operations:** a family of algorithms that are helpful for modifying object shapes

closing

~ fills small holes in foreground

```
[ ]: from skimage.morphology import binary_closing, disk
     binary_mask_sized = binary_closing(binary_mask,
                                        disk(1))
```

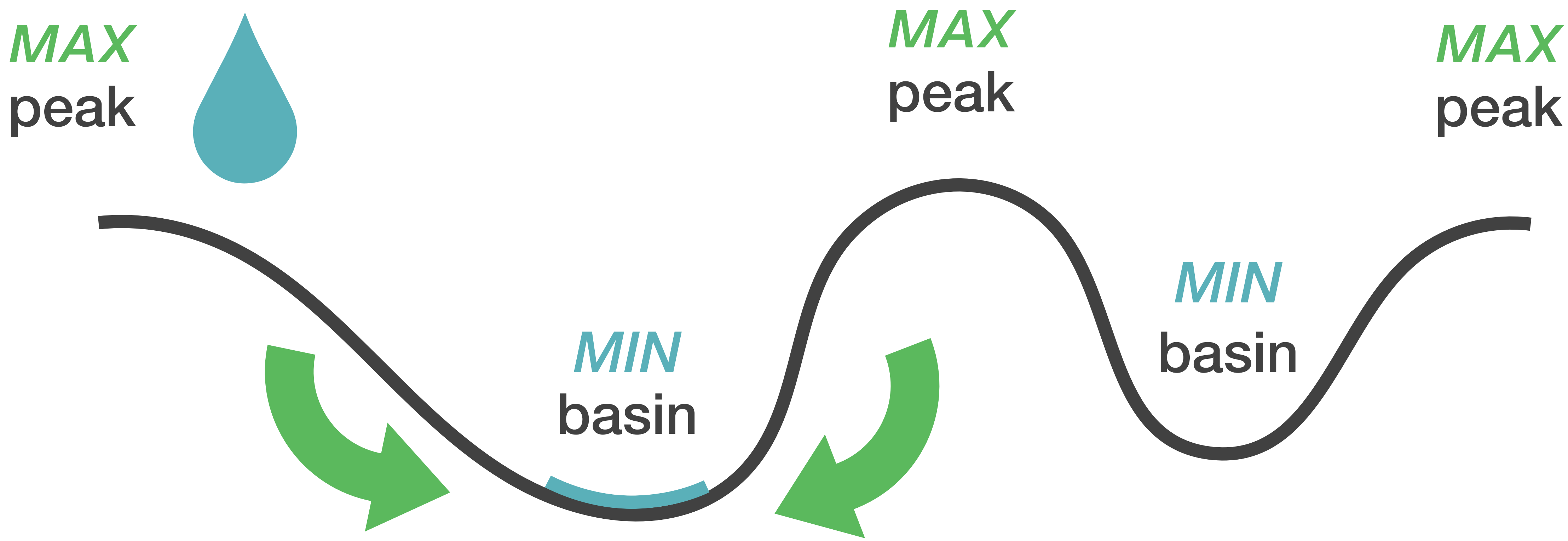


touching objects

**Watershed Algorithm:** useful for separating touching objects.

# What *is* the watershed algorithm?

The name **watershed** is inspired by how a drop of water falls along a surface

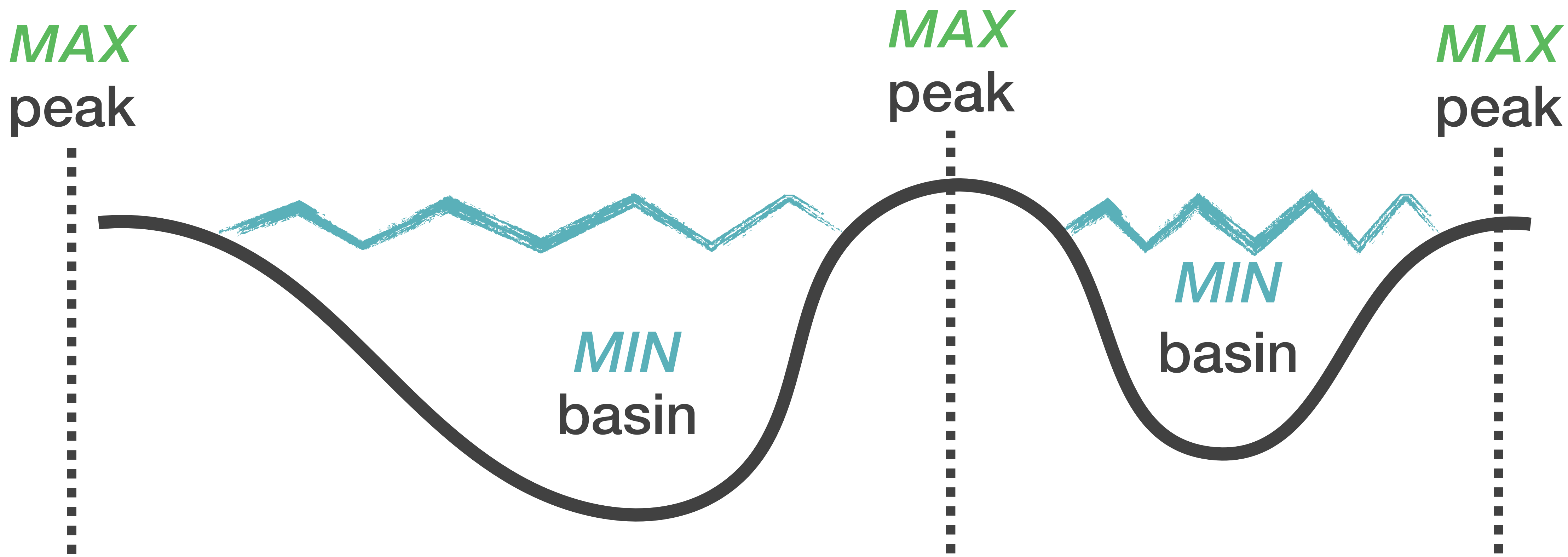



the drop flows to the nearest low point, called a **basin**

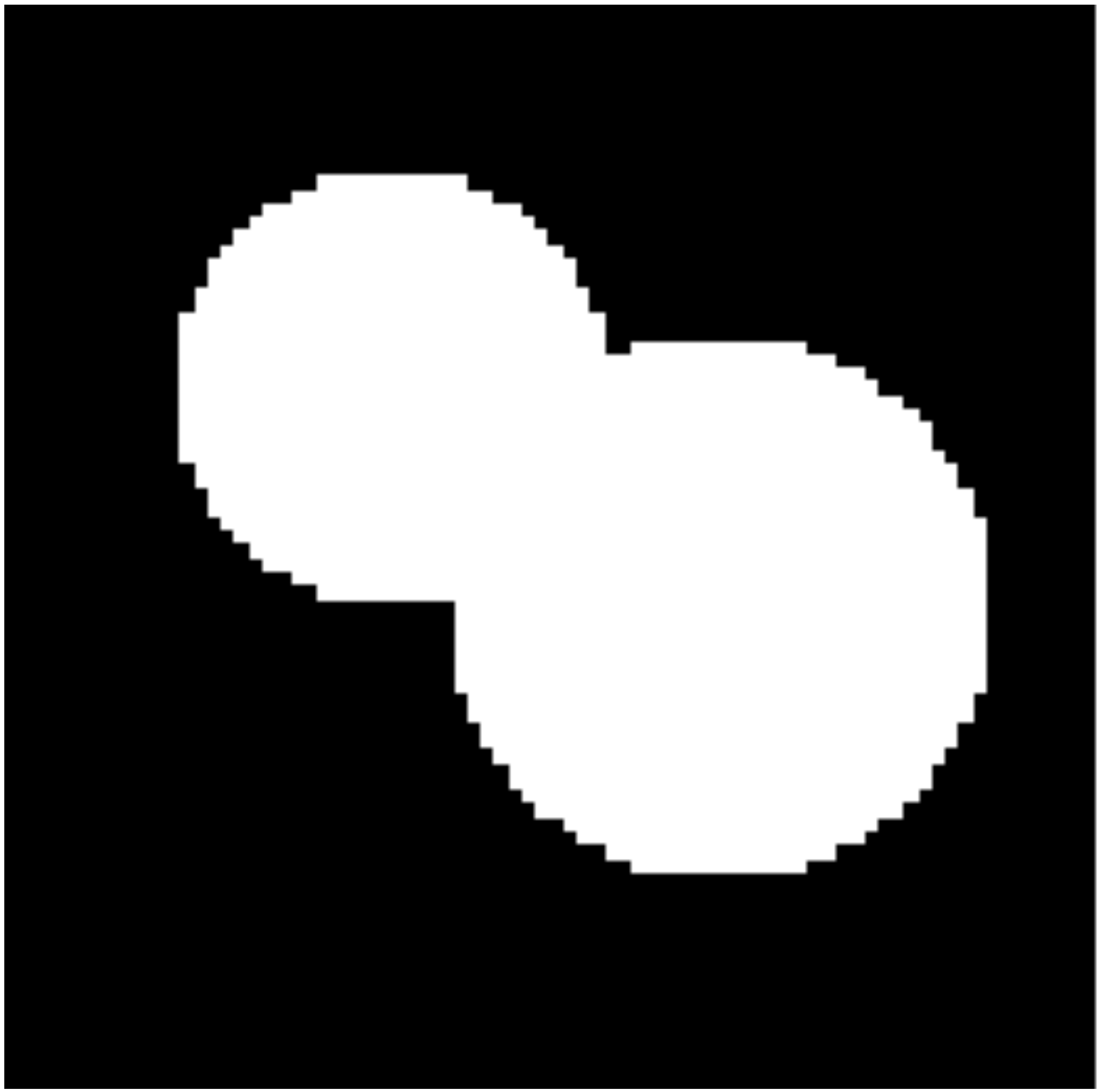
# What *is* the watershed algorithm?

The name **watershed** is inspired by how a drop of water falls along a surface

the **watershed line** separates which basin the water will go to

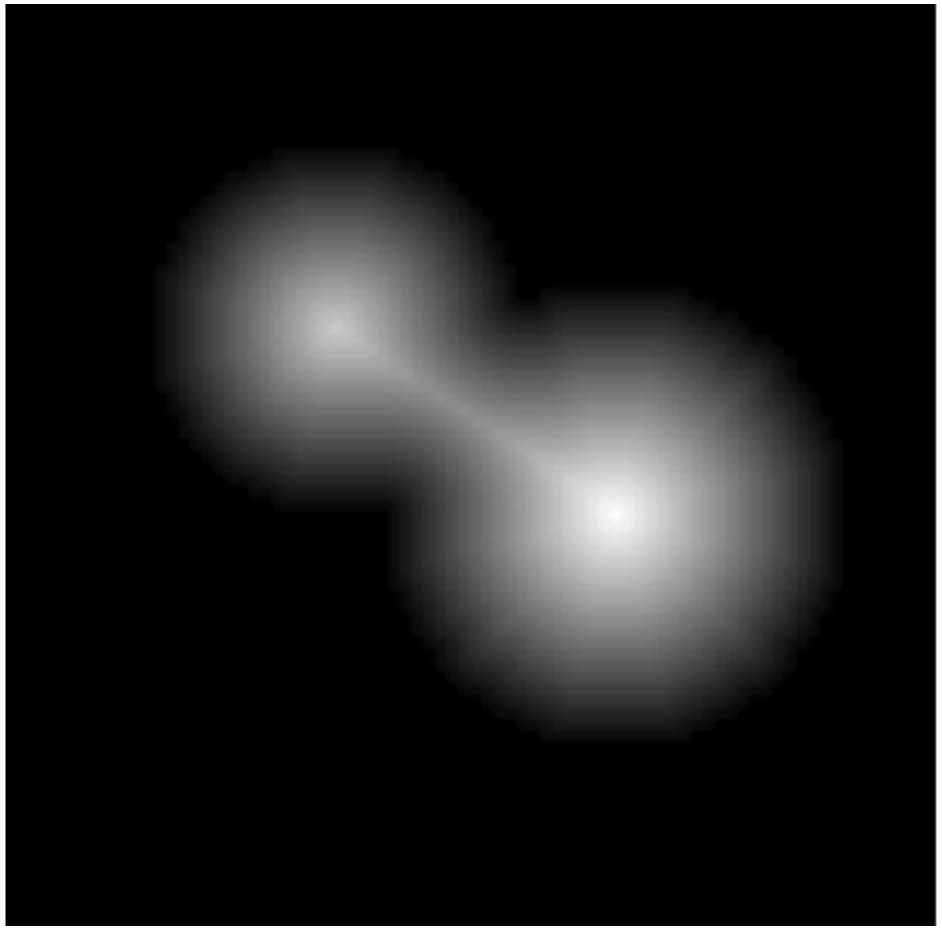


# What *is* the watershed algorithm?



from scikit-image.org

Calculate how far each white pixel is from the nearest black pixel



the resulting image is called a ***distance transform***

# What *is* the watershed algorithm?

Let's study this distance transform…

The largest distance values are in the ***centers*** of the 2 objects

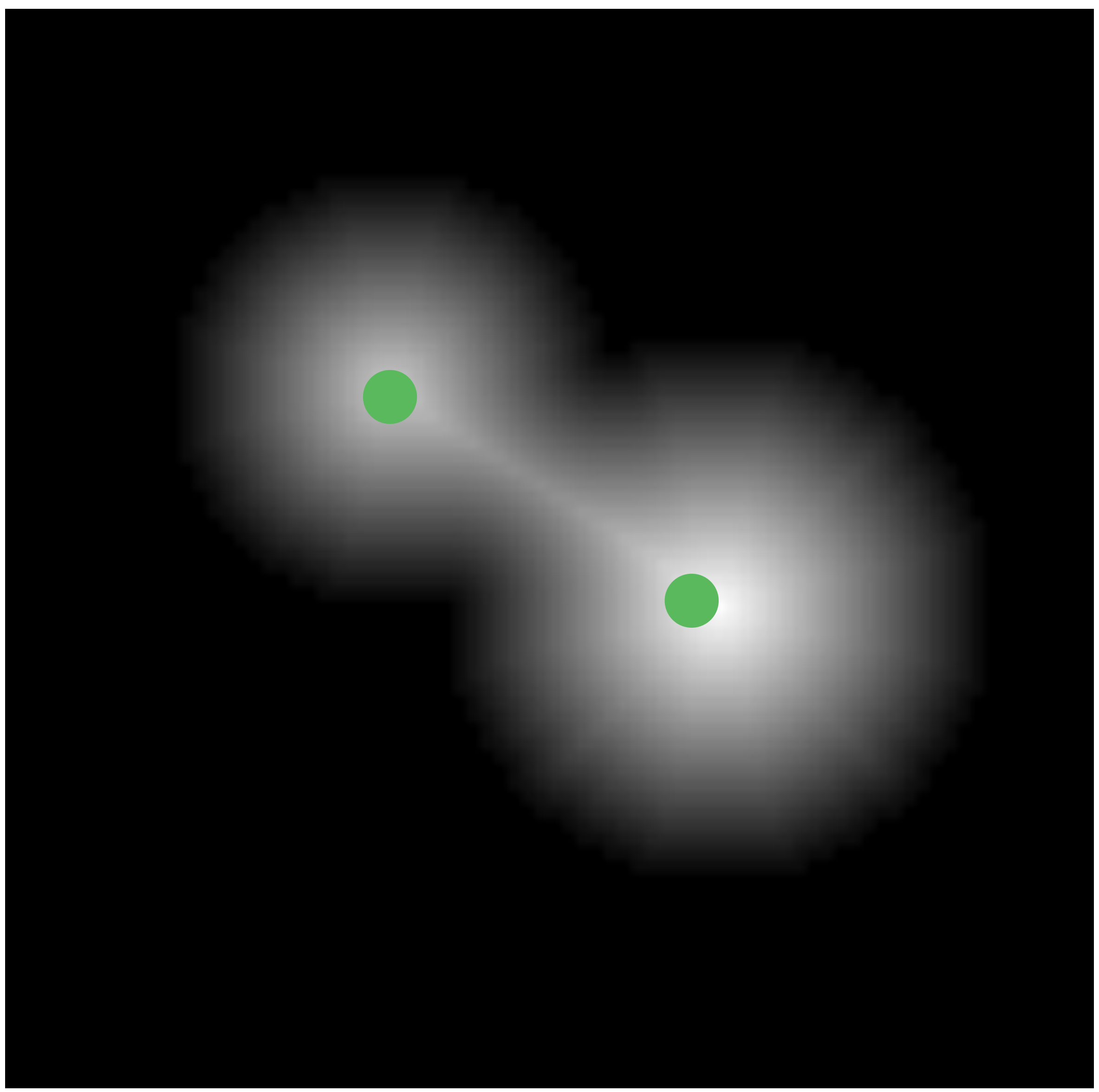
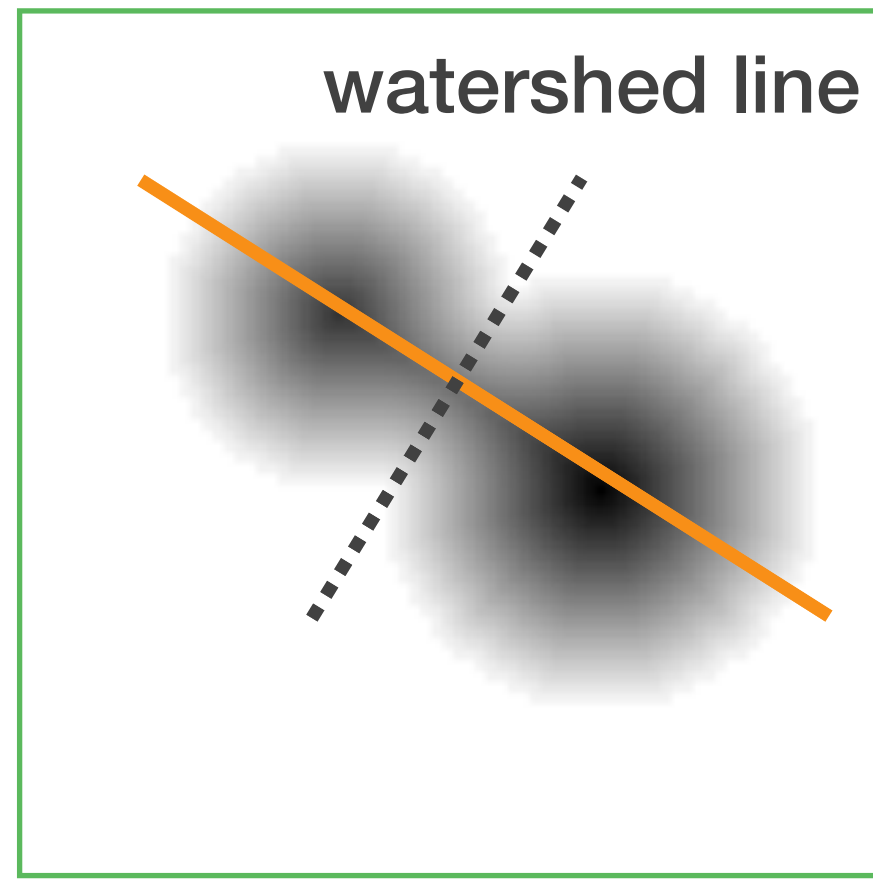
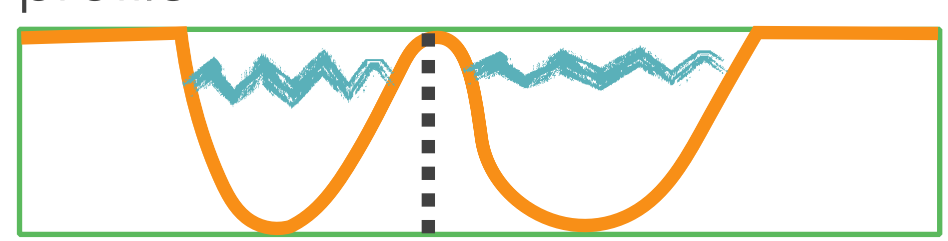


image of ***inverted distances***

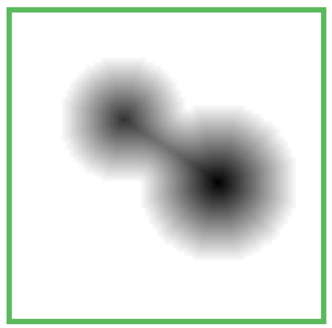
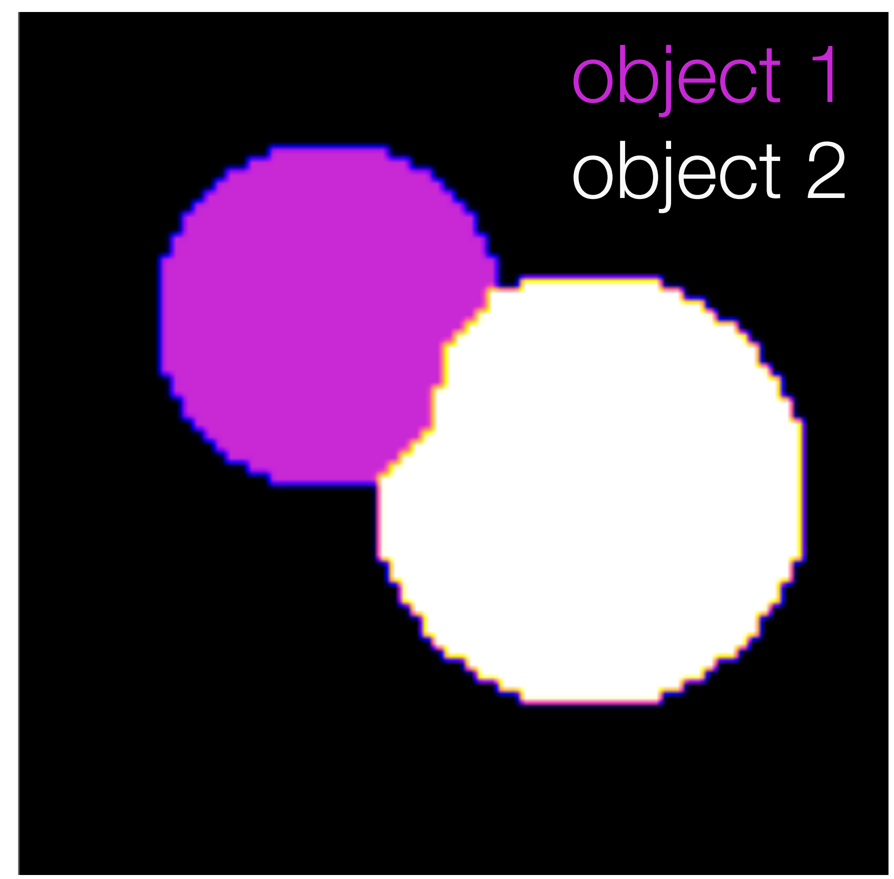
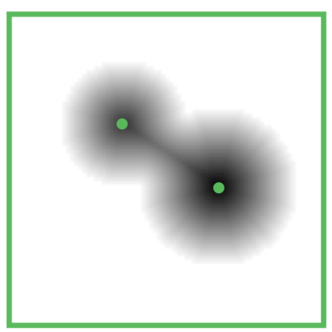



profile



# What *is* the watershed algorithm?

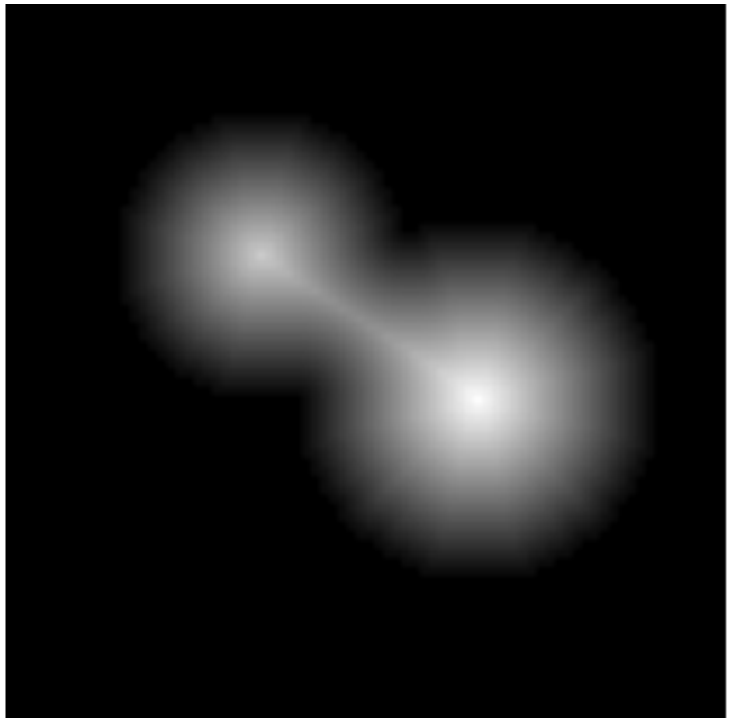
the **watershed algorithm**, in summary:

| **inputs** | **output** |
|---|---|
| -distance transform  | labeled image with separated objects   |
| labeled peaks (seeds)  | |
| binary mask  | |

# Watershed in Python

calculate the distance transform

[1]:
```
# calculate distance transform
from scipy.ndimage import distance_transform_edt
distance_transform =
                distance_transform_edt(binary_image)
```



# Watershed in Python

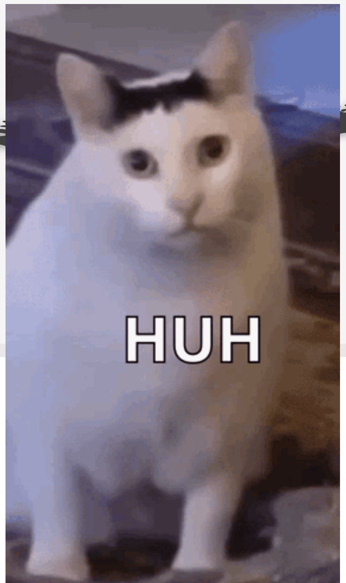
find the peak coordinates in the distance transform

[ ]:
```
# find the peak distances in distance_transform
from skimage.feature import peak_local_max
import numpy as np
peak_coords = peak_local_max(distance_transform,
                             footprint=np.ones((25,25)),
                             min_distance=10)
```

# Watershed in Python

create a labeled image with the peaks

[ ]:
```
# create img that's same size as binary_mask
local_maxima_image = np.zeros_like(
                        binary_mask, dtype=bool)
# add peak_coords to img
local_maxima_image[
          tuple(local_maxima_coords.T)] = True
```



# Watershed in Python

label the image with the peaks to create seeds

[ ]:
```
# label local_maxima_image
seeds = label(local_maxima_image)
```

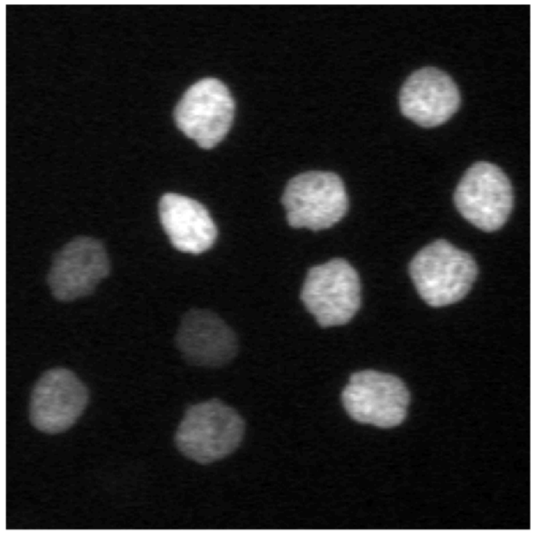
# Watershed in Python

give the watershed algorithm the distance transform, the seeds, and the binary mask

[ ]:
```
# perform watershed
from skimage.segmentation import watershed
labeled_image = watershed(
                          -distance_transform,
                           seeds,
                           mask = binary_mask)
```

# 2 types of segmentation: Semantic & Instance

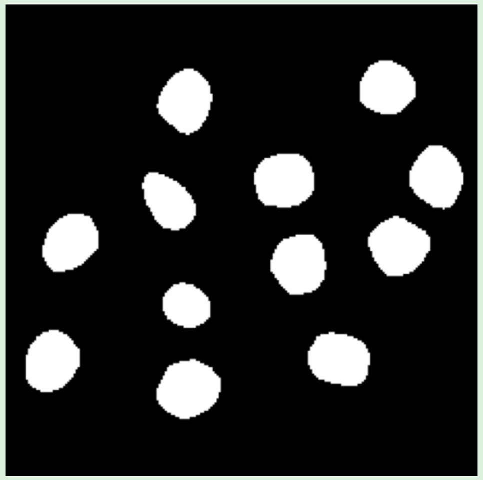
**Raw Image**



## Semantic Segmentation

All objects treated as the same category

**Example:**



**Categories:**

Nuclei

## Instance Segmentation

Each object is distinguished as separate and has a unique label

**Example:**

**Categories:**

Nucleus 1

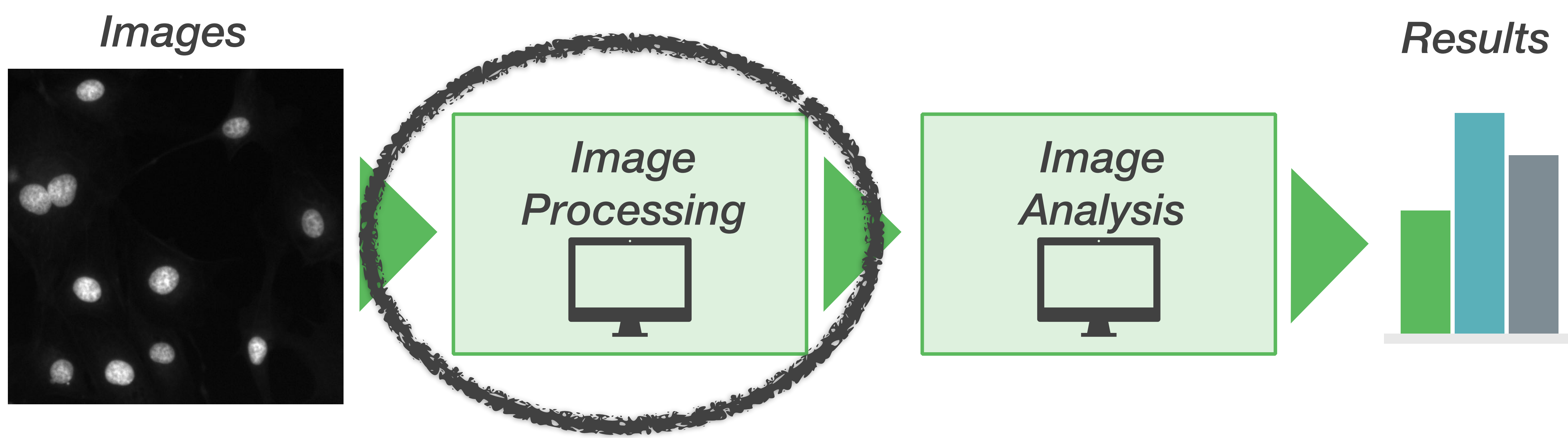
Nucleus 2

Nucleus 3

…

# Lab:

Classic Segmentation Notebook, Step 5




***Statistically relevant*** & ***reproducible*** measurements come from analyzing ***many fluorescence images.***

# We need to analyze enough images to represent an entire cell population

**25** widefield images to analyze

classic segmentation with Python

processing many images

# Processing many images in Python


We use a for loop to run same processing steps on each image!

# Processing many images in Python

Organize all images to process in 1 folder.

Use a **for** loop to loop through image paths.

[ ]:

```
# loop through image paths to get each image
from pathlib import Path
folder_dir = Path("/Users/edelase/bobiac")
for image_path in folder_dir.iterdir():
    # do classic processing steps
```

# Processing many images in Python

Use a **for** loop to loop through ***only tif*** image paths in a folder.

[ ]:
```
# loop through only tif image paths
from pathlib import Path
folder_dir = Path("/Users/edelase/bobiac")
for image_path in folder_dir.glob("*.tif"):
    # do classic processing steps
```

# what about saving images?

# Saving images in Python

Use a `tifffile.imwrite()` to save output images.

```
[ ]: # save an image
import tifffile
from pathlib import Path
output_dir = Path("/Users/edelase/output")
output_filepath = output_dir /
                  "output_file.tif"
tifffile.imwrite(output_filepath,
                 image.astype("uint32"))
```

Why ***won't*** this work in our image paths for loop?

# Saving images in Python

Use `f"{image_path.stem}_.tif"` to automatically generate file names for each loop

```
[ ]: # save an image
import tifffile
from pathlib import Path

input_dir = Path("/Users/edelase/input")
output_dir = Path("/Users/edelase/output")
for image_path in input_dir.glob("*.tif"):
   output_filepath = output_dir /
                       f"{image_path.stem}_.tif"
   tifffile.imwrite(output_filepath,
                       image.astype("uint32"))
```

**Lab:**

Classic Segmentation Notebook, Step 6

# We made it!

classic segmentation with Python

processing many images


Now what?

# General resources

### *Documentation*

scikit-image
image processing in python

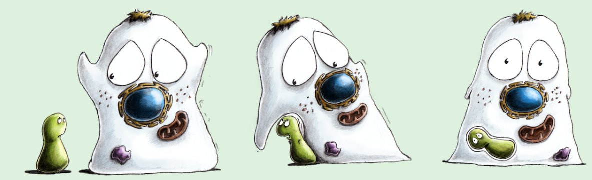
scikit-image.org

### *Image Analysis Forum*

image.sc

https://forum.image.sc/

### *Learning Resource*



https://bioimagebook.github.io/index.html

# Questions?