

# ZEN (blue edition) 3.1

## Image Analysis



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2019-11-04

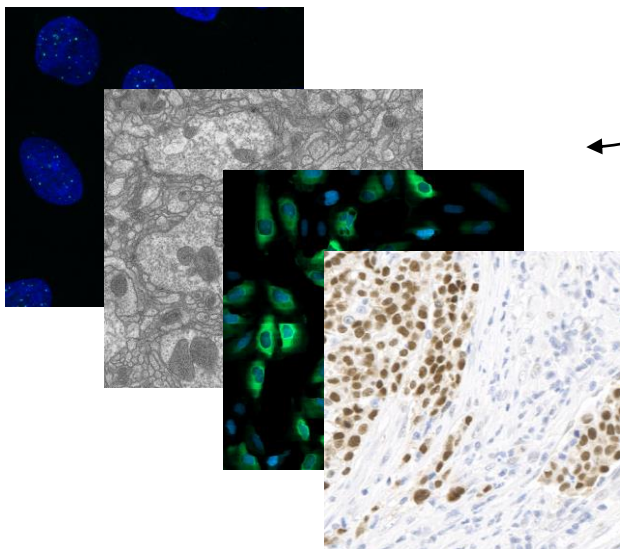
# Image Analysis

*It's all about Numbers!*

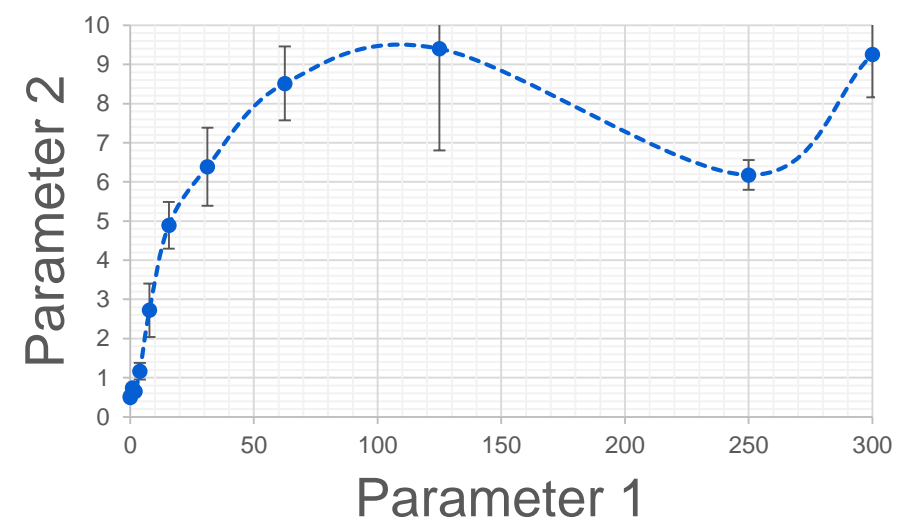
## Customer requirements:

- How many cells are positive/negative for...?
- How big is...?
- What percentage is...?
- What is the intensity of...?

## Solution: Automatic Quantification!



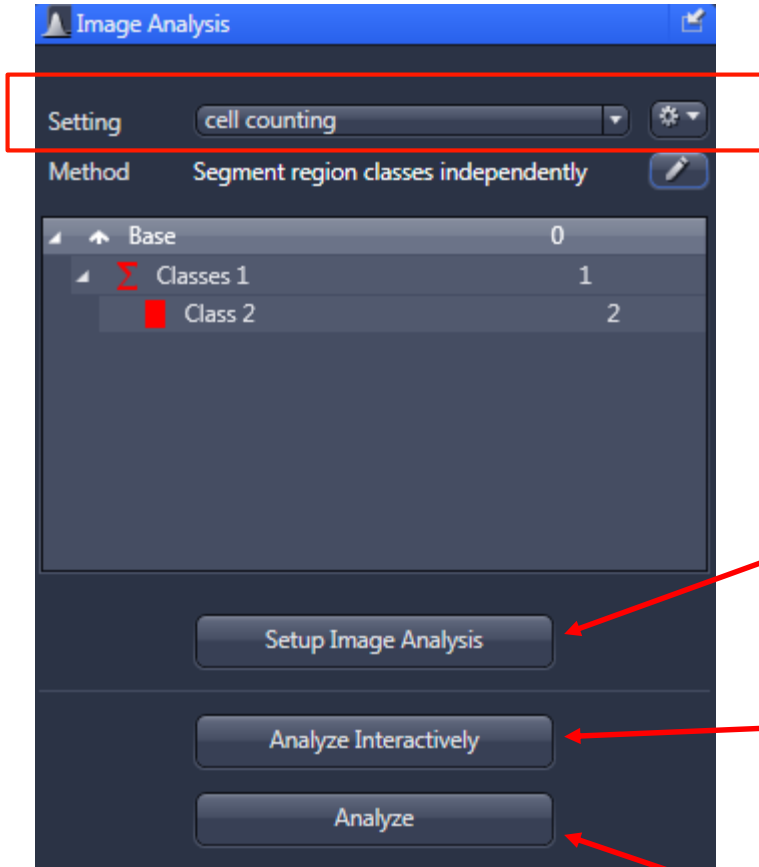
	ID	Area [µm²]	Intensity...	Circularity
	A	B	C	D
64	65	1.110	54.788	0.781
65	66	2.170		
66	67	2.340		
67	68	1.980		
68	69	1.180		
69	70	1.730		
70	71	1.630		
71	72	2.040		
72	73	2.130		
73	74	1.870		
74	75	2.170		
75	76	2.490		
76	77	1.900		
77	78	2.050		
78	79	2.500		
79	80	1.970		
80	81	2.360		
81	82	2.010		
82	83	2.150		
83	84	1.790		
84	85	1.770		
85	86	1.150	169.261	0.773
86	87	1.140	175.509	0.728
87	88	2.140	132.545	0.731



“Image analysis is the extraction of meaningful information from images.” --- Wikipedia

# Image Analysis with ZEN blue 3.1

## *Image Analysis Module*



Wizard to guide you through the **setup** step-by-step

### Analyze the images interactively

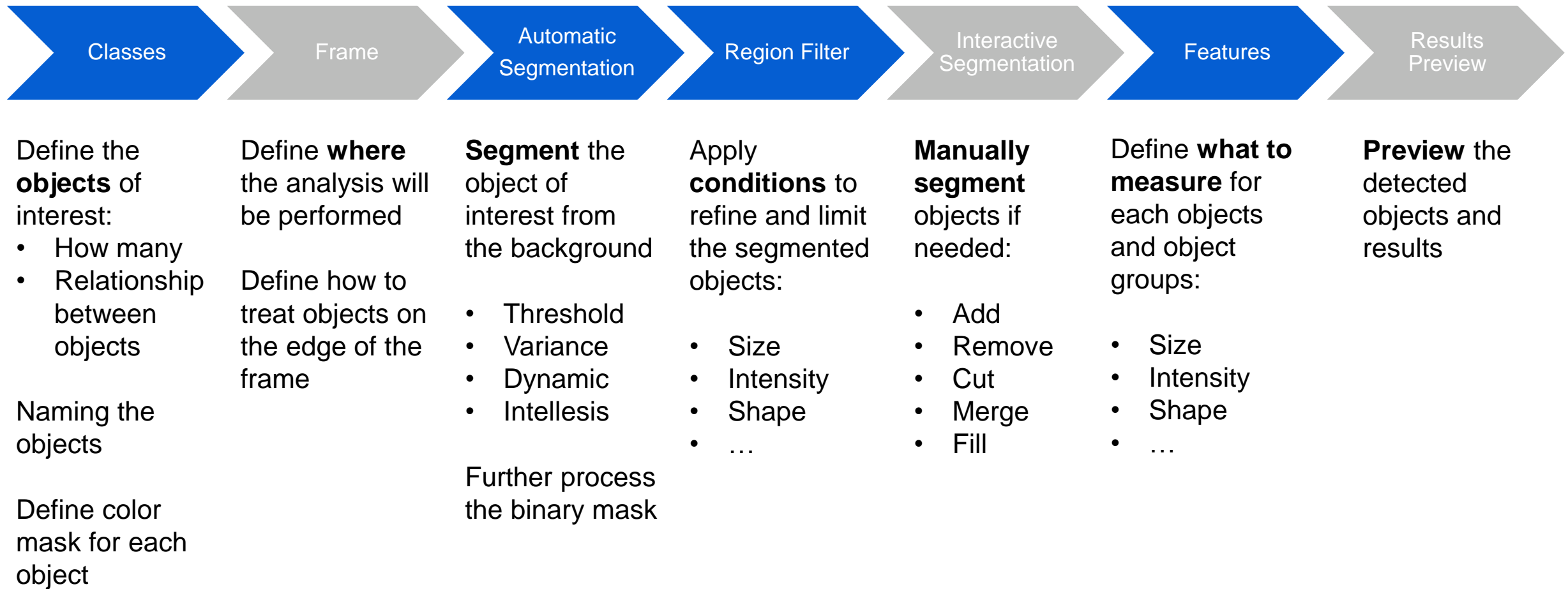
(if you have defined “Interactive” steps during setup)

When “Interactive” is active in a step, the analysis workflow will pause for you to interactively adapt parameters for the current image.

Analyze the complete .czi (without pause)

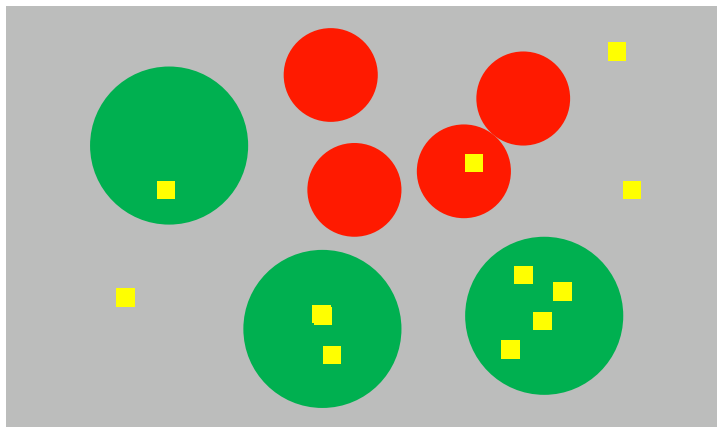
# Image Analysis using ZEN blue 2.6

## Image Analysis Wizard



# Image Analysis Step 1: Define Objects (Classes)

## Overview



### Classes vs. Class (always created in pairs!)

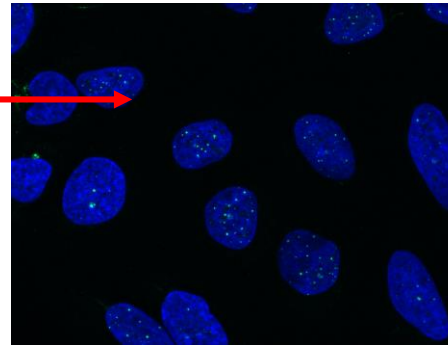
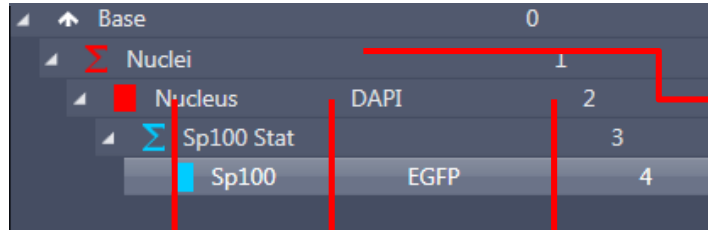
- **“Class” contains single objects:** e.g. a single nucleus.  
You can measure the mean intensity, area, compactness of this single nucleus.
- **“Classes” contains a group of objects:** e.g. all the nuclei in a frame.  
You can measure the number, total area, total intensity of nuclei inside a frame.

### Class vs Subclass:

- Each “Class” represents a object of interest, and **each Class is independent**  
E.g. white and red blood cells in a blood smear.
- **A “Subclass” is a object that is part of the primary “Class”:**  
E.g. a FISH image where the nucleus is stained with DAPI, as the primary “Class”; while the HER2 dots is a “Subclass” for each nucleus.  
You can specifically count how many HER2 dots “Subclass” are there in each nucleus “Class”.

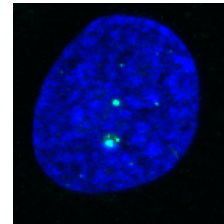
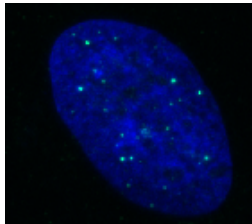
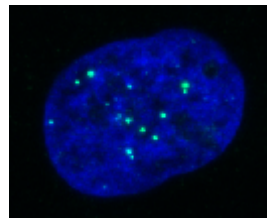
# Image Analysis: Class/Classes

## *Classes vs. Class*



### Nuclei: Statistical Features of all single nuclei

Number of all objects  
Mean Area of all objects  
Mean Intensity of all objects  
....



### Nucleus: Features of each individual nucleus

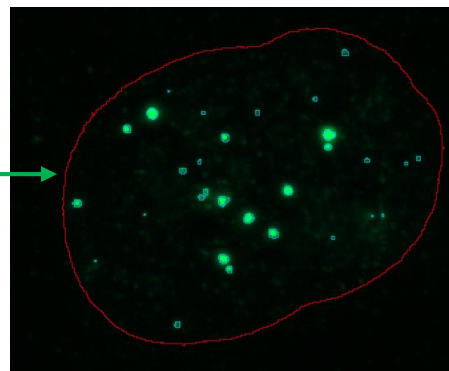
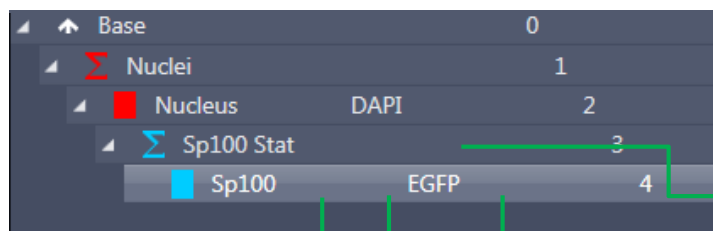
Area 1  
Intensity 1  
Circularity 1  
...

Area 2  
Intensity 2  
Circularity 2  
...

Area n  
Intensity n  
Circularity n  
...

# Image Analysis: Class/Classes

## Sub-class



**Sp100 Stat: Statistical features of all green spots in one cell**

Number of Spots  
Mean Area of Spots  
Mean Intensity of Spots  
...



Area 1  
Intensity 1

...



Area 2  
Intensity 2

...



Area n  
Intensity n

...

**Sp100: Features of each individual spot**

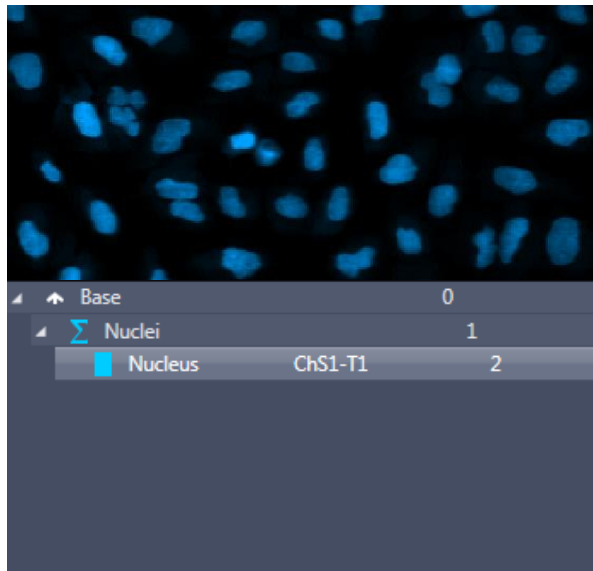


# Image Analysis Step 1: Define Objects (Classes)

## Classes examples

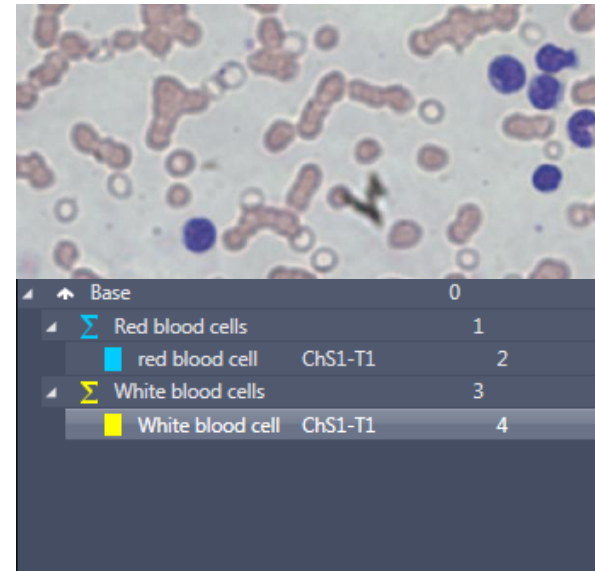


### One class



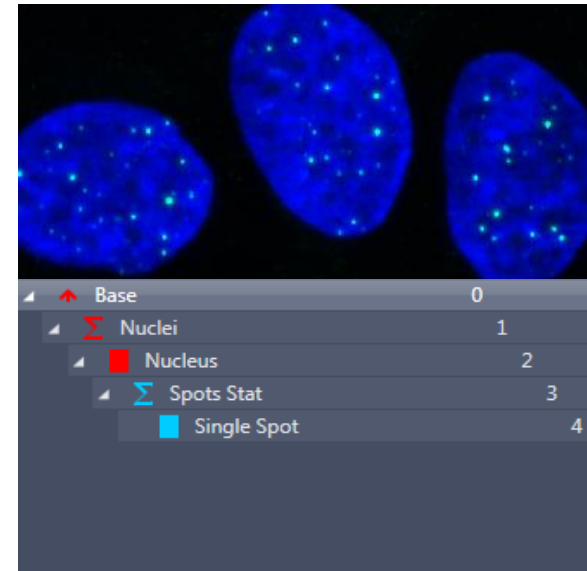
- Cell counting
- Wound healing

### Two independent classes



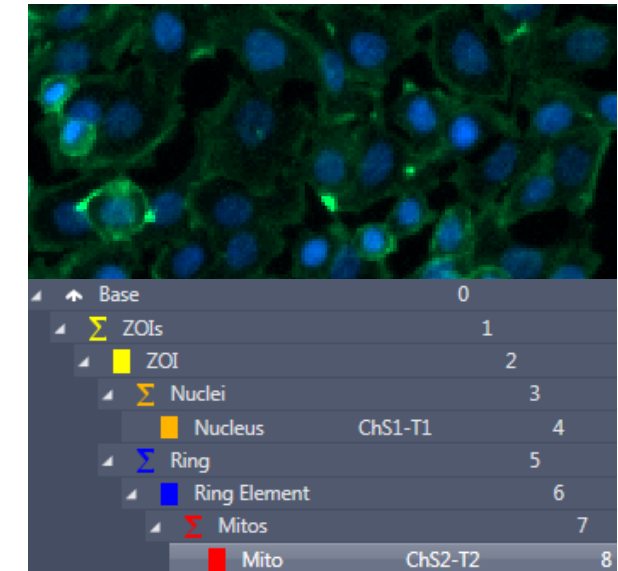
- Calculate white blood cell/red blood cell ratio
- Calculate ratio tissue vs. blood vessels

### One class and one subclass



- Quantify HER2/PML bodies/SP100 dots per cell nucleus

### ZOI (Zone Of Influence)

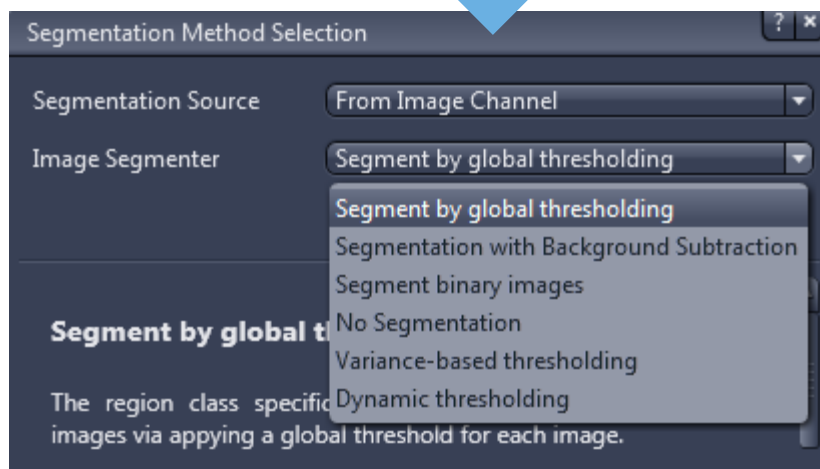
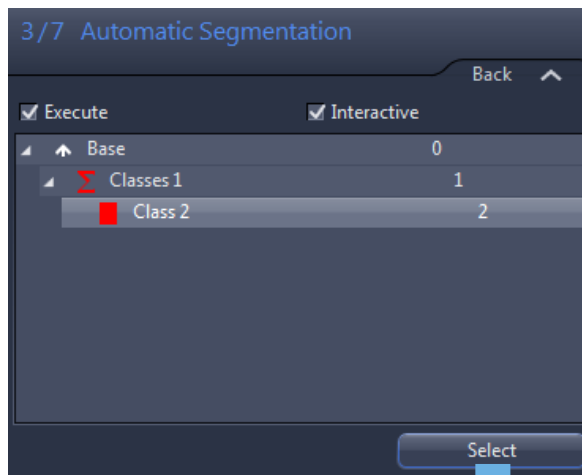


- Measure mitochondria expression level per cell
- Calculate translocation ratio



# Image Analysis Step 3: Segmentation

*The most critical step*



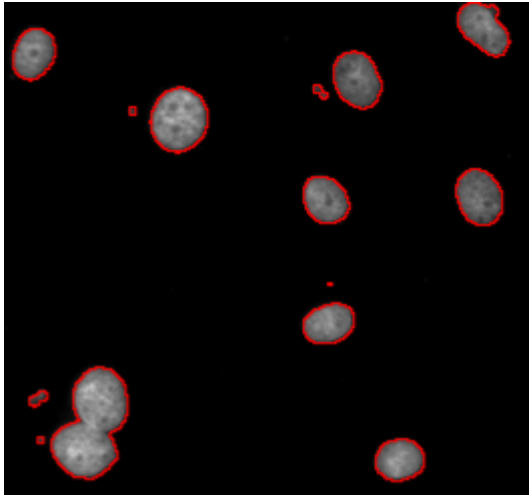
Segmentation: partitioning a digital image into multiple segments.  
Binary process: the object of interest is 1, the rest is 0.  
The result is a “mask” of the segmented objects.

## Available segmentation methods:

- Segmentation by global thresholding  
*One global thresholding is performed for the whole image*
- Segmentation with background subtraction  
*background subtraction is performed before thresholding*
- Variance-based thresholding  
*edge detection: detects changes in pixel intensities, independent of the absolute intensity*
- Segment binary images  
*simple binary segmentation*
- Dynamic thresholding  
*adaptive thresholding to the surrounding of the object*
- Intellesis  
*machine learning*

# Image Analysis Step 3: Segmentation

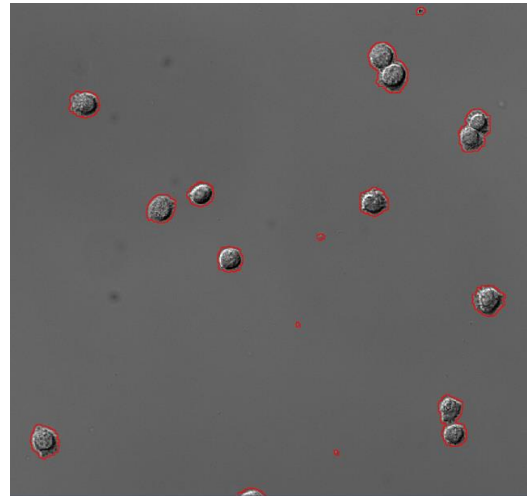
*Examples for different segmentation methods*



## Threshold

(intensity based)

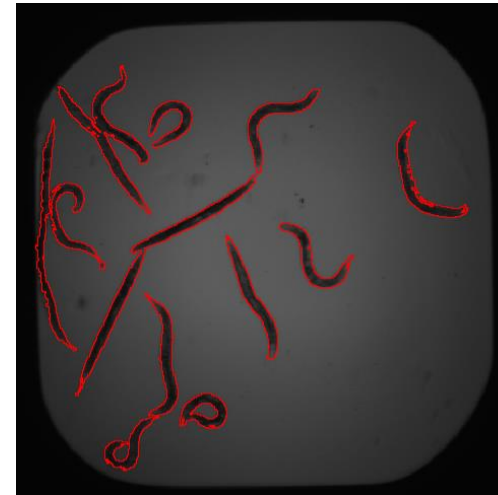
- Fluorescence images



## Variance

(change in intensity)

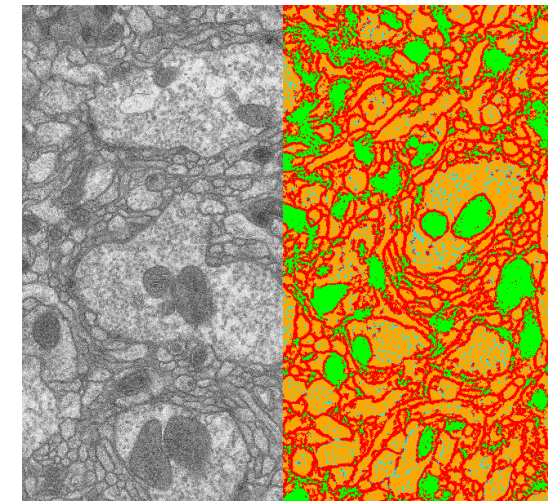
- Brightfield images



## Dynamic

(local threshold)

- Inhomogeneous shading



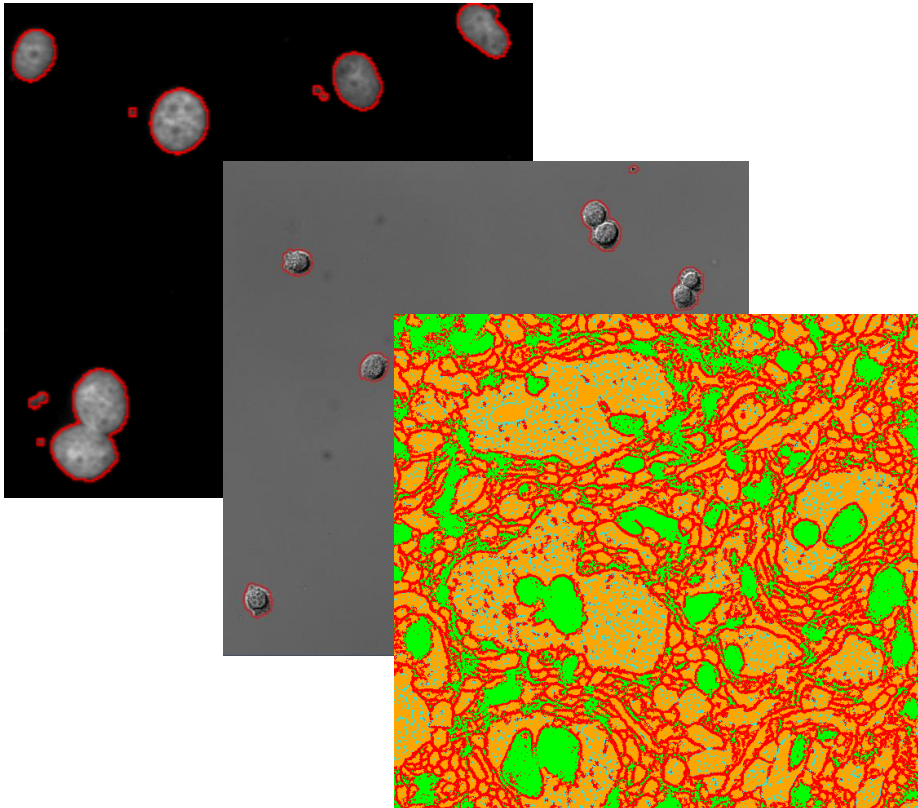
## Intellesis

(machine learning)

- Everything (but slow)
- Nothing else works
- Ease of use

# Image Analysis Step 3: Segmentation

*The most critical step*



Further refine the segmentation mask

- **Min. Area:** Remove small objects
- **Binary Options**
  - Dilate
  - Erode
  - Open
  - Close
- **Separate Objects**
  - Watershed
  - Morphology

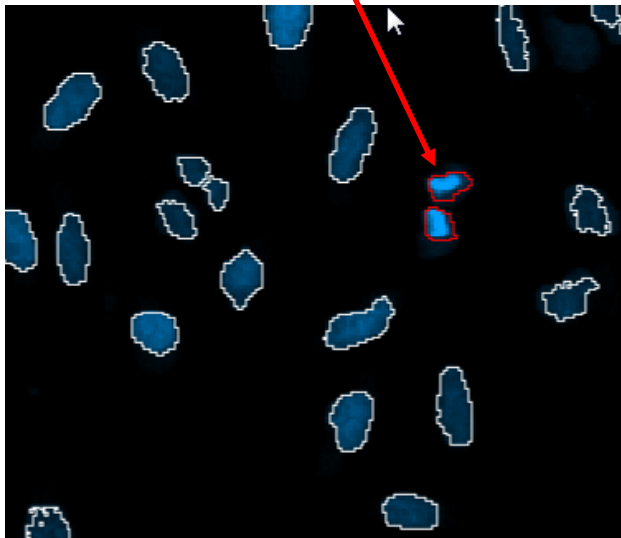
# Image Analysis Step 4: Region Filter

*Refine/Limit detected objects by conditions*

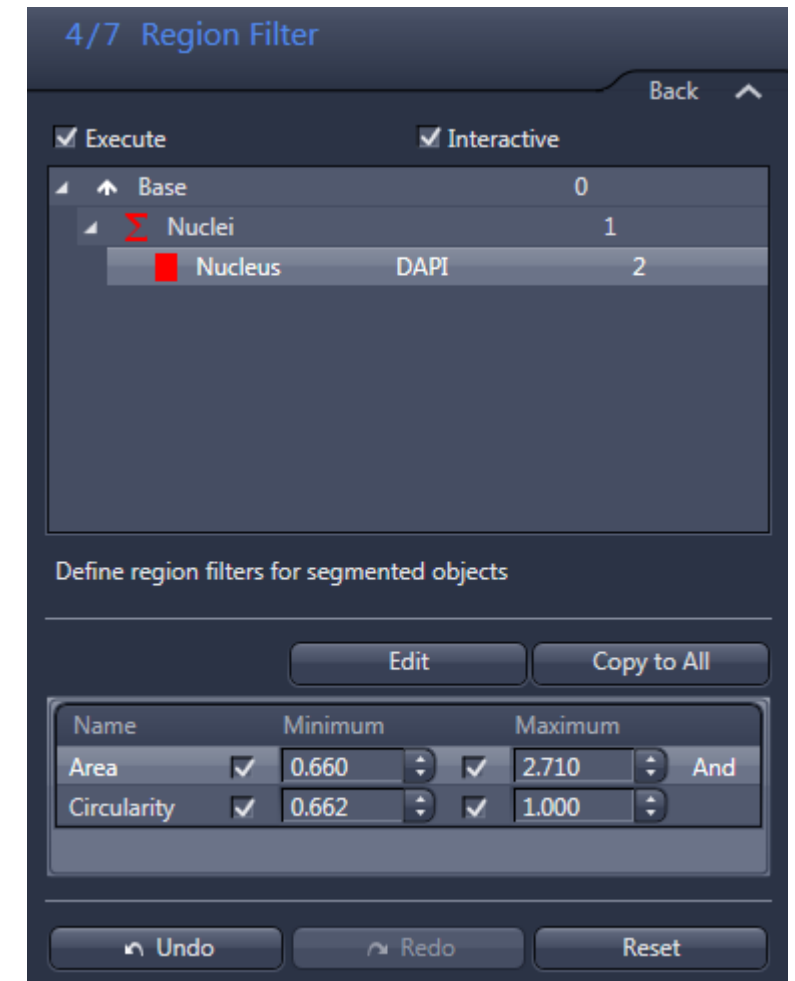
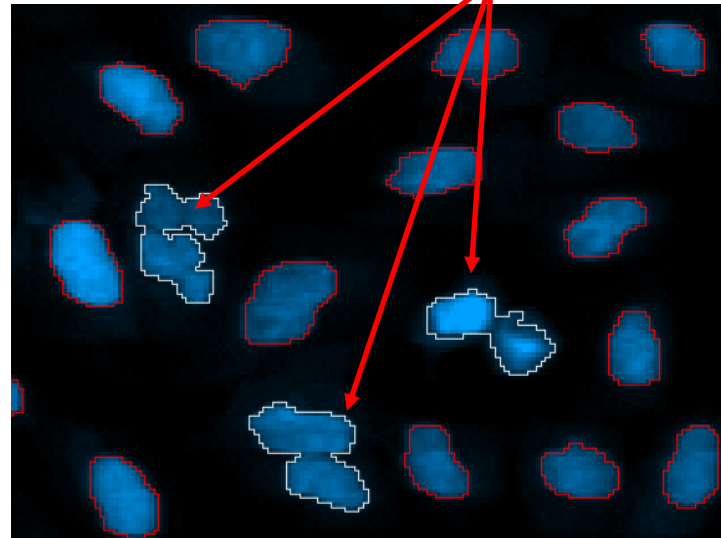


- Region Filters allow to further refine/limit the segmented objects.
- Detect only specific objects, that fulfill certain criteria rather than all segmented objects
  - Only mitotic cells
  - Only nicely separated cells, not clusters
  - ...

Mitotic cells

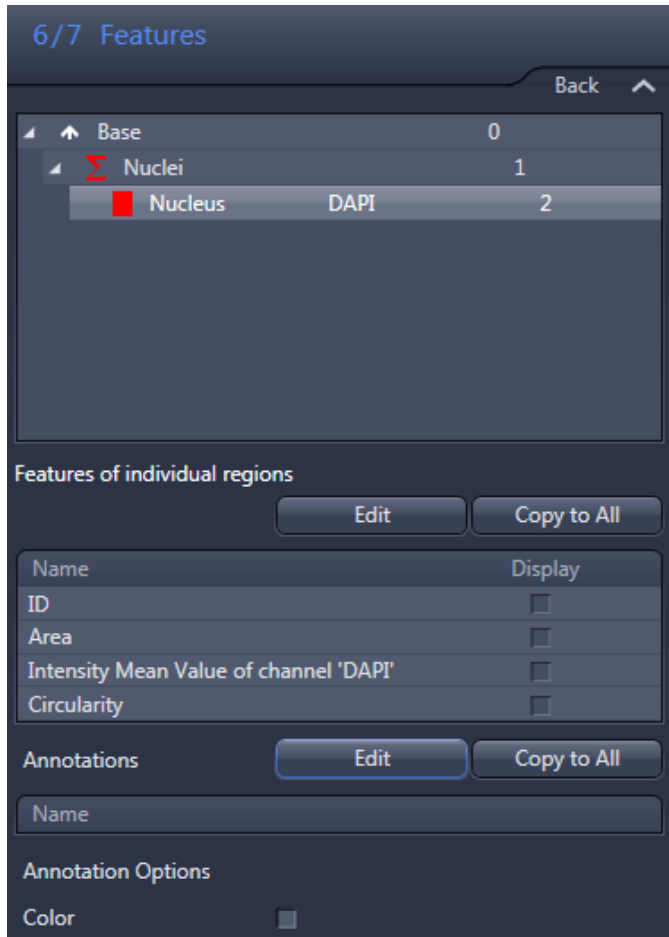


Exclude clusters



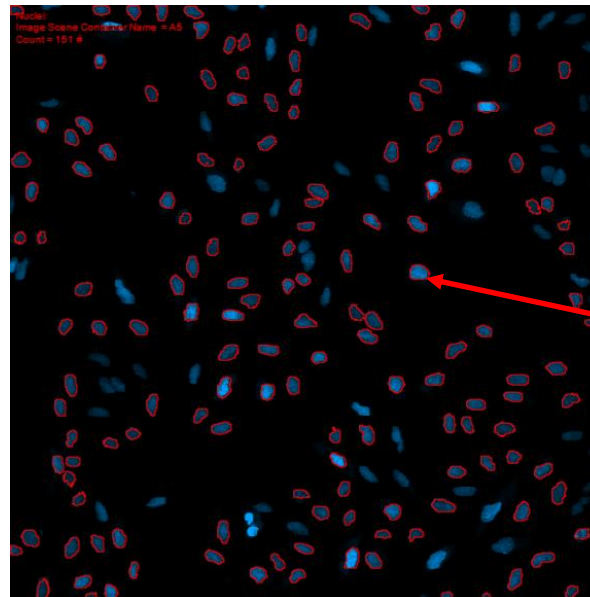
# Define Features

*Define what to measure for the detected objects*



You can define features for “Nuclei” and “Nucleus” independently  
Select features from a list of ~ 100 features

- Geometry
- Intensity
- Image
- Position



“Nuclei”

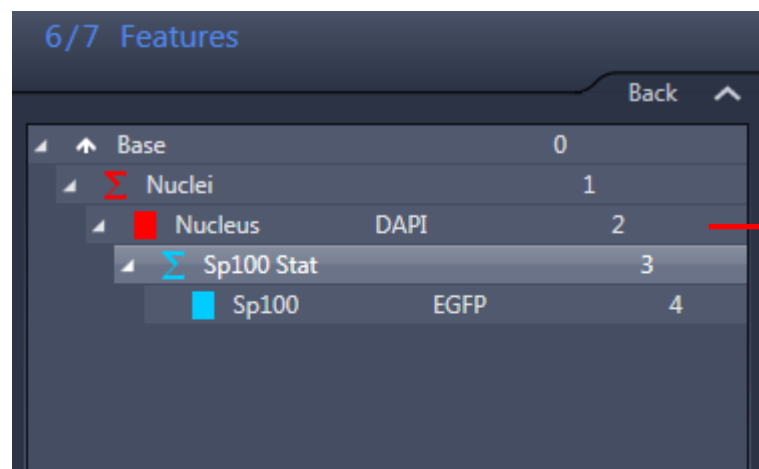
- Image Scene Container Name: A5
- Count: 151

“Nucleus”

- Area: 2.49  $\mu\text{m}^2$
- Mean Intensity: 154
- Roundness: 0.8

# Define Features

*Define what to measure for the detected objects*

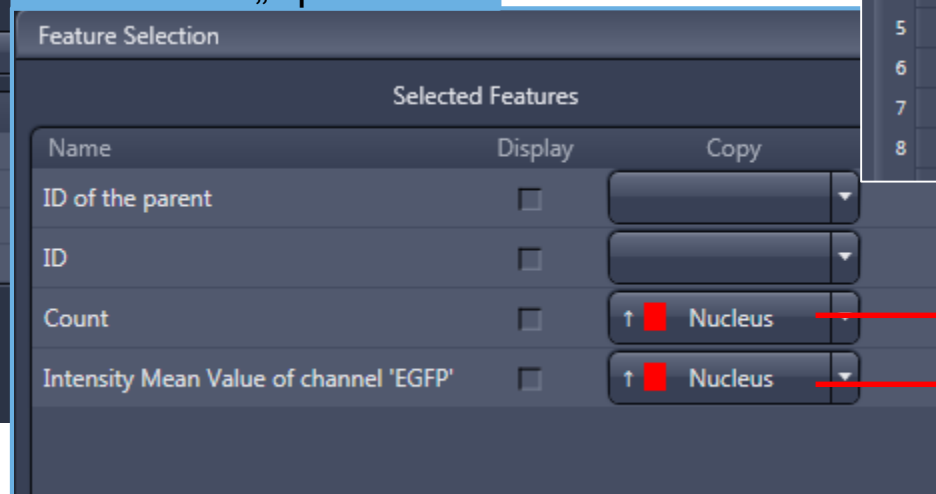


In case of more complex Classes/Class structures, use the “copy-to” functionality to copy features from one class to another (collect all results in one table)

Result table for „Nucleus“

	ID		Area [ $\mu\text{m}^2$ ]	Sp100 Stat...	Sp100 Stat...
	A	B	C	D	
1	14	319.654	14	1,532.777	
2	12	212.405	30	1,513.664	
3	16	247.973	52	1,350.677	
4	18	183.832	27	1,207.857	
5	4	27.415	2	1,206.605	
6	19	295.594	41	1,036.691	
7	7	193.159	32	1,000.144	
8	9	195.454	41	931.038	
	12	261.783	30	863.518	

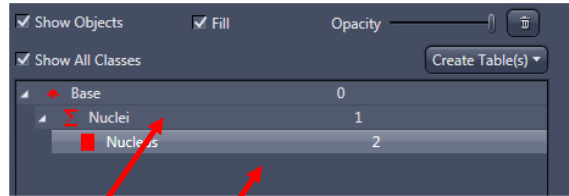
Features for „Sp100 Stat“



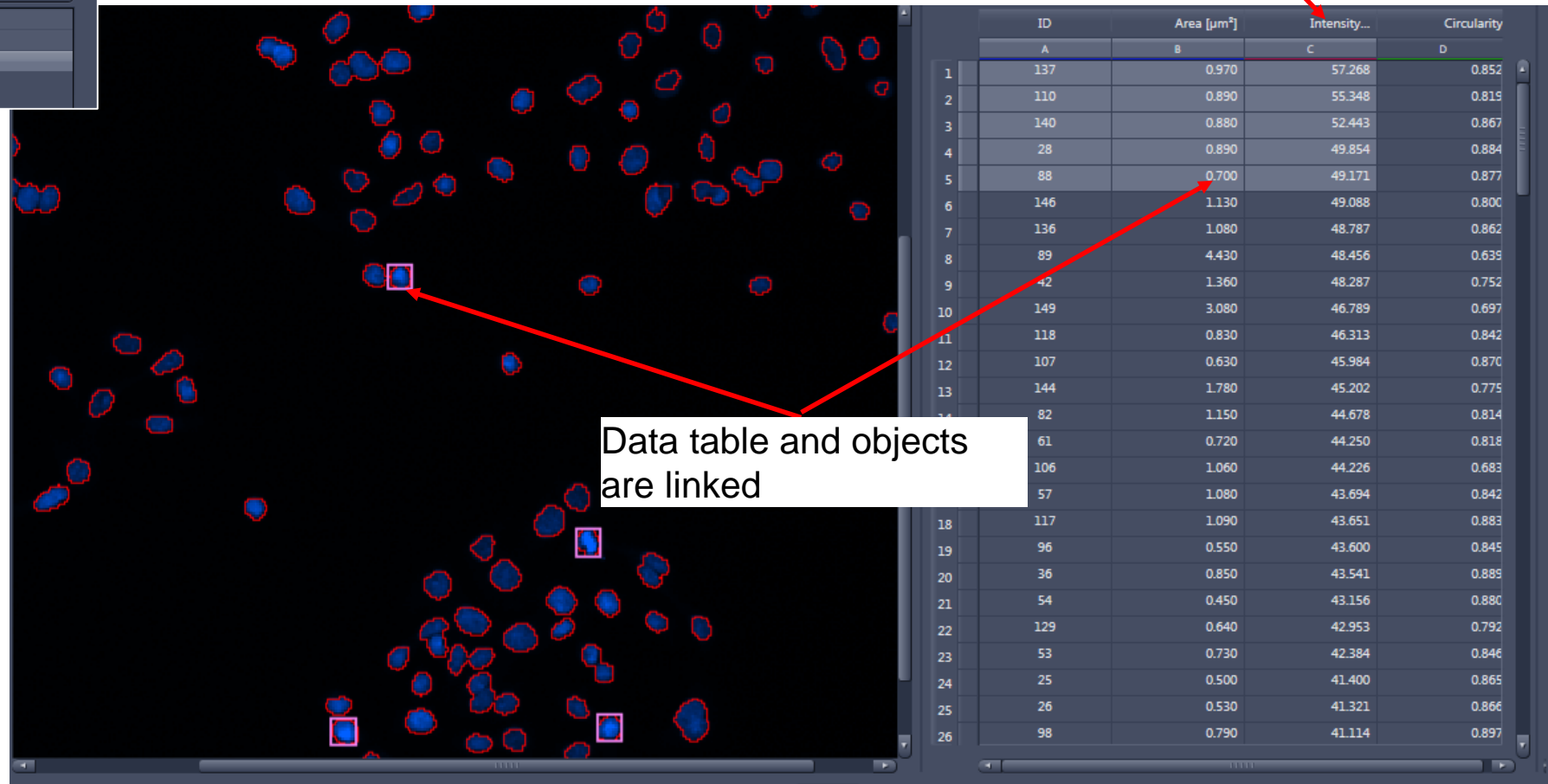


# View results after Image Analysis

## *Link between table and detected objects*



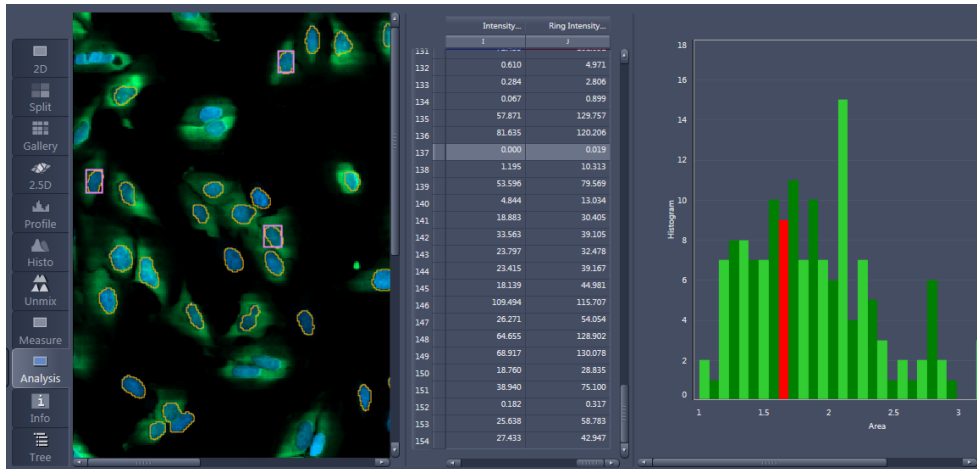
Highlight „Nucleus“ / „Nuclei“ to see corresponding tables



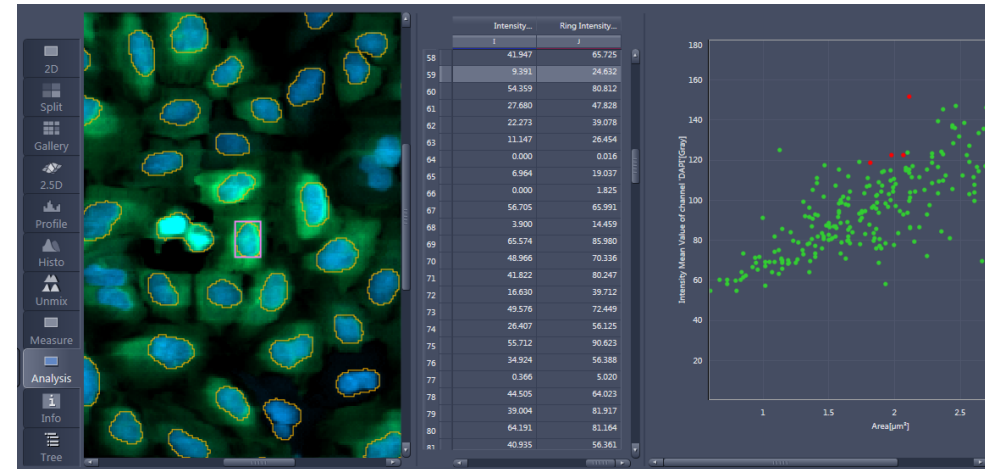


# ZEN Module Image Analysis: Plotting

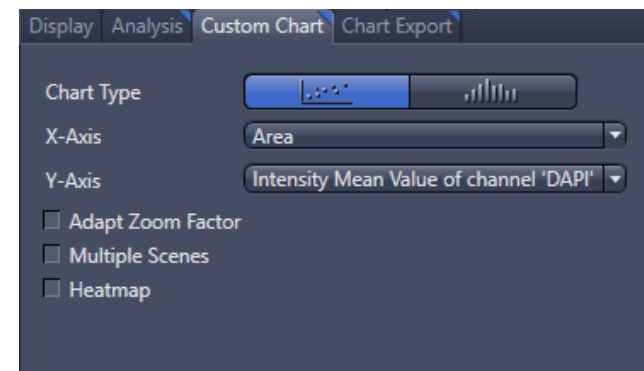
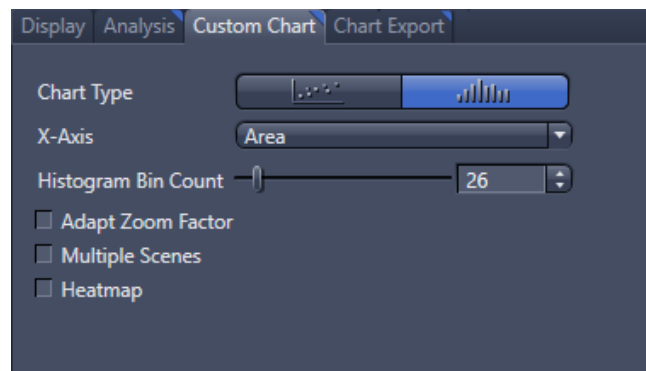
## *Histogramm and Scatterplot*



Histogram „Area“ of cell nuclei

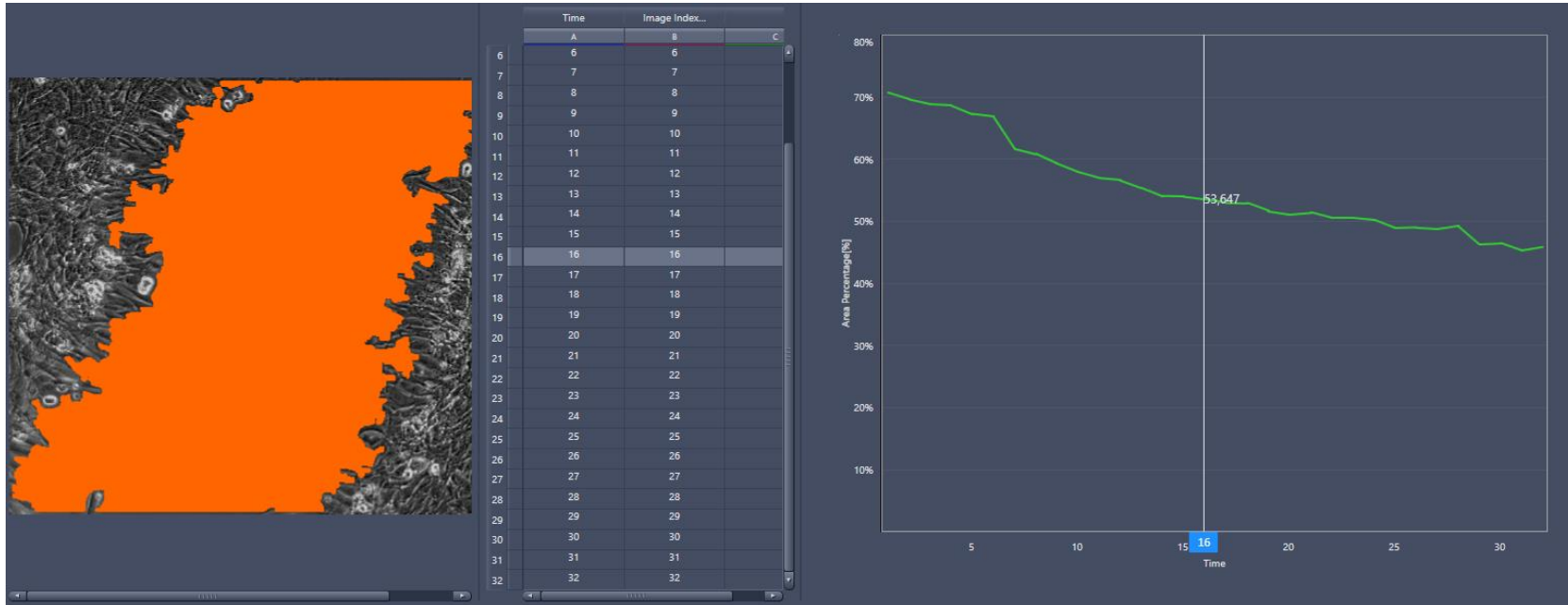


Scatter Plot „Area“ vs „Intensity Mean DAPI“ of cell nuclei

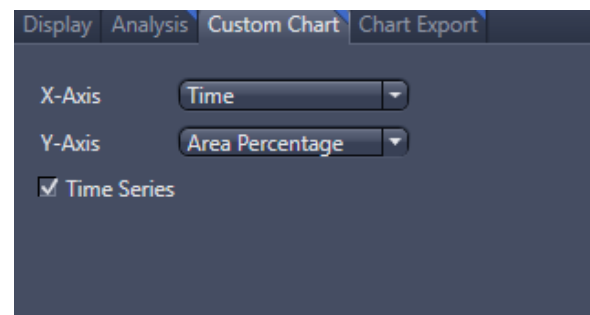


# Display of Image Analysis Results

## *Time Series*

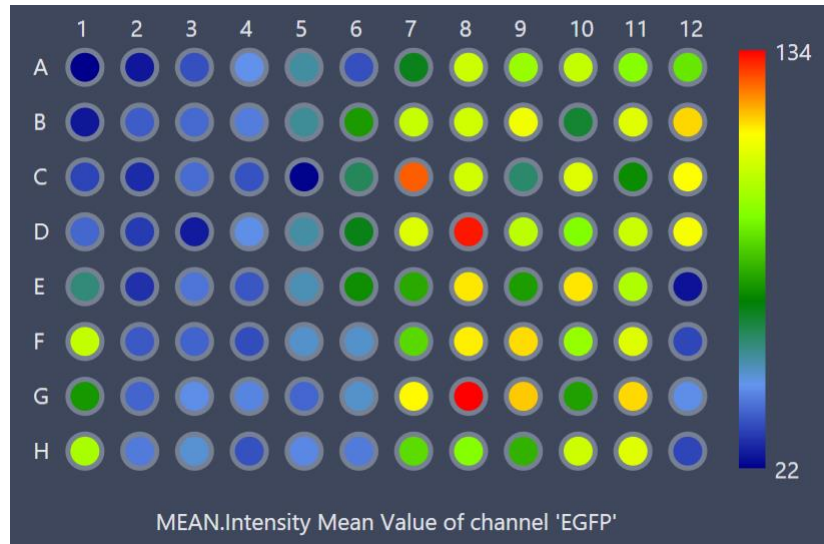


Time-series plot: Area percentage over Time

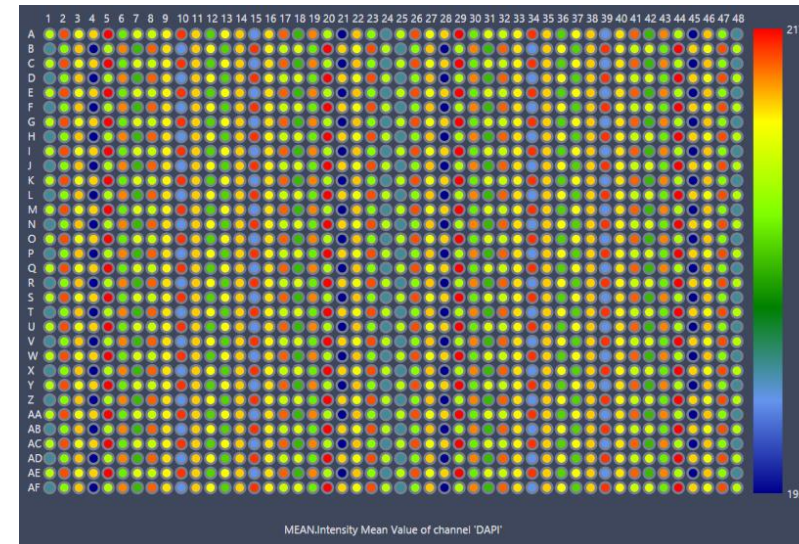


# Display of Image Analysis Results

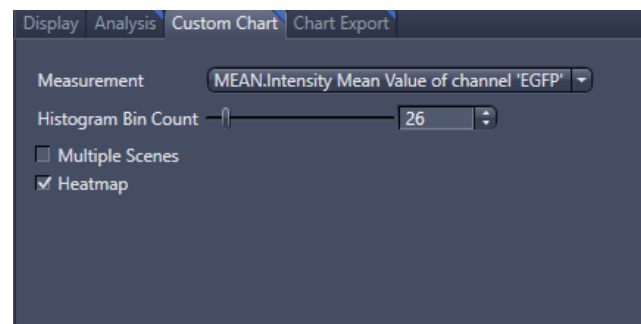
## Heatmaps



96-well plate:  
Mean Intensity value of „EGFP“

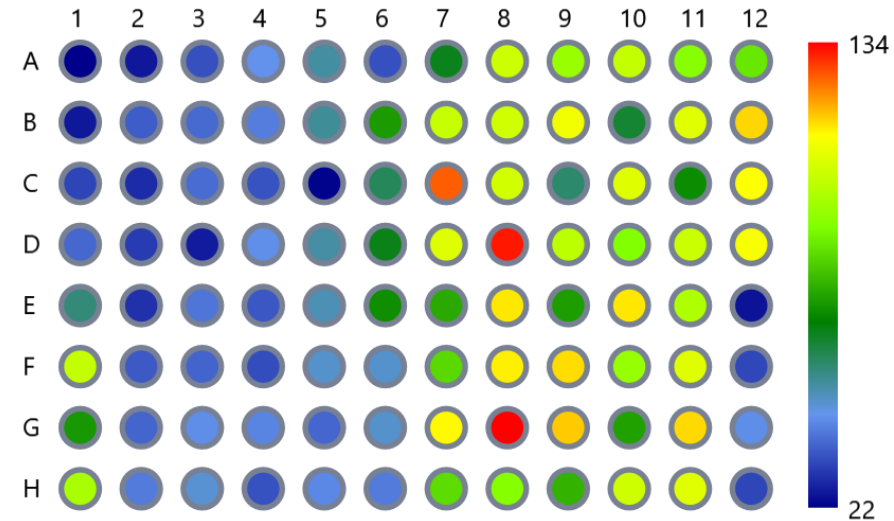
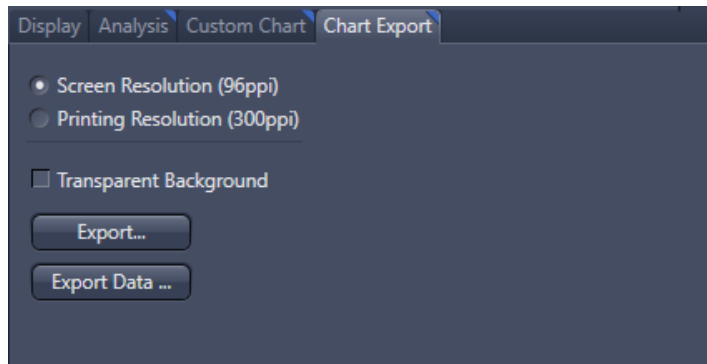


1586-well plate



# Display of Image Analysis Results

## *Export plots*



MEAN.Intensity Mean Value of channel 'EGFP'

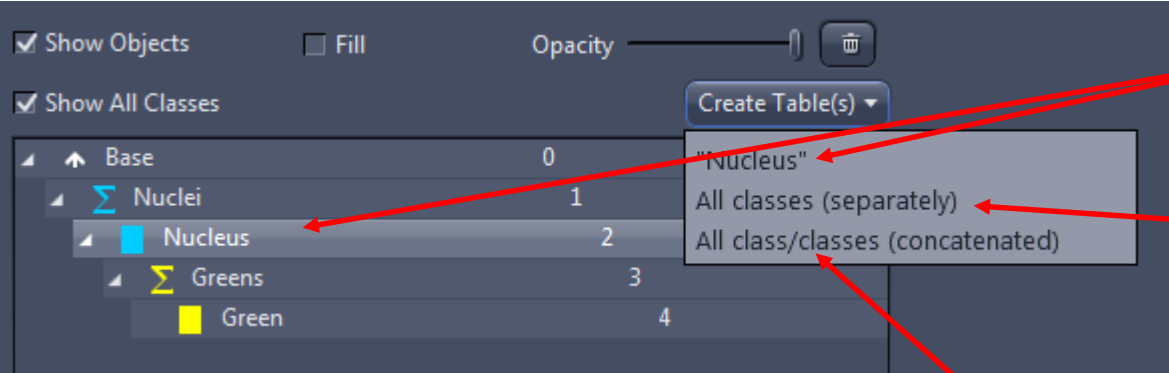
Export plots as \*.png, \*.tiff, \*.bmp, \*.jpg

Choose between Screen Resolution (96 ppi) or printing resolution (300 ppi)

Either with transparent background or as-is.

# Export data after Image Analysis

## Generate result tables



Highlight a specific “Classes”/”Class” to export data for this “Classes”/”Class” only (one table only)

Export data all individual “Classes” and “Class” in separate tables.

In this case, 4 separate data sets:

ID	Count [#]
1	10

The subsequent data can be saved as .csv  
If the input data has higher dimension, e.g. time series, multi-positions, the final data will be concatenated!

Export two data sets where all tables for “Classes” and “Class”, respectively, are concatenated (2 data tables)

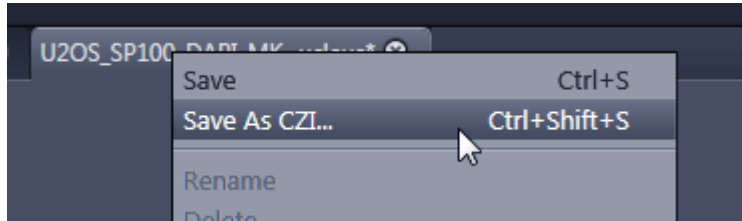
ID	Count [#]	Area [µm²]	Intensity Sum...
1	10	22.36	260,913.00

# Export data after Image Analysis

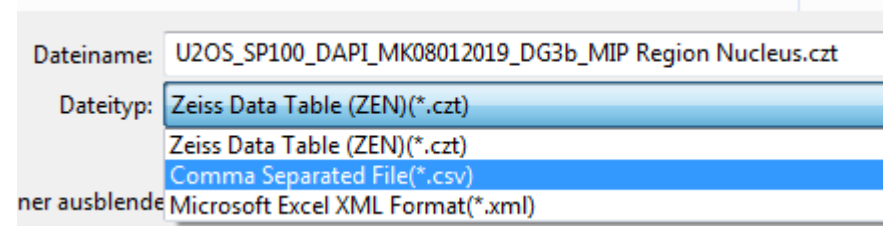
*Export into \*.csv format*



Right-click on tab containing the table



Select “Save As CZI...”



Select \*.csv

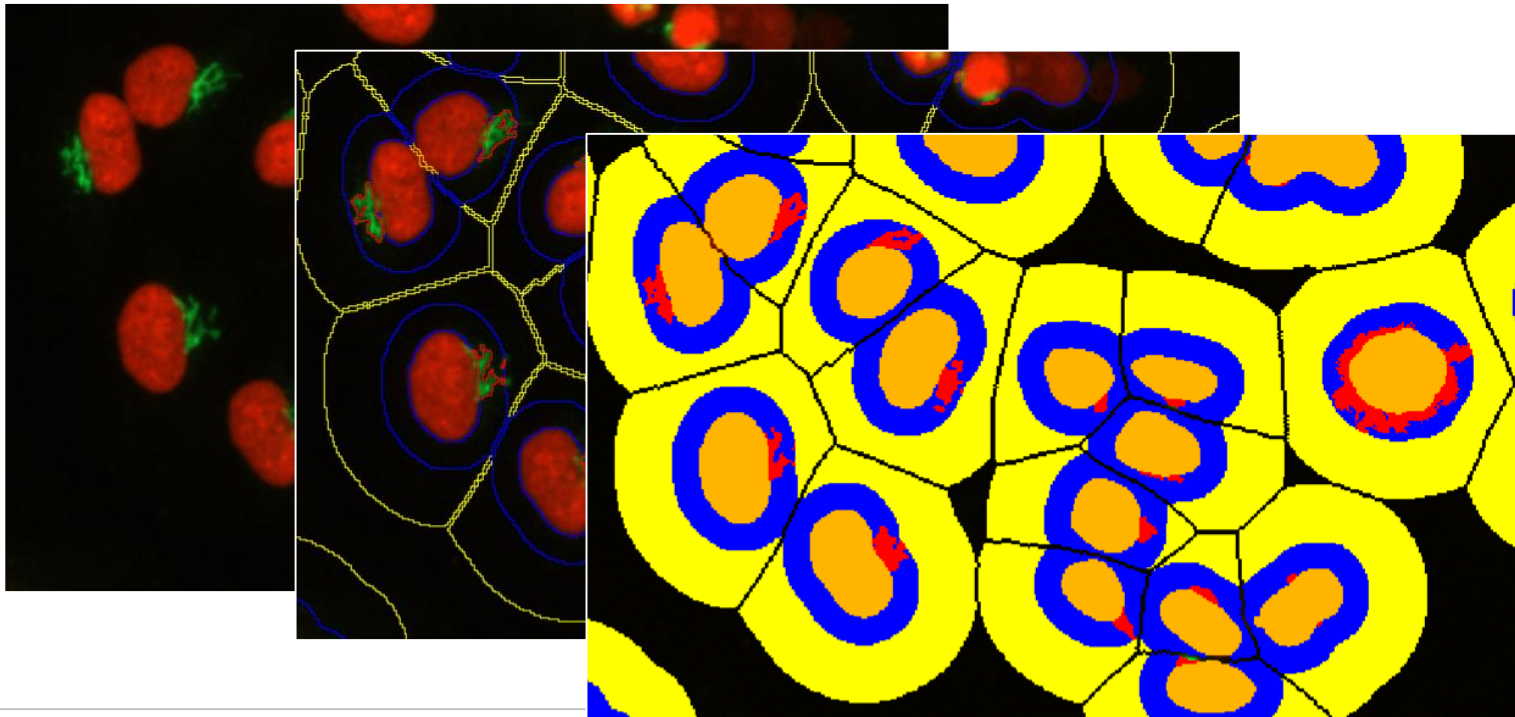
# Zone-of-Influence (ZOI) Image Analysis

## *Typical ZOI Applications*



Applications in cell biology, drug discovery, in-vitro assays, endpoint assays,  
That require to detect objects outside of the object used for segmentation, e.g.:

- Cytoplasm-Nucleus Translocation
- Protein Localization
- Actin, Mitochondria....

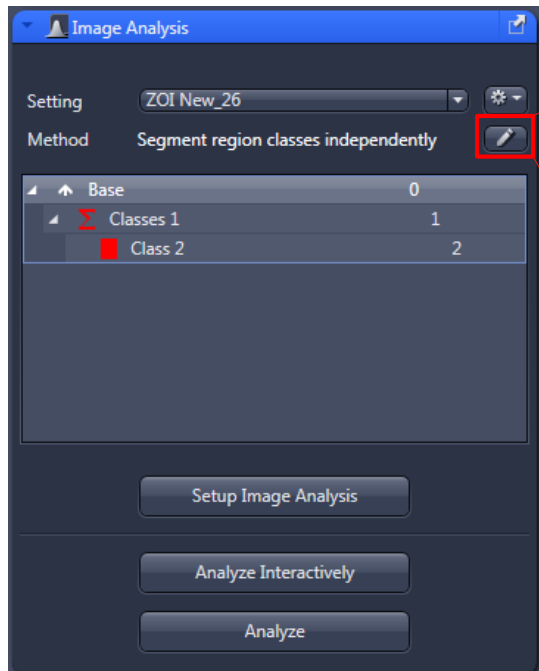


e.g. quantify the total green structures (area and intensity) per cell

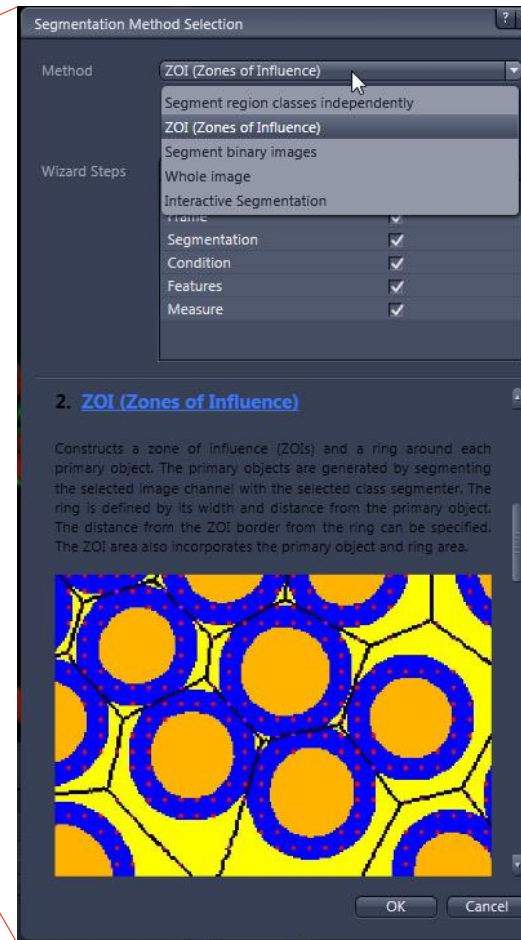


# Creating an Image Analysis Setting using ZOI

*Select the ZOI (Zones of Influence) Method*



Create a new Image Analysis Setting and choose ZOI (Zones of Influence) as Segmentation Method

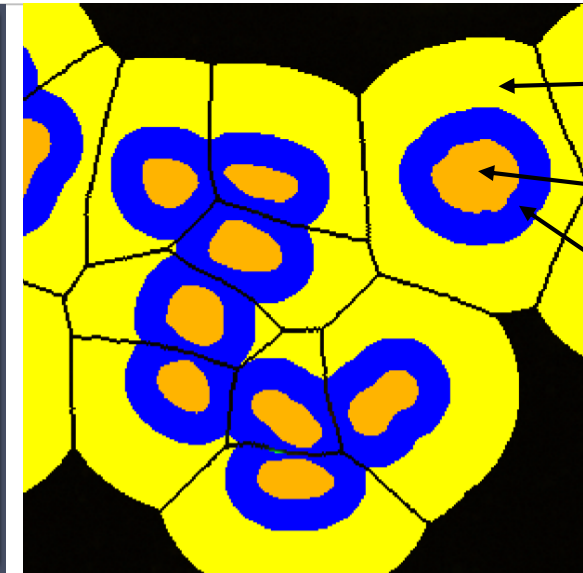
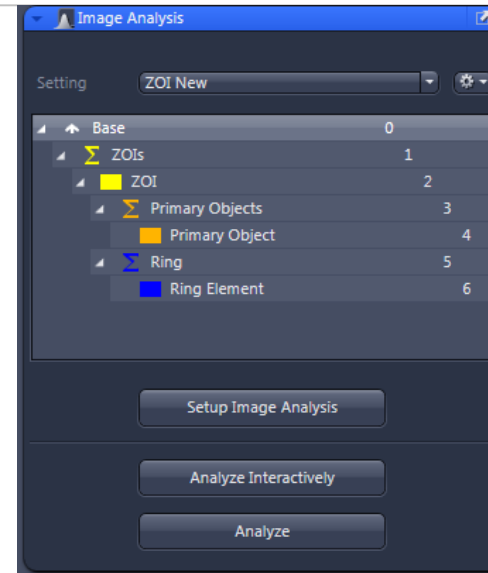


# Set up an Image Analysis Setting using ZOI

## *Classes Setup*

The ZOI-method will create the necessary classes automatically:

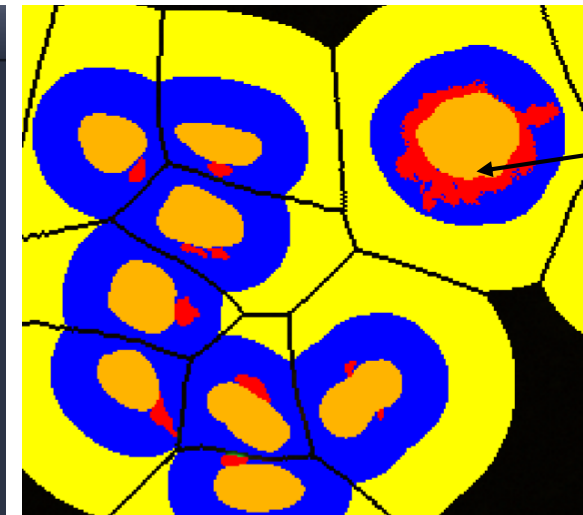
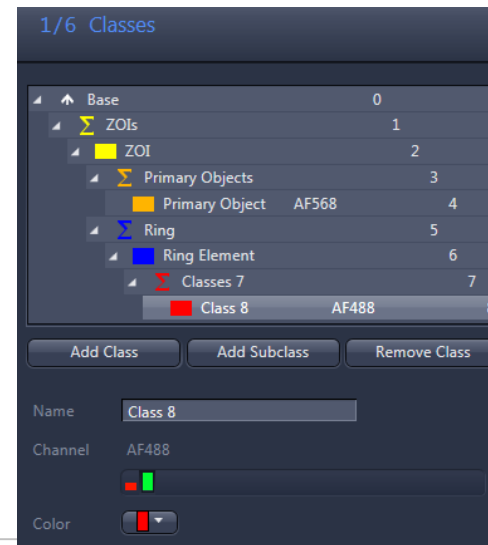
- **ZOIs/ZOI:** the area (zone of influence) that is attributed to the primary objects
- **Primary Objects/Primary Object:** the objects that identify the cell (e.g. nuclei)
- **Ring/Ring Element:** automatically generated around each primary object to measure parameters or to detect sub-objects
- **Optional: Ring sub-object:** objects you want to measure per cell other than the nucleus



ZOI

Primary Object

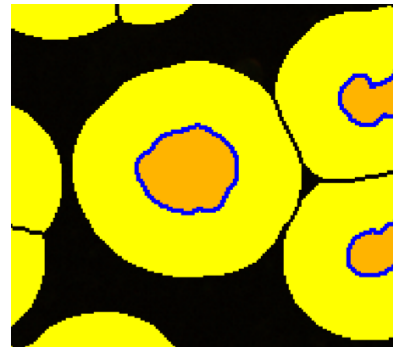
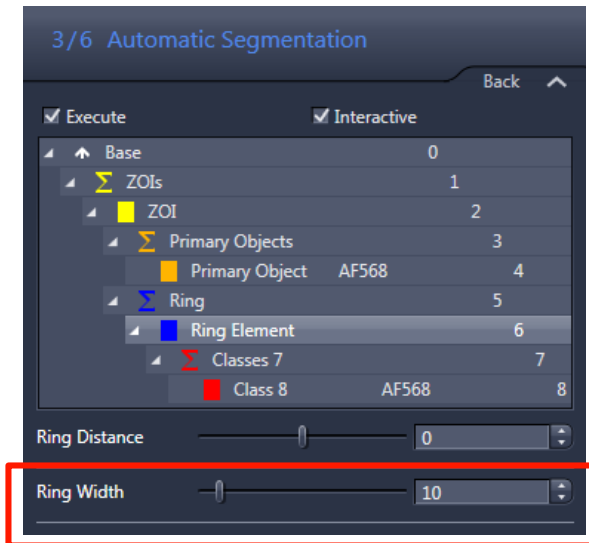
Ring



Sub-Object

# Adjust Ring Parameters

## *Set Width and Distance*



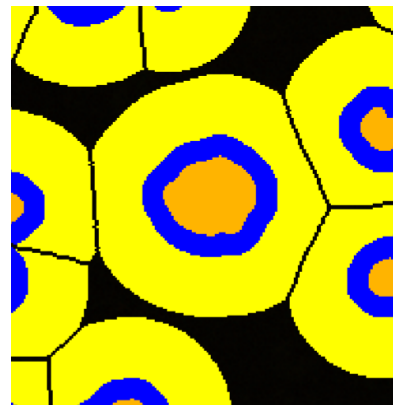
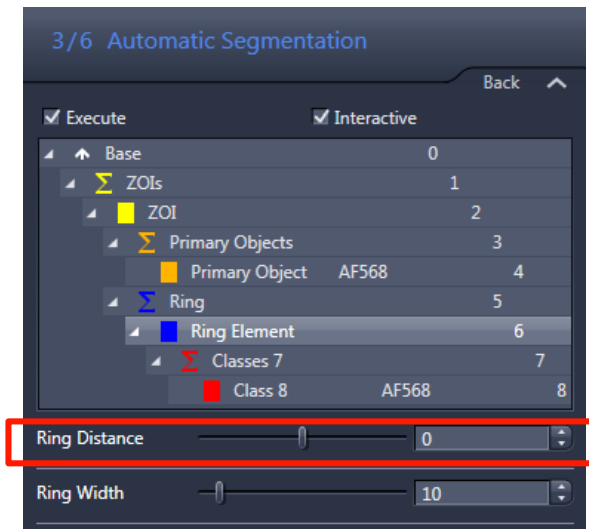
Width: 2



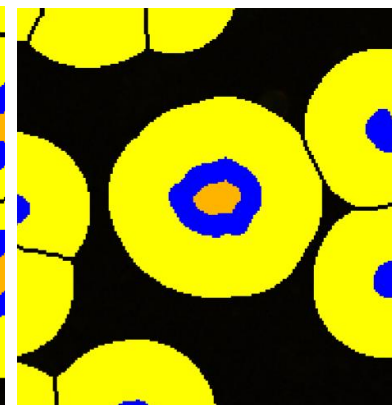
Width: 10



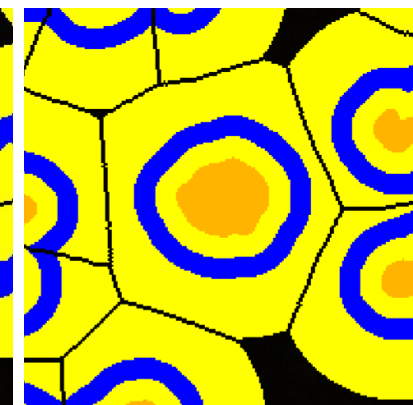
Width: 20



Distance: 0



Negative values



Positive values

# Attribute Ring-Features to Primary Object

*Use the „copy“-Function*



Copy „Ring“/“Actin\_stat“ features to the nucleus

The screenshot shows the 'Features' panel in the Zeiss software. The left pane displays a hierarchy of objects: Base (0), ZOI (1), ZOI (2), Primary Objects (3), Nucleus (H3258, 4), Ring (5), Ring Element (6), Actin\_stat (7), and Actin (EGFP, 8). The right pane shows the 'Features Ring' and 'Features Actin\_stat' panels. The 'Features Ring' panel has a 'Copy' column with dropdown menus for 'Nucleus' and 'Nucleus'. The 'Features Actin\_stat' panel has a 'Copy' column with a dropdown menu for 'Nucleus'. A blue arrow points from the 'Ring' object in the left pane to the 'Features Ring' panel. A red arrow points from the 'Actin\_stat' object in the left pane to the 'Features Actin\_stat' panel. The 'Features Actin\_stat' panel is highlighted with a red border.

**Features Ring**

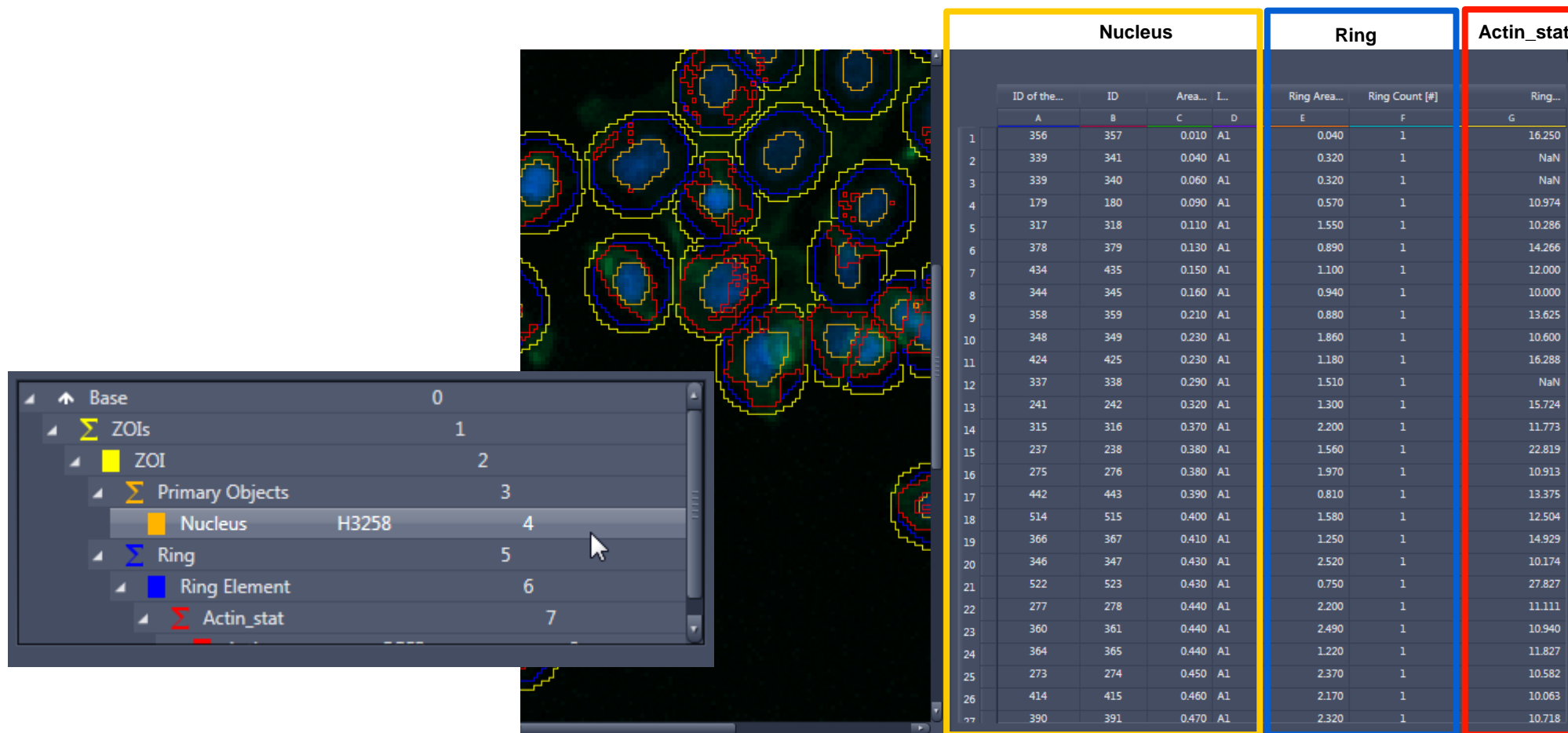
Name	Display	Copy
ID of the parent	<input type="checkbox"/>	
ID	<input type="checkbox"/>	
Area	<input type="checkbox"/>	→ Nucleus
Count	<input type="checkbox"/>	→ Nucleus

**Features Actin\_stat**

Name	Display	Copy
ID of the parent	<input type="checkbox"/>	
ID	<input type="checkbox"/>	
Area	<input type="checkbox"/>	
Intensity Mean Value of channel 'EGFP'	<input type="checkbox"/>	→ Nucleus

# Results

## Features for „Nucleus“



The results table for „Nucleus“ also contains the copied features of „Ring“ and „Actin\_stat“

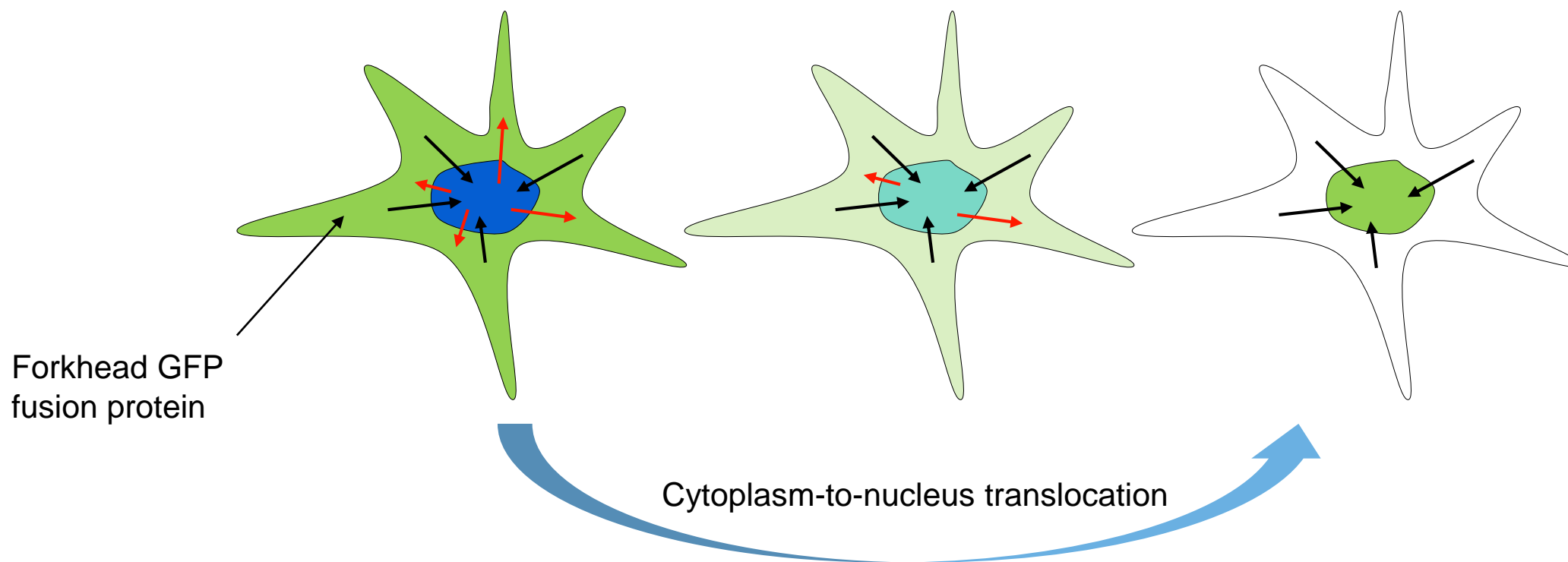
# Application Example: Translocation

## *Inhibition of nuclear export of Forkhead*

Unhibited export

Add Wortmannin / LY294002

Inhibited export

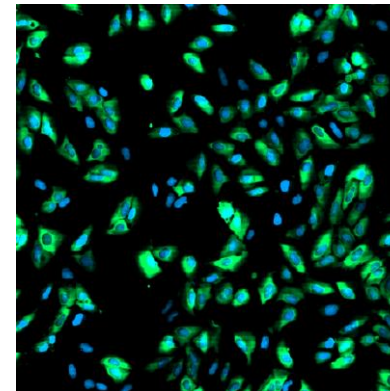


### *Inhibition of nuclear export of Forkhead*

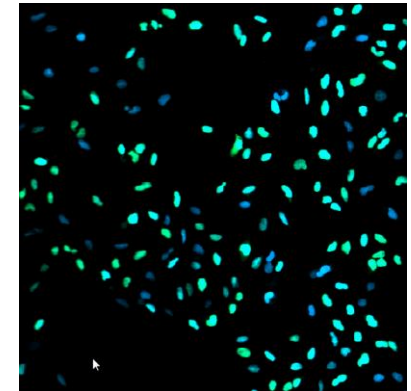
- 96-well plate, human osteosarcoma cells (U2OS), nuclei stained with DRAQ
- Cytoplasm-to-nucleus translocation of the Forkhead (FKHR-EGFP) fusion protein
- In proliferating cells, FKHR is localized in the cytoplasm (constantly moving into the nucleus, but is transported out again by export proteins).
- Upon inhibition of nuclear export, FKHR accumulates in the nucleus
- Export is inhibited by blocking PI3 kinase / PKB with Wortmannin or LY294002.

96-well plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	Neg. Ctrl	39688	0.977	1.95	3.91	7.81	15.63	31.25	62.5	125	250	Pos. Ctrl
B	Neg. Ctrl	empty	0.977	1.95	3.91	7.81	15.63	31.25	62.5	125	250	Pos. Ctrl
C	Neg. Ctrl	empty	0.977	1.95	3.91	7.81	15.63	31.25	62.5	125	250	Pos. Ctrl
D	Neg. Ctrl	empty	0.977	1.95	3.91	7.81	15.63	31.25	62.5	125	250	Pos. Ctrl
E	Pos. Ctrl	empty	0.31	0.63	1.25	2.5	5	10	20	40	80	Neg. Ctrl
F	Pos. Ctrl	empty	0.31	0.63	1.25	2.5	5	10	20	40	80	Neg. Ctrl
G	Pos. Ctrl	empty	0.31	0.63	1.25	2.5	5	10	20	40	80	Neg. Ctrl
H	Pos. Ctrl	empty	0.31	0.63	1.25	2.5	5	10	20	40	80	Neg. Ctrl
	Wortmannin in nM			LY294.002 in $\mu$ M								



Negative Ctrl



Positive Ctrl

Data set [BBBC013v1](#) by Ilya Ravkin, available from the Broad Bioimage Benchmark Collection [[Liosa et al., \*Nature Methods\*, 2012](#)]



# Software Demo

## *Set up an Image Analysis for Translocation Assay*



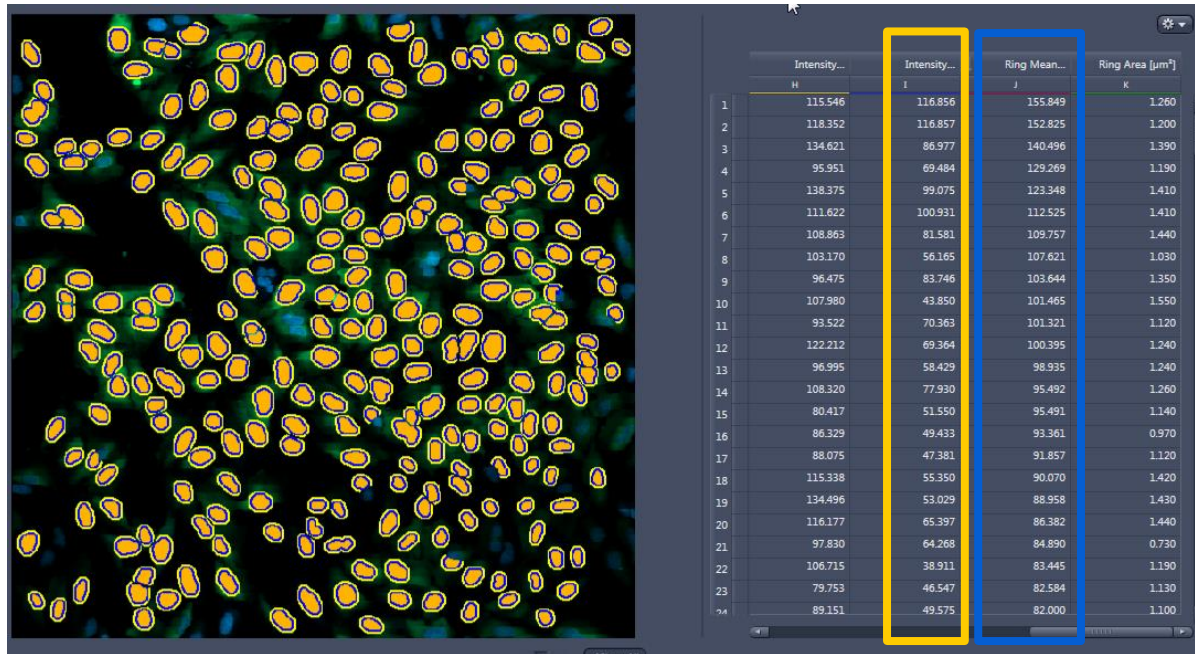
Set up an image analysis and check the results:

- Image Data: Translocation\_comb\_96\_5ms.czi
- Image Analysis Setting: Translocation\_26.czas

# Human U2OS cells cytoplasm–nucleus translocation

## Calculate Translocation Ratio

- Create tables for „Primary Object“ and export to \*.csv format
- Calculate the Translocation-Ratio (T) for each cell
- Calculate the mean value of the Translocation-Ratio for each well



Translocation Ratio (per cell):

$$T = \frac{I_{\text{Mean, Nucleus}}}{I_{\text{Mean, Ring}}}$$

Data set [BBBC013v1](#) by Ilya Ravkin, available from the Broad Bioimage Benchmark Collection [[Ljosa et al., Nature Methods, 2012](#)]

# OAD Script for automatization

## *Start Python Script for Calculation and Plotting*



Tasks to perform:



1. Load load the image file (\*.csv) and image anlaysis setting (\*.czias)
2. Run the image analysis
3. Extract the image analysis results as \*.csv
4. Start the python script (test\_wellplate\_from\_ZEN.PY)



5. Read in data
6. Calculate the translocation Ratio
7. Generate heatmaps for different features (e.g. Translocation Ratio)
8. Save heatmaps as PNG files



9. Load PNG files in ZEN

# Plot Results

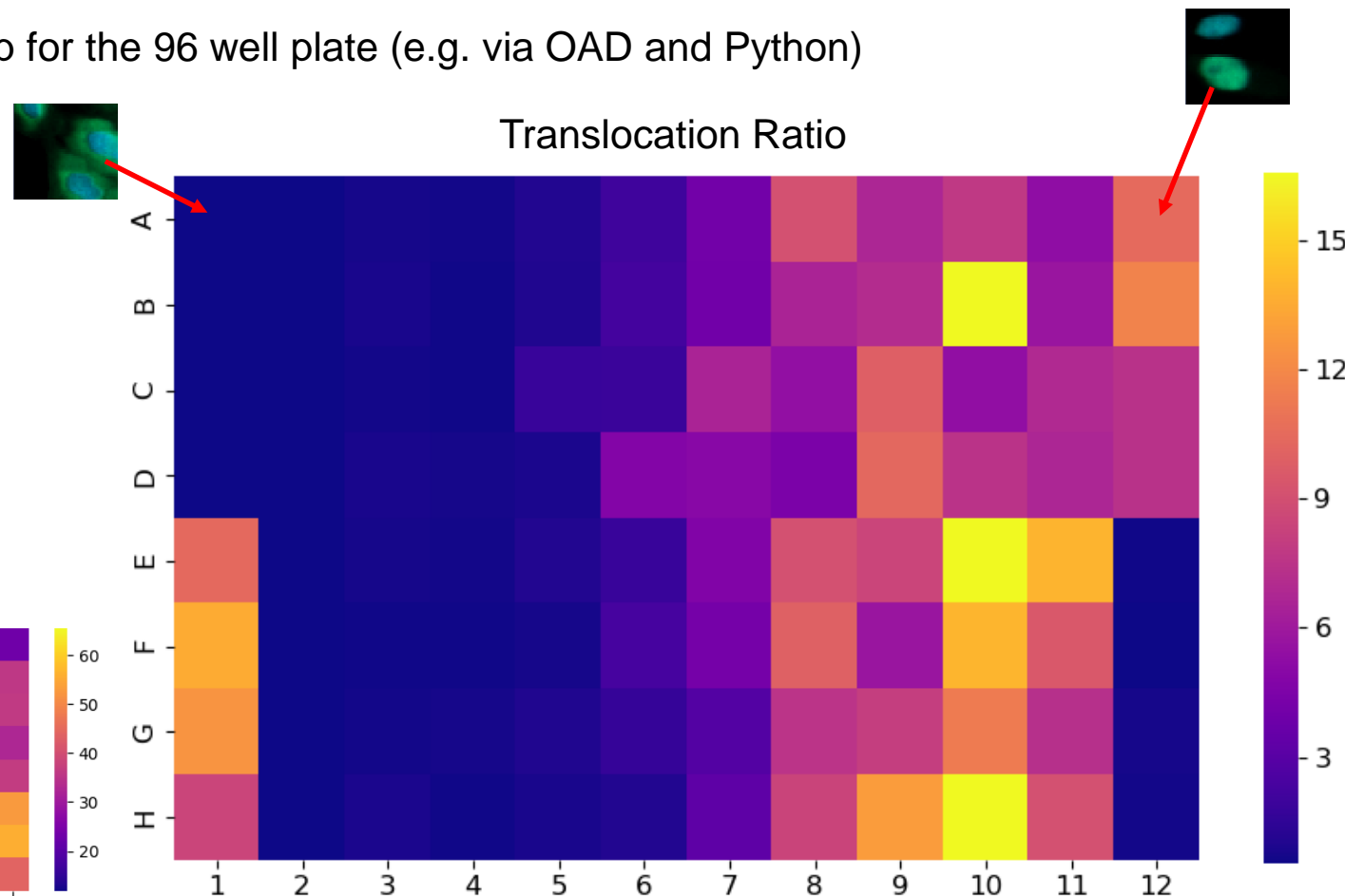
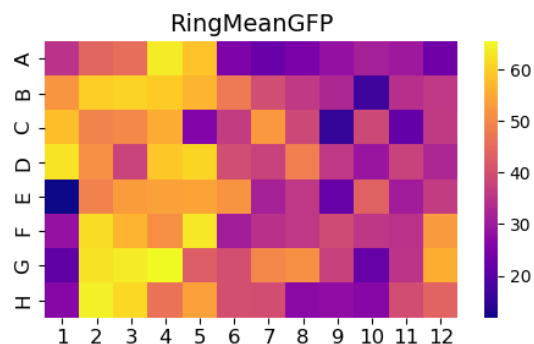
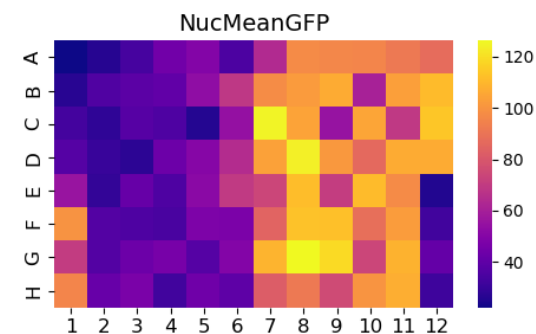
## Heatmap of the 96-well plate



Extract relevant features and plot them as a heatmap for the 96 well plate (e.g. via OAD and Python)

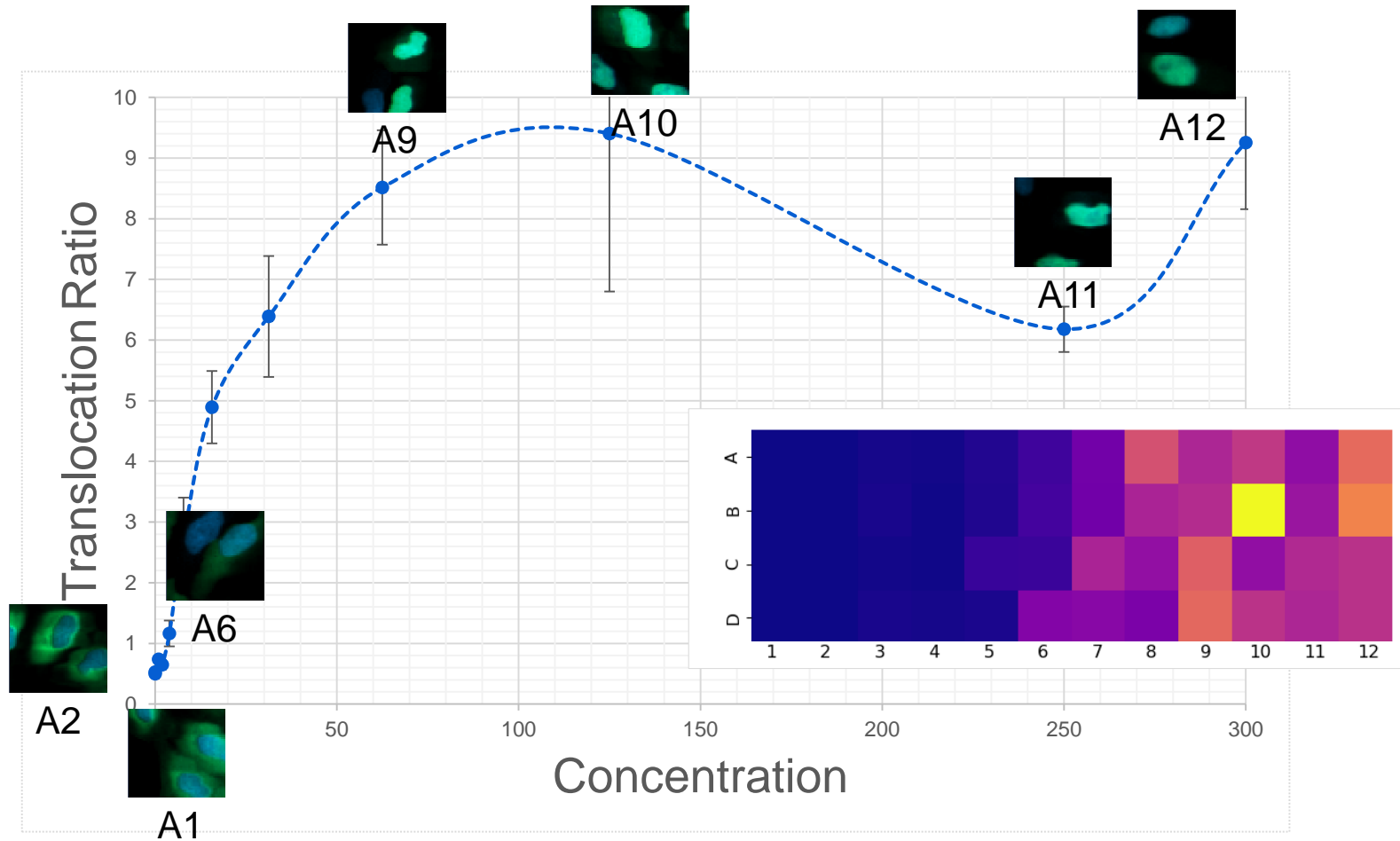
### 96-well plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	Neg. Ctrl	39688	0.977	1.95	3.91	7.81	15.63	31.25	62.5	125	250	Pos. Ctrl
B	Neg. Ctrl	empty	0.977	1.95	3.91	7.81	15.63	31.25	62.5	125	250	Pos. Ctrl
C	Neg. Ctrl	empty	0.977	1.95	3.91	7.81	15.63	31.25	62.5	125	250	Pos. Ctrl
D	Neg. Ctrl	empty	0.977	1.95	3.91	7.81	15.63	31.25	62.5	125	250	Pos. Ctrl
E	Pos. Ctrl	empty	0.31	0.63	1.25	2.5	5	10	20	40	80	Neg. Ctrl
F	Pos. Ctrl	empty	0.31	0.63	1.25	2.5	5	10	20	40	80	Neg. Ctrl
G	Pos. Ctrl	empty	0.31	0.63	1.25	2.5	5	10	20	40	80	Neg. Ctrl
H	Pos. Ctrl	empty	0.31	0.63	1.25	2.5	5	10	20	40	80	Neg. Ctrl
	Wortmannin in nM			LY294.002 in μM								



# Results (mean translocation ratio)

*Treatment with different concentrations of Wortmannin*



- Plot results (e.g. via Excel)
- Mean translocation ratio (for rows A to D)

