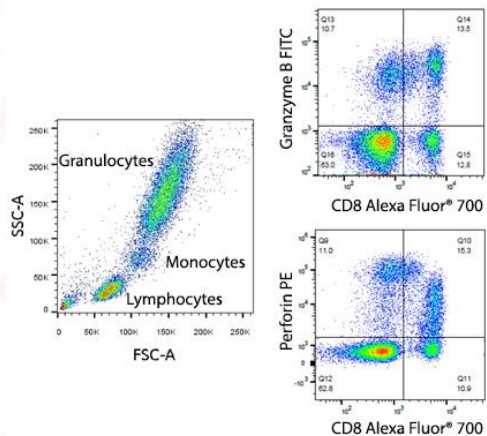
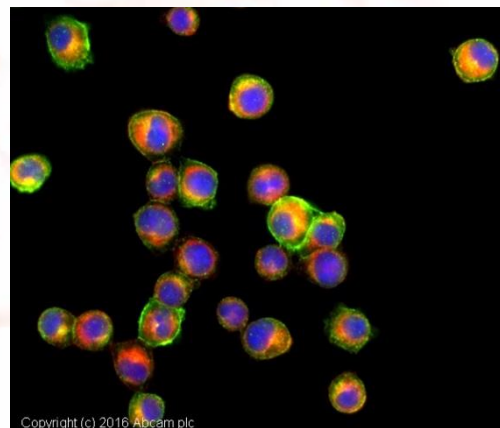


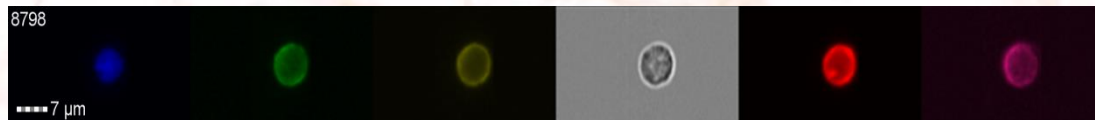
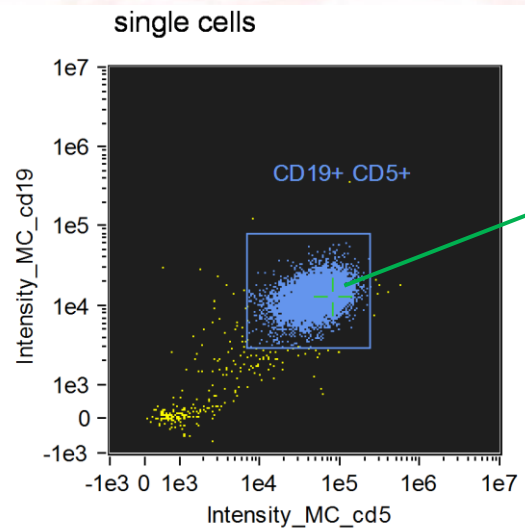
# Flow cytometry



# Microscopy



# Imaging Flow Cytometry



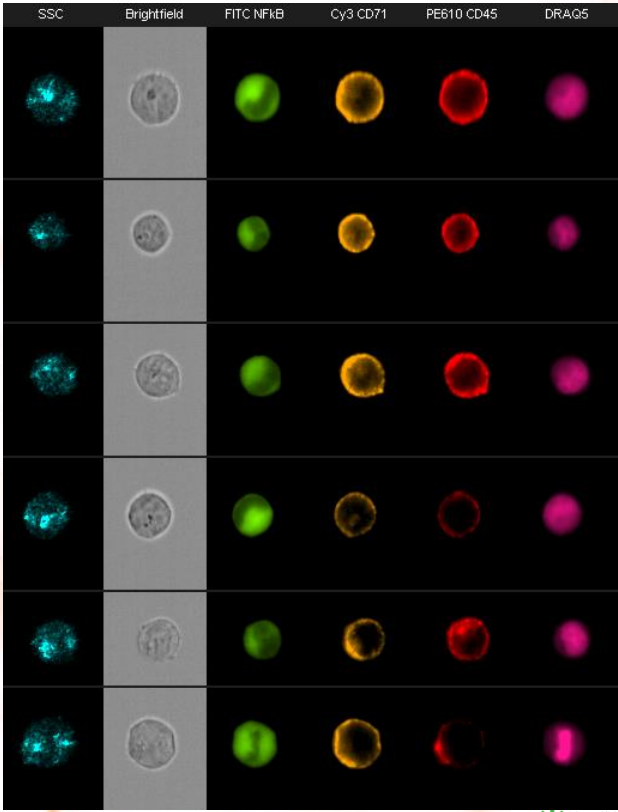
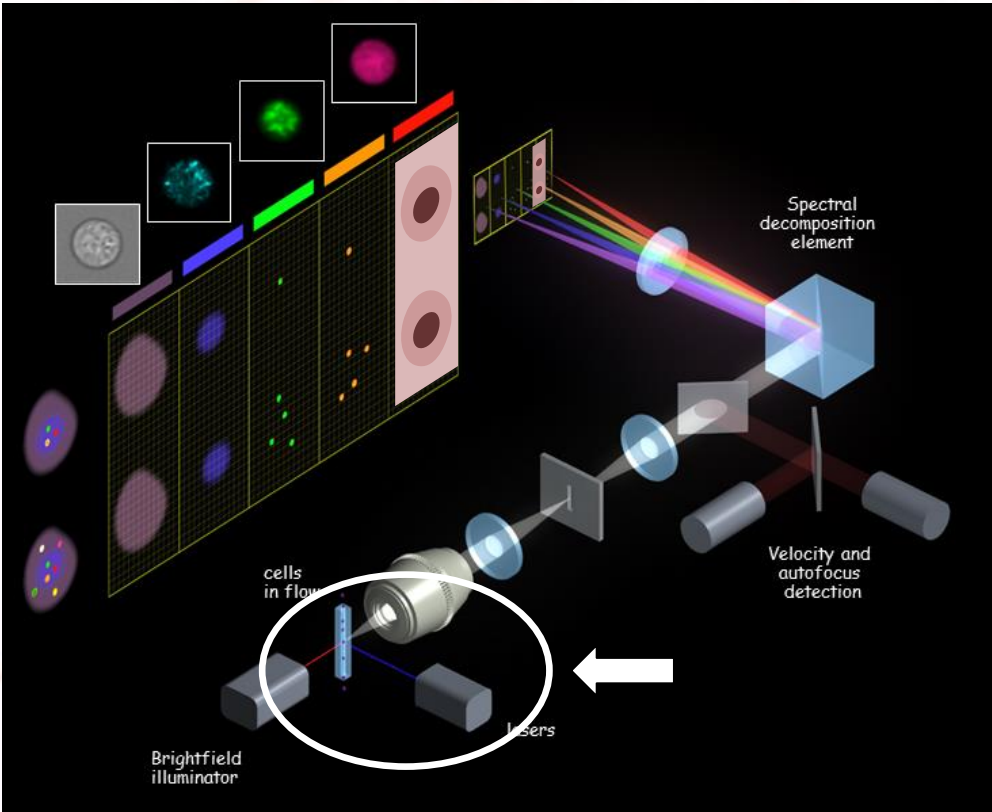
the cell under each single dot !

# ImageStream MKII

Imagery of cells in suspension



Identification and localization of each single signal in the cell



# ImageStream MKII

## Magnifications:

20x → 120um Field of view, 8um depth of field

40x → 60um Field of view, 4um depth of field

60x → 40um Field of view, 2.5um depth of field



Cell diameter up to 120um  
Endothelial, epithelial,  
large cancer cells



Cell diameter up to 60um  
Cell culture line



Cell diameter up to 2.5 um  
PBMCs, lymphocytes, RBC,  
small vesicles, exosomes

Source: Merck

# ImageStream MKII

## Sample Preparation Guide

Ch	Band (nm)	Excitation Laser (nm)							Used	Ch	
		375	405	488	561	592	642	730			785
1	435-505 (457/45)	AF350, DAPI, Hoechst, PacBlue, eFluor490	DAPI, Hoechst, PacBlue, CascadeBlue, AF405, eFluor490, DyLight405, CFP, LIVE/DEAD Molet								1
2	505-560 (533/55)	PacOrange, CascadeYellow, AF430, QD525, eFluor525	PacOrange, CascadeYellow, AF430, BDHorizonV550, QD525, eFluor525	FITC, AF488, GFP, YFP, DyLight488, PKH67, Syto13, SpectrumGreen, LysoTrackerGreen, MitoTrackerGreen							2
3	560-595 (577/35)	QD565, QD585, eFluor565	QD565, QD585	PE, PKH26, DSRed, mOrange, CellMask/CellTracker, SYTOX Orange, Cy3	PE, AF546, Cy3*, DyLight550, PKH26, DSRed, SpectmOrg, MTOrg						3
4	595-642 (610/30)	QD625, eFluor625	QD625, eFluor625	PE-TexRed, ECD, PE-AF610, 7AAD, PI, RFP, QD625, eFluor625	AF568, Cy3*, PE- TexRed, DyLight594, ECD, TexRed, PE- AF610, RFP, mCherry, 7AAD, PI	TexRed, AF594, DyLight594, mCherry, SpectrumRed, PI, 7AAD					4
5	642-745 (702/85)	QD705, eFluor650	QD705, eFluor650	PE-Cy5, PE-AF647, PerCP, PerCP-Cy5.5, DRAQ5, QD705, eFluor650, FuraRedo	PE-Cy5, PE- AF647, DRAQ5	AF647, AF660, AF680, APC, Cy5, DyLight649, PE-AF647, PE-Cy5, DRAQ5	APC, AF647, AF660, AF680, DRAQ5, Cy5, DyLight649, DyLight680, PE- AF647, PE-Cy5, PerCP, PerCP-Cy5.5				5
6	745-780 (762/35)	QD800	QD800	PE-Cy7, PE-AF750, QD800	PE-Cy7, PE- AF750	APC-Cy7, APC-AF750, APC-H7, APC- eFluor750	APC-Cy7, APC- AF750, APC-H7 APC- eFluor750, Cy7, AF750, DyLight750, PE-Cy7, PE-AF750	AF750, Cy7, SSC DyLight750, PE-Cy7, PE- AF750			6

# ImageStream applications



## Field of Study

## Example from Imagestream

 Cell signaling:

NFkB Translocation, HIV induced NFAT,  
FoxP3 localization

 Internalization &  
phagocytosis

CpGB, Internalization, phagocytosis of  
Bacteria by monocytes

 Intracellular co-  
localization

Ligand colocalization to lysosomes

 Shape change &  
chemotaxis

MCP-1 activation of monocytes,  
Differentiation of FDCP cells

 Cell-cell interaction

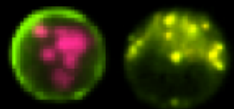
Immune synapse formation,  
NFkB activation from T-cell APC conjugation

# ImageStream applications



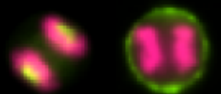
## Field of Study

## Example from Imagestream



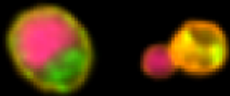
Cell death & autophagy

Apoptosis, nuclear fragmentation,  
caspase3 activation



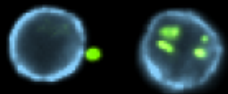
Cell cycle & mitosis

Morphological classification of mitosis



Stem cell biology

Erythroid differentiation



Microbiology

Bacterial phagocytosis in PBMC



Parasitology

Babesia infection in RBCs



# ISX acquisition software

The screenshot displays the INSPIRE for the ISX mkII software interface, which is used for microscopy data acquisition and analysis. The interface is divided into several main sections:

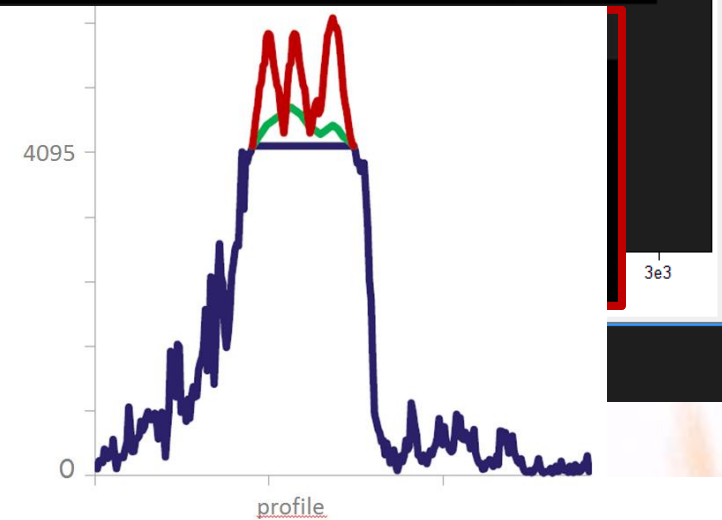
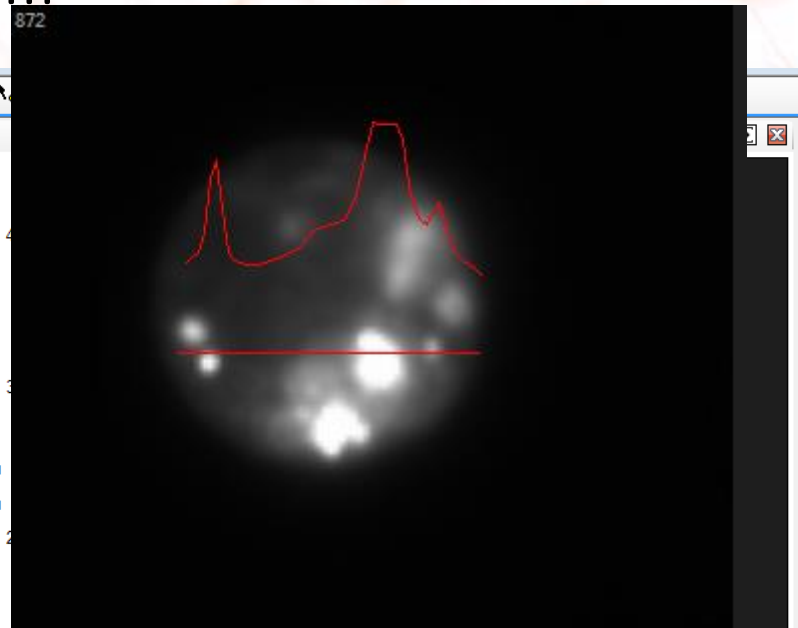
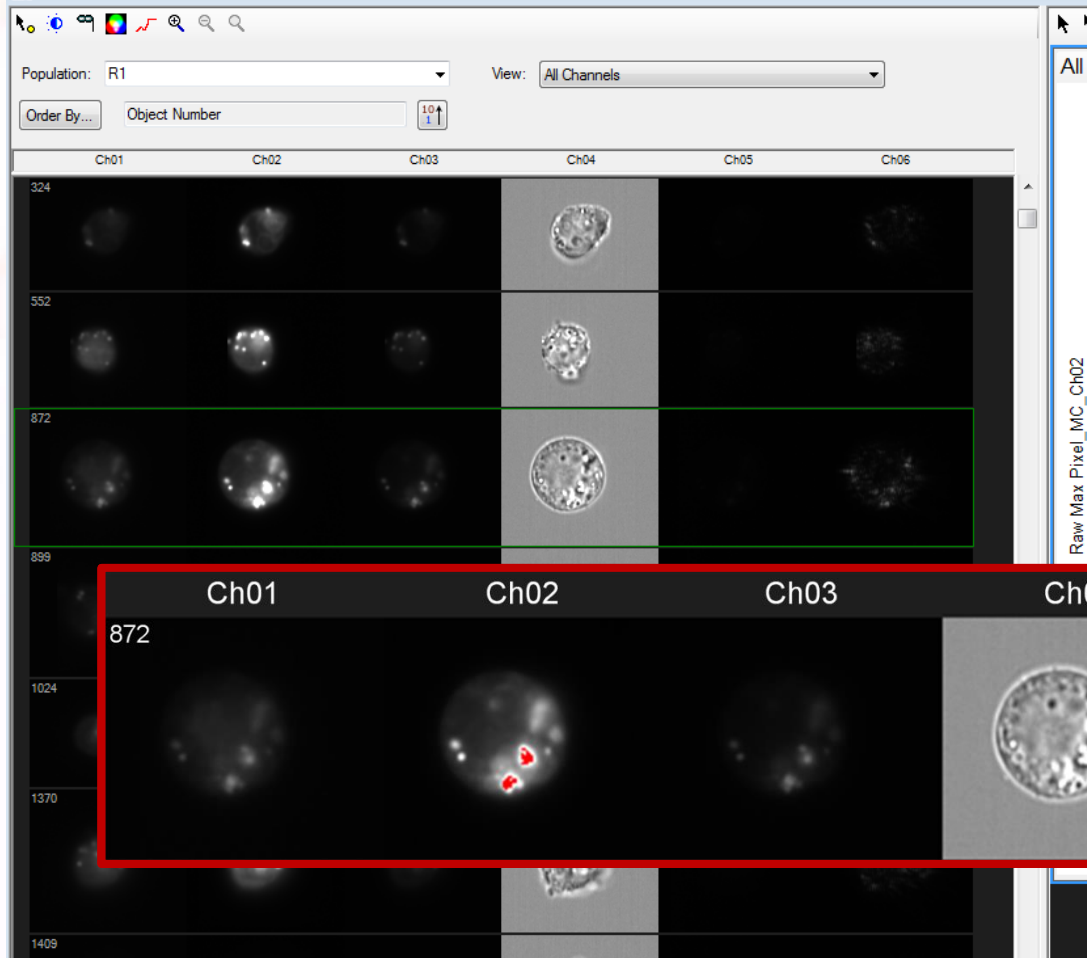
- Top Panel:** Contains a menu bar (File, Instrument, Analysis, Compensation, Layout, Advanced, Help) and a toolbar with various icons for file operations and analysis.
- Left Panel:** Shows a grid of microscopy images. The top row displays raw data for channels Ch01 through Ch06. Below this, there are several rows of processed images, including a grayscale image and a color image with red and green channels.
- Center Panel:** Features three scatter plots at the top, each showing 'Raw Max Pixel\_MC' for different channels (Ch01, Ch03, Ch05). Below these are two histograms: one for 'Aspect Ratio\_M04' (with regions R1 and R2 highlighted) and another for 'Normalized Frequency' vs 'Gradient RMS\_M04\_Ch04'. A red circle highlights the top three scatter plots.
- Right Panel:** Contains a 'Sample' section with 'Load' and 'Return' buttons, a 'File Acquisition' section with 'Acquire' button, and an 'Illumination' section with various intensity and wavelength settings (405, 488, 642, SSC).
- Bottom Panel:** Includes a status bar with 'Switch Core Mode' completion time, 'Sheath', 'Flush', and 'Beads' sliders, and a 'Processing Load' indicator.

The software is running on a Windows operating system, as indicated by the taskbar at the bottom showing the Start button and system tray icons.

**amnis**<sup>®</sup>  
part of EMD Millipore

# ISX acquisition software

Avoid saturation !!!





# IDEAS software

IDEAS - [Ko intra-TOT.daf]

File Guided Analysis Analysis Compensation Tools Options Reports Windows Help

Population: high internalization & R1 & neutrofil & single cells & fc View: Internalization\_Ch04\_Ch03\_Ch05\_Ch01\_Ch03/Ch1

Order By... Object Number

CH04 CH03 CH05 CH01 CH03/CH05

800 901 1260 1312 1474 2049 2342 2517 2577 2762 3240 3509 3736

Wizards

Select the wizard to use for analysis:

	Open File	Creates a template to facilitate analysis.
	Display Properties	Automatically sets image display properties.
	Begin Analysis	Identifies single, focused, fluorescent positive cells.
	Feature Finder	Assists the user in picking relevant features for separating populations. The file must contain members of each population.
	Apoptosis	Creates an analysis template for identifying apoptotic events based on brightfield and nuclear morphology.
	Cell Cycle - Mitosis	Creates an analysis template that distinguishes mitotic and apoptotic events.
	Co-localization	Creates an analysis template for measuring the co-localization of two probes on, in, or between cells in your sample.
	Internalization	Creates an analysis template for measuring the internalization of a probe.
	Nuclear Localization	Creates an analysis template for measuring the nuclear localization of a probe.
	Shape Change	Creates an analysis template for measuring circular morphology.
	Spot	Creates an analysis template for measuring texture based on spot counting.

OK Cancel

focus

single cells

Intensity\_MC\_Ch03

Intensity\_MC\_Ch01

monociti neutrofilii

internalization

Max Pixel

Intensity\_MC\_Ch05

Normalized

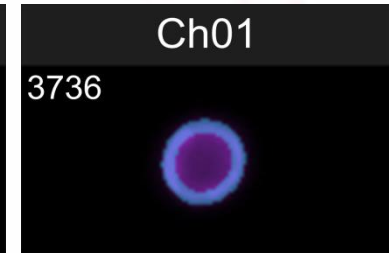
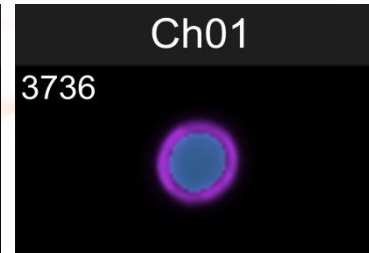
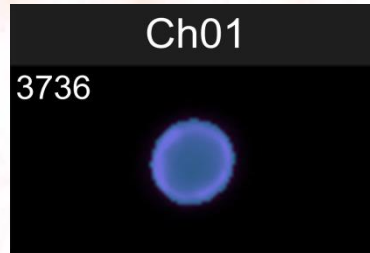
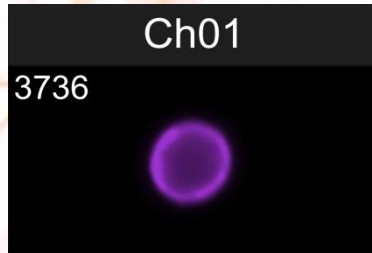
internalization erode2 ch3

surface outside high internalization

5:58 PM 2/24/2019

# IDEAS software

Cell membrane  
mask



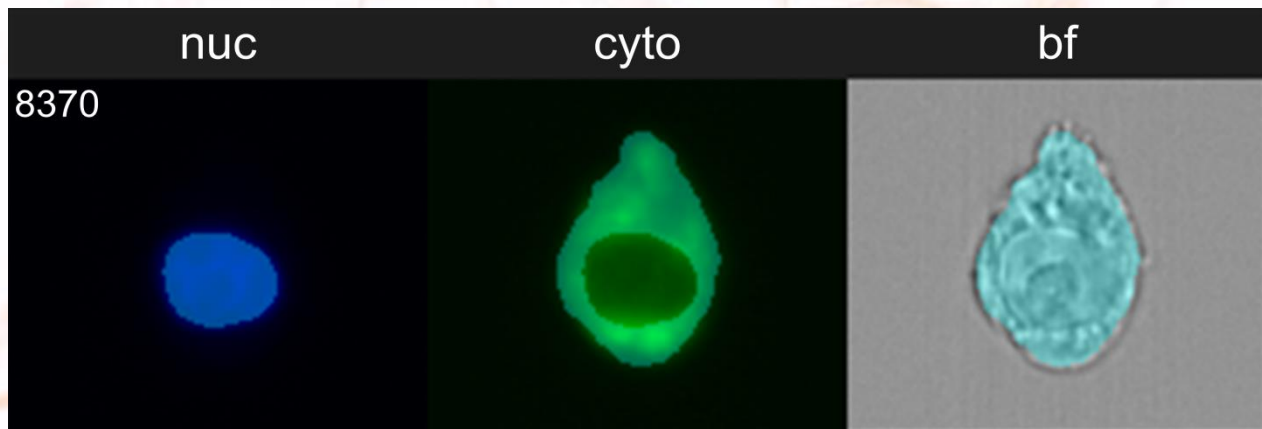
Nuc mask not needed

Whole cell mask

Whole cell mask-eroded

Whole cell mask AND NOT  
Whole cell mask-eroded

Cytoplasm mask



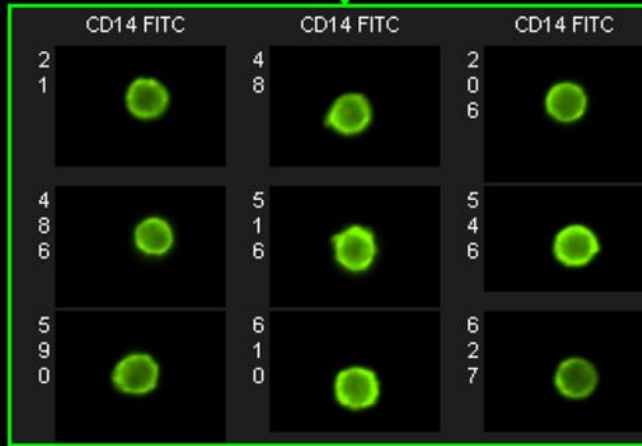
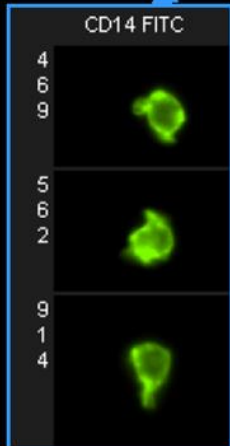
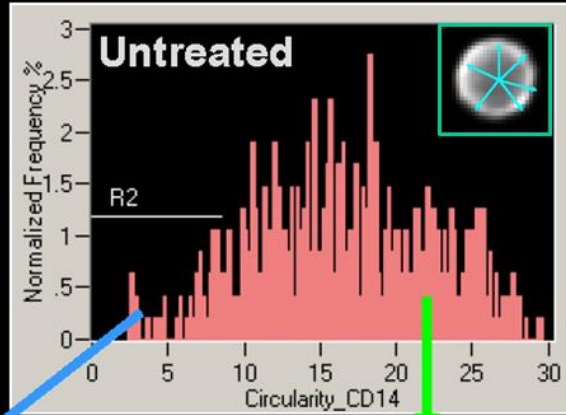
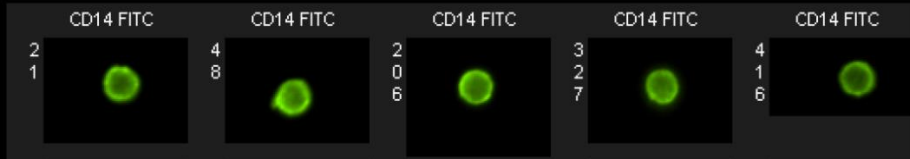
Nuc mask not needed

BF mask AND NOT nuc mask

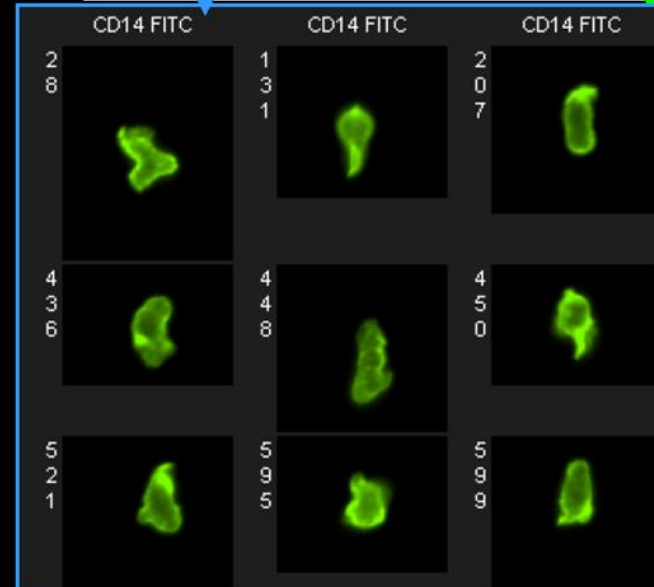
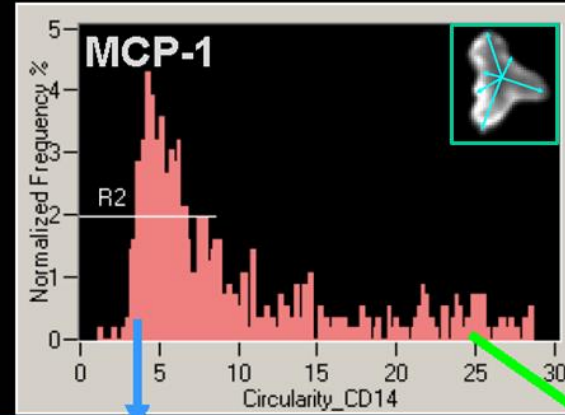
# ImageStream MKII: Shape Change

Shape change in human monocytes

**Untreated**



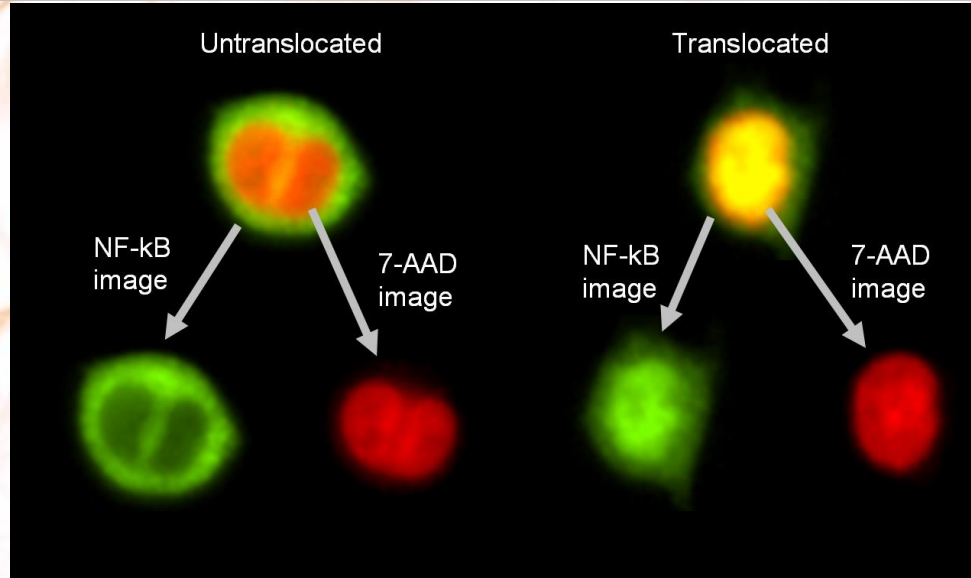
**MCP-1**



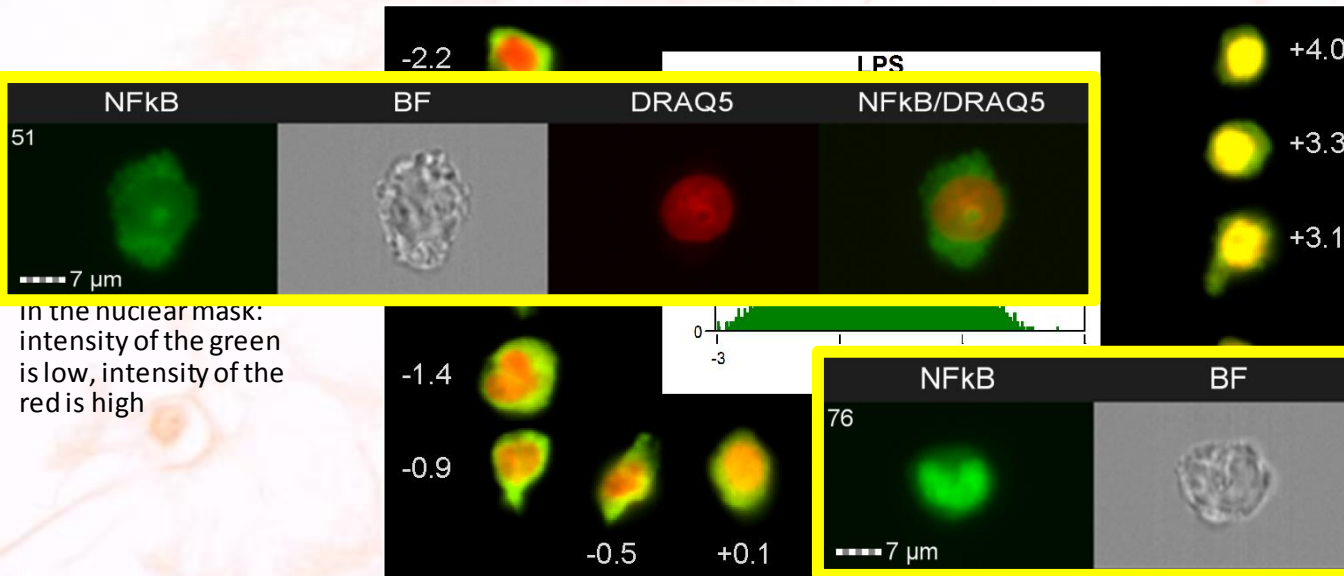
Circularity measures the degree of the mask's deviation from a circle.



# ImageStream MKII: NF-kB Translocation

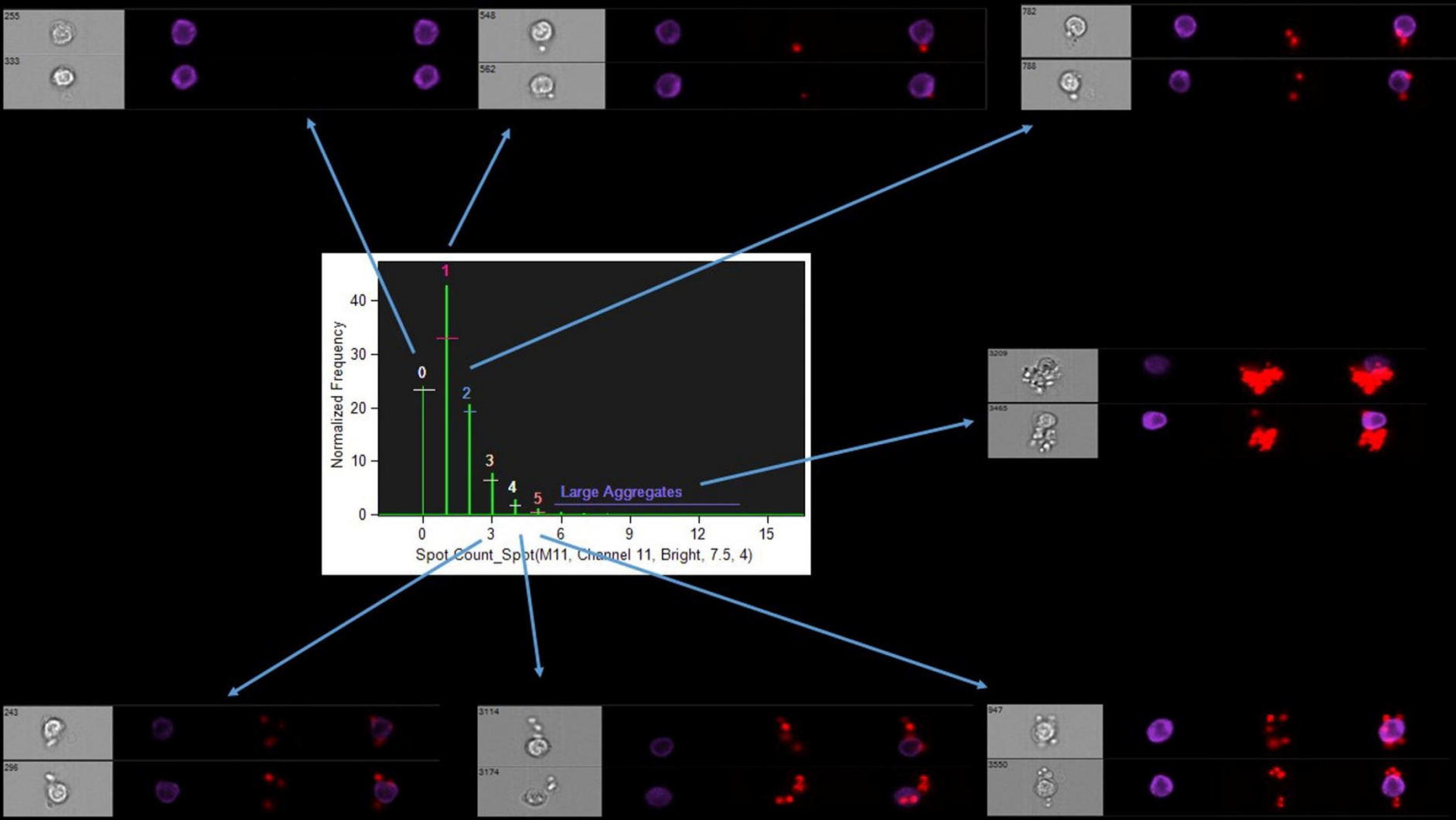


The **Similarity feature** is a measure of the degree to which two images are linearly correlated within a masked region.



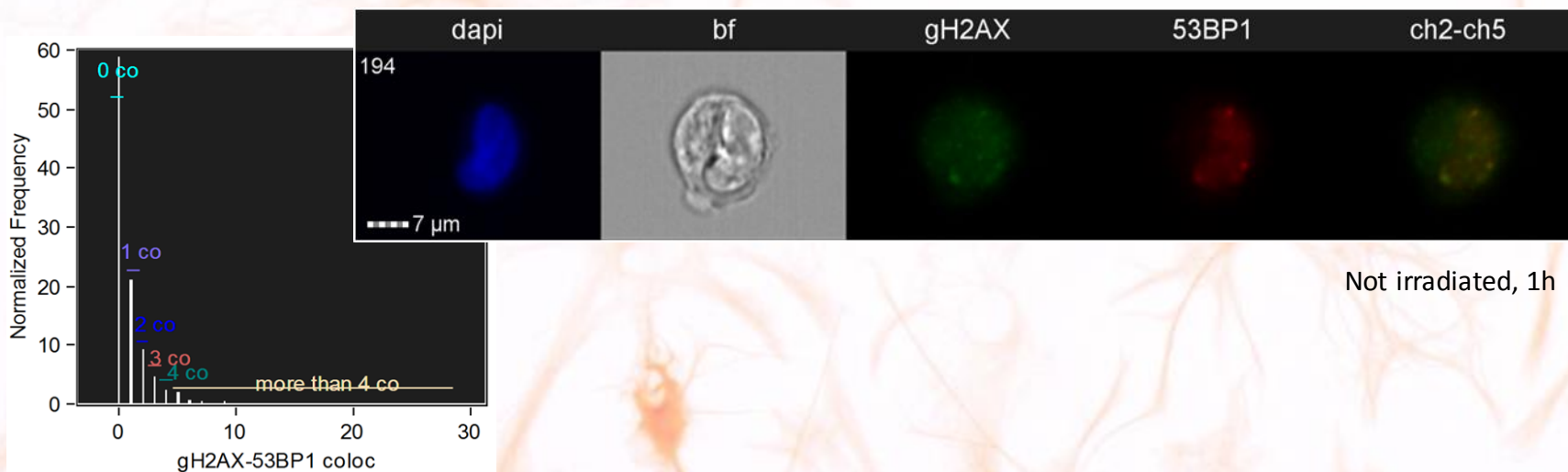
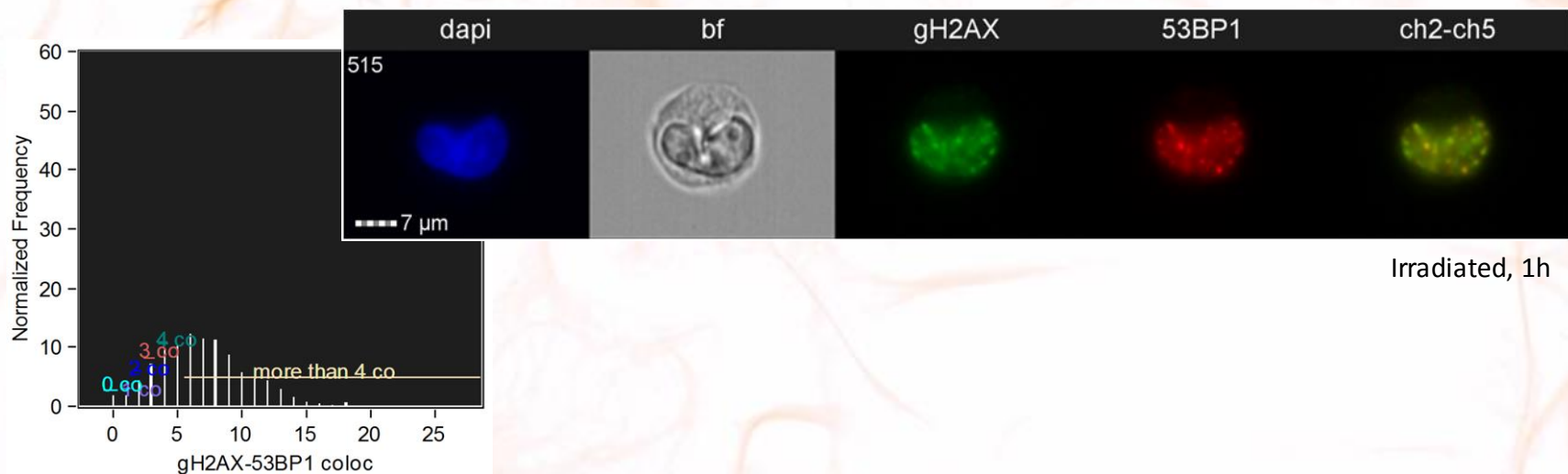
In the nuclear mask:  
intensity of the green

# ImageStream MKII: Mixed Populations



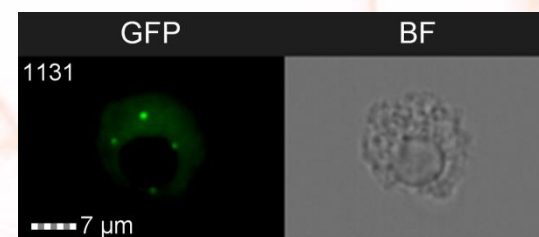
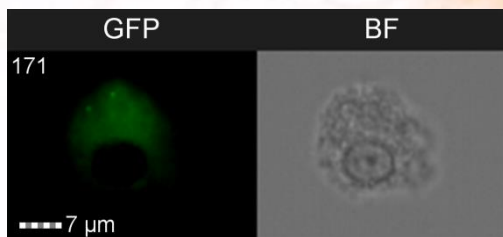
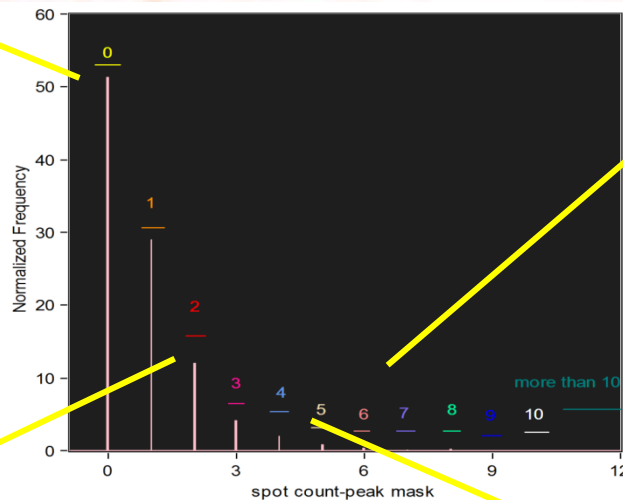
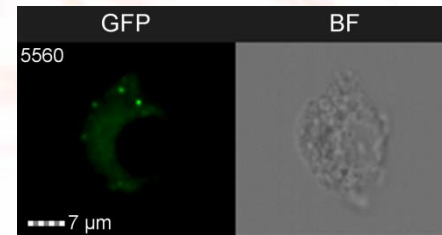
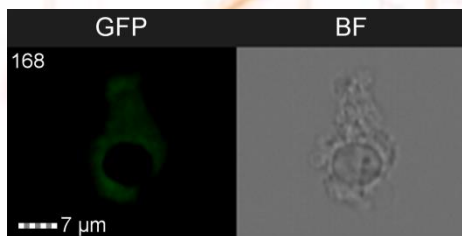


# ImageStream MKII: Spot Counting-DNA damage and repair



Count of **gH2AX** and **53BP1 foci** and quantification of their colocalization in irradiated and not irradiated K562 cells, at 1h or 48h after irradiation (Courtesy of: Della Volpe Lucrezia, Raffaella Di Micco's lab)

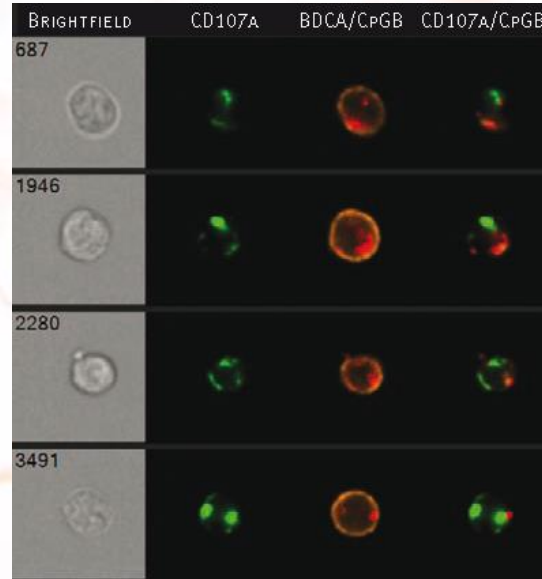
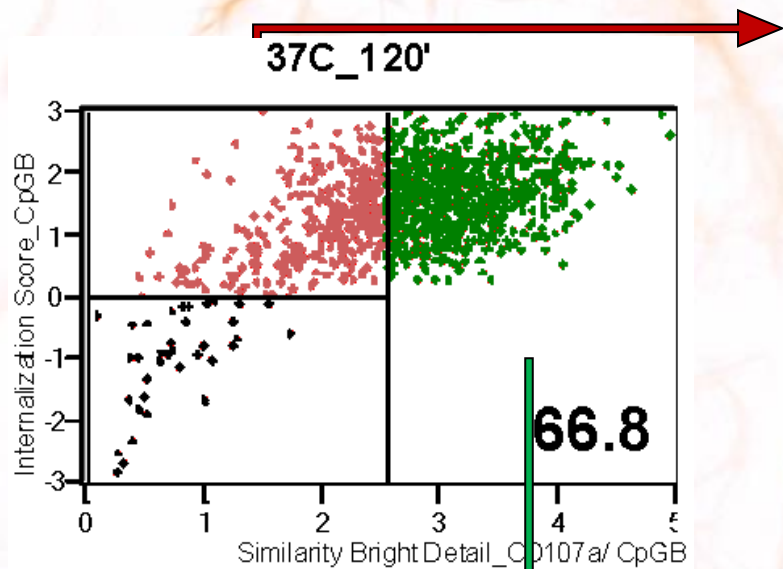
# ImageStream MKII: Spot Counting-*in vitro* phagocytosis assay



spot count-peak mask		
Population	Count	%Gated
macrophages	4755	100
0 spot	2439	51.3
1 spot	1379	29
2 spots	568	11.9
3 spots	198	4.16
4 spots	95	2
5 spots	40	0.84
6 spots	15	0.32
7 spots	7	0.15
8 spots	6	0.13
9 spots	3	0.06
10 spots	2	0.04
more than 10 spots	3	0.06

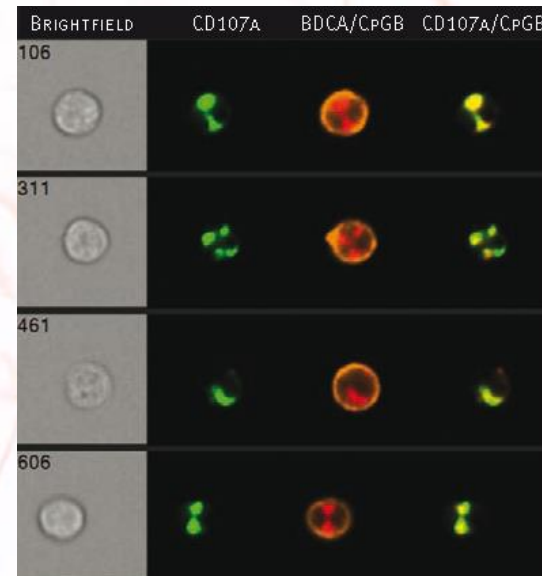
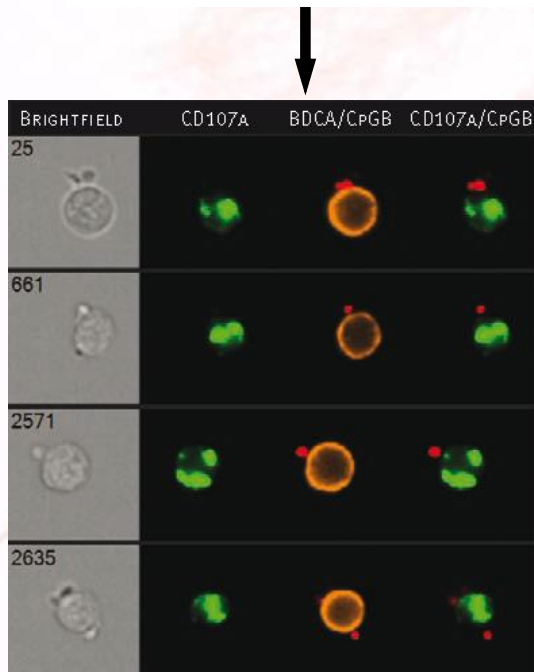
Study of the phagocytosis process of lentiviral vectors (LV) by primary human macrophages by counting intracellular GFP-positive vectors (Courtesy of: Michela Milani, Luigi Naldini's lab)

# ImageStream MKII: Internalization and Trafficking



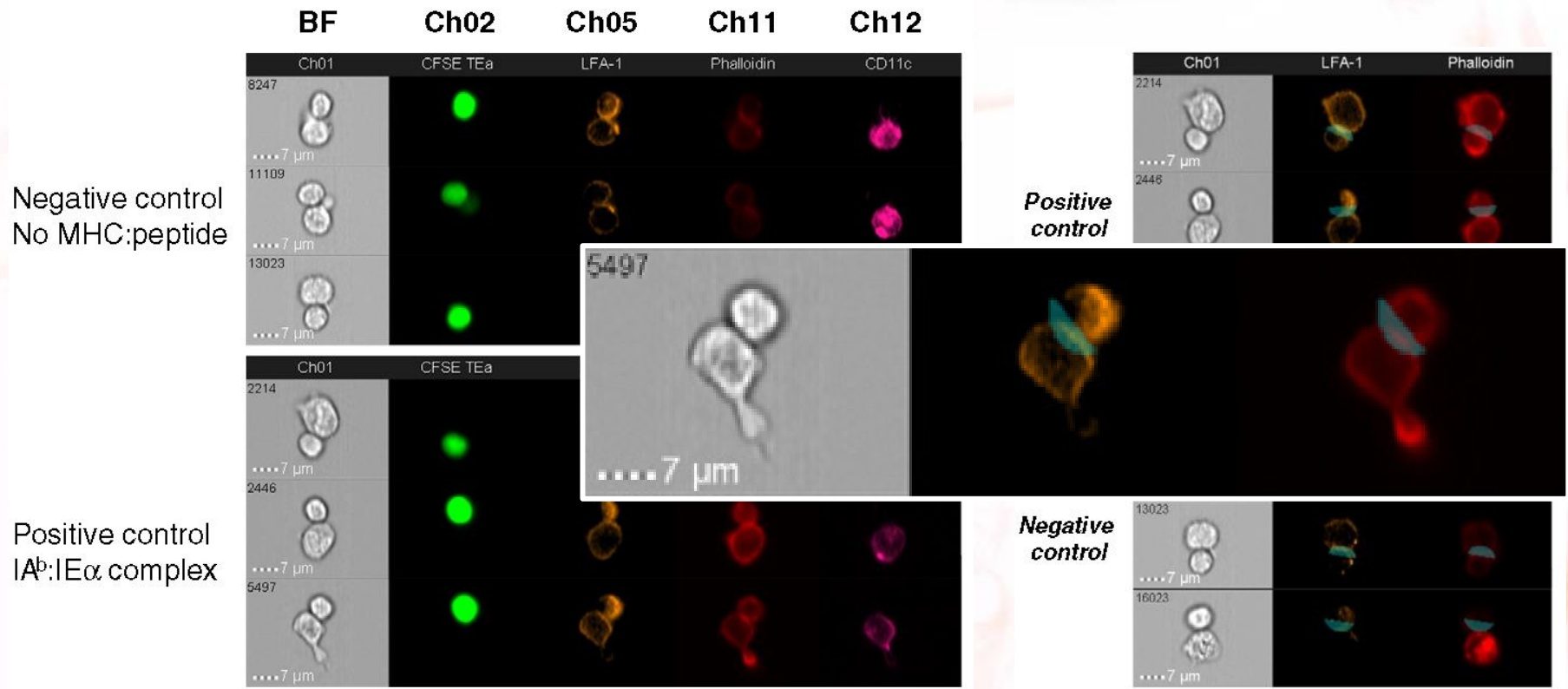
Kinetics of CpGB internalization and subcellular organelle co-localization within circulating human plasmacytoid dendritic cells (pDC)

Source:Amnis



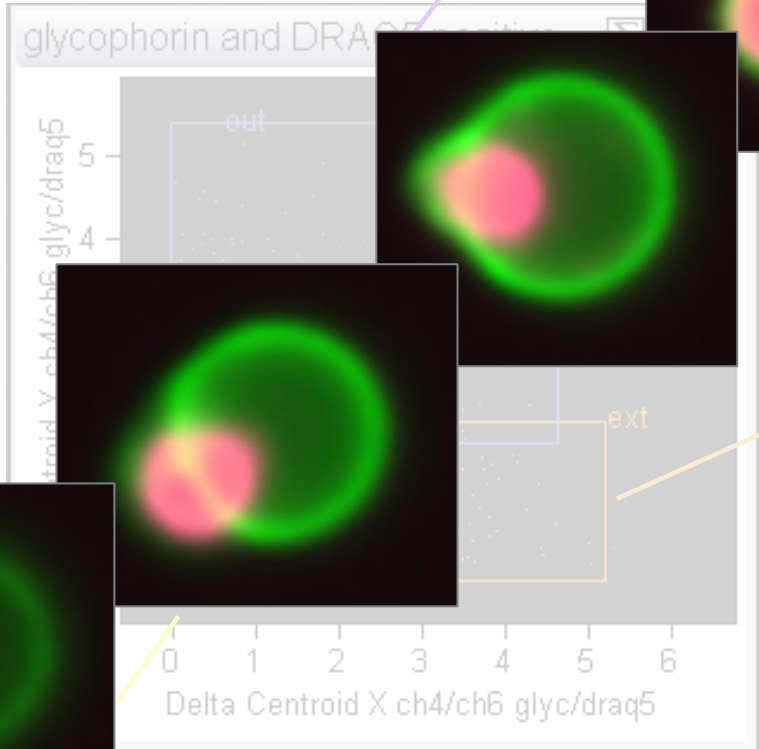
# ImageStream MKII: Immunological Synapses

## DC and Tcell immunological synapse formation



Markey K.A. et al, 2015

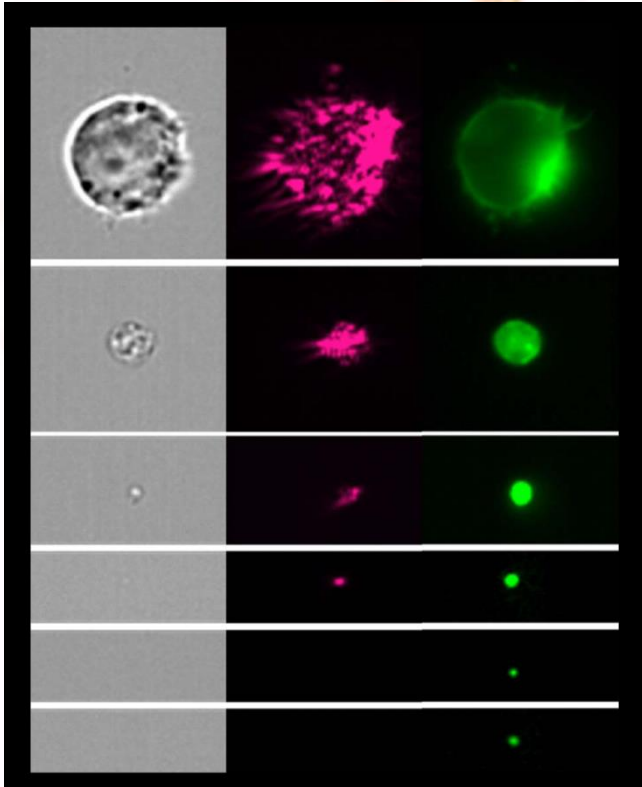
# ImageStream MKII: Eucleation process in human erithr



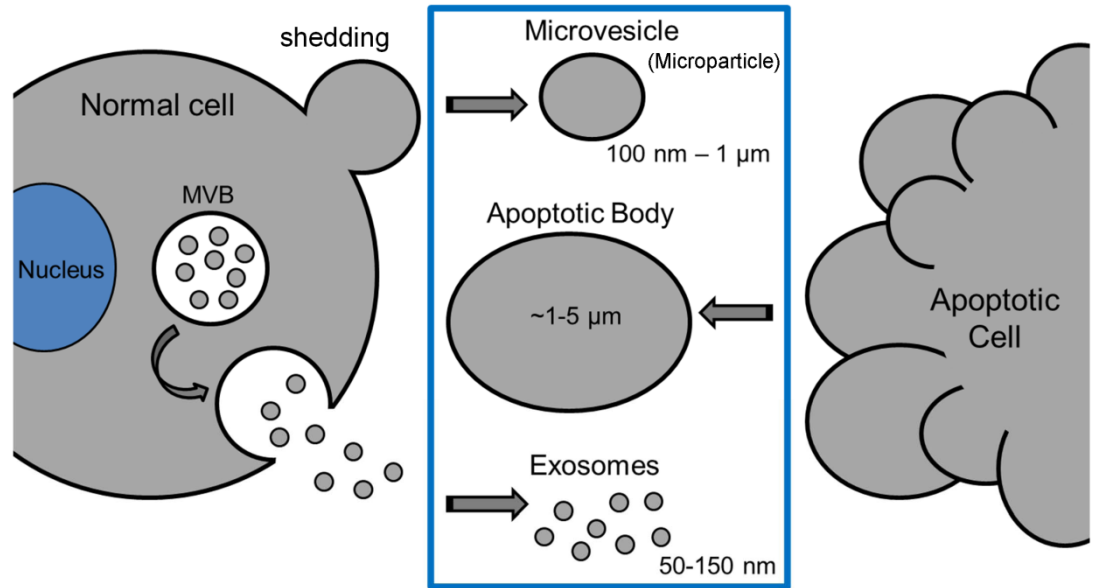
Courtesy of: Samantha Scaramuzza, Giuliana Ferrari's lab



# ImageStream MKII: analysis of Extracellular Vesicles



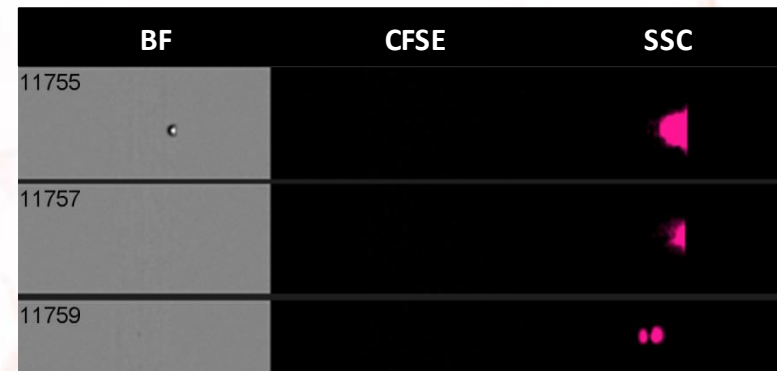
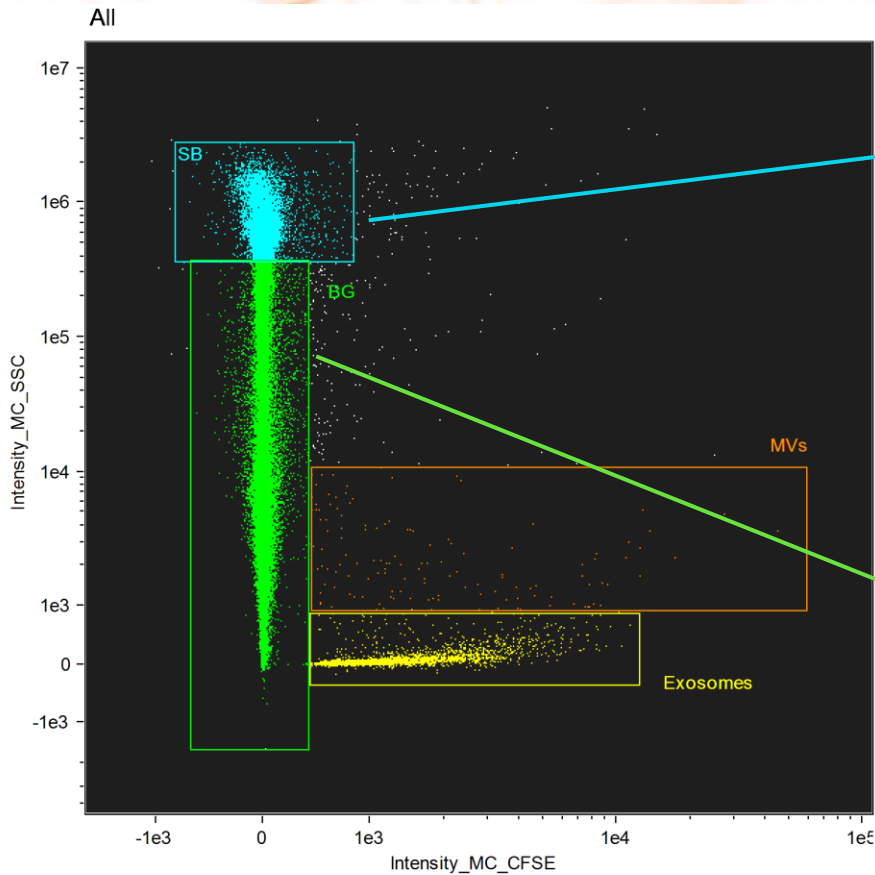
## Extracellular Vesicles (EVs)



EVs are secreted by a wide range of cells from different species.  
EVs can be found in nearly any body fluid.

(Görgens A., Science webinar 2016)

# ImageStream MKII: analysis of extracellular particles

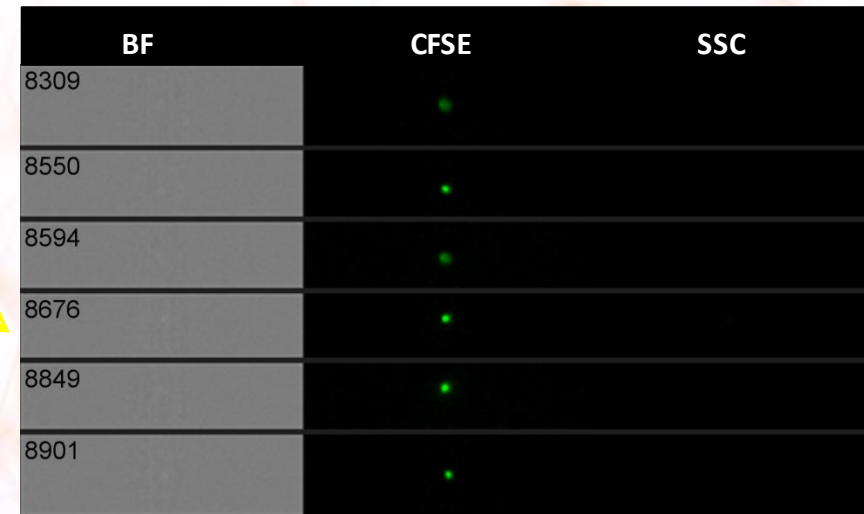
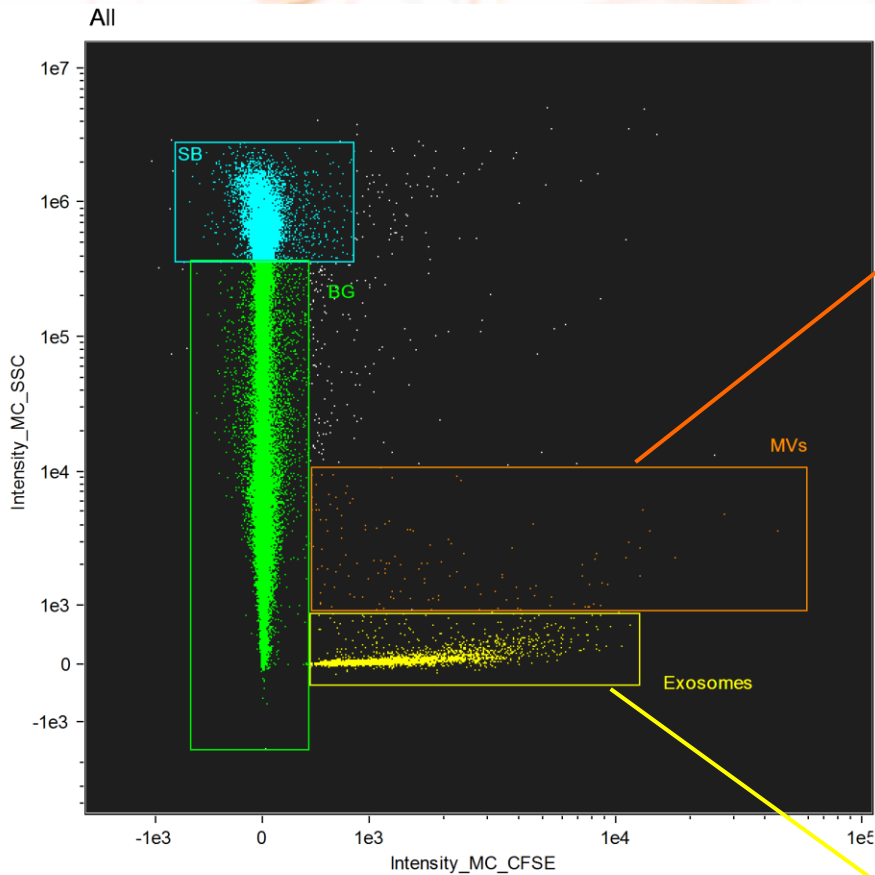


Intensity\_MC\_CFSE, Intensity\_MC\_SSC

Population	Count	%Total	%Gated	Objects/mL
All	59658	100	100	50753598.38
SB	9062	15.2	15.2	7709428.89
BG	47765	80.1	80.1	40635717.36
Exosomes	2488	4.17	4.17	2116647.44
MVs	132	0.22	0.22	112298.02

(Courtesy of Chiara Villa, Università degli Studi di Milano)

# ImageStream MKII: analysis of extracellular particles



Intensity\_MC\_CFSE, Intensity\_MC\_SSC

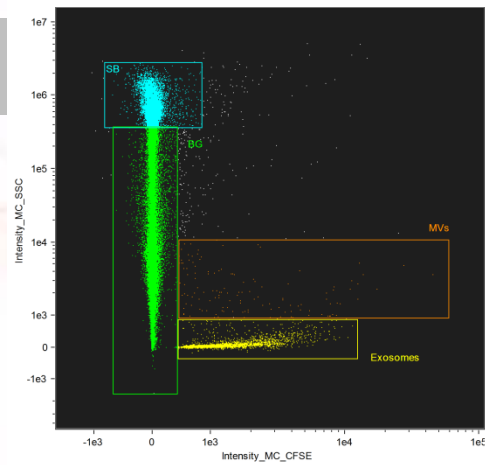
Population	Count	%Total	%Gated	Objects/mL
All	59658	100	100	50753598.38
SB	9062	15.2	15.2	7709428.89
BG	47765	80.1	80.1	40635717.36
Exosomes	2488	4.17	4.17	2116647.44
MVs	132	0.22	0.22	112298.02

(Courtesy of Chiara Villa, Università degli Studi di Milano)

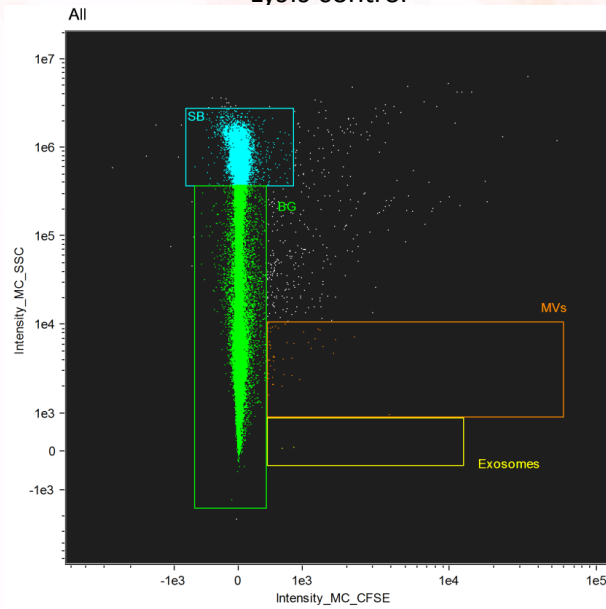
# Stream MKII: analysis of extracellular particles

Controls are essential !

- PBS alone
- PBS plus antibody alone or dye alone
- Lysis control
- Unstained Evs



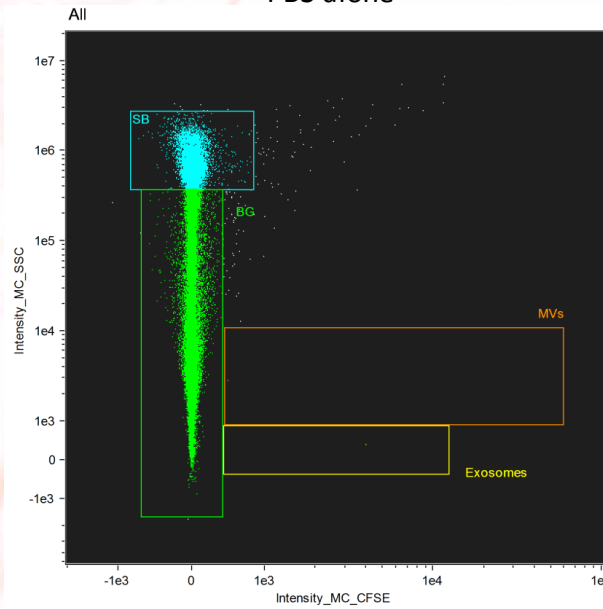
Lysis control



Intensity\_MC\_CFSE, Intensity\_MC\_SSC

Population	Count	%Total	%Gated	Objects/mL
All	55545	100	100	47575953.46
SB	10698	19.3	19.3	9163156.9
BG	44492	80.1	80.1	38108728.44
Exosomes	2	0	0	1713.06
MVs	45	0.08	0.08	38543.85

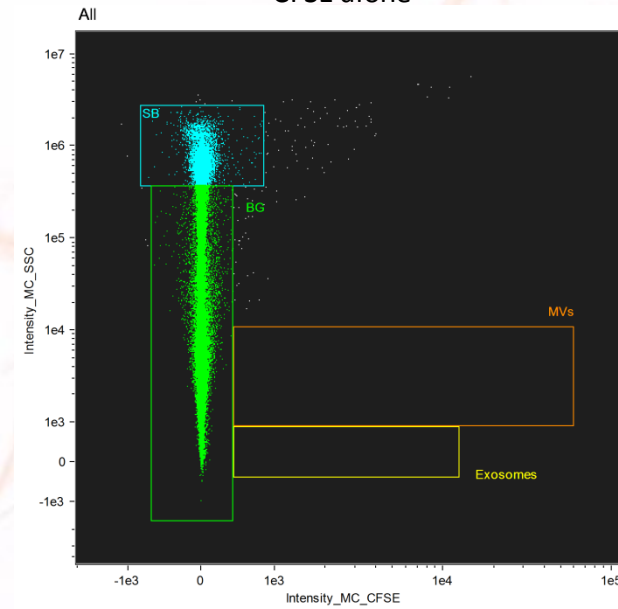
PBS alone



Intensity\_MC\_CFSE, Intensity\_MC\_SSC

Population	Count	%Total	%Gated	Objects/mL
All	57007	100	100	49331583.68
SB	9877	17.3	17.3	8547161.79
BG	47032	82.5	82.5	40699616.6
Exosomes	1	0	0	865.36
MVs	1	0	0	865.36

CFSE alone



Intensity\_MC\_CFSE, Intensity\_MC\_SSC

Population	Count	%Total	%Gated	Objects/mL
All	48155	100	100	40556461.73
SB	6958	14.4	14.4	5860073.94
BG	41099	85.3	85.3	34613851.54
Exosomes	0	0	0	0
MVs	0	0	0	0

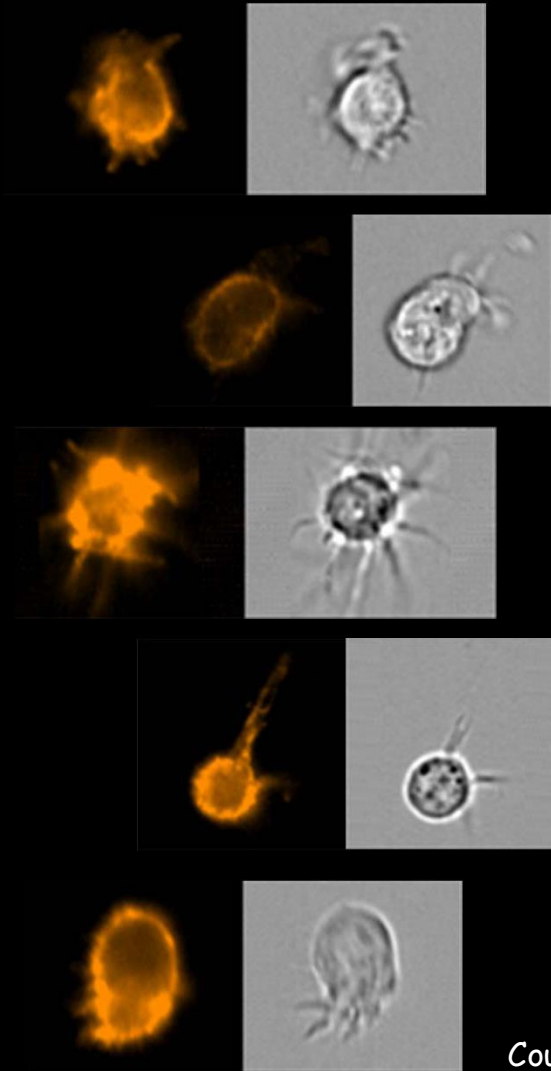
# Summary

ImageStream system delivers:

- Imagery of cells in suspensions
- Identification and characterization of cell population and even of rare events
- Image-based analysis



High throughput approach combined  
with an high content technique



Courtesy of  
Cristina Scielzo



# ALEMBIC

The Advanced Light and Electron Microscopy Bio-Imaging Center (ALEMBIC) started its activities in the field of microscopy in 2002.

It is a multimodal imaging facility that offers a wide spectrum of approaches to provide a comprehensive solution to scientific demands requiring optical or electronic imaging.

The staff instructs researchers in the most effective and independent use of microscopes, provides support to users (from the experimental design throughout the workflow phases of the project, up to data analysis) and performs, when required, full service

Numbers:

- about 1200 people trained
- >400 active users (>250 different users/year)
- >20,000 hours/year

# ALEMBIC offers access to external users



Providing access, service  
and training to state-of-the-art  
imaging technologies



<https://www.eurobioimaging-interim.eu>

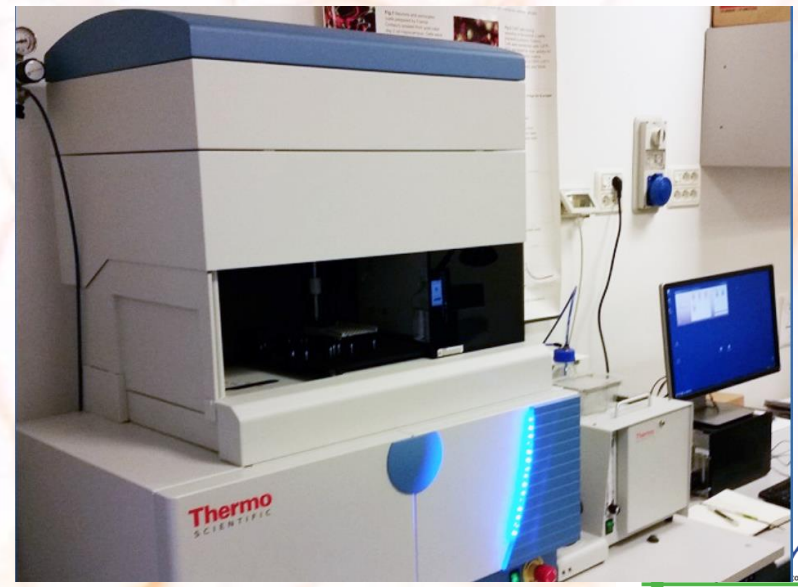
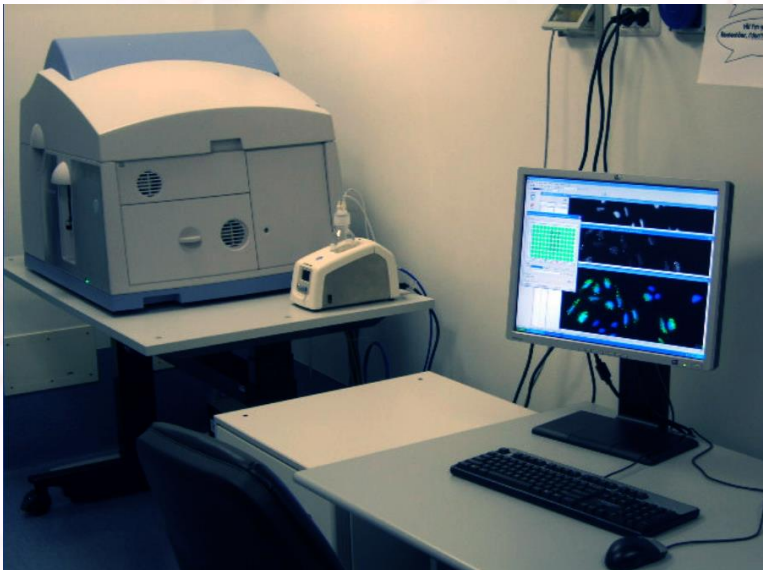


# HIGH CONTENT MICROSCOPY for adherent cells

High Content Screening (HCS) allows the quantitative multiparametric analysis of a large number of cells, by coupling high-throughput technology with fluorescence microscopy.

HCS shows a lot of advantages with respect to traditional microscopy and provides a wider spectrum of information that spans from single-cell (identification of distinct phenotypic sub-populations) to sub-cellular level (localization of identified molecules in different micro-domains).

ALEMBIC has two different HCS instruments that cover a wide range of applications: the **IN Cell Analyzer 1000** and the **Arrayscan XTI**.





# Cell count

IN Cell Analyzer 1000 Workstation

File Edit View Image Mode Settings Sample Application Help

Summary by fields

Image Stack: \\Alembic-master\alembic\Wir... \angelina 2.xdc

View - Ch 1 - \\Alembic-master\alembic\Wiriam\angelina 060411\F - 8(fid 22).tif

File

Preview

	1	2	3	4	5	6	7	8	9	10	11	12	Analyzed:
A	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	
B	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	
C	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	
D	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	
E	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	
F	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	
G	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	
H	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	

Cell Count

max

min

no images acquired not analyzed analysis in progress

Image

Well: F - 8 Field: 22 Time: 1 Z: 1

λ: D360\_40X-HQ460\_40M

Protocol

<lorenzo prolif 2>(from file)

Selection: F - 8

Analyze View thumbnails Done Help

F - 9(fid 1)	8.000
F - 9(fid 1)	31.000
F - 9(fid 1)	178.000
F - 9(fid 1)	74.000
F - 9(fid 1)	0.000
F - 9(fid 1)	184.000
F - 9(fid 2)	2.000
F - 9(fid 2)	198.000
F - 9(fid 2)	529.000
F - 9(fid 2)	159.000
F - 9(fid 2)	2.000
F - 9(fid 2)	262.000
F - 9(fid 2)	5.000
F - 9(fid 2)	59.000
F - 9(fid 2)	176.000
F - 9(fid 2)	42.000
F - 9(fid 3)	10.000
F - 9(fid 3)	78.000
F - 9(fid 3)	17.000
F - 9(fid 3)	14.000
F - 9(fid 3)	17.000
F - 9(fid 3)	0.000
F - 9(fid 3)	15.000
F - 10(fid)	116.000
F - 10(fid)	24.000
F - 10(fid)	14.000

Ch 1 - \\Alembic-master\alembic... \F - 8(fid 22).tif

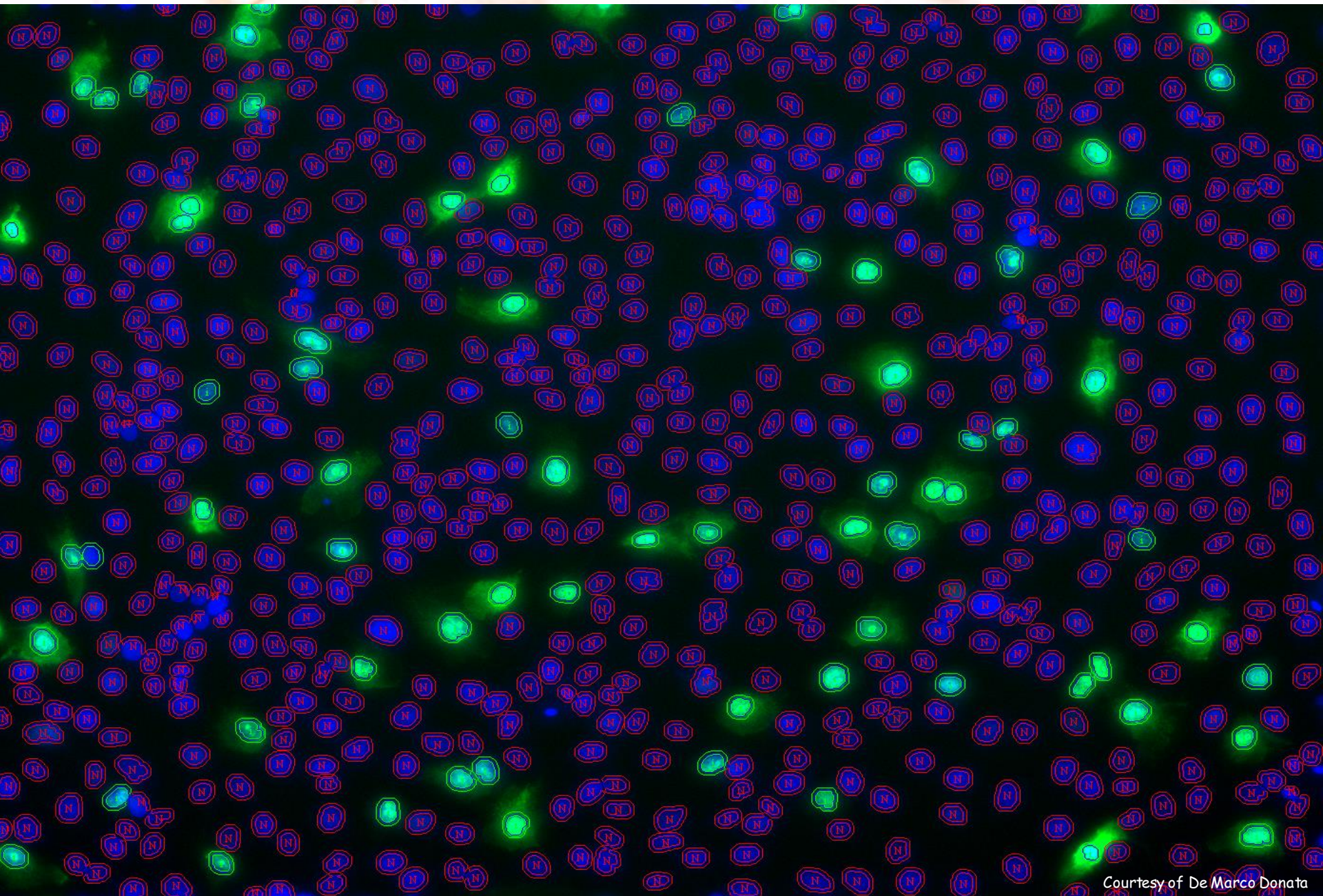
Mode: Assay Development

Display: 12 bit Mono / 1

Protocol: <lorenzo prolif 2>(from file)

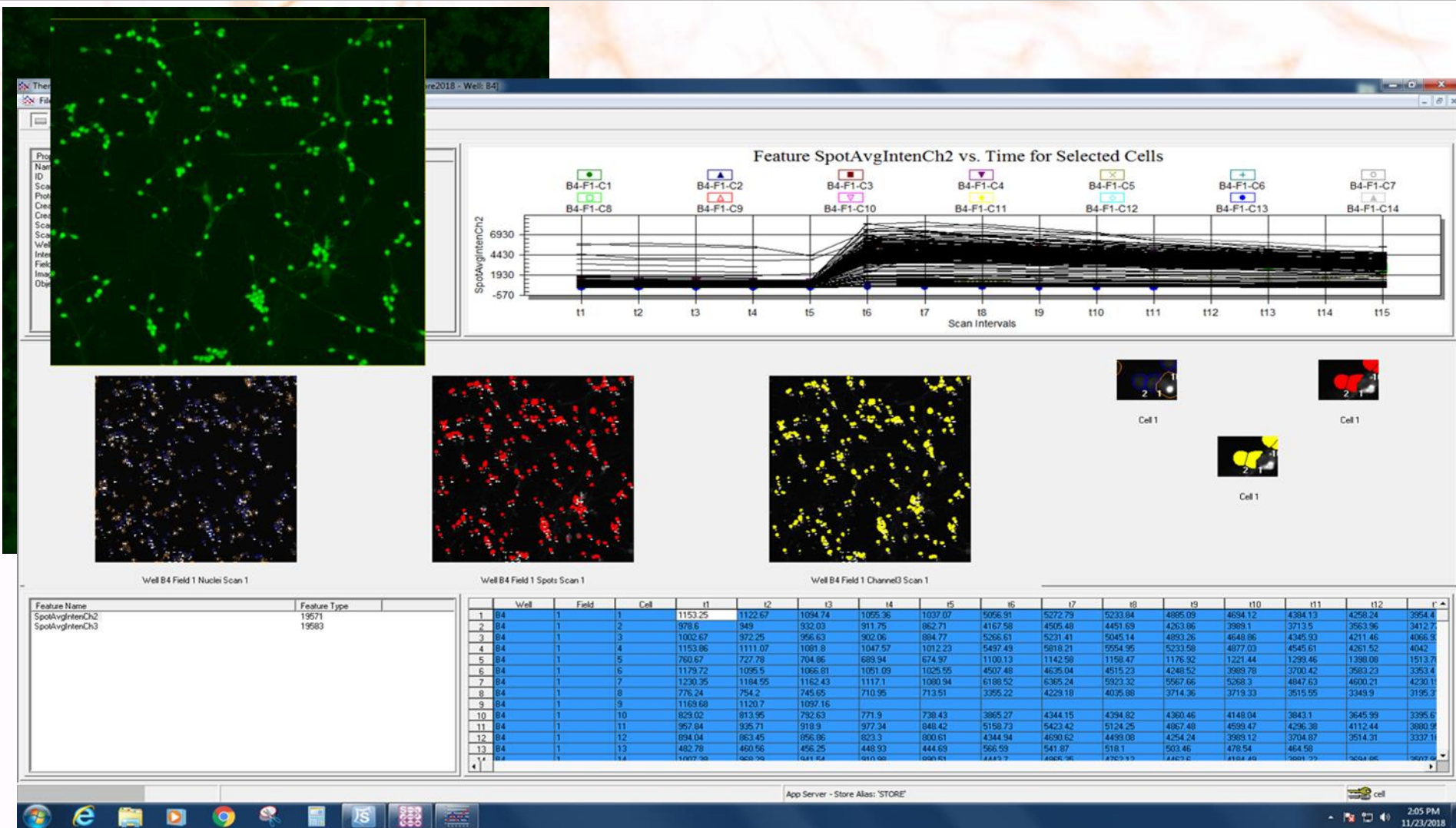


# Identification of cell subpopulations



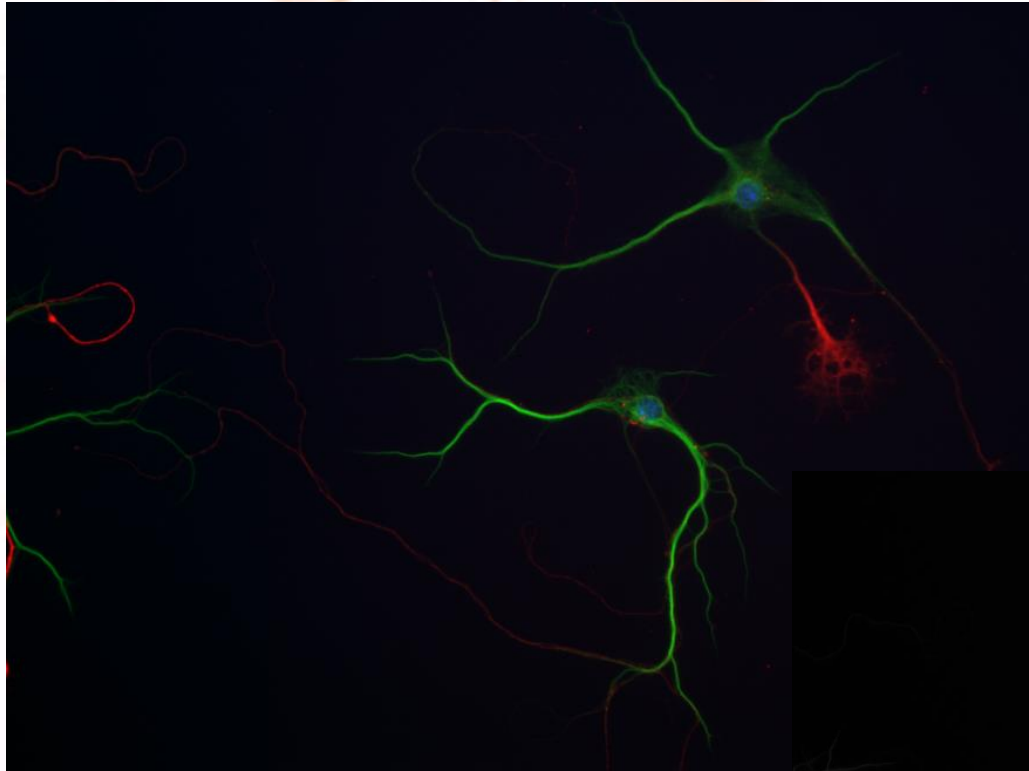


# Liquid Handling Module: Calcium flux



Calcium flux in cerebellar granules loaded with Fluo-8 and stimulated with Glutamate  
(Courtesy of : Franca Codazzi)

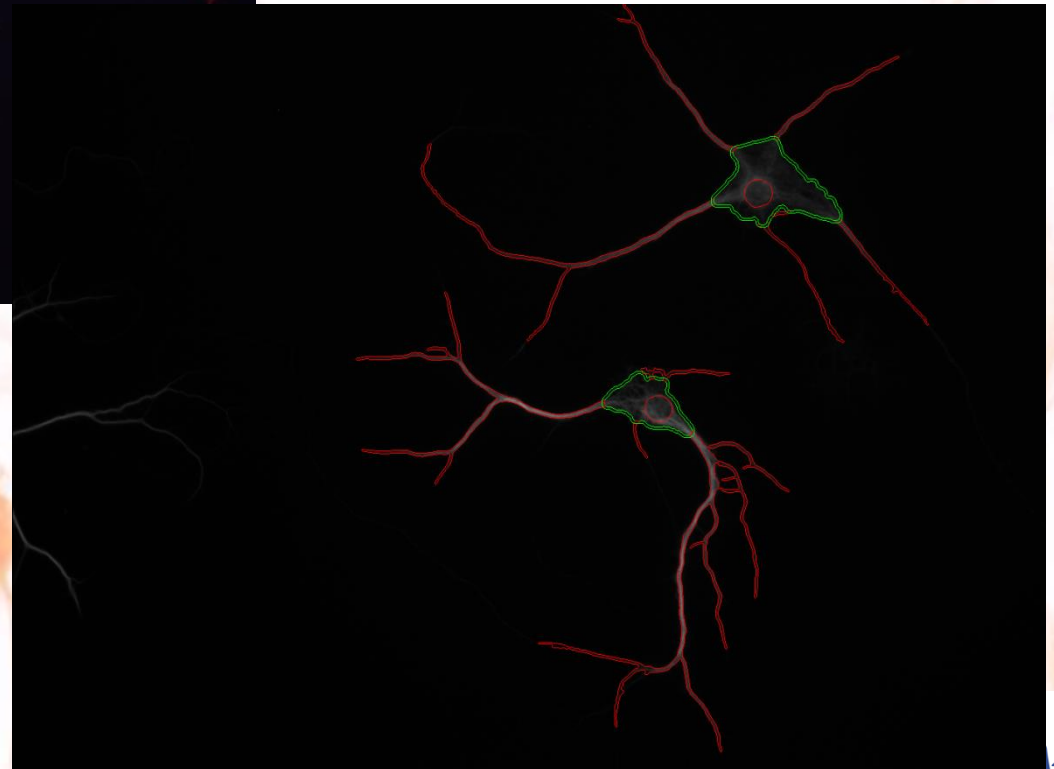
# Neurite outgrowth analysis



Dendritic  
arborization

mouse neurons\_div10

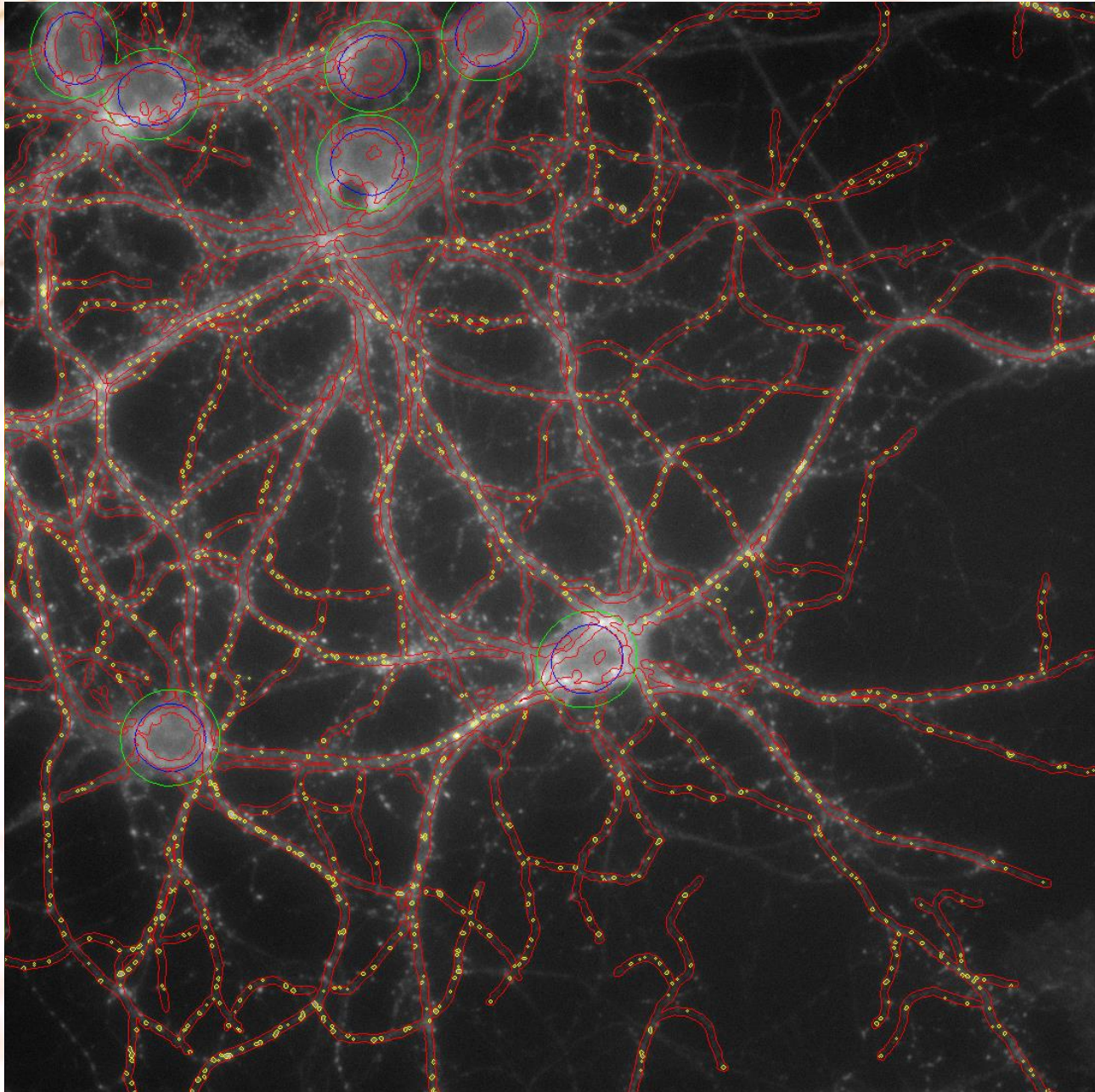
DAPI (blue)  
TAU (red)  
MAP2 (green)



- Total neurite length
- Neurite count
- Neurite length/cell
- Neurite count/cell
- Neurite branching
- Length of the longest neurite
- Cell body area
- Intensity measurements



# Neurite outgrowth analysis and spot count

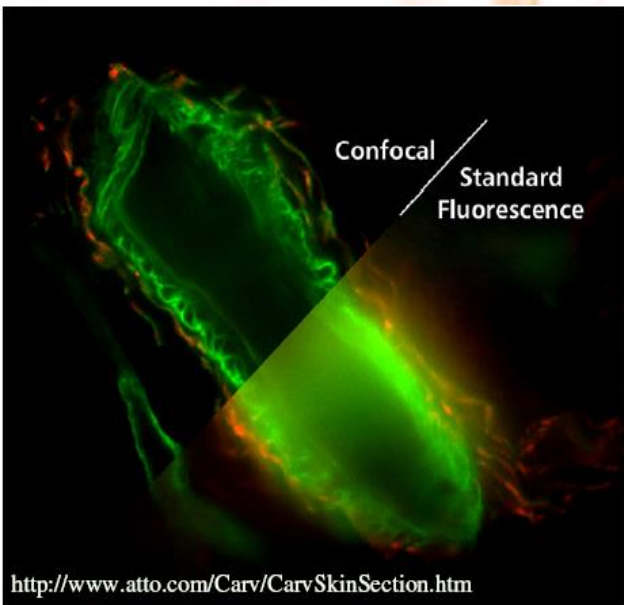


Primary hippocampal  
neurons

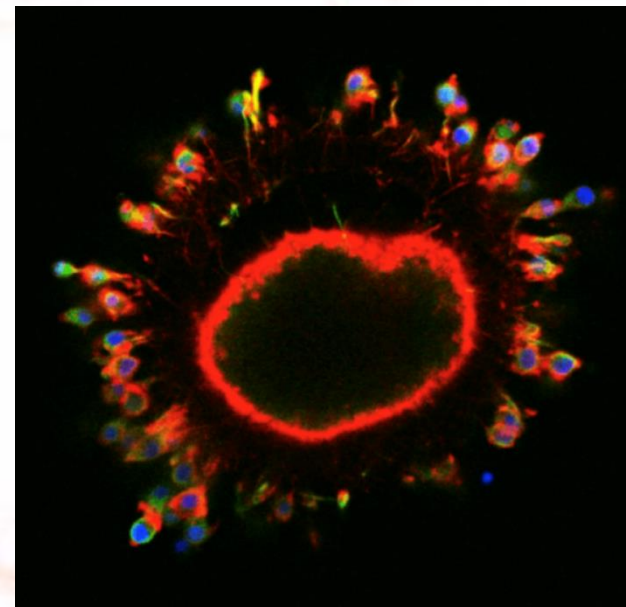
DAPI (blue)  
MAP2 (green)  
PSD-95 (red)

# OPTICAL SECTIONING BY REDUCING IMAGE VOLUME

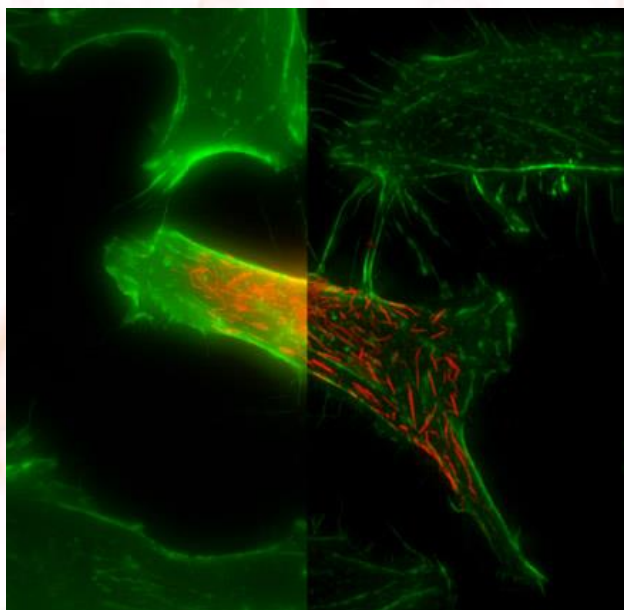
## Point-scan confocal microscopy



## Spinning disk confocal microscopy



## Deconvolution microscopy



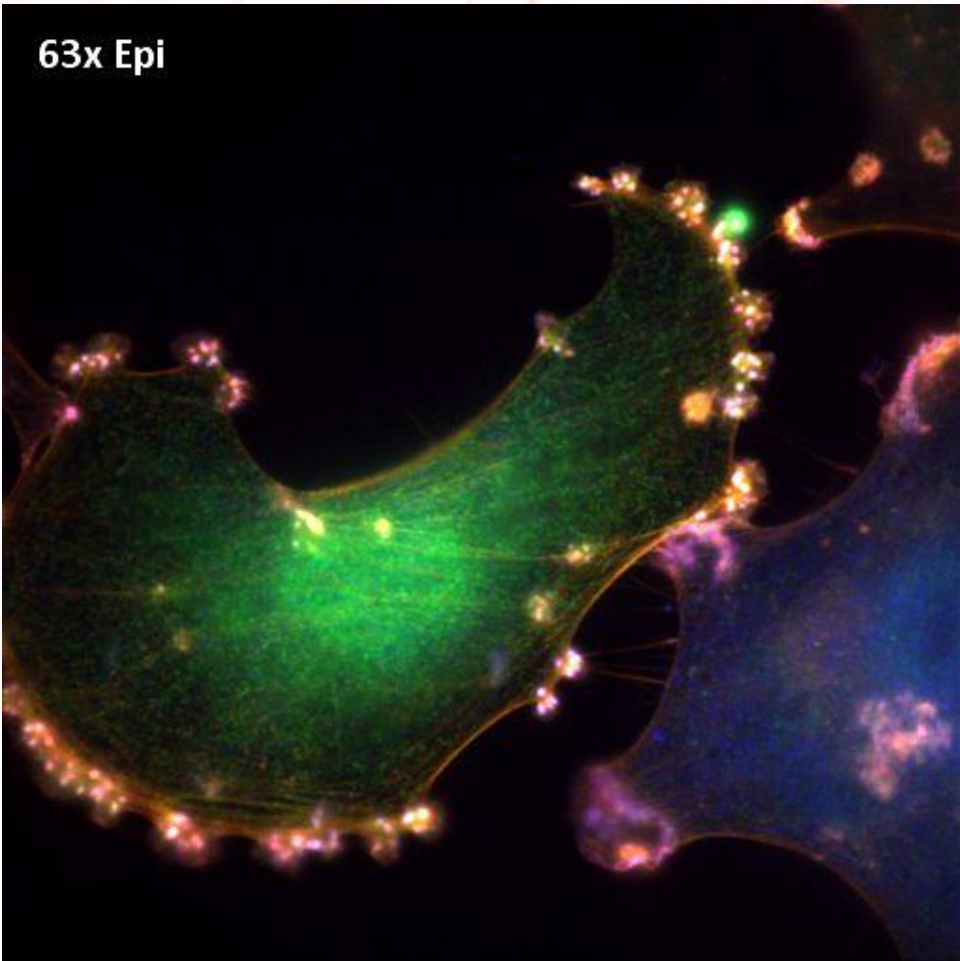
HELA cells, Actin 757Δ MYC  
(Courtesy of Cesare Covino and  
Cristina Sironi, OSR)

Human oocyte dapi actin tubulin  
(Courtesy of Maria Cristina Guglielmo,  
Ist. Clinici Zucchi-Monza)

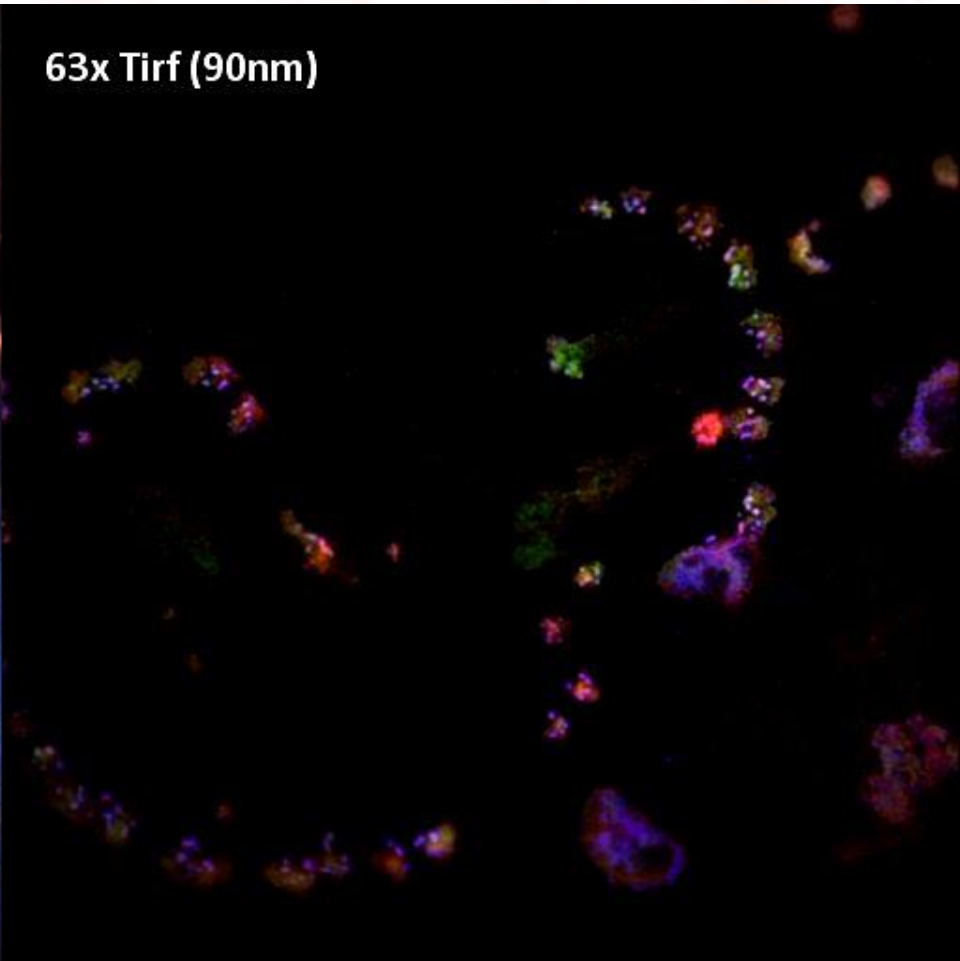


# OPTICAL SECTIONING BY SELECTIVE ILLUMINATION: Total Internal Reflection Microscopy

63x Epi



63x Tirf (90nm)



NIH/3T3 Src stably transfected cell

Green: GFP, Red: Phalloidin, Blue: MT1-MMP (transmembrane matrix metallo protease)

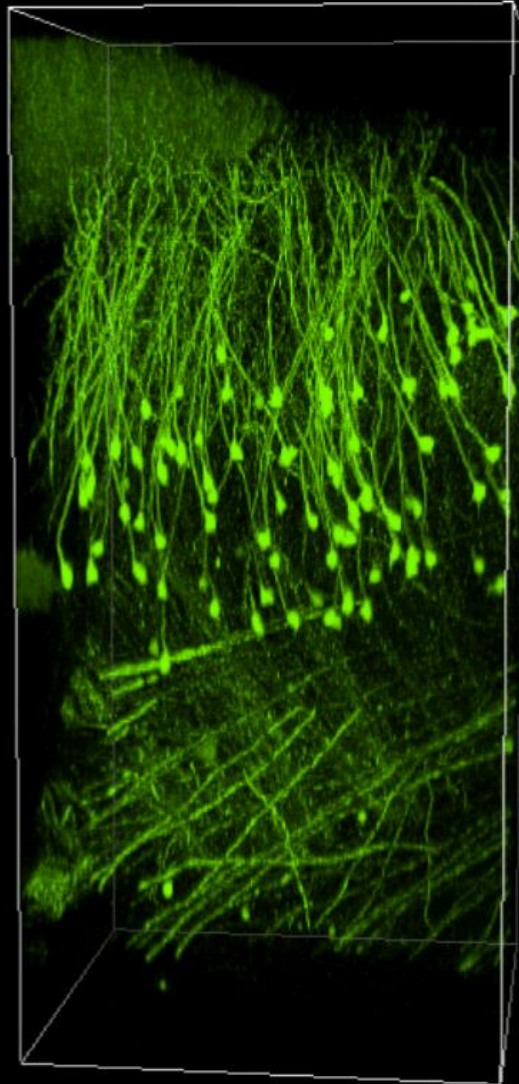
(Courtesy of: Cesare Covino and Kristyna Hanusova, OSR)



# OPTICAL SECTIONING BY SELECTIVE ILLUMINATION: 2P and Light Sheet Microscopy

## Hippocampus

CA1, GFP, 1.1 mm slice



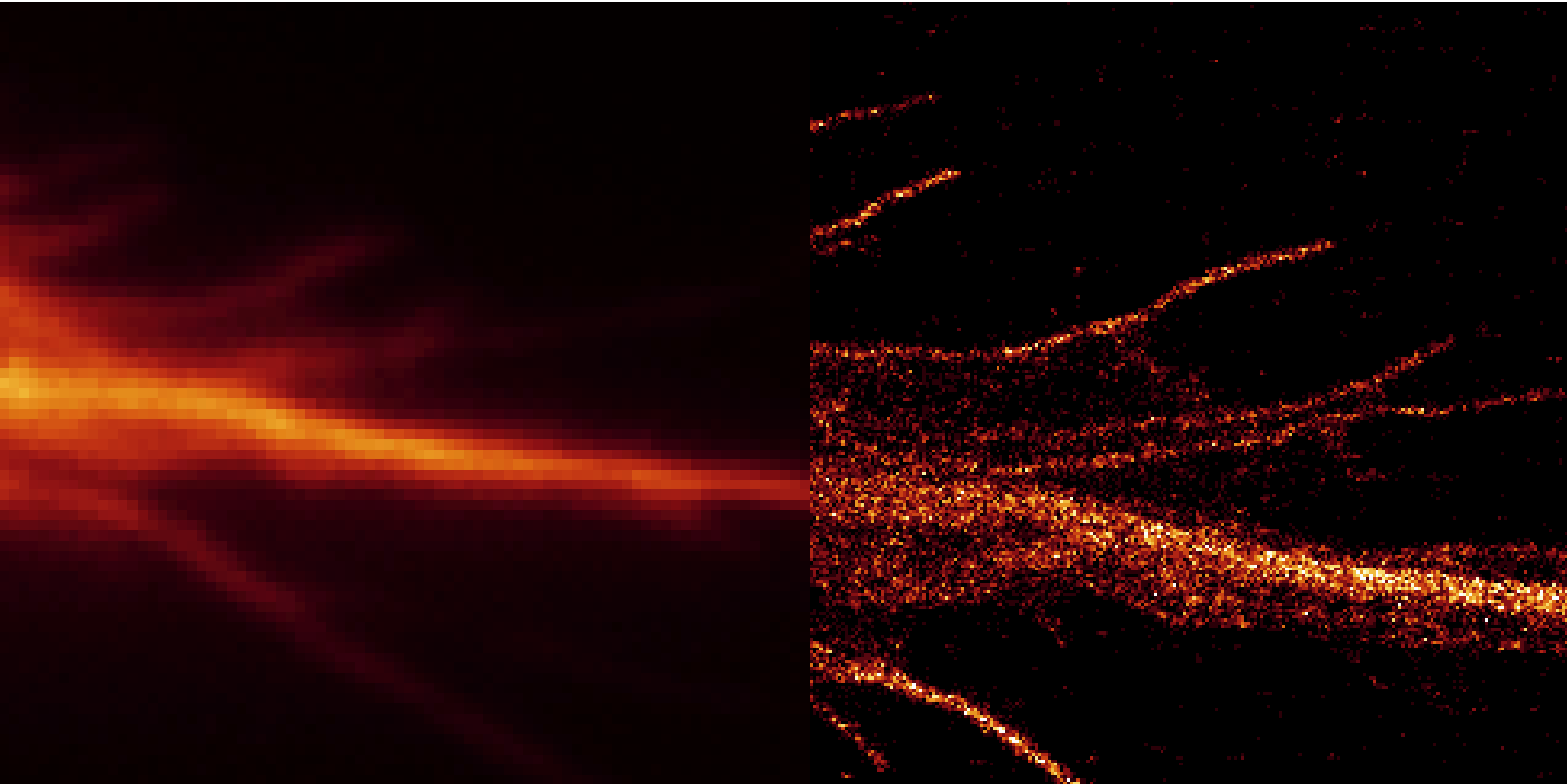
Calibration x:0.99  $\mu\text{m}$ , y:0.99  $\mu\text{m}$ , z:2.50  $\mu\text{m}$   
Width:509.12  $\mu\text{m}$  Height:509.12  $\mu\text{m}$  Depth:1105.00  $\mu\text{m}$

Courtesy of: Gianluca  
Verlengia and Cesare  
Covino - ALEMBIC

# Super-resolution (down to 30 nm)

Widefield

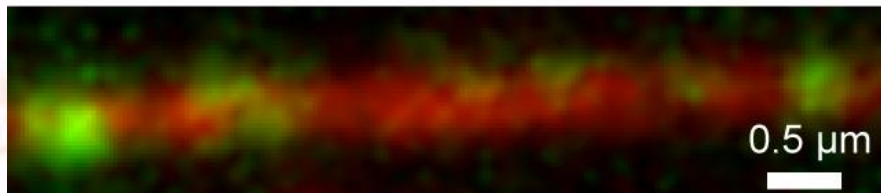
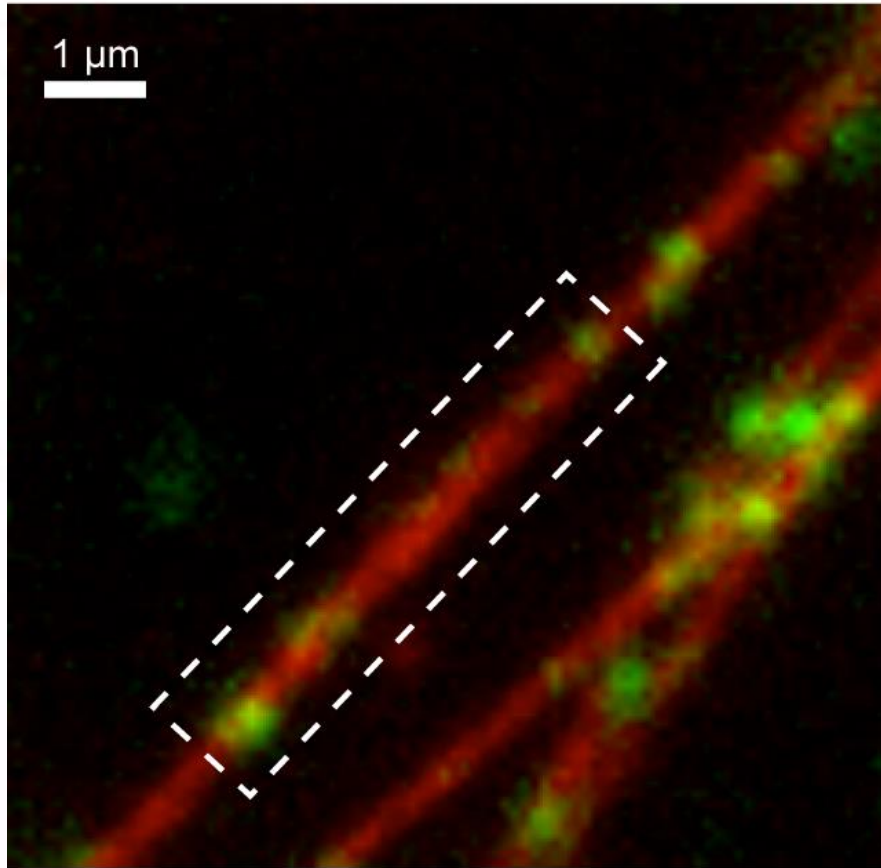
GSD microscopy



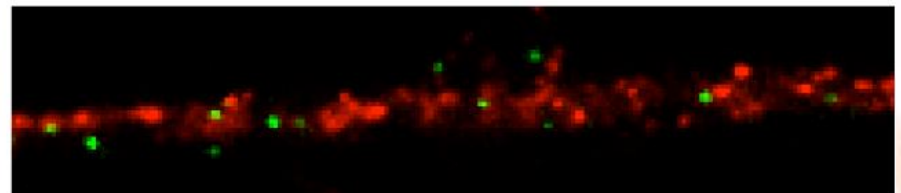
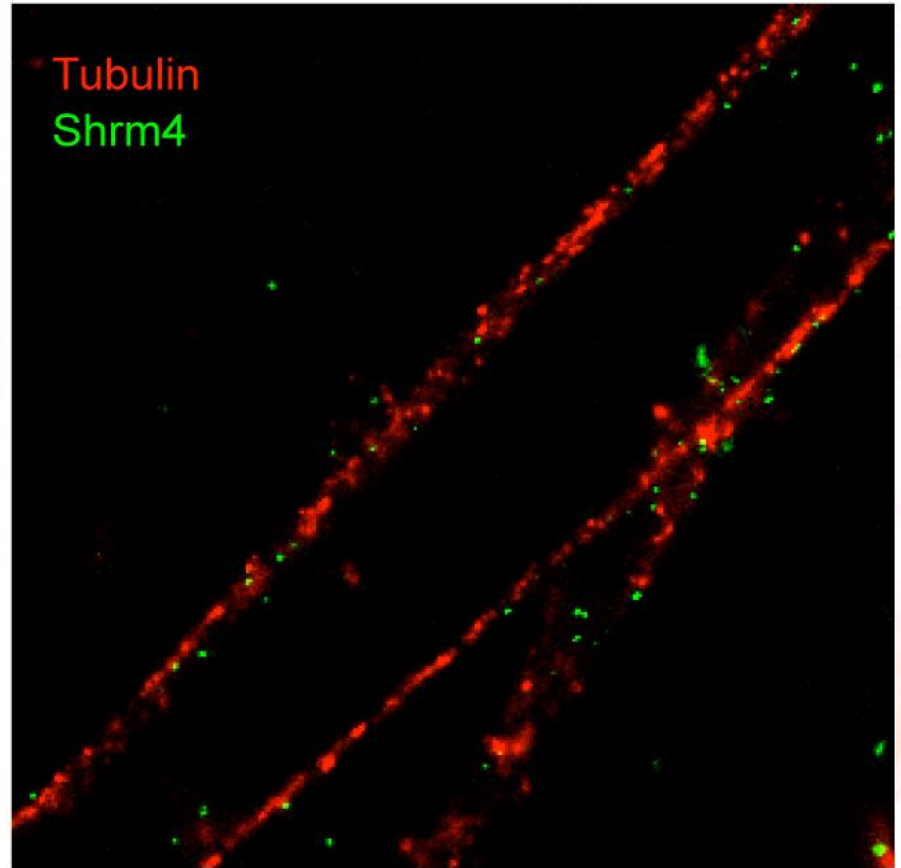
H1299 small lung human carcinoma cells - Actin: Atto 488.  
(Courtesy of: Cesare Covino and Davide Mazza, OSR)

# Super-resolution (down to 30 nm)

Widefield



GSD microscopy

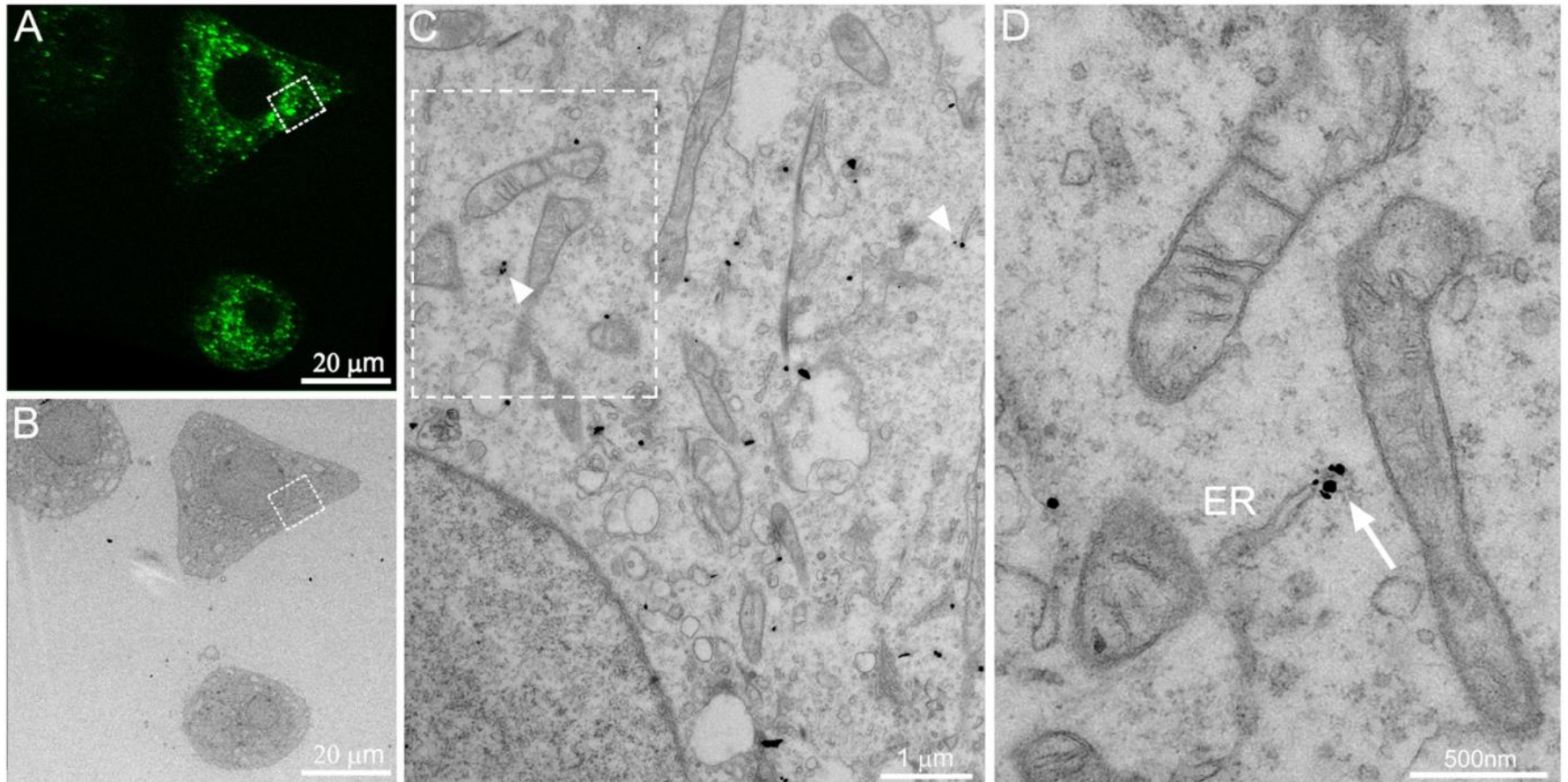


Mouse neurons (Tubulin-Alexa647, Shrm4-Alexa488) – (Courtesy of: Davide Mazza, OSR, and Maura Francolini, UniM)



# Correlative Light Electron Microscopy (CLEM)

Correlative Light Electron Microscopy (CLEM) is a powerful tool that can combine the resolution of electron microscopy with the possibility to observe the molecule of interest *in vivo* by means of fluorescence microscopy; thereby allowing to disclose the molecular machinery involved in biological processes and, at the same time, observing their ultrastructure features. Numerous methodological CLEM approaches, each designed to address a specific scientific question, have been recently developed. Here is reported a CLEM approach used to localize an ER membrane protein (IRE1-GFP).



(Courtesy of : Andrea Raimondi ALEMBIC)



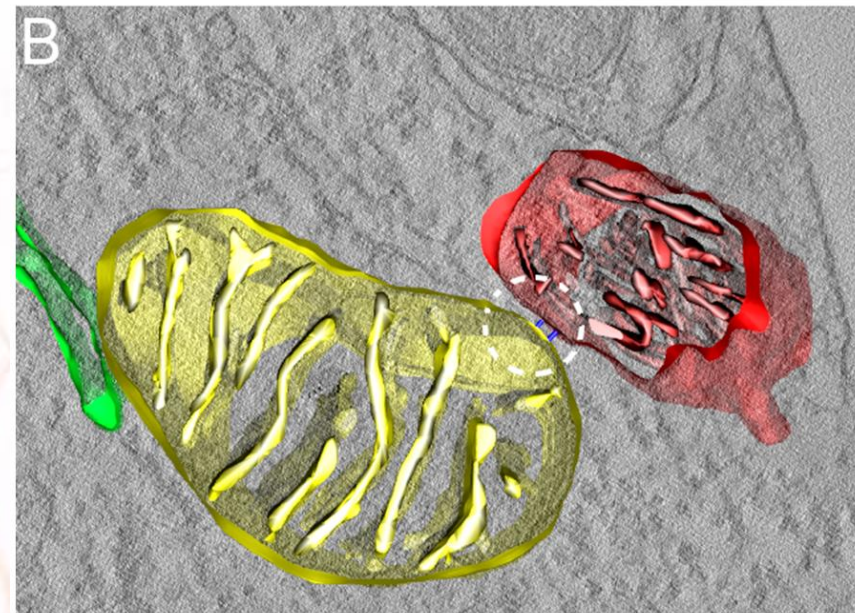
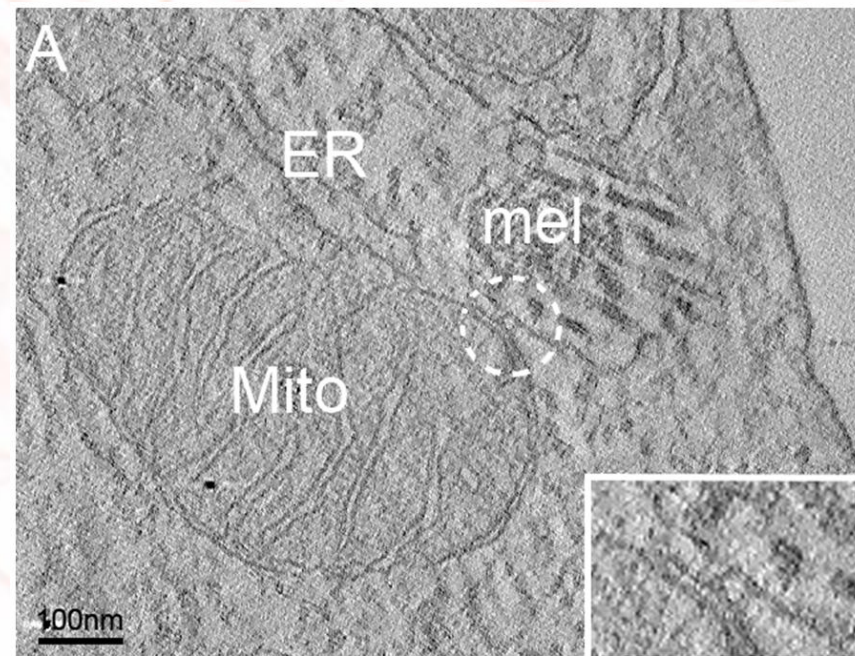
# 3D EM Tomography

**ET** is an EM technique that combines the 3D visualization of the cells and organelles with high spatial resolution allowing to appreciate fine ultrastructural details.

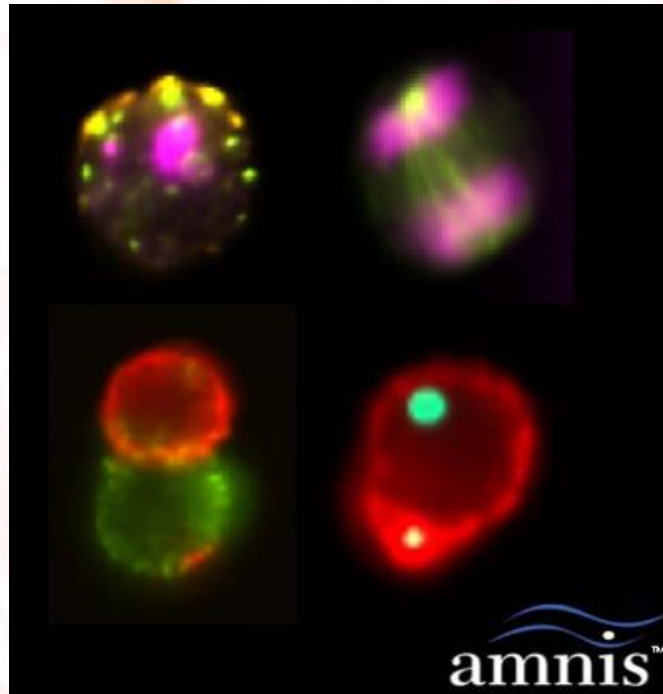
Thanks to ET it was possible to show the fine structures that bridge mitochondria (mito) and melanosomes (mel) together.

(A) A slice of the 3D tomographic reconstruction showing mitochondria-melanosomes connections;

(B) 3D rendering of the two organelles and the contact.







*Thank you for your attention !*