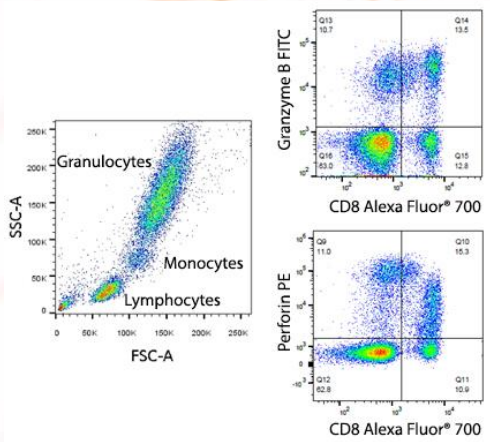
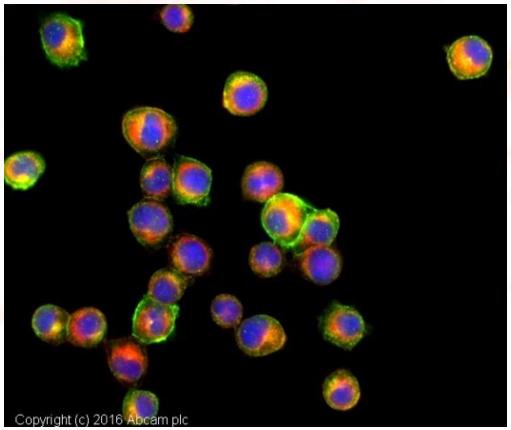
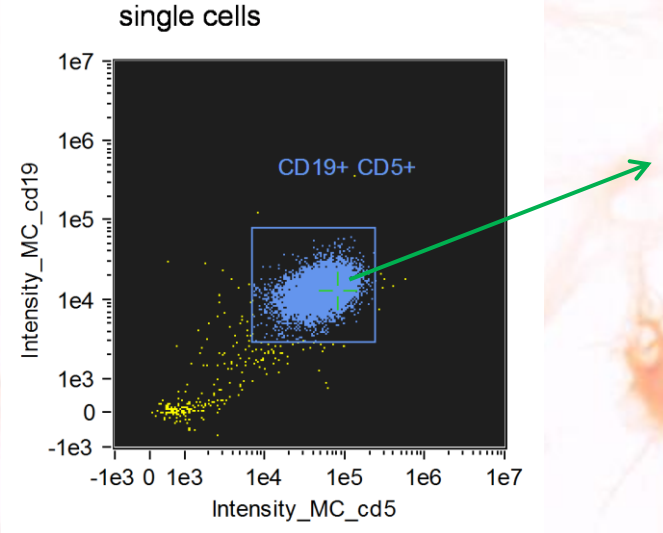
Flow cytometry



Microscopy



Imaging Flow Cytometry



the cell under each single dot !

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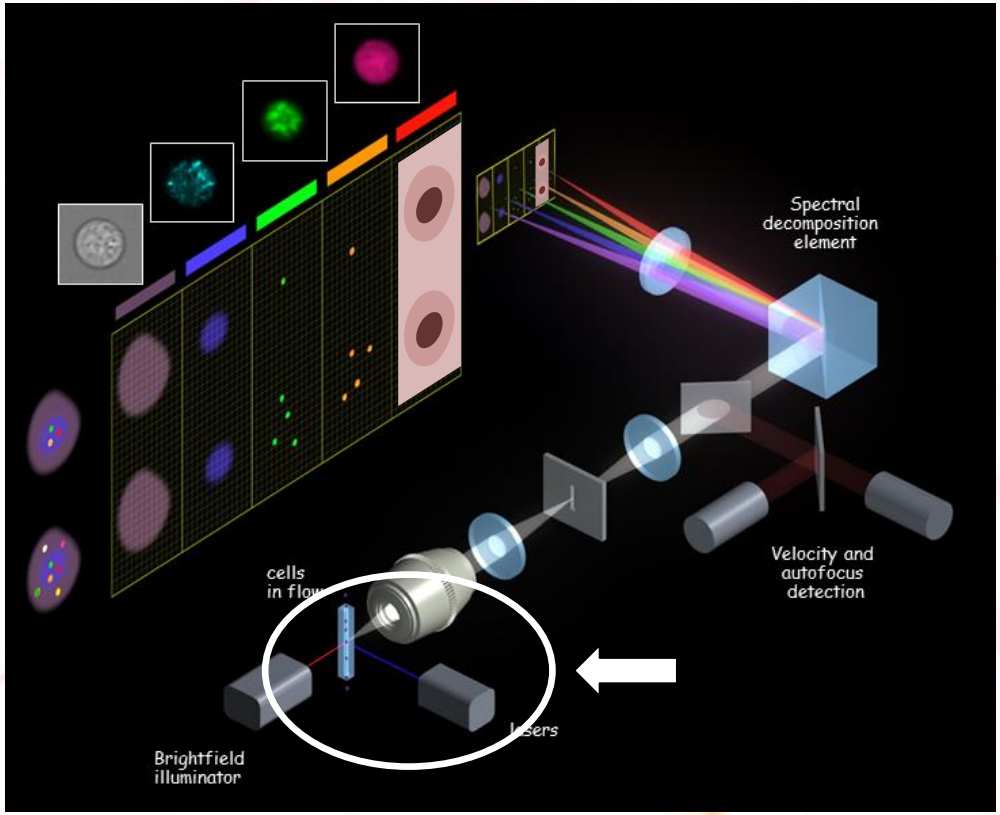
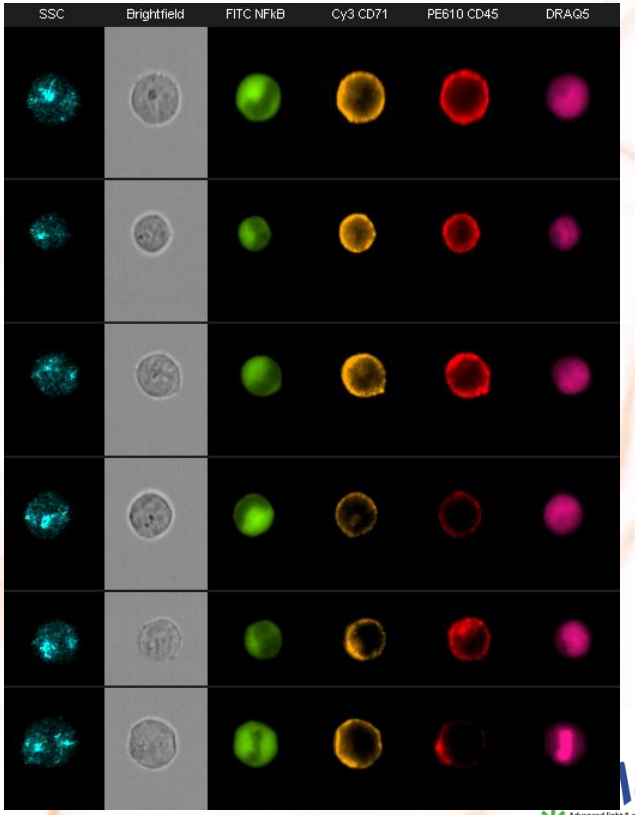
Imagery of cells in suspension

Identification and localization of each single signal in the cell





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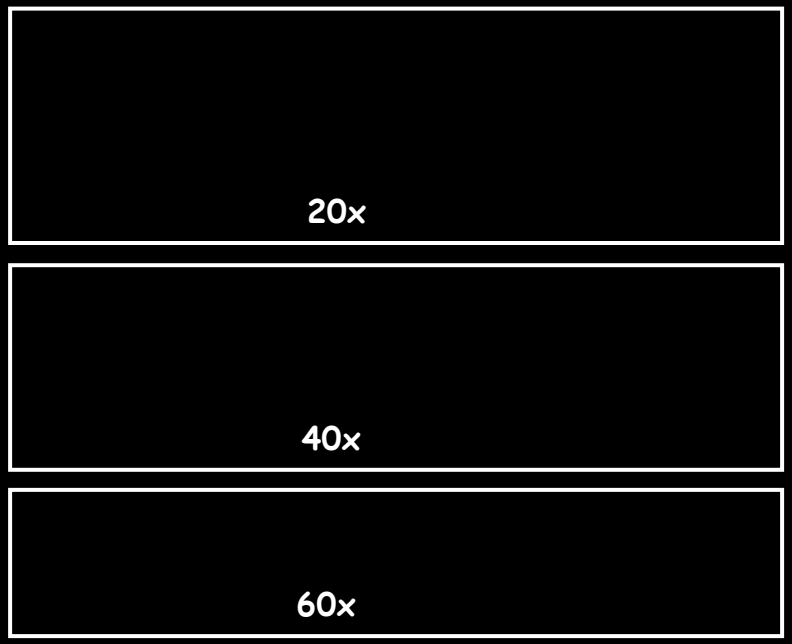
Magnifications:
20x → 120um Field of view, 8um depth of field
40x → 60um Field of view, 4um depth of field
60x → 40um Field of view, 2.5um depth of field



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

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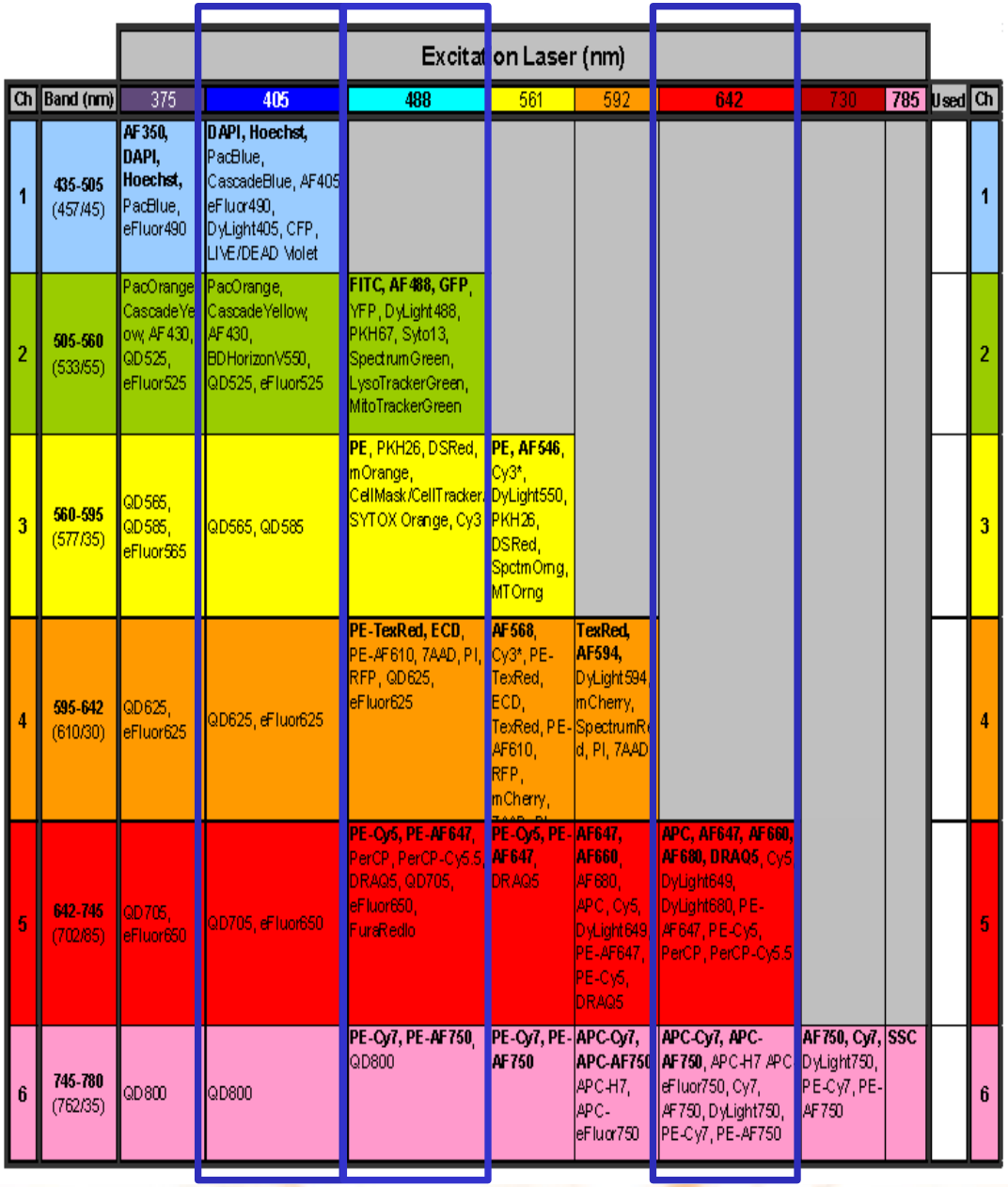
Sample Preparation Guide



Ch	Band (nm)	Excitation Laser (nm): 375	405	488	561	592	642	730	785	Used	Ch
1	435-505 (457/45)	**AF350, DAPI, Hoechst,** PacBlue, eFluor490	**DAPI, Hoechst,** PacBlue, CascadeBlue, AF405 eFluor490, DyLight405, CFP, LIVE/DEAD Violet								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)	QD585, QD585, eFluor565	QD565, QD585	**PE,** PKH26, DSRed, mOrange, CellMask/CellTracker, SYTOX Orange, Cy3	**PE, AF546,** Cy3*, DyLight550, PKH26, DSRed, SpctrmOrng, MTOrng						3
4	595-642 (610/30)	QD625, eFluor625	QD625, eFluor625	**PE-TexRed, ECD,** PE-AF610, 7AAD, PI, RFP, QD625, eFluor625	**AF568,** Cy3*, PE-TexRed, ECD, TexRed, PE-AF610, RFP, mCherry,	**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, FuraRedIo	**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,** DyLight750, PE-Cy7, PE-AF750	**SSC**		6

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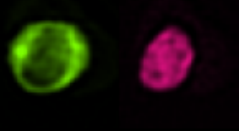
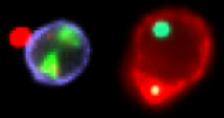
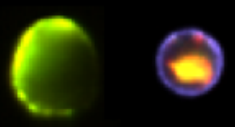
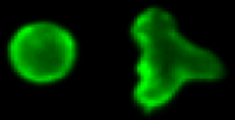
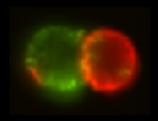
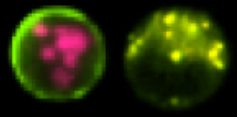
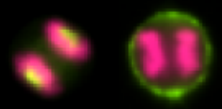
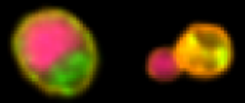
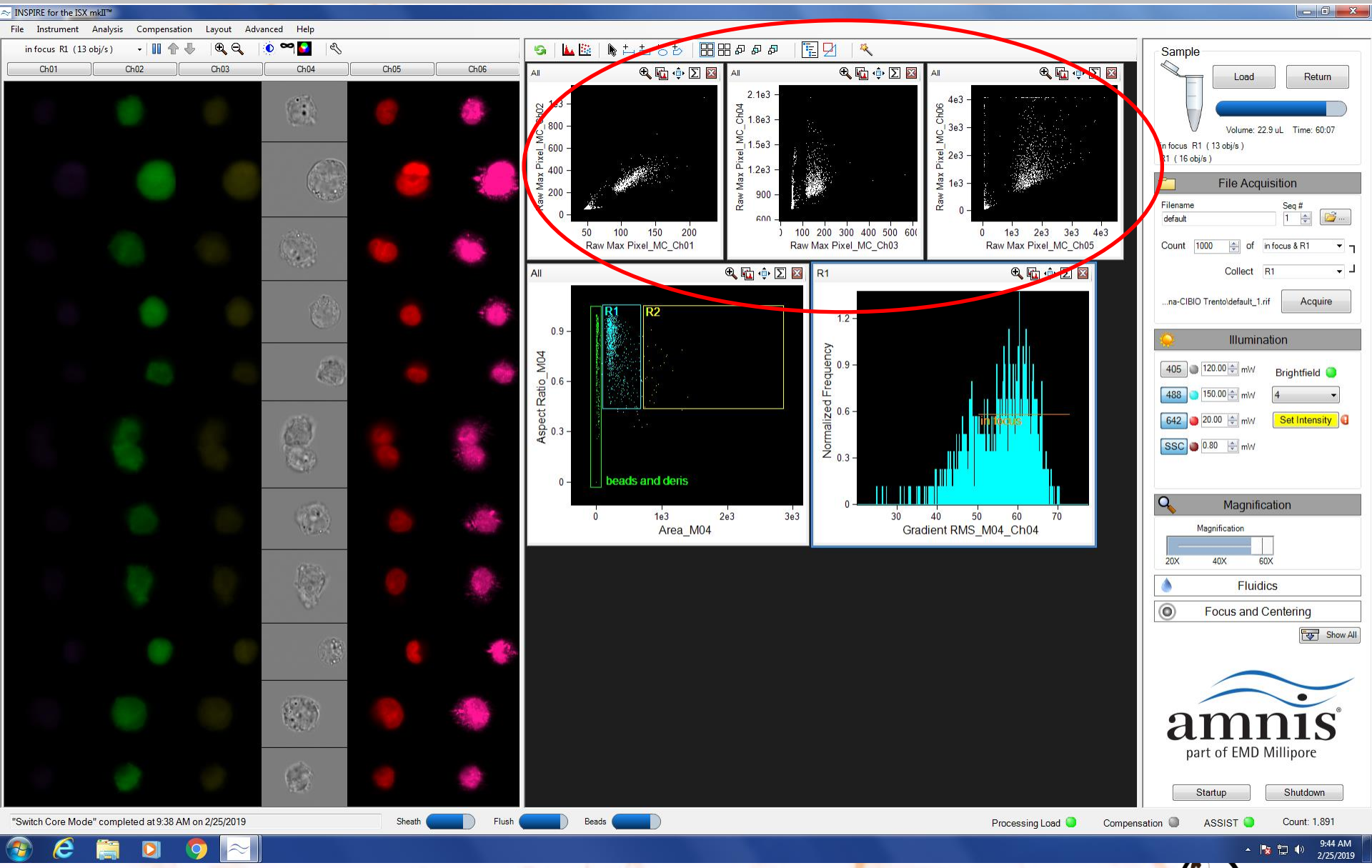
ImageStream applications

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	Eryithroid differentiation
 Microbiology	Bacterial phagocytosis in PBMC
 Parasitology	Babesia infection in RBCs

ISX acquisition software



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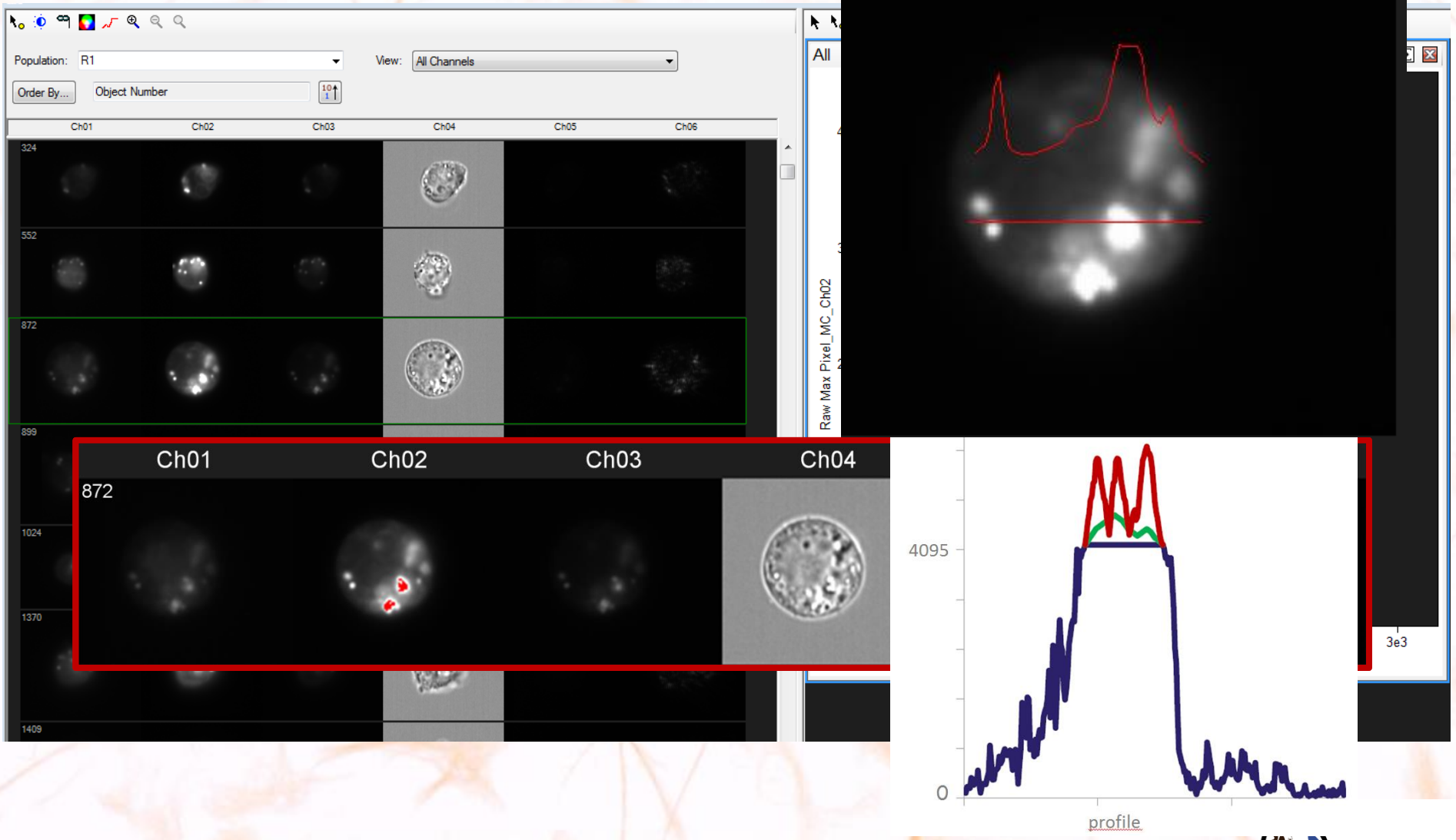
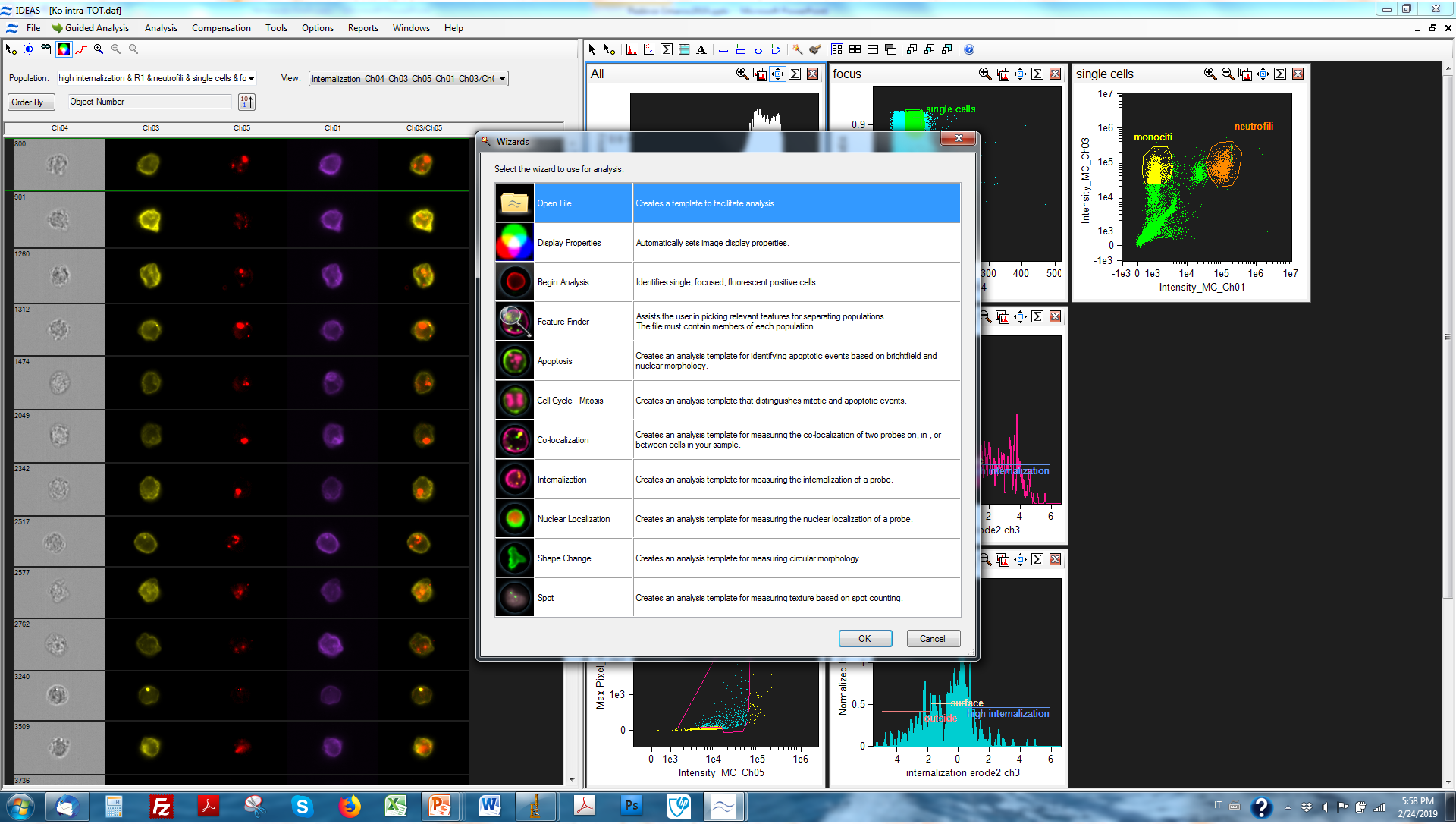
ISX acquisition software

Avoid saturation !!!



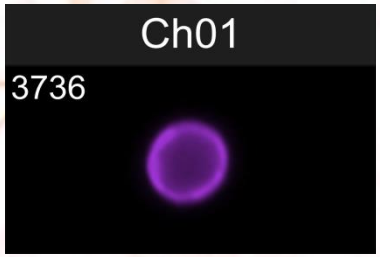
IDEAS software

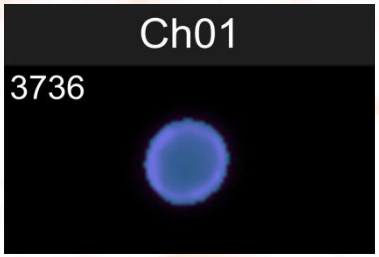


IDEAS software

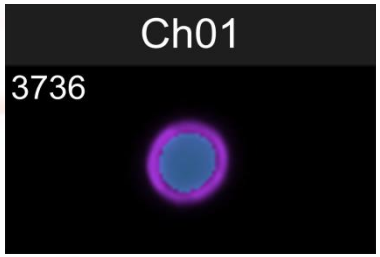
Cell membrane mask

Nuc mask not needed

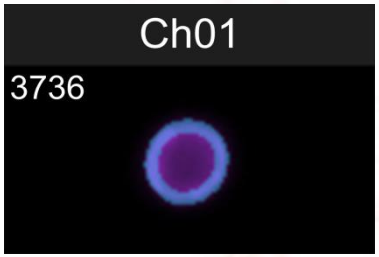




Whole cell mask



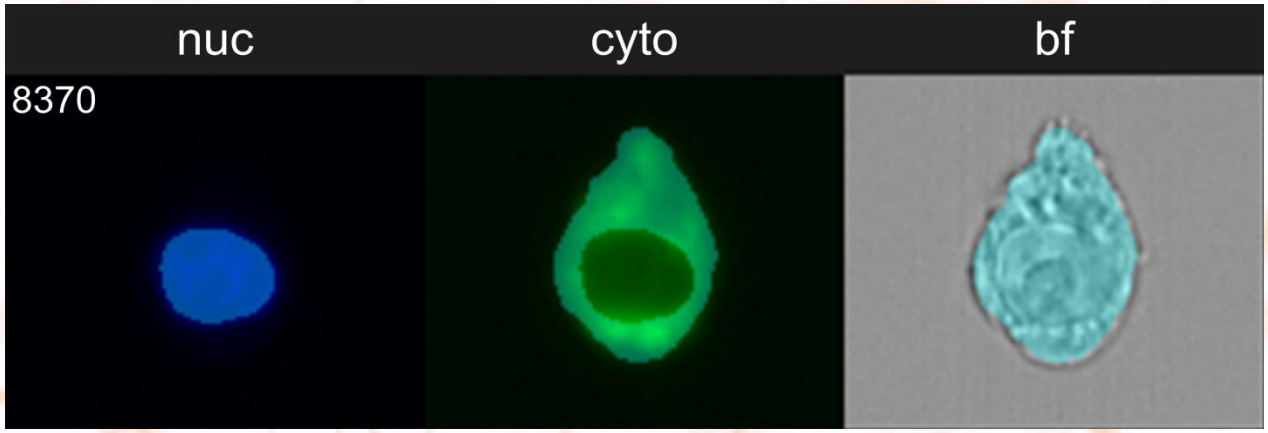
Whole cell mask-eroded



Whole cell mask AND NOT Whole cell mask-eroded

Cytoplasm mask

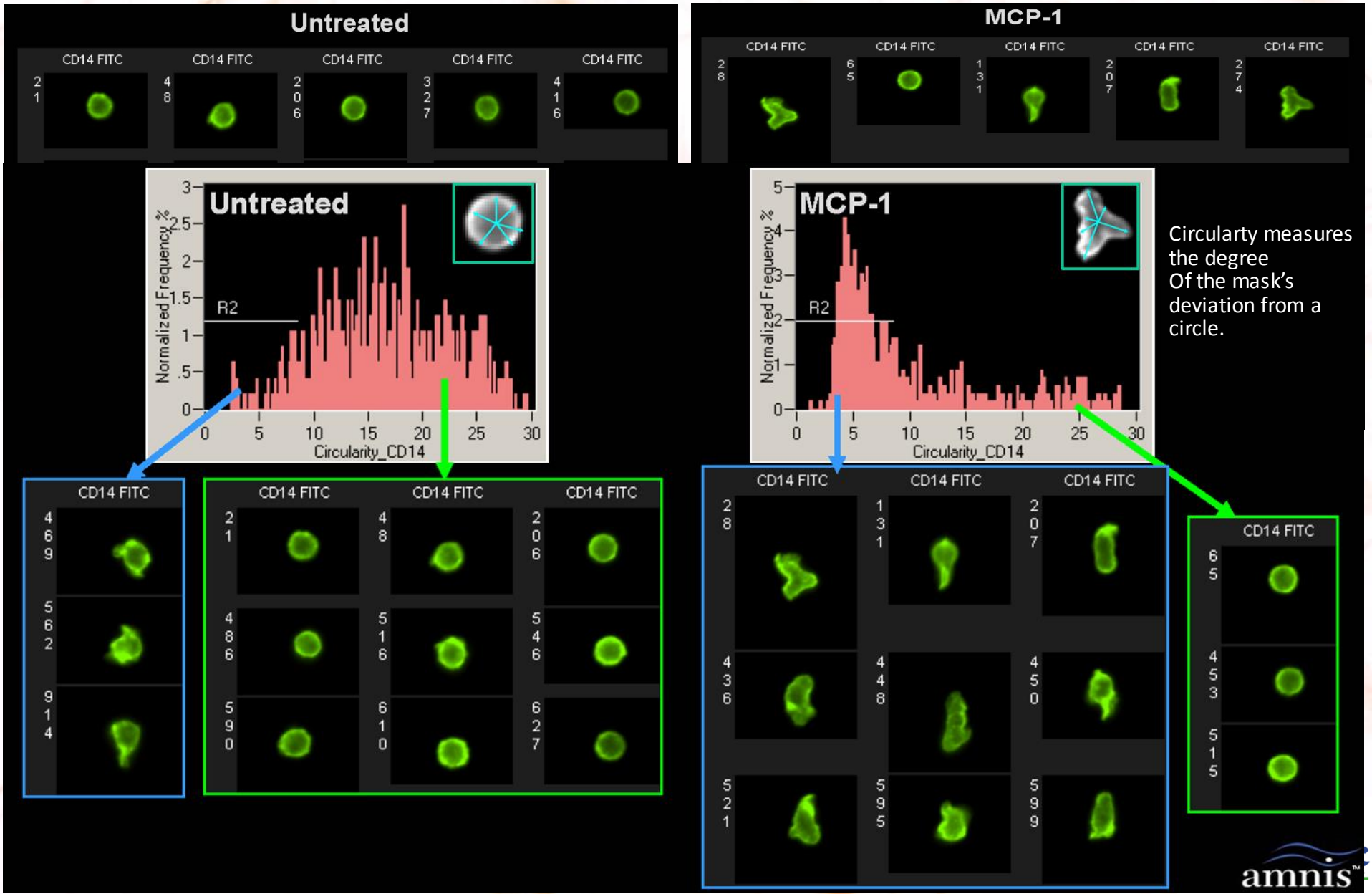
Nuc mask not needed



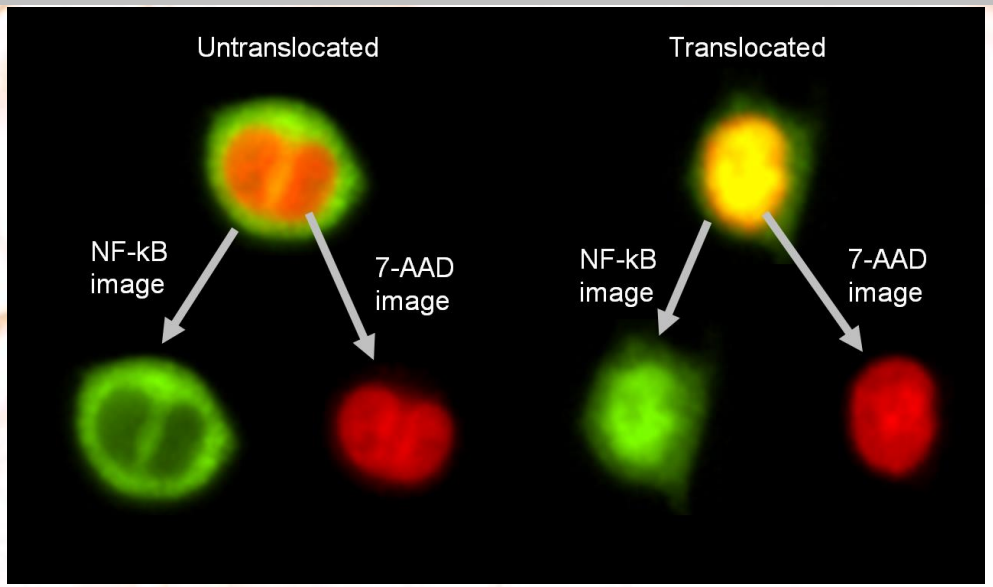
BF mask AND NOT nuc mask

ImageStream MKII: Shape Change

Shape change in human monocytes



ImageStream MKII: NF-kB Translocation

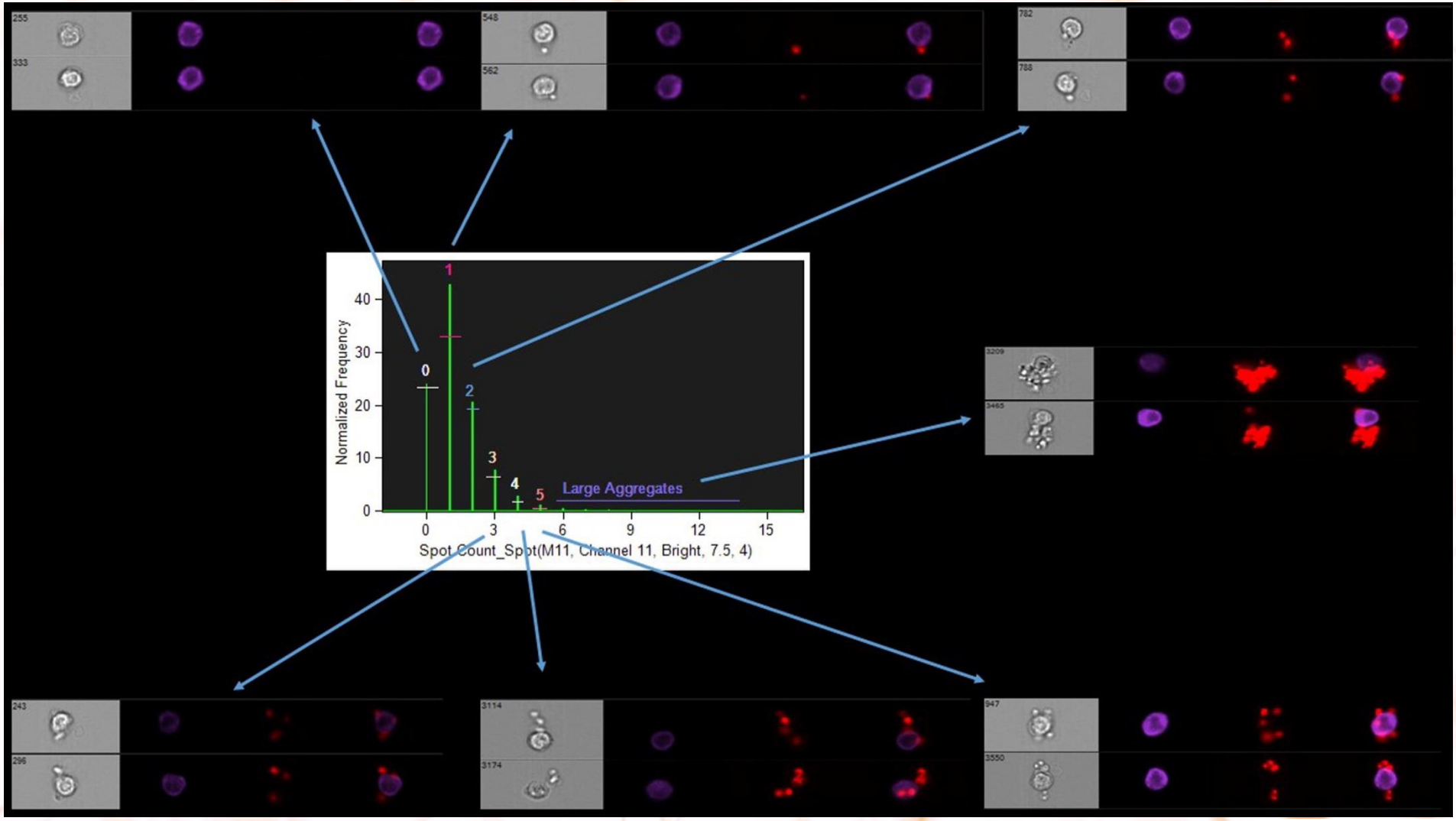


The **Similarity feature** is a measure of the degree to which two images are linearly correlated within a masked region.

In the nuclear mask: intensity of the green is low, intensity of the red is high

In the nuclear mask: intensity of the green

ImageStream MKII: Mixed Populations



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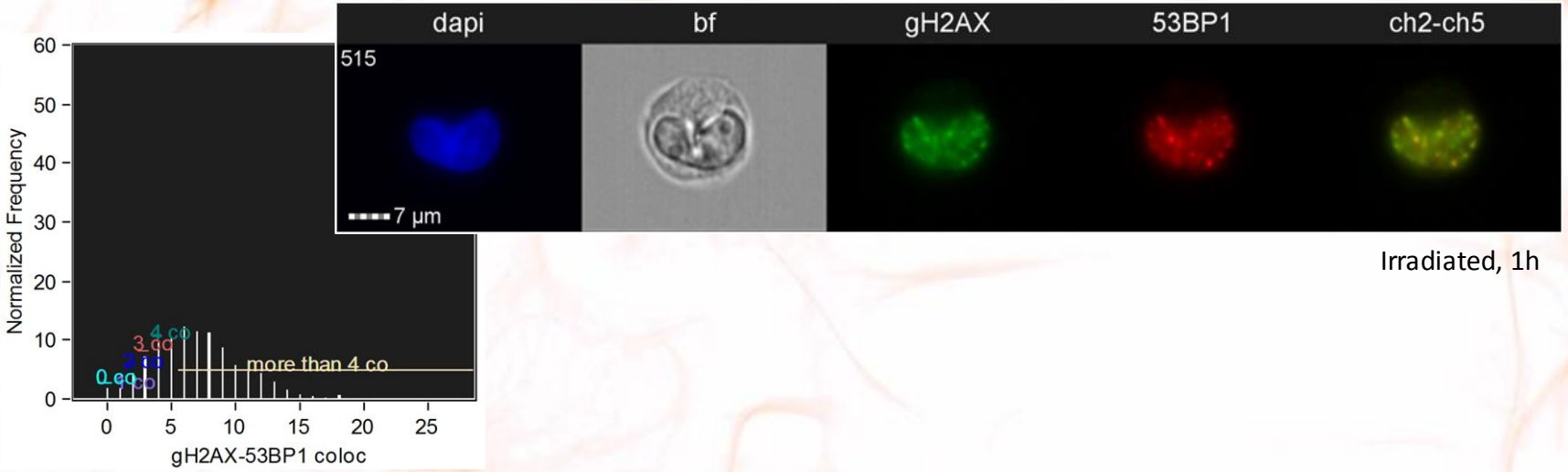
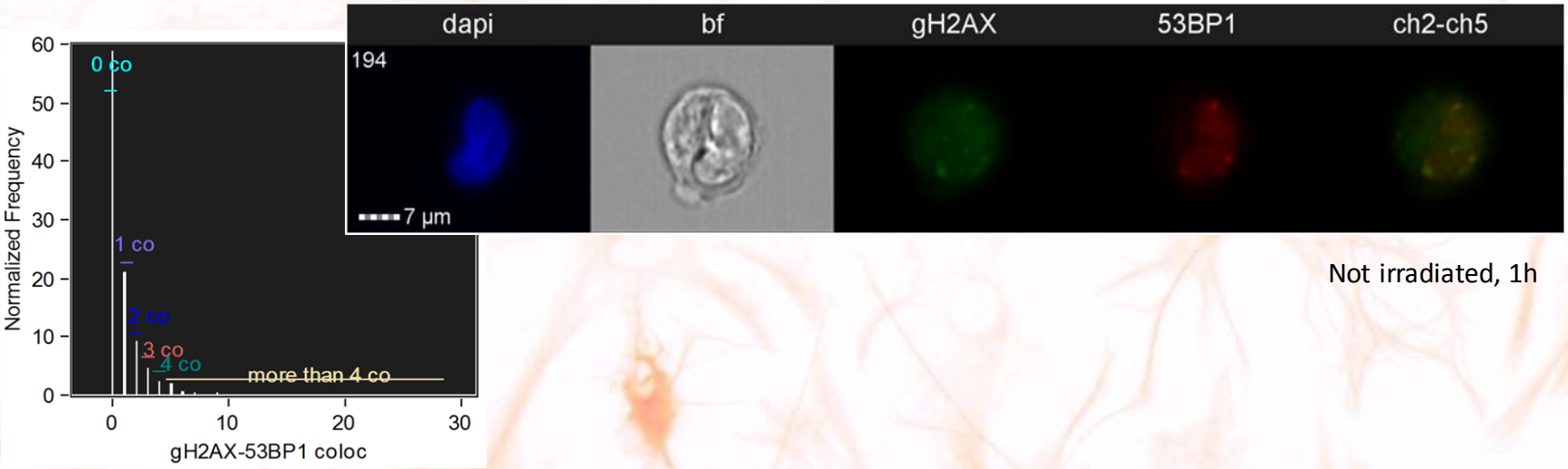
ImageStream MKII: Spot Counting-DNA damage and repair



Irradiated, 1h



Not irradiated, 1h

Count of **γH2AX** 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)

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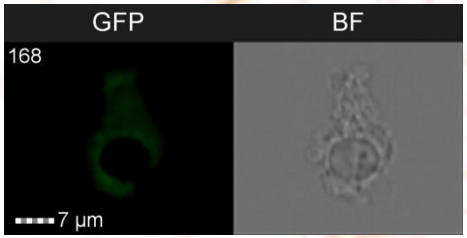
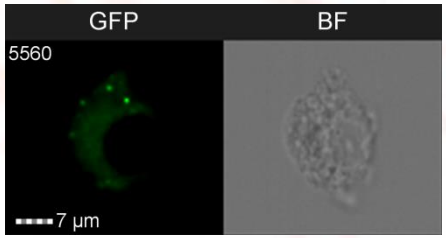
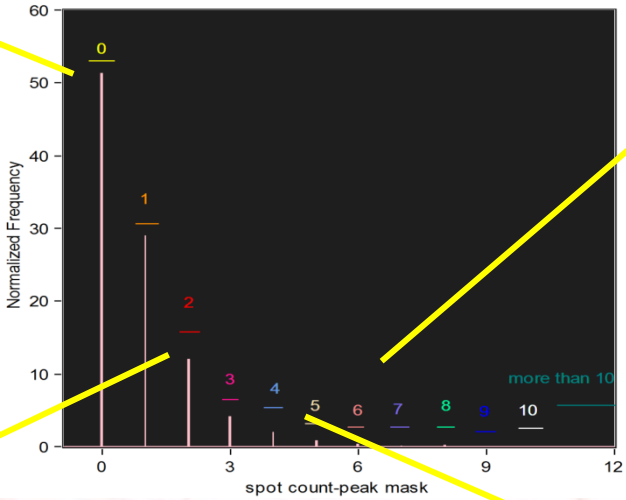
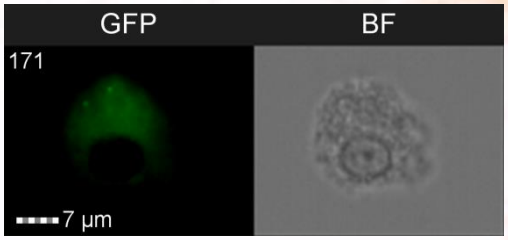
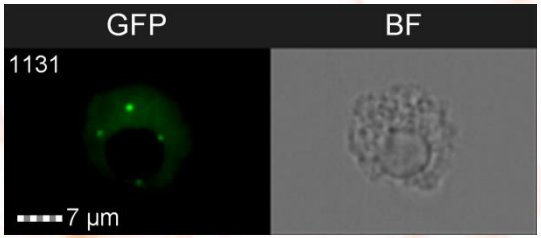
ImageStream MKII: Spot Counting-*in vitro* phagocitosis assay











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)

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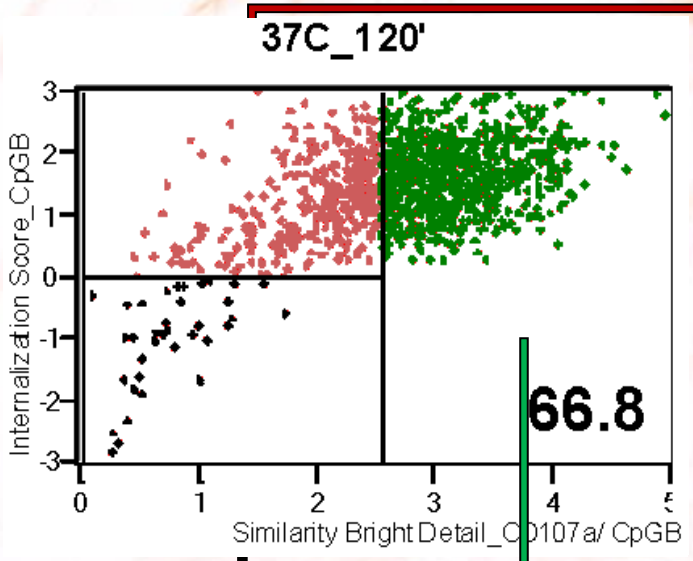
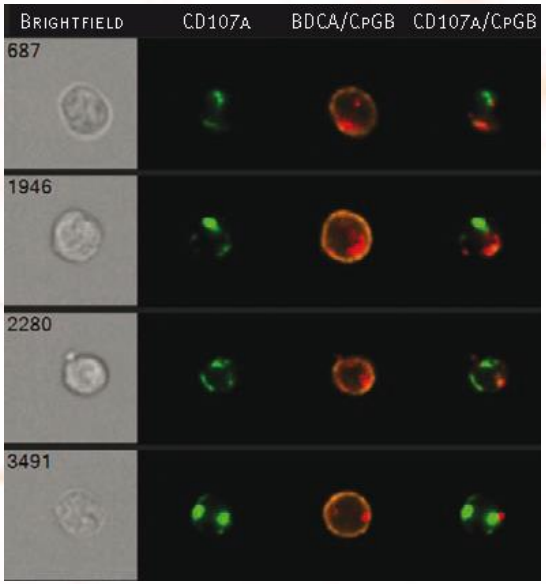
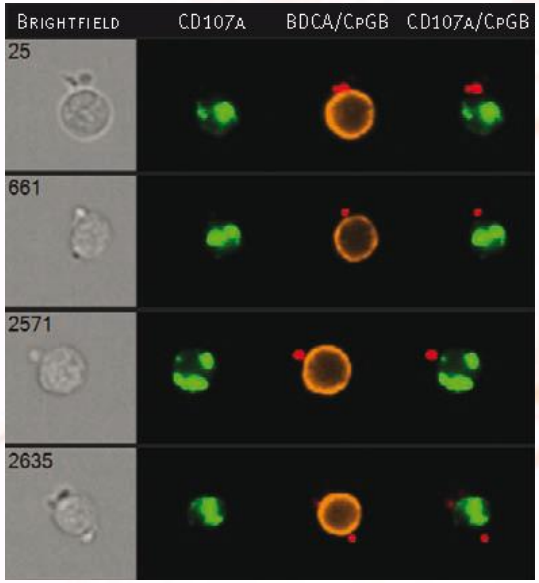
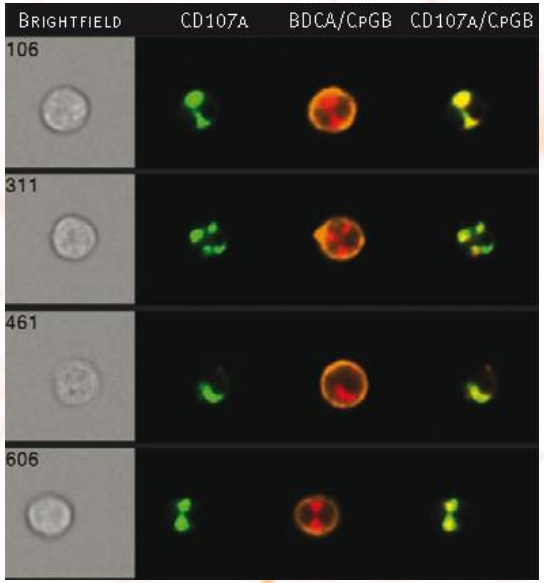
ImageStream MKII: Internalization and Trafficking









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

Source:Amnis

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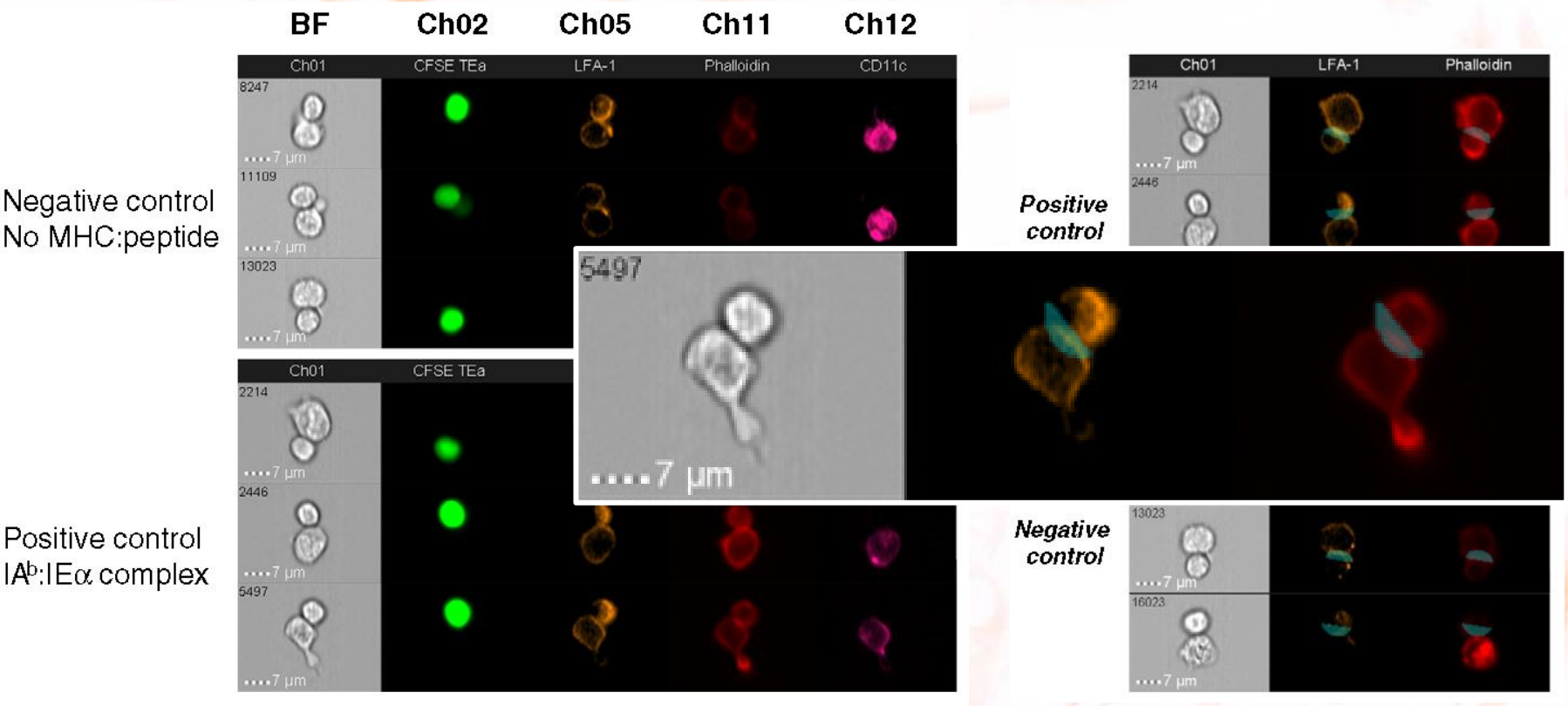
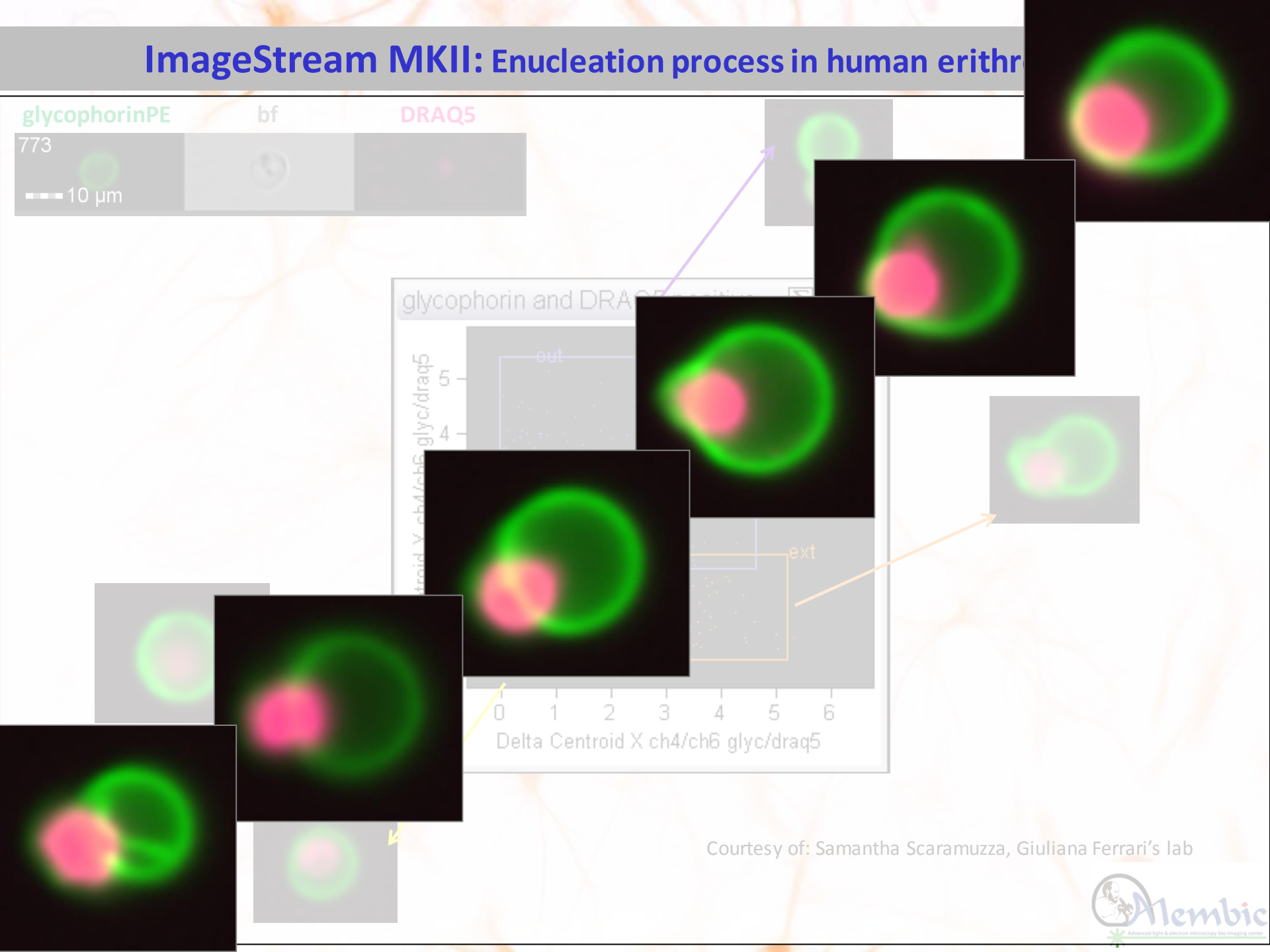
ImageStream MKII: Immunological Synapses

DC and Tcell immunological synapse formation



Markey K.A. et al, 2015

ImageStream MKII: Enucleation process in human erithr



Courtesy of: Samantha Scaramuzza, Giuliana Ferrari's lab

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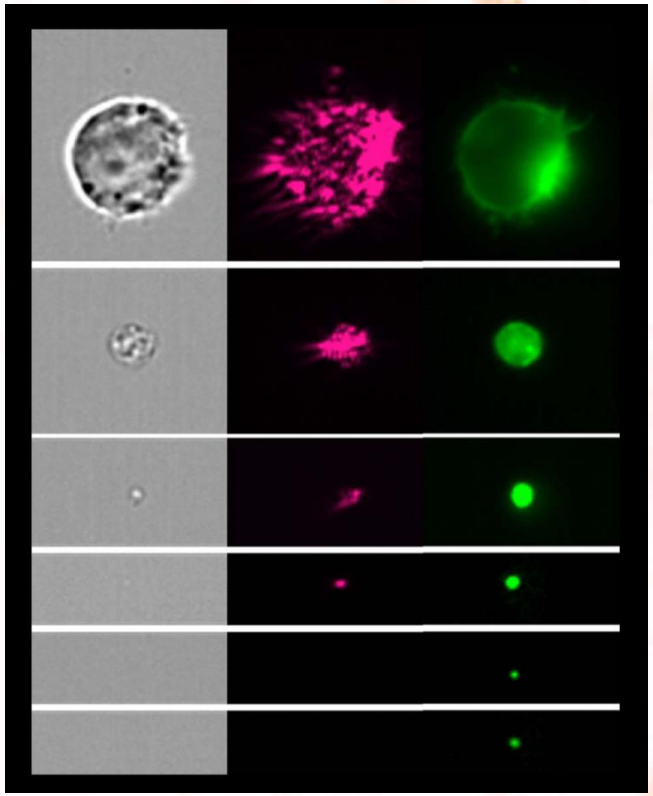
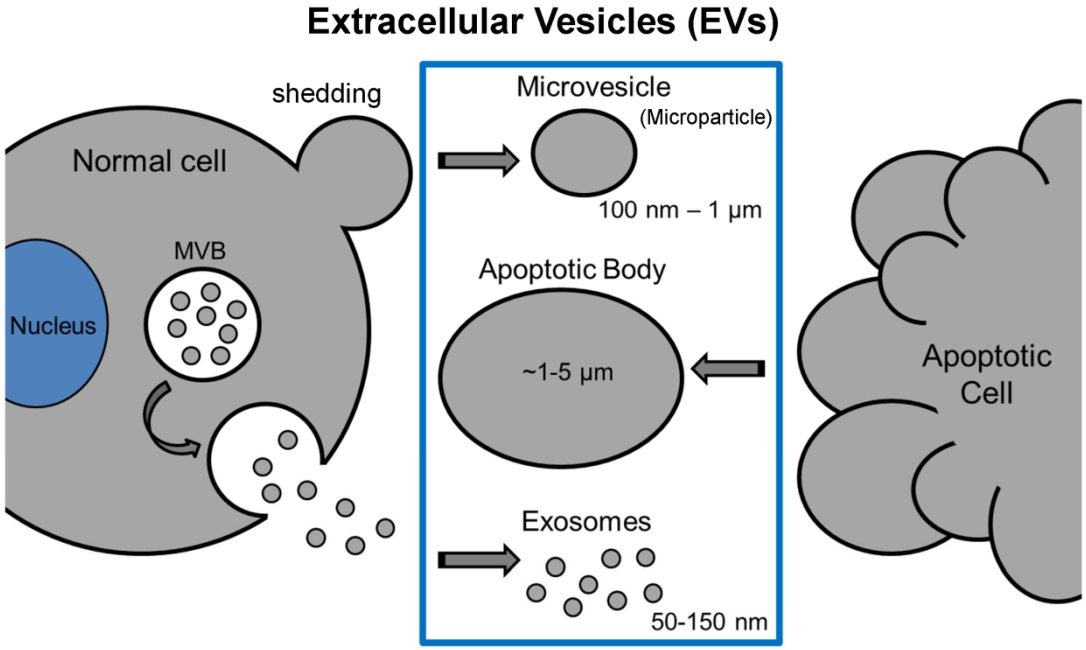
ImageStream MKII: analysis of Extracellular Vesicles

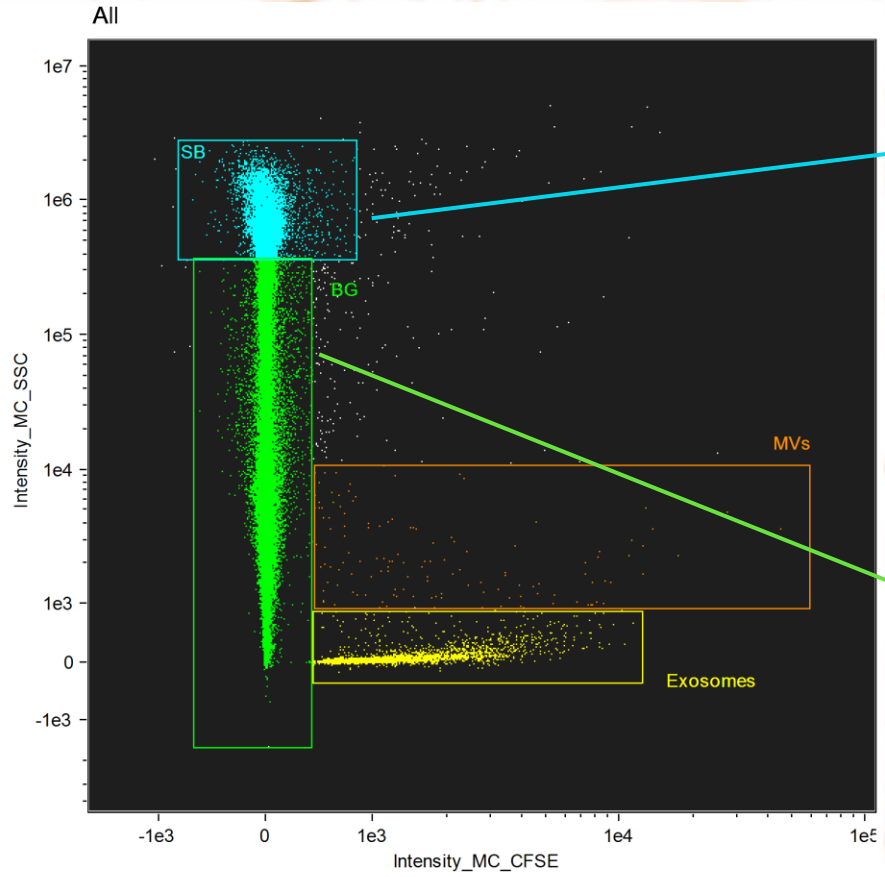




EVs are secreted by a wide range of cells from different species.
EVs can be found in nearly any body fluid.

(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)

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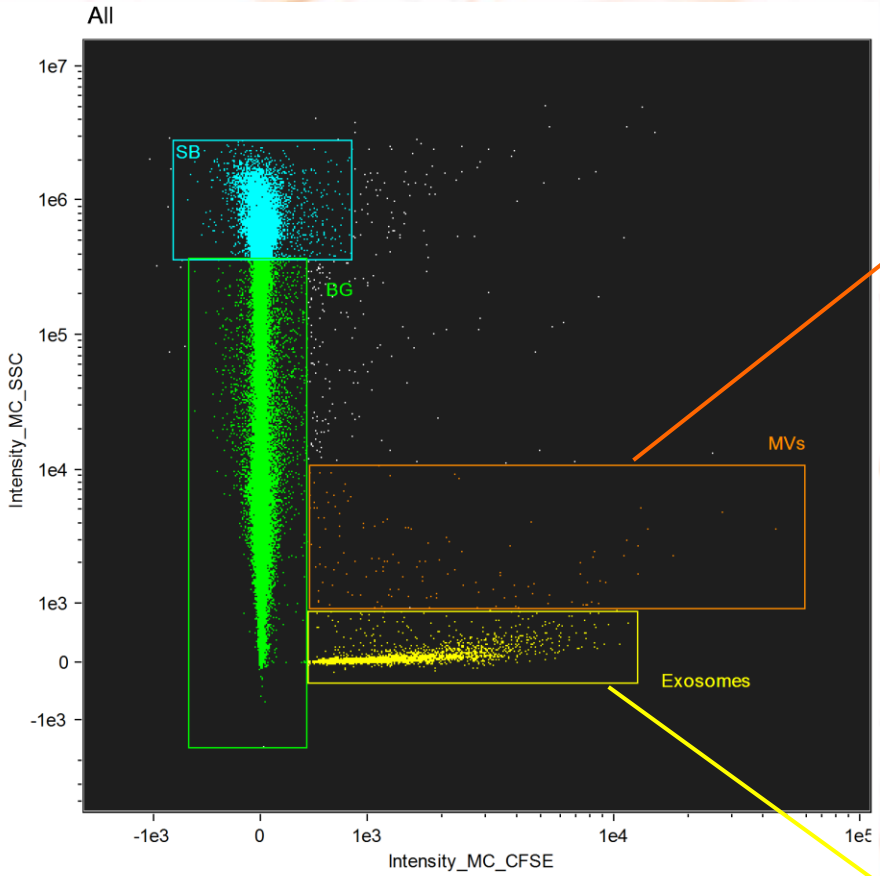
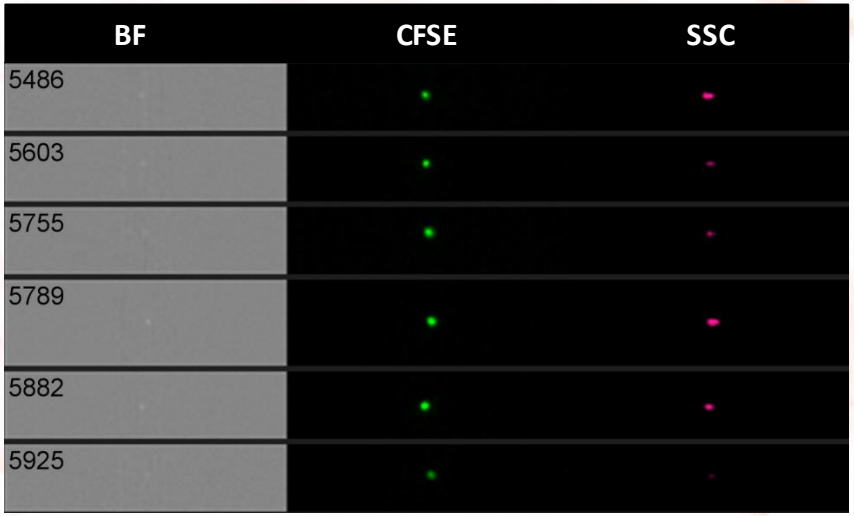
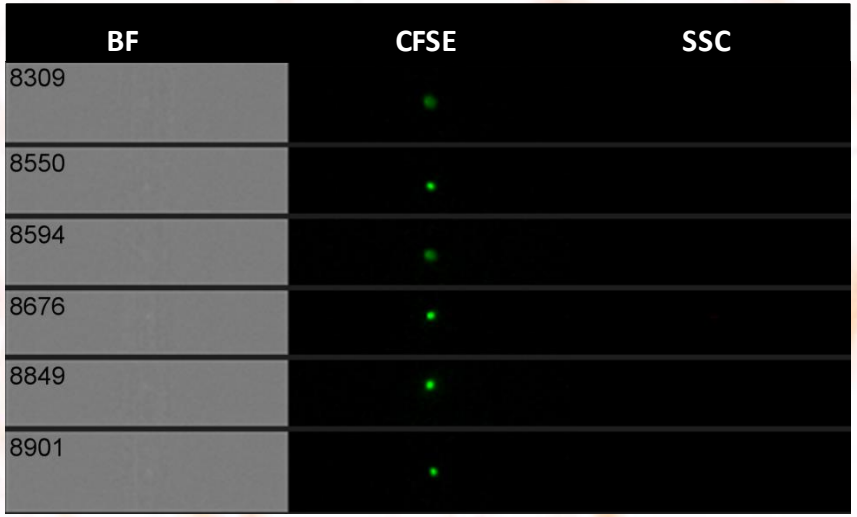
ImageStream MKII: analysis of extracellular particles



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)

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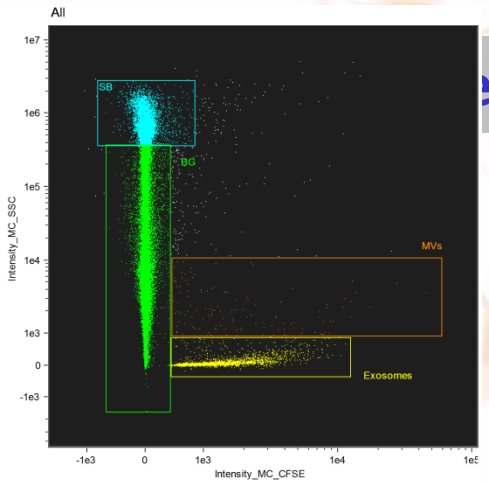
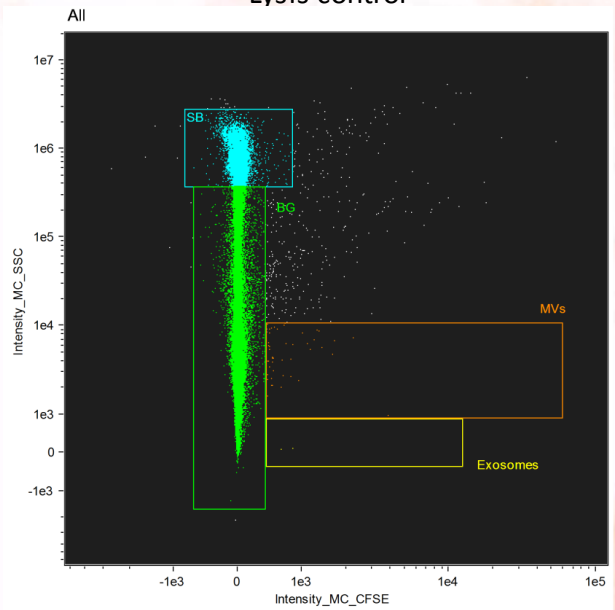
eStream MKII: analysis of extracellular particles



Controls are essential !

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

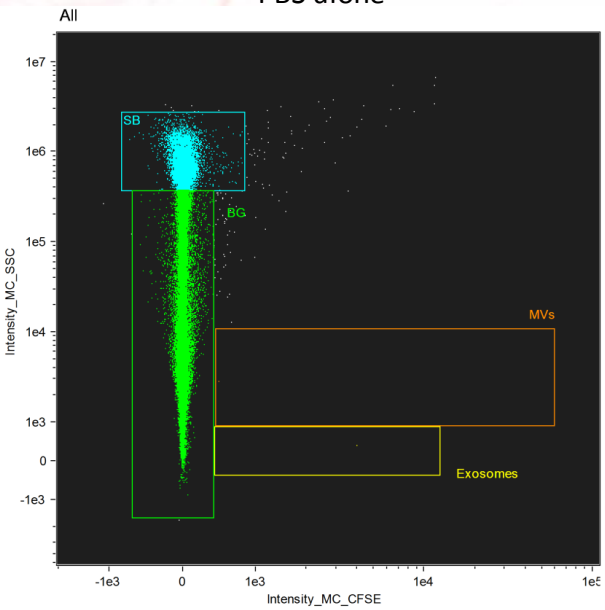
Lysis control



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

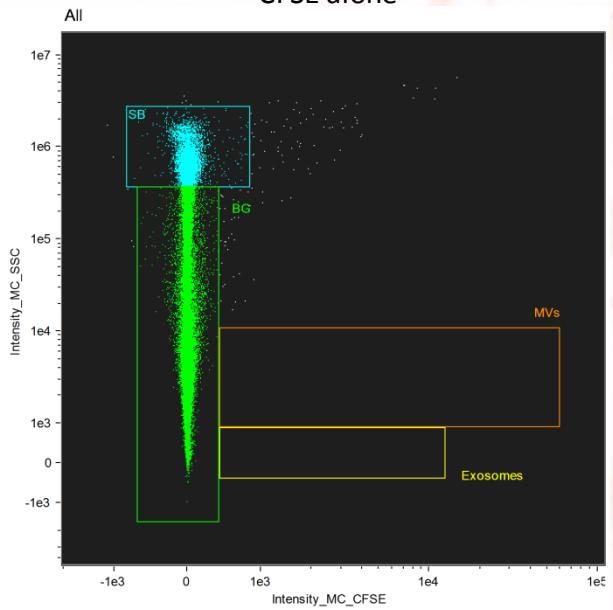
PBS alone



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

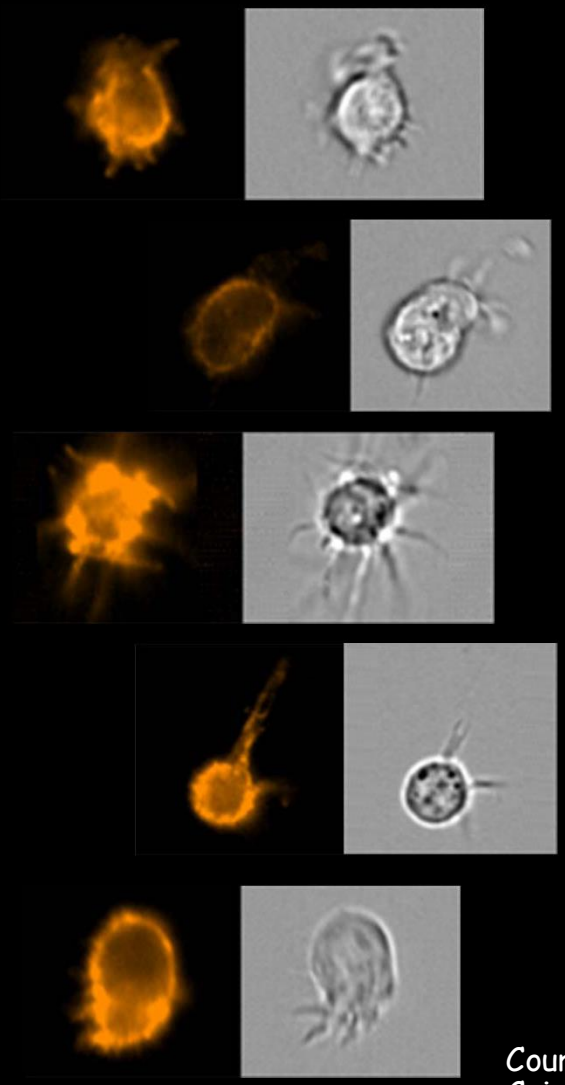
Summary

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

Welcome to the Italian Community for Bioimaging

ItaBI

Italy BioImaging

https://www.eurobioimaging-interim.eu

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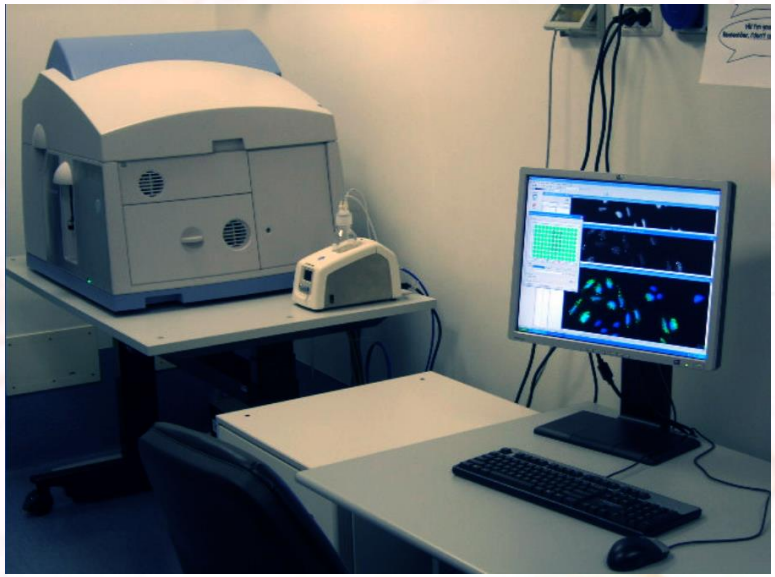
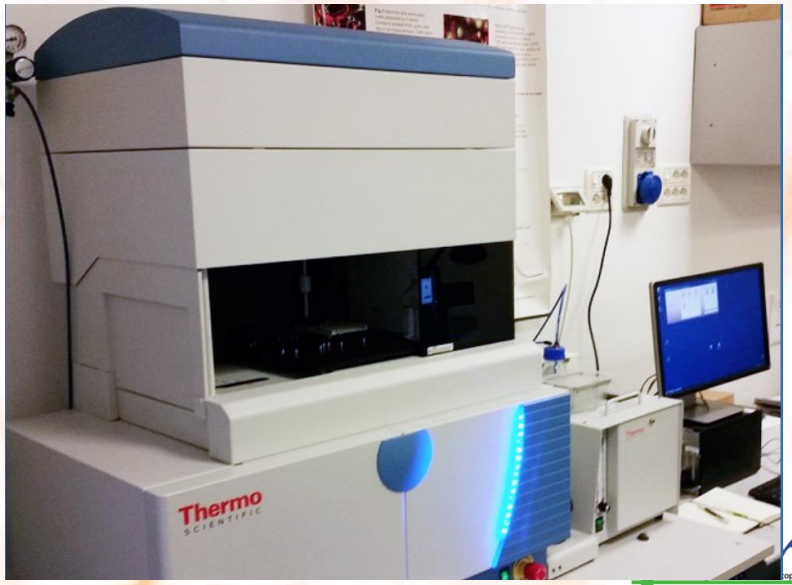
HIGH CONTENT MICROSCOPY for adherent cells

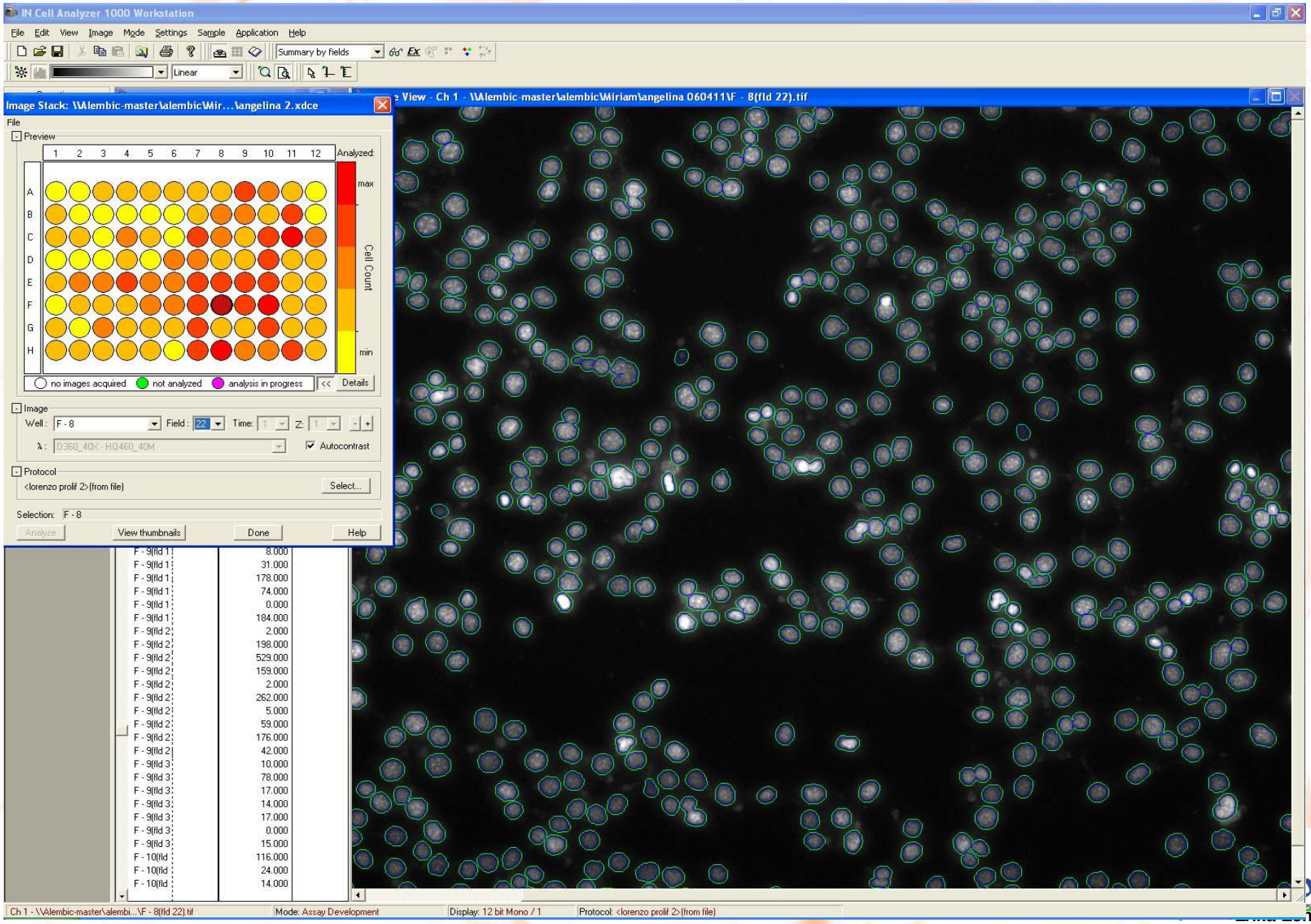
High Content Screening (HCS) allows the quantitative multiparametric analysis of a large number of cells, by coupling high-throughput technology with fluorescence microscopy.
HCS shows a lot of advantages with respect to traditional microscopy and provides a wider spectrum of information that spans from 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
Linear
Image Stack: \\Alembic-master\alembic\Wir...\angelina 2.xdce
File
Preview
1 2 3 4 5 6 7 8 9 10 11 12
Analyzed:
max
Cell Count
min
A B C D E F G H
no images acquired
not analyzed
analysis in progress
<<
Details
Image
Well : F - 8
Field : 22
Time:
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<lorenzo prolif 2> (from file)
Select...
Selection: F - 8
Analyze
View thumbnails
Done
Help
2 View - Ch 1 - \\Alembic-master\alembic\Miriam\angelina 060411\F - 8(fld 22).tif
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Display: 12 bit Mono / 1
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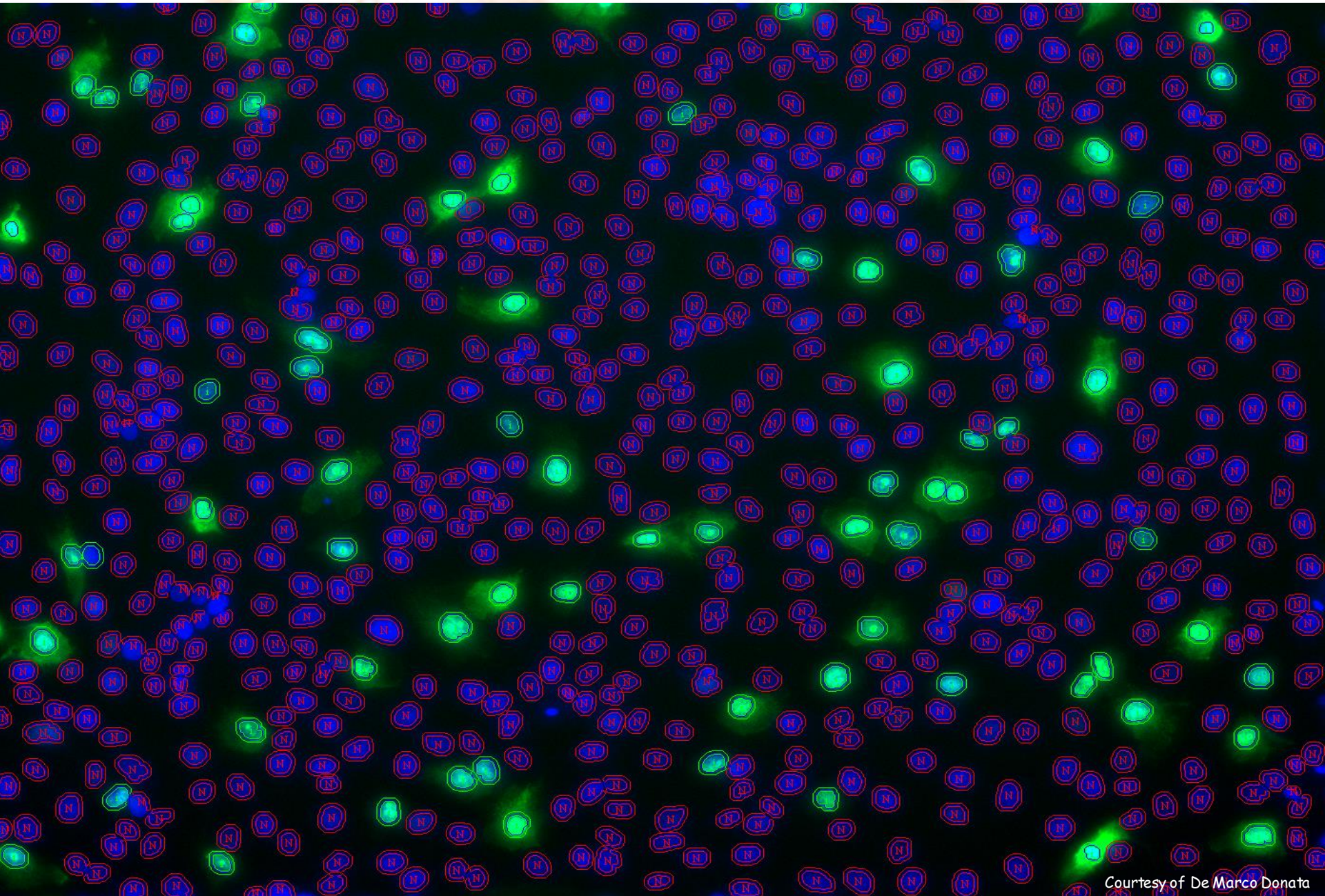
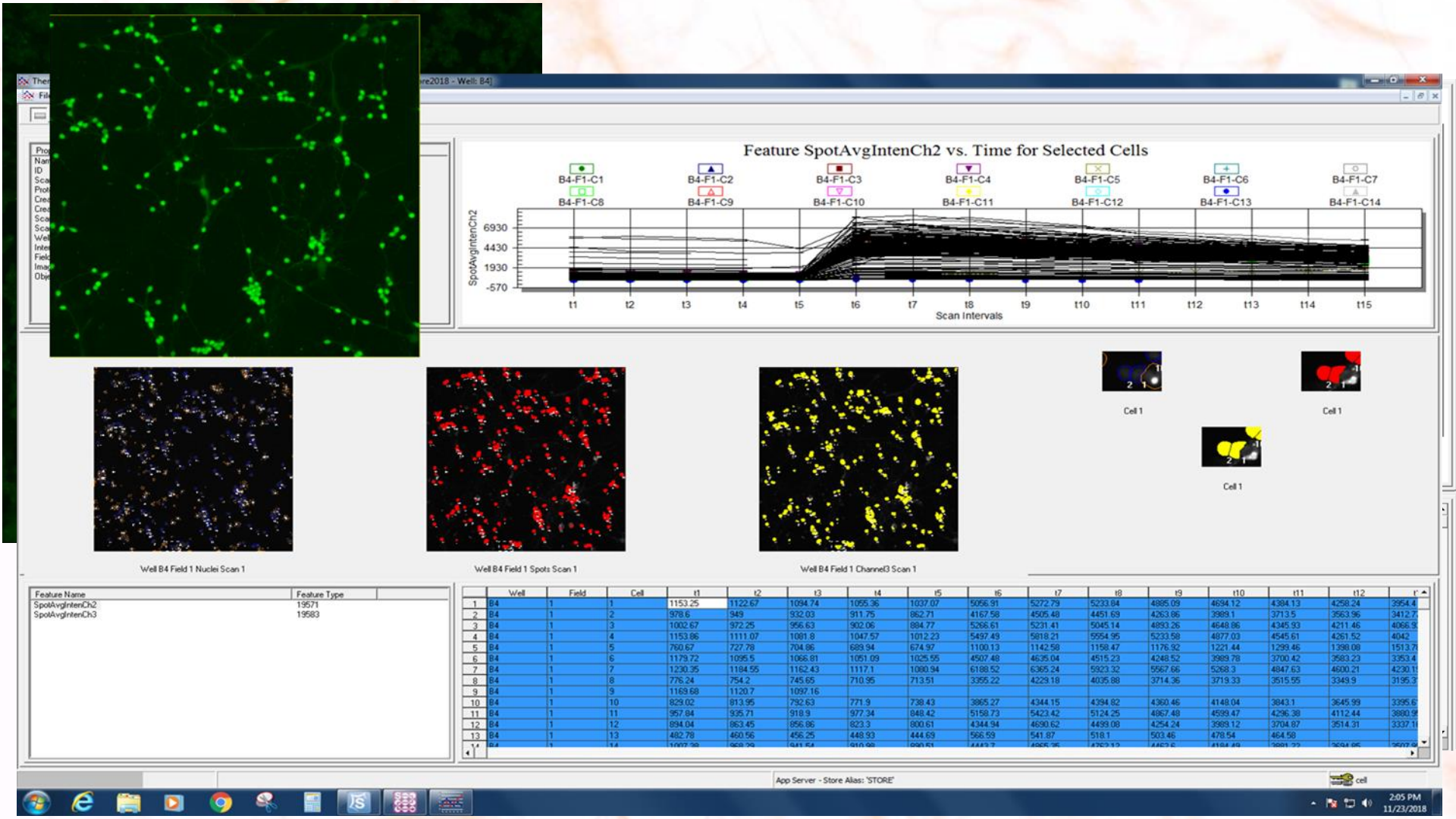
Identification of cell subpopulations



Courtesy of De Marco Donata

Liquid Handling Module: Calcium flux



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

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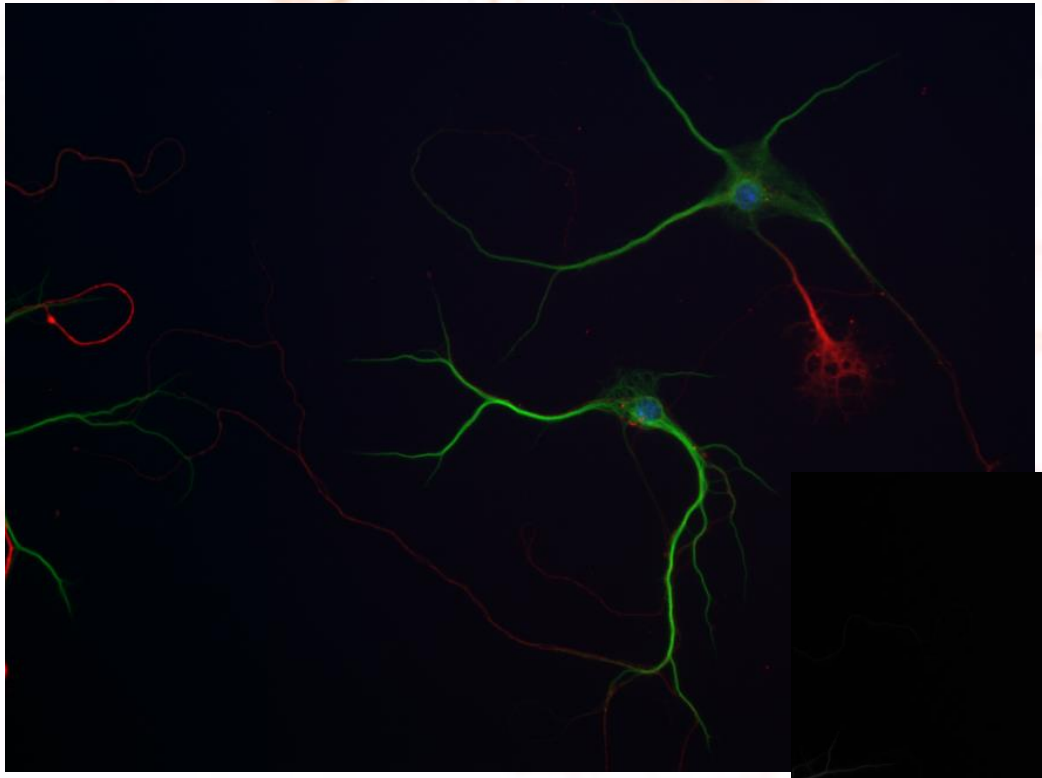
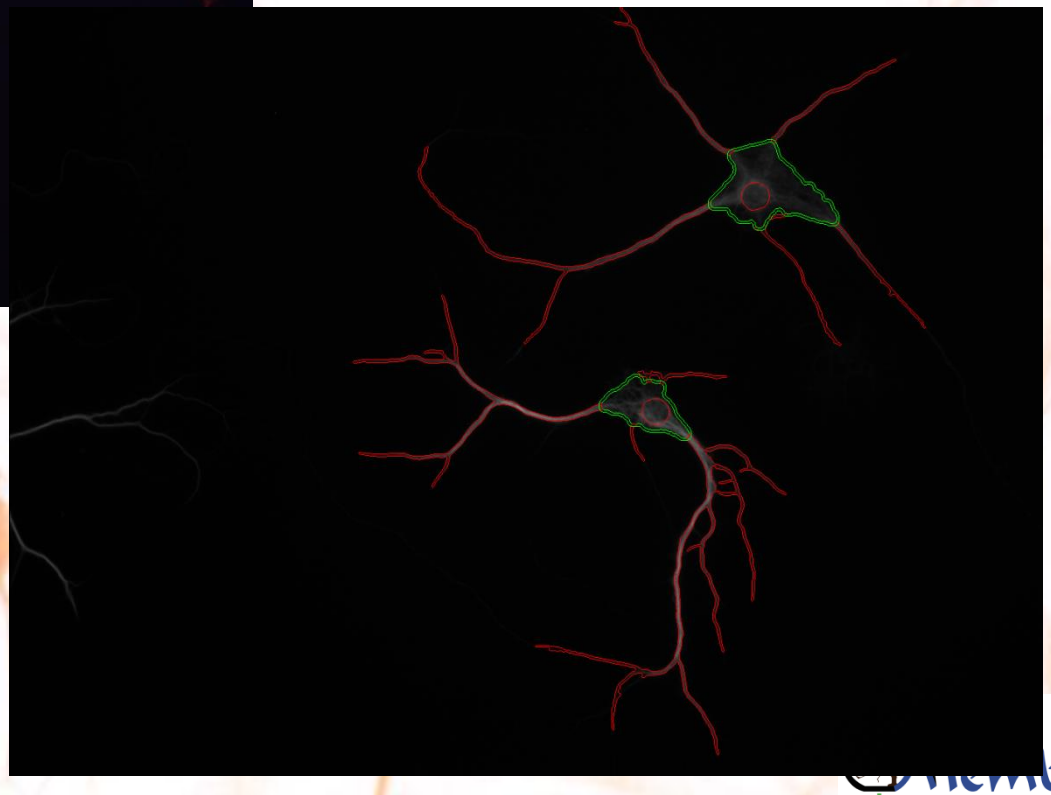
Neurite outgrowth analysis



Dendritic arborization

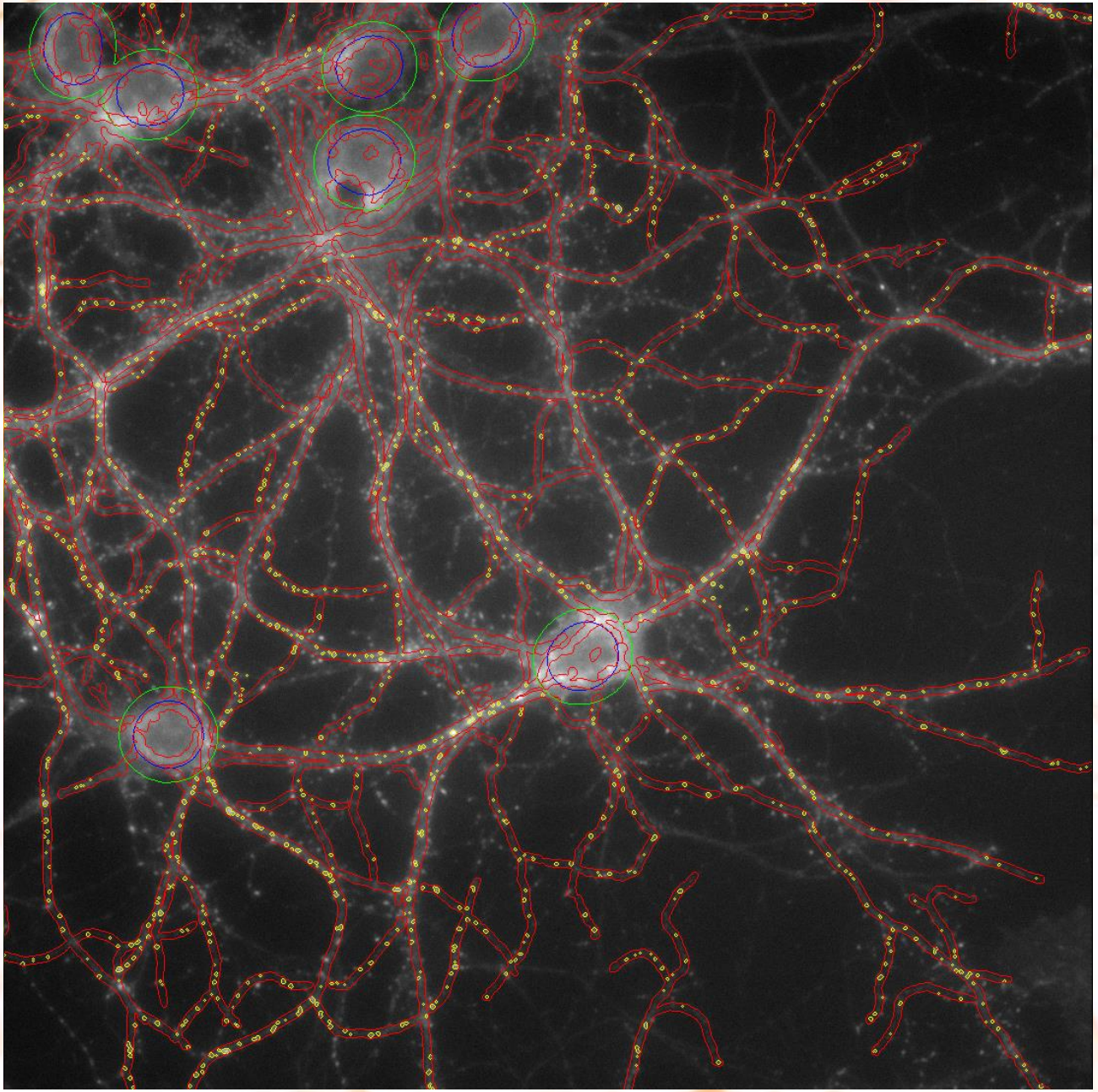
mouse neurons_div10

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



- Total neurite lenght
- Neurite count
- Neurite lenght/cell
- Neurite count/cell
- Neurite branching
- Lenght 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)

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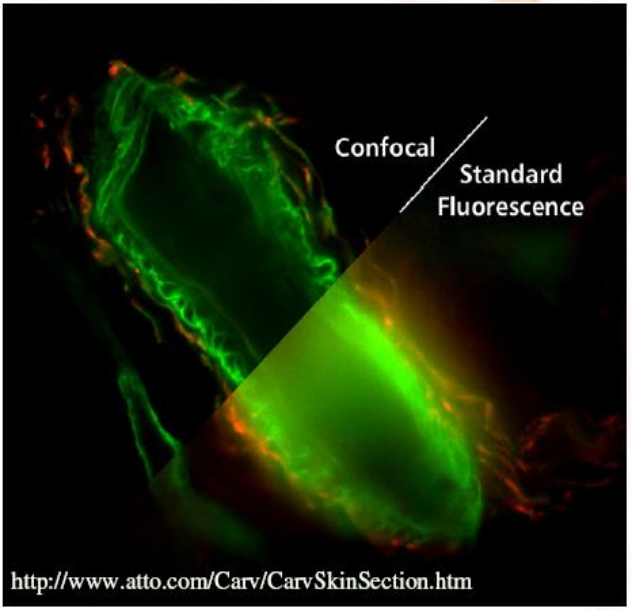
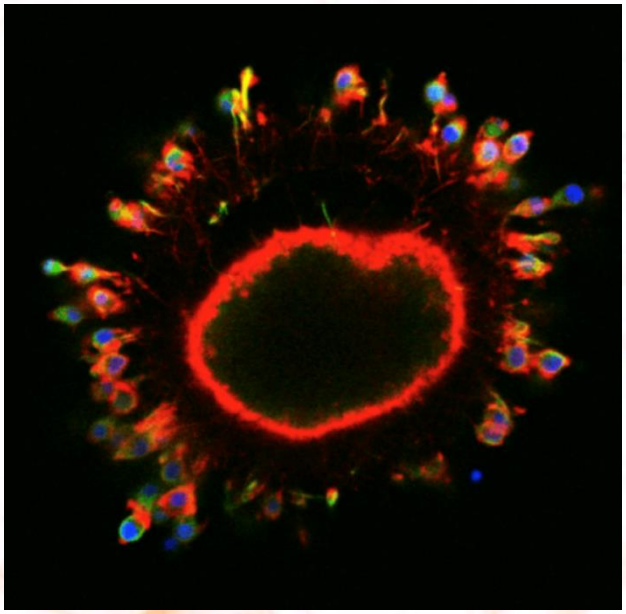
OPTICAL SECTIONING BY REDUCING IMAGE VOLUME

Point-scan confocal microscopy

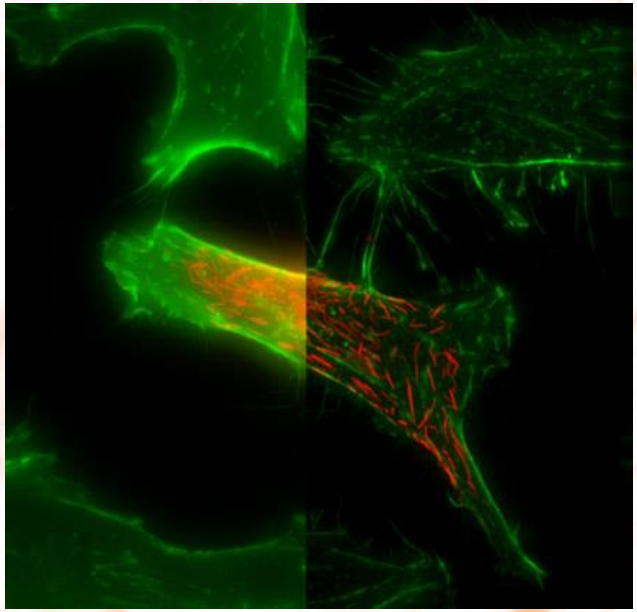


Spinning disk confocal microscopy



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

Deconvolution microscopy



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

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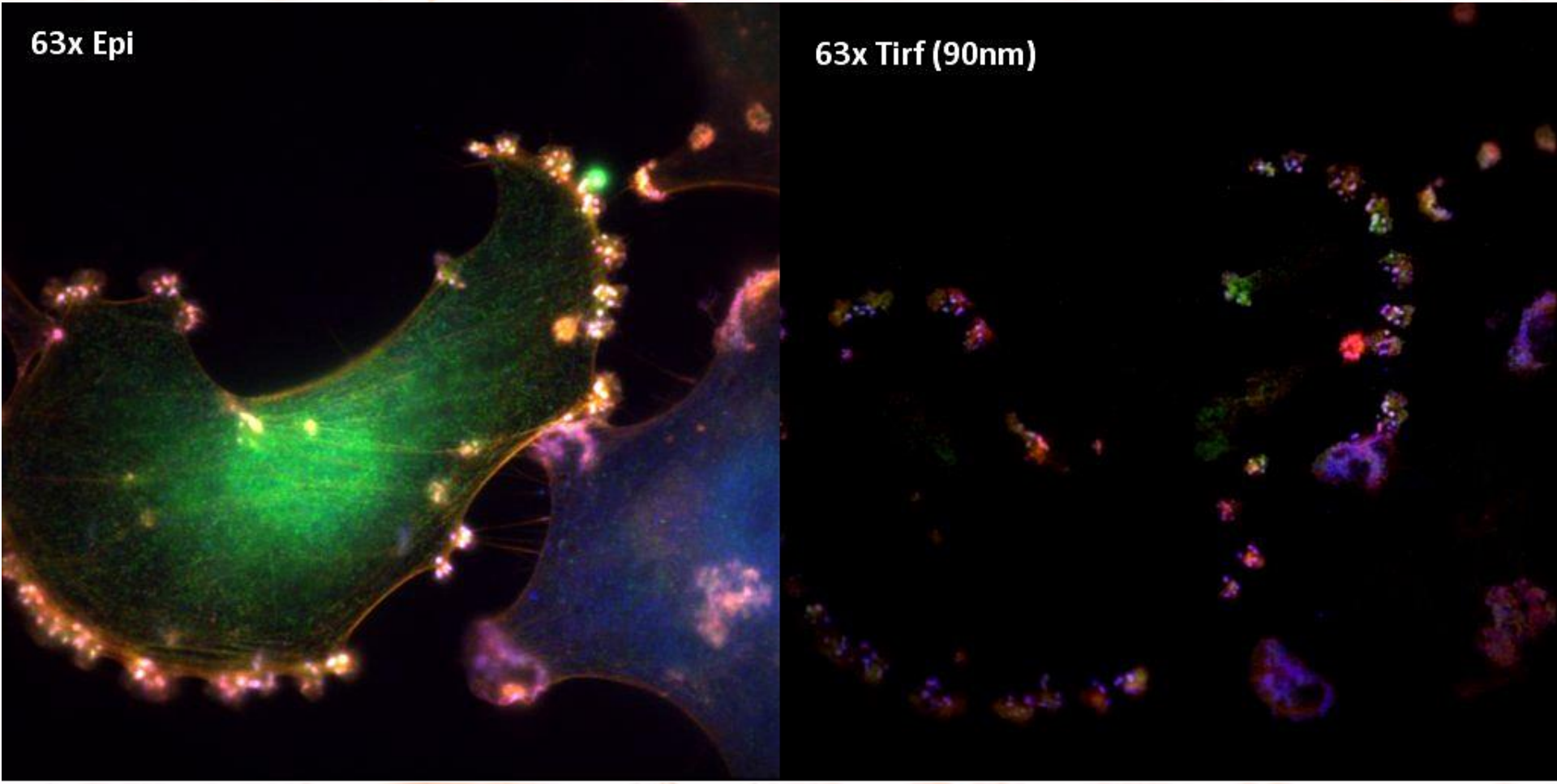
OPTICAL SECTIONING BY SELECTIVE ILLUMINATION: Total Internal Reflection Microscopy



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)

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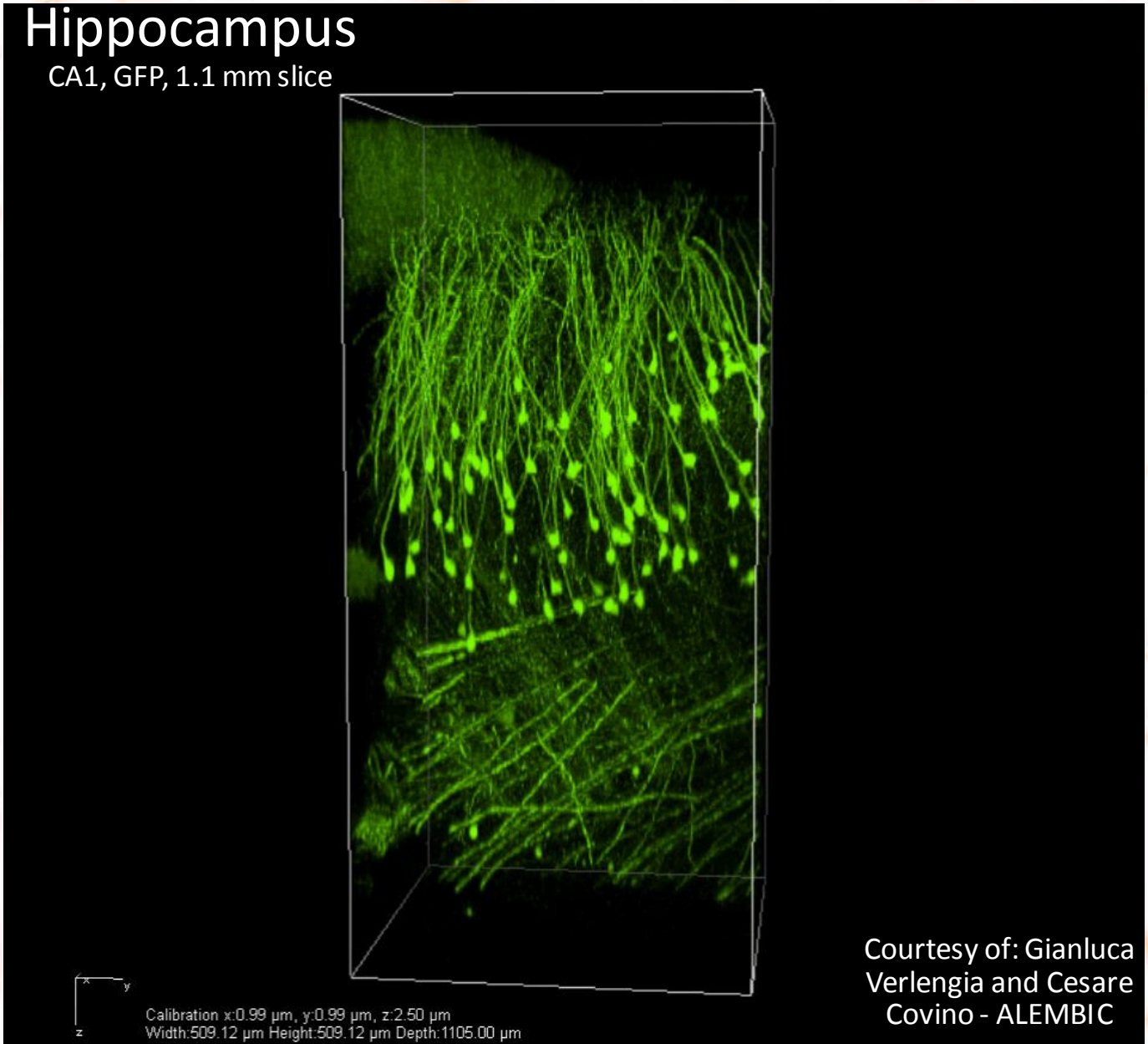
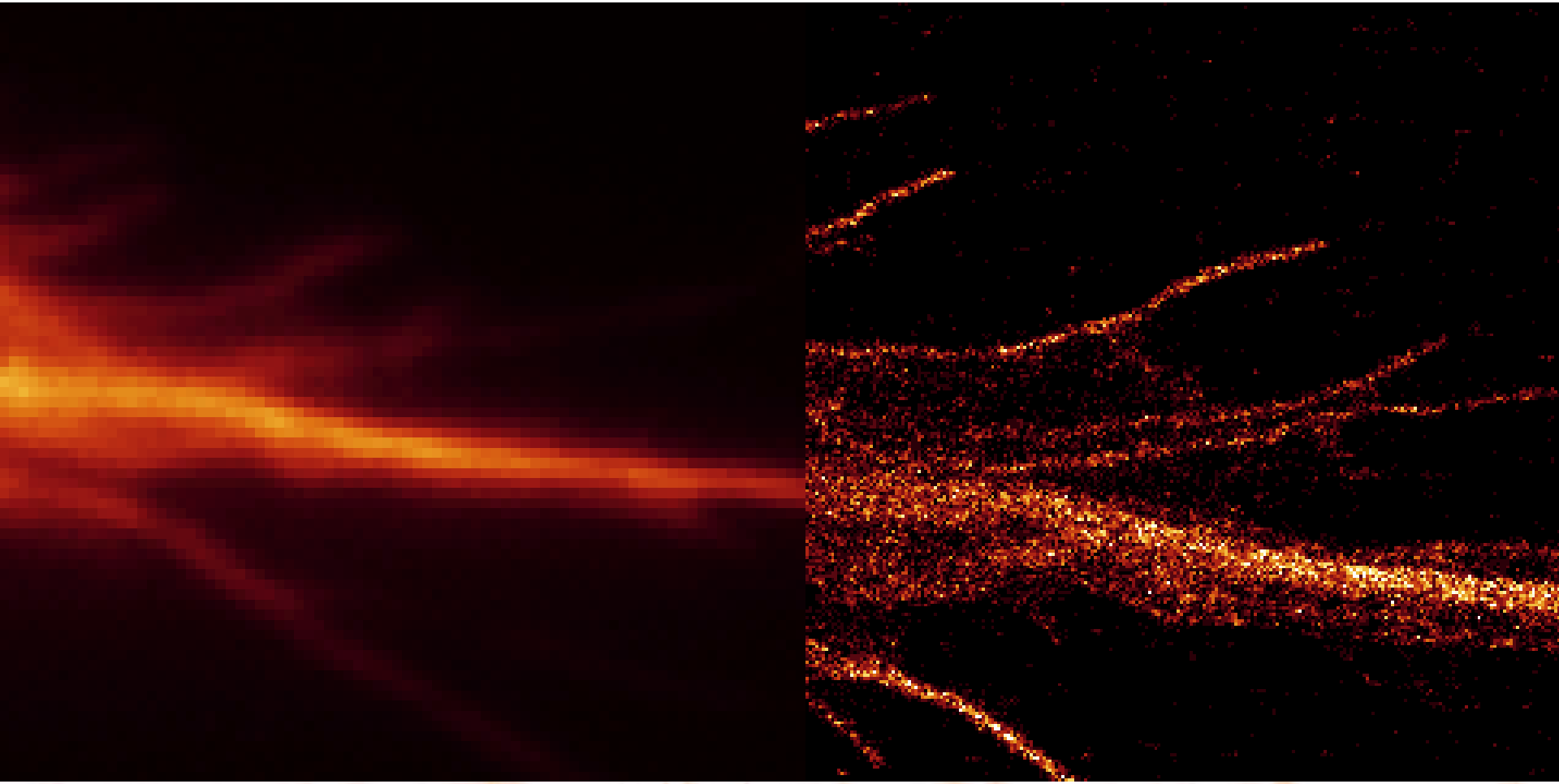
OPTICAL SECTIONING BY SELECTIVE ILLUMINATION: 2P and Light Sheet Microscopy



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)

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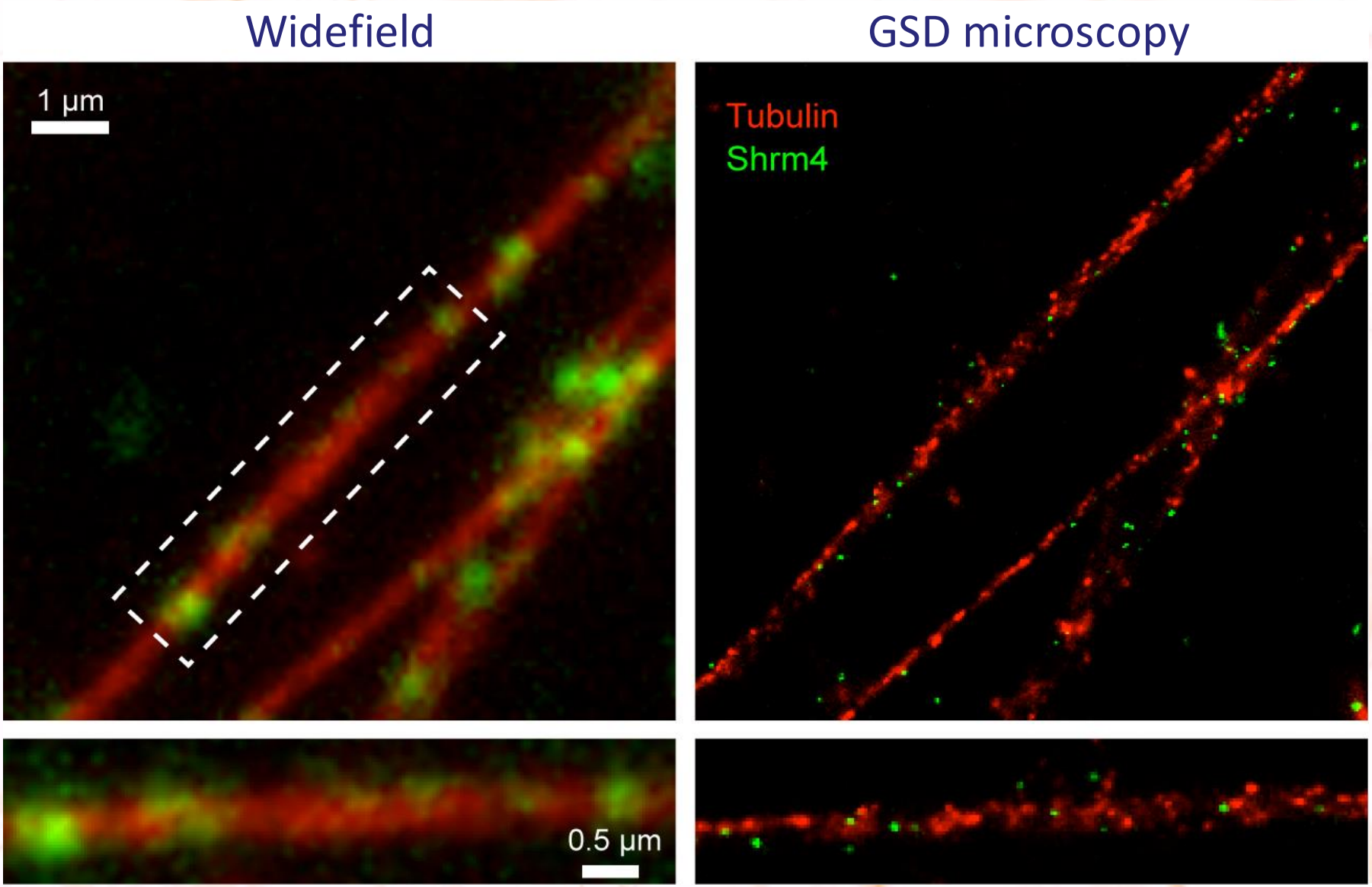
Super-resolution (down to 30 nm)



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

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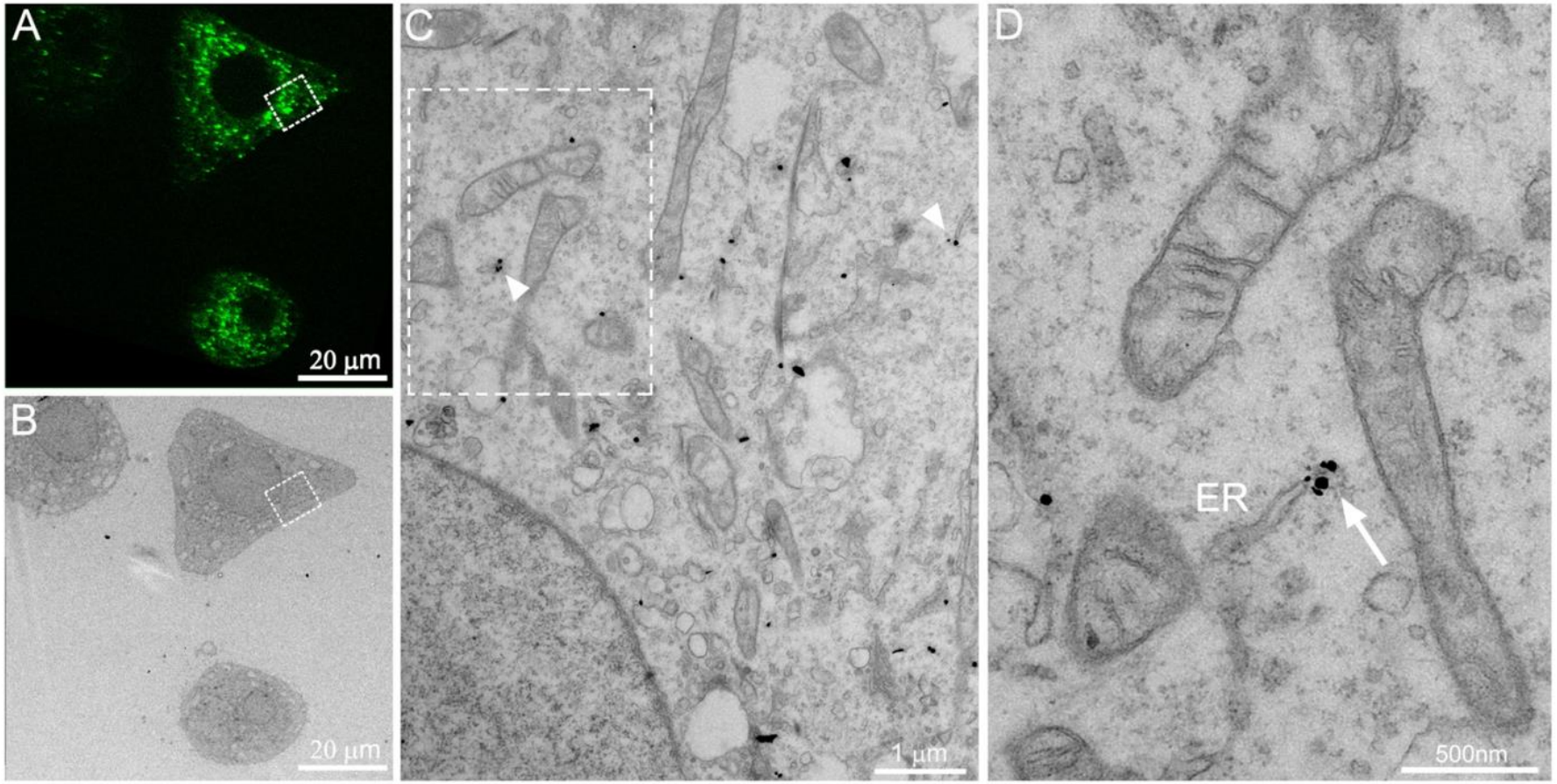
Correlative Light Electron Microscopy (CLEM)

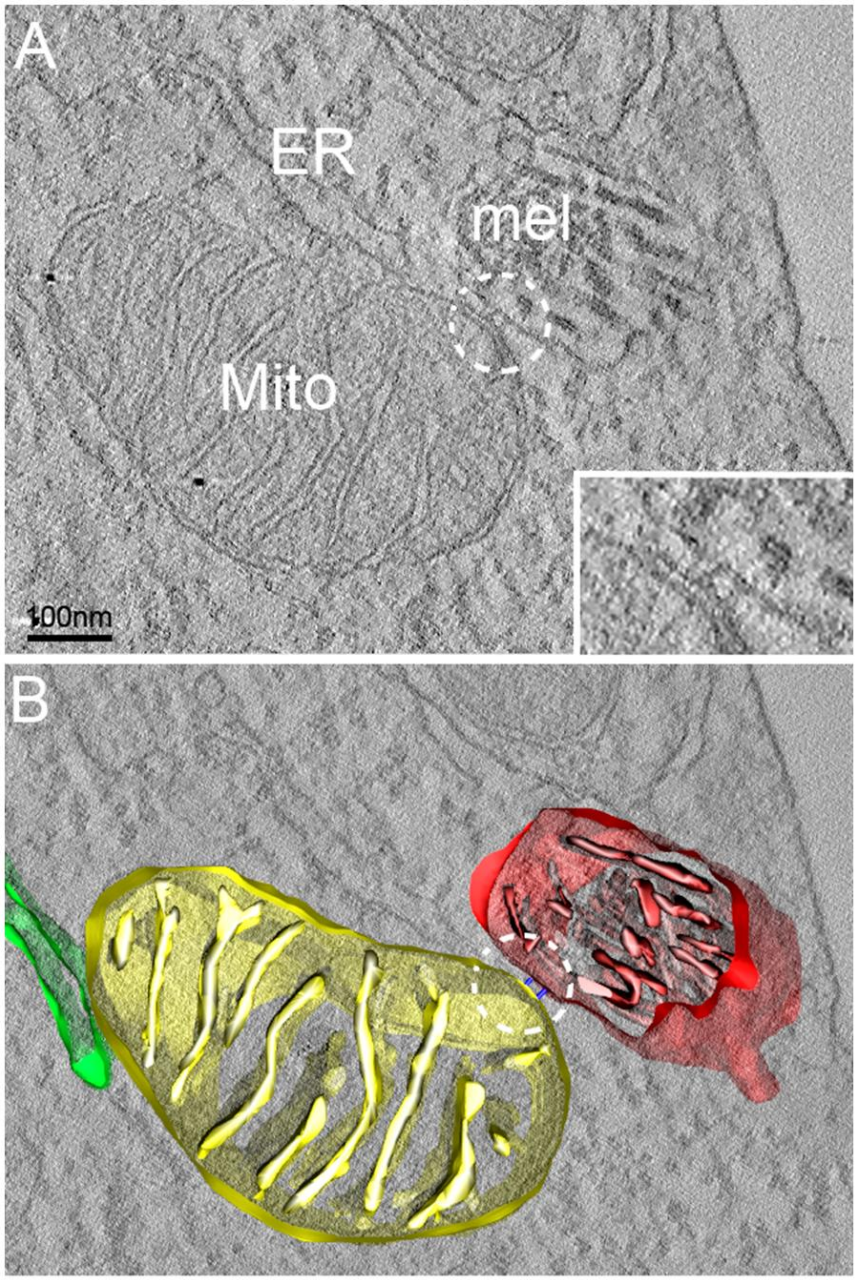
Correlative Light Electron Microscopy (CLEM) is a powerful tool that can combine the resolution of electron microscopy with the possibility to observe the molecule of interest in vivo by means of fluorescence microscopy; thereby allowing to disclosure the molecular machinery involved in biological processes 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 tecnique 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 recostruction showing mitochondria-melanosomes connections;
(B) 3D rendering of the two organelles and the contact.



Maria Vittoria Schiaffino and
Andrea Raimondi
San Raffaele Scientific Institute

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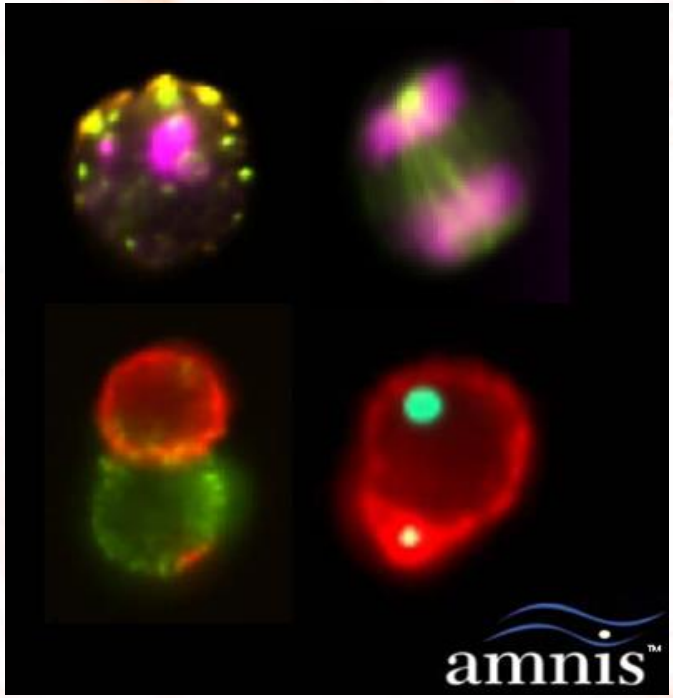

Thank you for your attention !

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