ImageStream X
imaging flow cytometry application

Technical journal club

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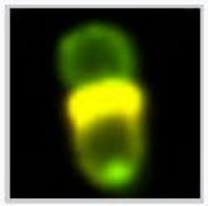
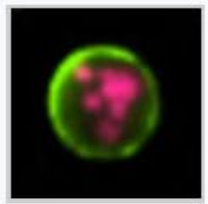
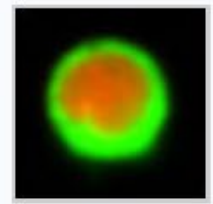
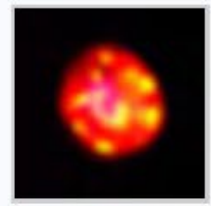
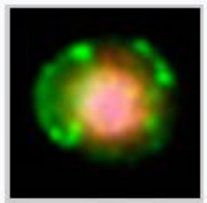
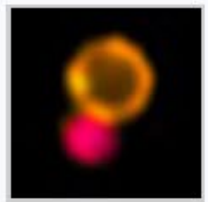
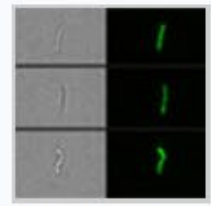
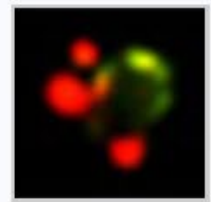
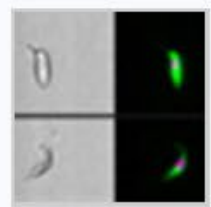
2022.02.15

What is ImageStreamX?

- An instrument which integrates the features of *flow cytometry* and *fluorescence microscopy* combined with a modern methodology for image analysis

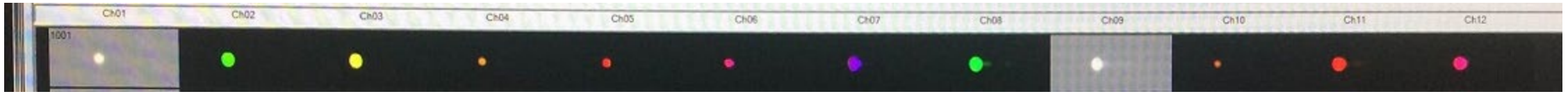
 Immunology	 Oncology	 Biochemistry	 Drug Discovery
 Stem Cell Biology	 Hematology	 Microbiology	 Virology
 Nanotechnology	 Toxicology	 Oceanography	 Parasitology

6 lasers

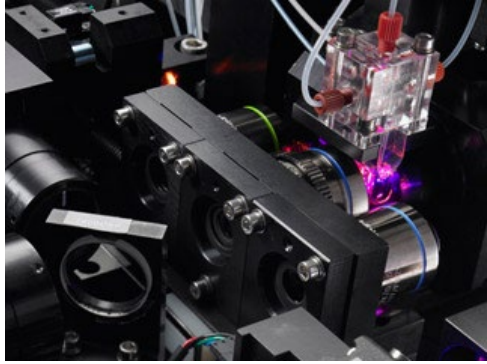
Laser	375 nm	405 nm	488 nm	561 nm	592 nm	642 nm
Example Dyes	DAPI Hoechst 33258 Alexa Fluor 405 Marina Blue Pacific Blue Cascade Blue LIVE/DEAD Violet DyLight 405 eFluor 450 Spectrum Aqua DyeCycle Violet Alexa Fluor 430 Pacific Orange Cascade Yellow Lucifer Yellow Qdot 525 Qdot 545 Qdot 565 Qdot 585 Qdot 605 Qdot 625 eFluor 605 Qdot 705 eFluor 650 Qdot 800	CFP DAPI Hoechst 33258 Alexa Fluor 405 Marina Blue Pacific Blue Cascade Blue LIVE/DEAD Violet DyLight 405 eFluor 450 Spectrum Aqua DyeCycle Violet Alexa Fluor 430 Pacific Orange Cascade Yellow Lucifer Yellow Qdot 525 Qdot 545 Qdot 565 Qdot 585 Qdot 605 Qdot 625 eFluor 605 Qdot 705 eFluor 650 Qdot 800	FITC GFP YFP Acridine Orange Alexa Fluor 488 Alexa Fluor 500 Alexa Fluor 514 SYTO Spectrum Green LysoTracker Green DyeCycle Green Calcium Green-1 MitoTracker Green DyLight 488 DsRed DiI Cy3 R-phycoerythrin OFP 7-AAD PE-Texas Red (ECD) PE-Alexa Fluor 610 Propidium Iodide PerCP PerCP-Cy5.5 PE-Alexa Fluor 647 PE-Alexa Fluor 680 PE-Cy5 PE-Cy5.5 DRAQ5 PE-Cy7 PE-Alexa Fluor 750	DsRed DiI Cy3 R-phycoerythrin OFP Alexa Fluor 546 Alexa Fluor 555 DyLight 549 Calcium Orange PE-Texas Red (ECD) PE-Alexa Fluor 610 Propidium Iodide Spectrum Orange MitoTracker Red LysoTracker Red RFP mCherry Alexa Fluor 568 Alexa Fluor 594 Alex Fluor 610 DyLight 594 Texas Red PE-Alexa Fluor 647 PE-Alexa Fluor 680 PE-Cy5 PE-Cy5.5 DRAQ5 Nile Blue PE-Cy7 PE-Alexa Fluor 750	mCherry Alexa Fluor 568 Alexa Fluor 594 Alexa Fluor 610 DyLight 594 Texas Red Spectrum Red Calcium Crimson Nile Blue APC APC-Cy5.5 DyLight 649 MitoTracker Deep Red APC-Cy7 APC-Alexa Fluor 750 APC-eFluor780	Nile Blue APC APC-Cy5.5 DyLight 649 MitoTracker Deep Red Alexa Fluor 647 Alexa Fluor 660 Alexa Fluor 680 DRAQ5 Cy5 Cy5.5 APC-Cy7 APC-Alexa Fluor 750 APC-eFluor780 DyLight 750

12 image channels

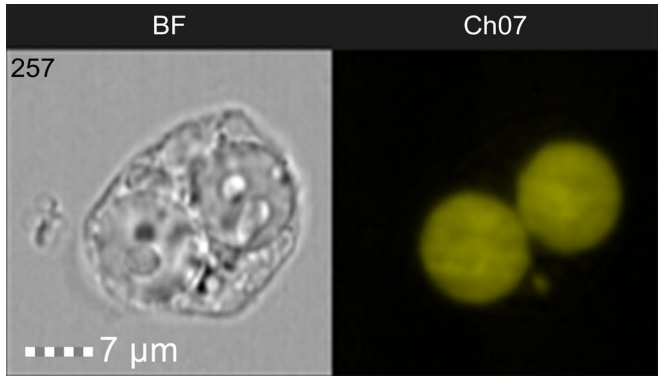
- Increase experimental capacity by utilizing a broader palette of fluorescent markers
- Improve data quality by reducing or eliminating spectral crosstalk between detection channels
- Improve throughput via multiplexing of experiments in a single tube



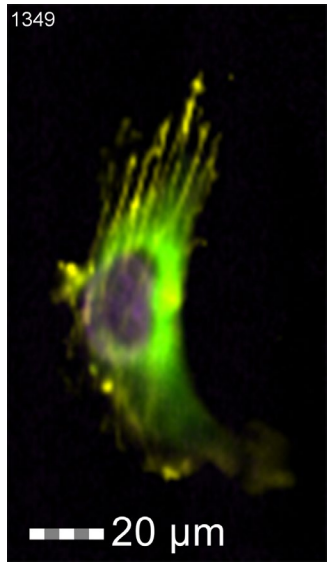
60X/40X/20X Magnification



- Superior quality, high numerical aperture (NA) objectives yield crisp images
- Convenient magnification changes via motorized objective moun



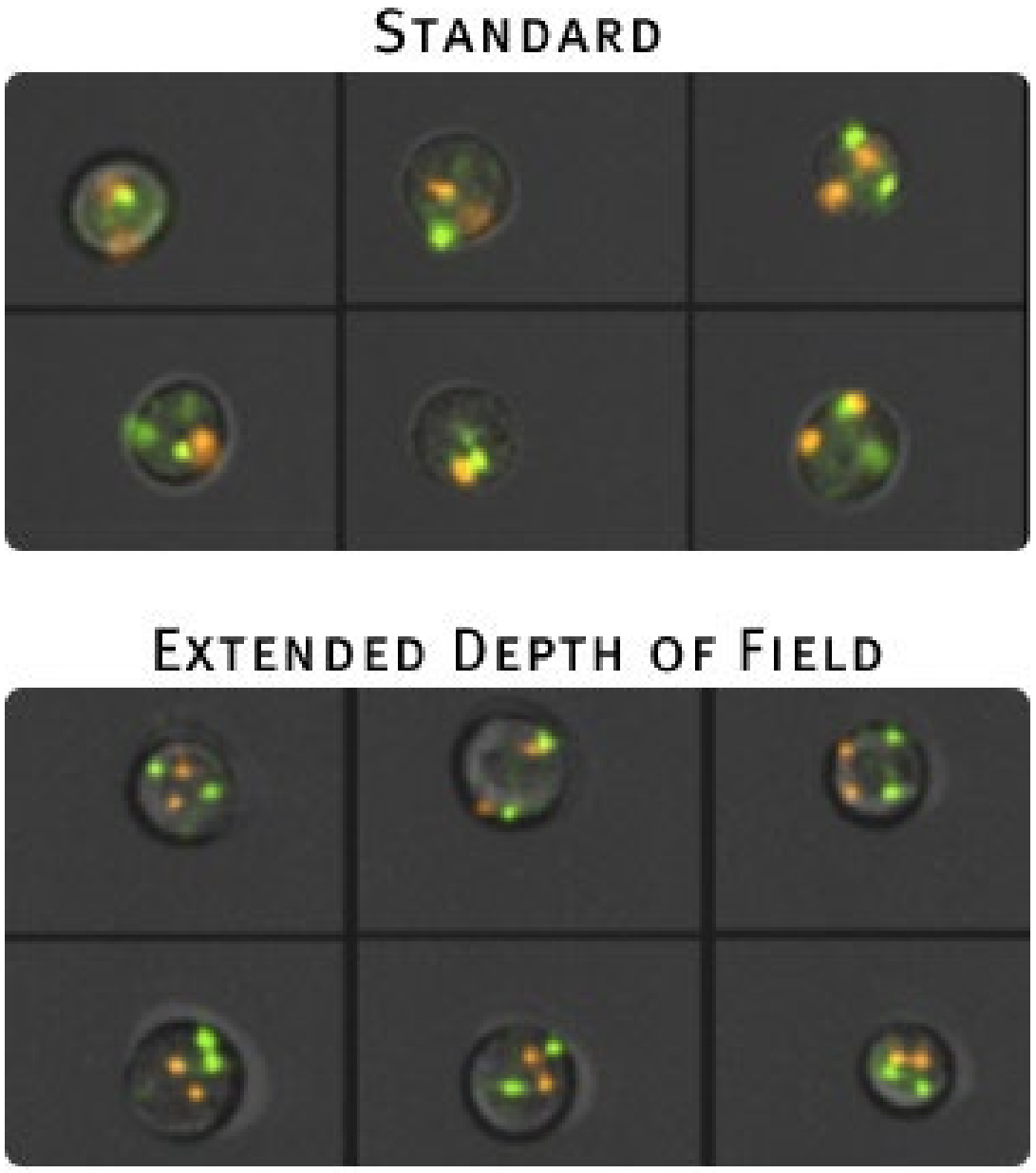
60X magnification for higher resolution



20X magnification for large cells

Extended Depth of Field

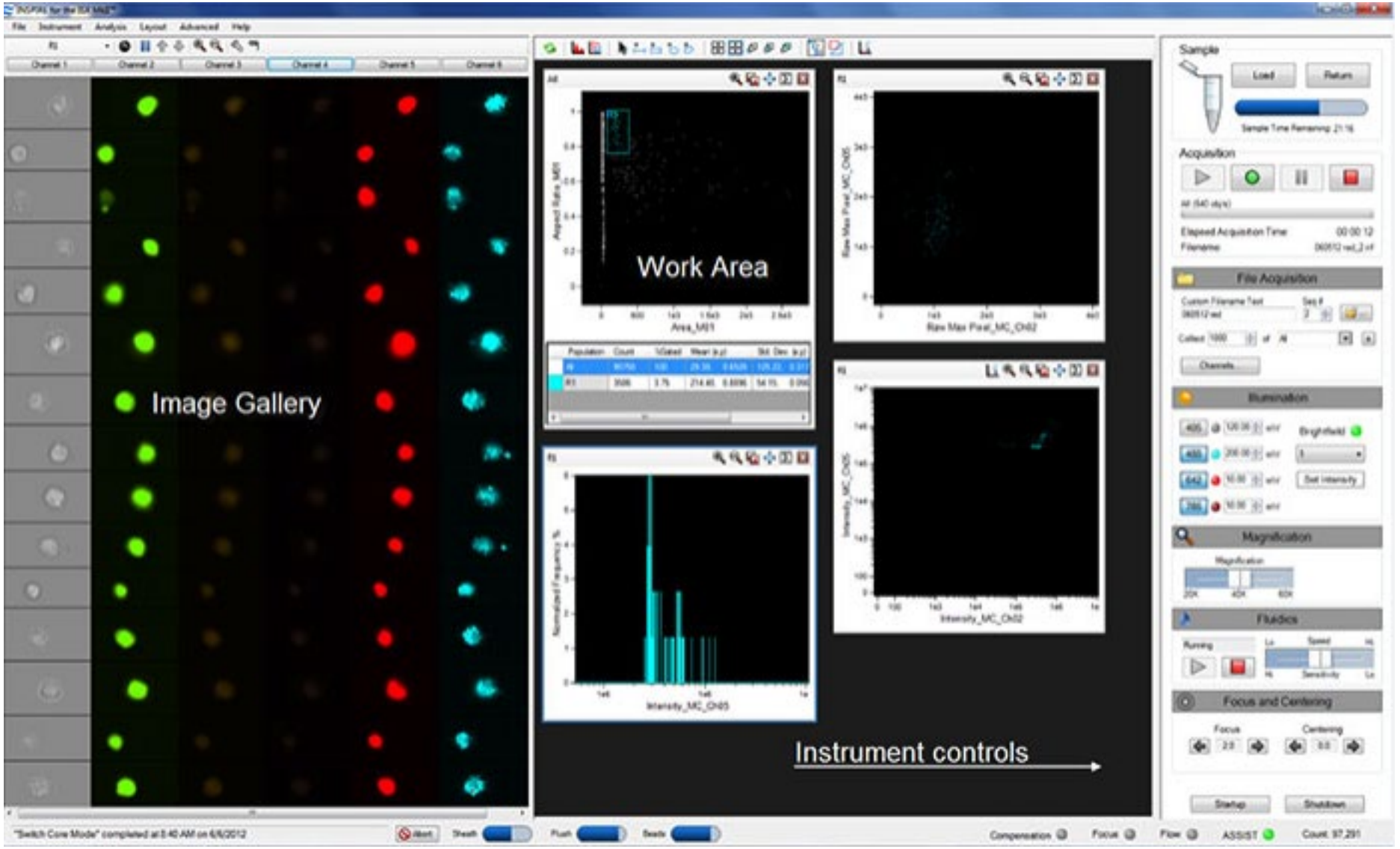
- Projects all features of a cell into a single plane of focus



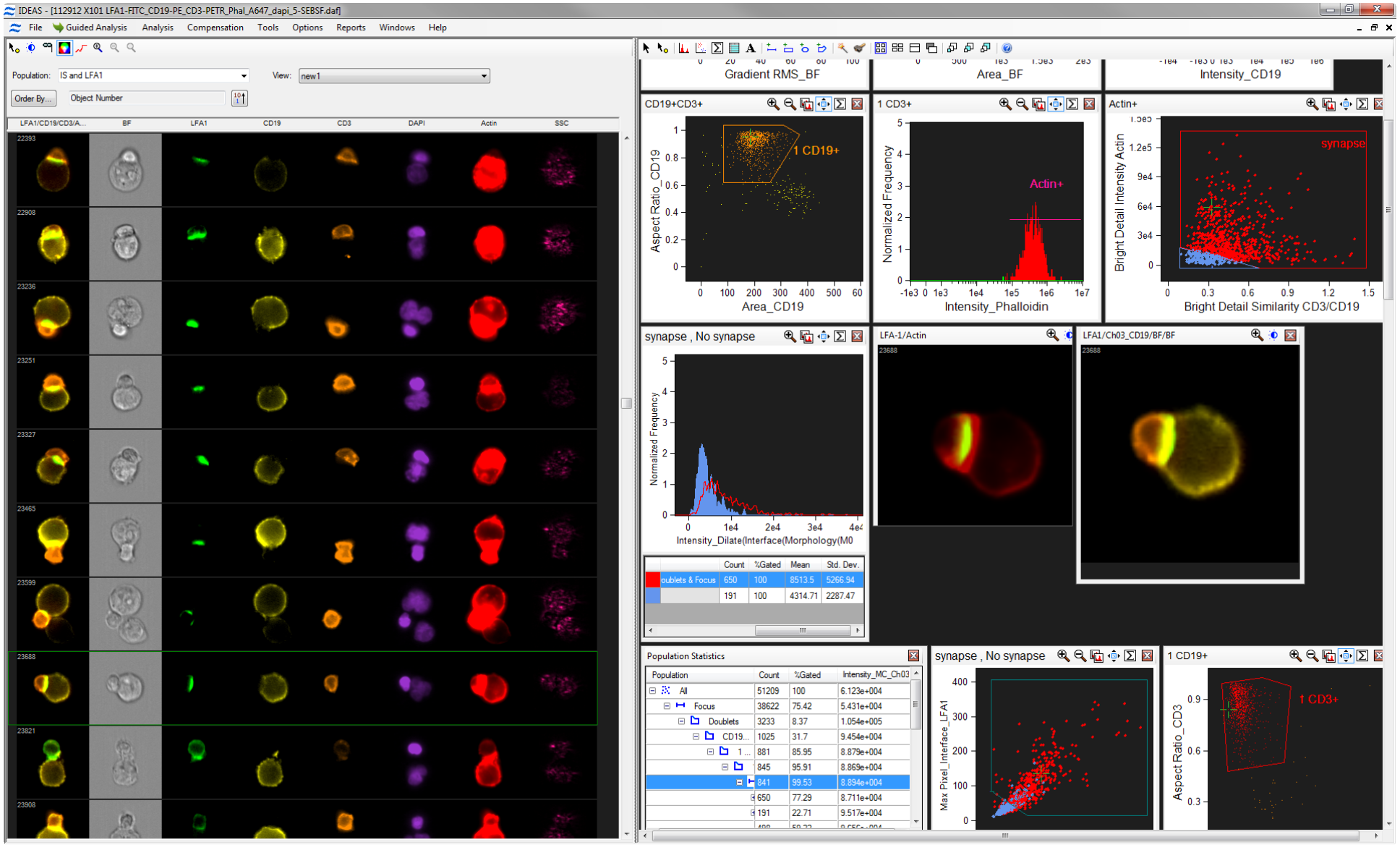
AutoSampler for Multiwell Plates

- Ideal for high throughput analysis of suspended cells
- Automatic process logging and error notification
- Automatic sipper rinse between samples and <0.5% carryover
- Automatic sample resuspension via aspiration
- Automated data analysis

INSPIRE™ Software

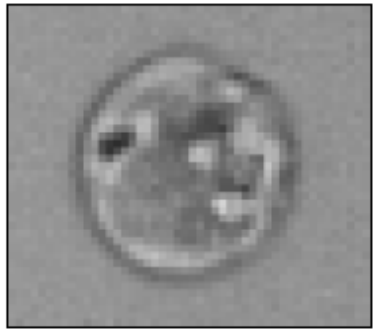


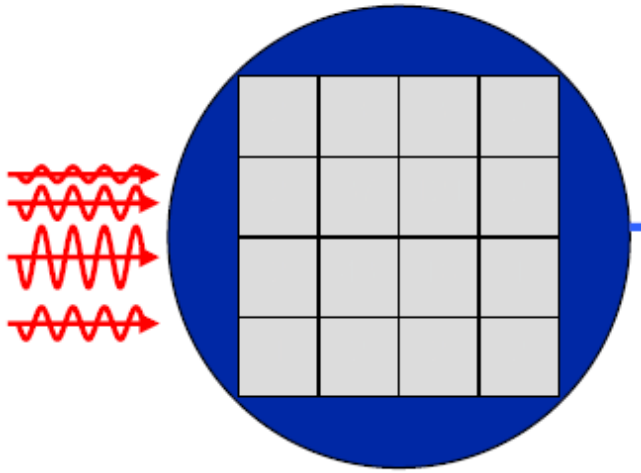
IDEAS® Software



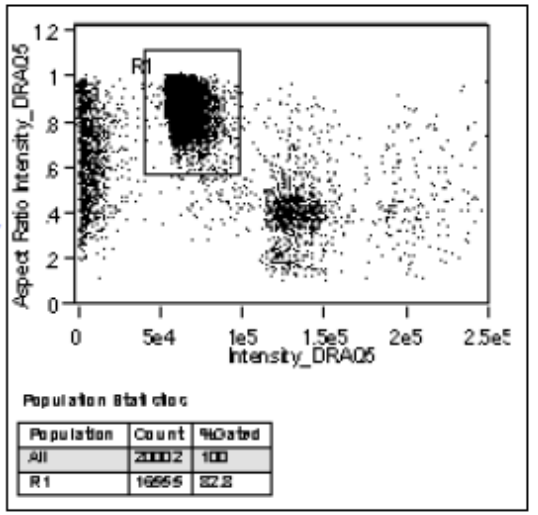
How data is generated?

Convert cells into photons → Convert photons into digits → Convert digits into features → Plot features of cell populations



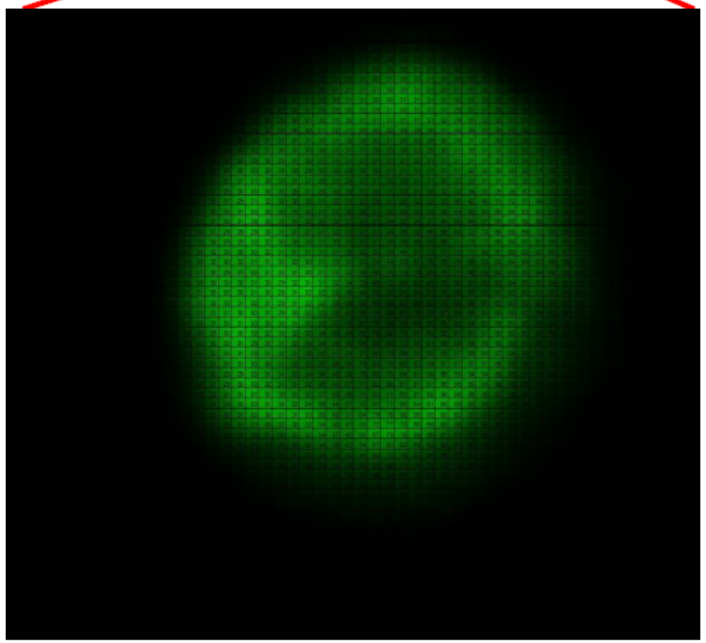


Area = 753
Aspect Ratio = 0.84
Centroid X = 43
Intensity = 153,119



@ cytometry facility UZH

How data is generated?



- Images are collected using a CCD camera.
- Pixel values from a 12 bit detector range from 0 to 4096.
- Each image is a grey scale two dimensional image of the cell.
- A mask is applied that determines the region of interest.
- Features are calculated based on the pixel values underneath the mask.

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How data is generated?

Converting Digits into Features

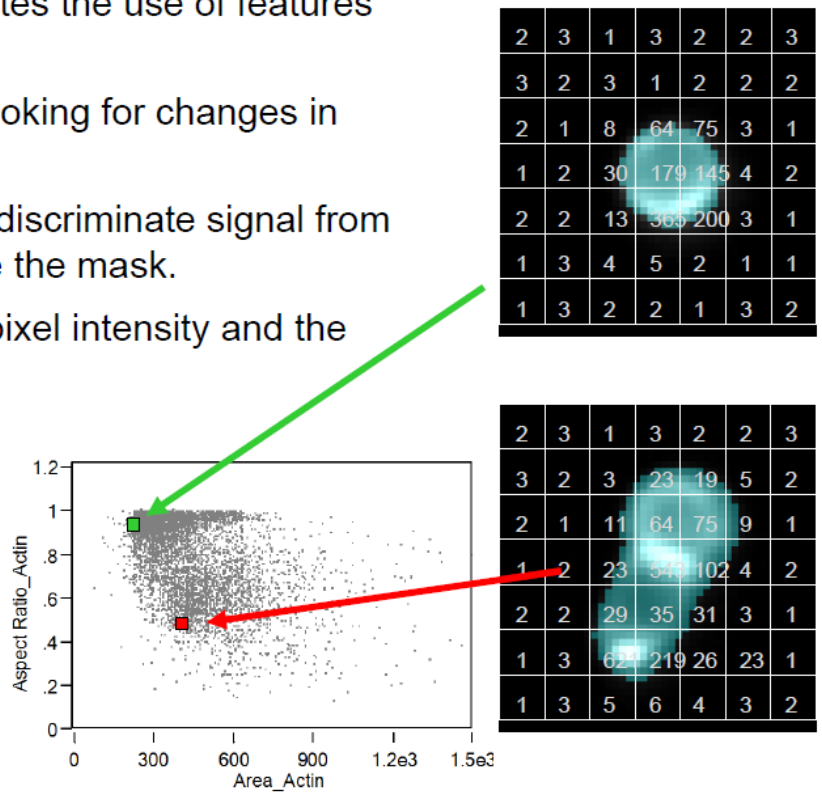
This example of singlet gating demonstrates the use of features and masks….

1) The computer first scans the image looking for changes in pixel values from the background
2) An automated threshold is applied to discriminate signal from background and uses this to generate the mask.
3) Finally features are calculated using pixel intensity and the location under the mask.

Features for singlet identification:

Area = square microns of the mask

Aspect ratio = minor axis/major axis



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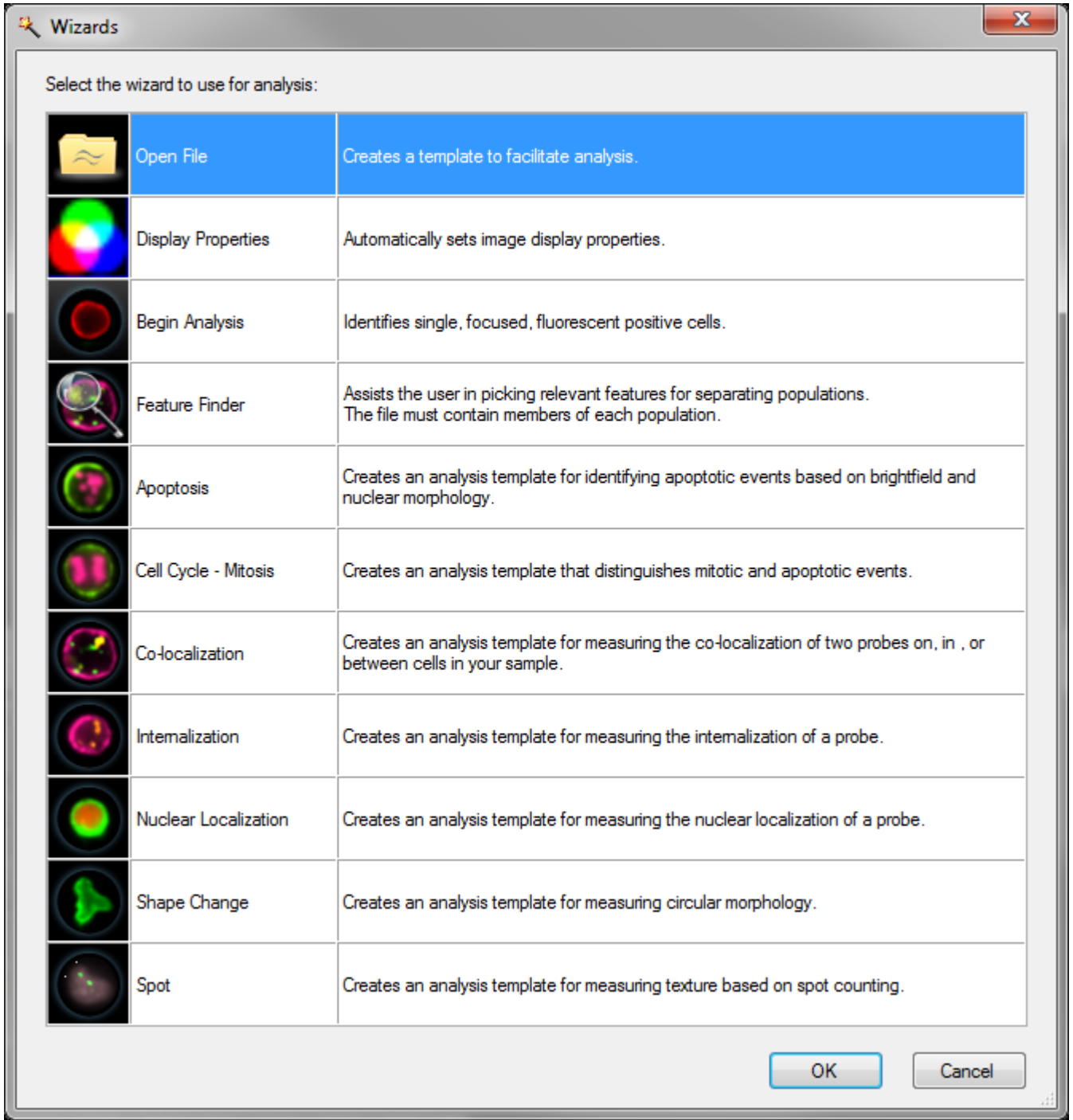
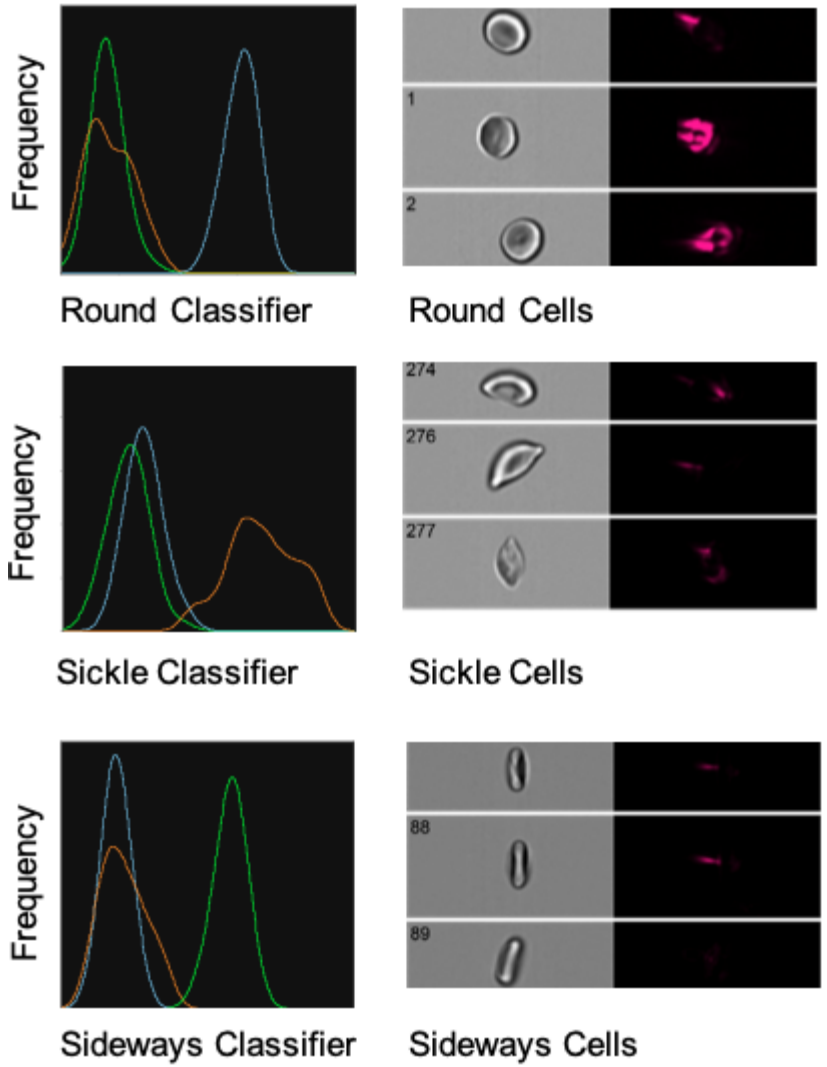


A wide range of features

Size
Shape
Texture
Signal strength
Comparison
Location
……

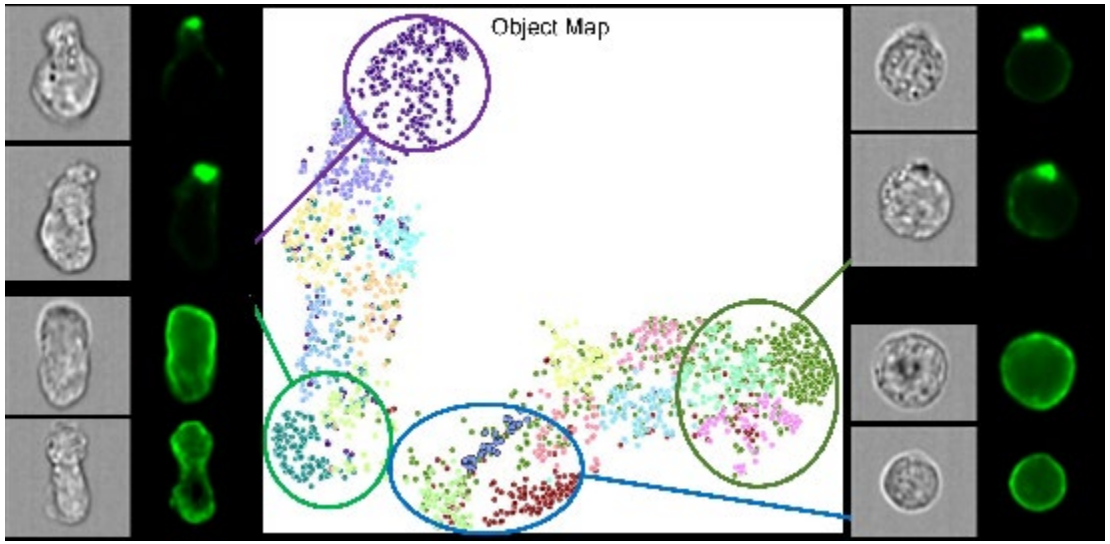
Machine Learning for IDEAS 6.3

- With Machine Learning, you can simply click on a cell of interest and the software will identify other cells with the same features.



Amnis® AI Image Analysis Software

- Visually categorize multiple cell classes in their experiment, which in turn trains the computer to identify those populations in unknown samples.
- Can combined with Amnis high-throughput imaging systems.



frontiers in Immunology

METHODS
published: 18 February 2020
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Imaging Flow Cytometry Protocols for Examining Phagocytosis of Microplastics and Bioparticles by Immune Cells of Aquatic Animals

*Youngjin Park[1], Isabel S. Abihssira-García[1], Sebastian Thalmann[2], Geert F. Wiegertjes[3], Daniel R. Barreda[4], Pål A. Olsvik[1] and Viswanath Kiron[1]**

[1] Faculty of Biosciences and Aquaculture, Nord University, Bodø, Norway, [2] Luminex B.V., 's-Hertogenbosch, Netherlands, [3] Aquaculture and Fisheries Group, Wageningen University & Research, Wageningen, Netherlands, [4] Department of Biological Sciences, University of Alberta, Edmonton, AB, Canada

High-Sensitivity Assessment of Phagocytosis by Persistent Association-Based Normalization

Therese de Neergaard, Martin Sundwall, Sebastian Wrighton and Pontus Nordenfelt

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Check for updates

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Phagocytosis measurements

- Direct observation with electron or light microscopy.
- Indirect methods in which the prey is labeled with a marker such as radiation or fluorescence.
- Differential double labeling, quenching, or by fluorescent color change induced by the intracellular environment are used to discriminate between prey that are on the outside or the inside of the phagocyte.

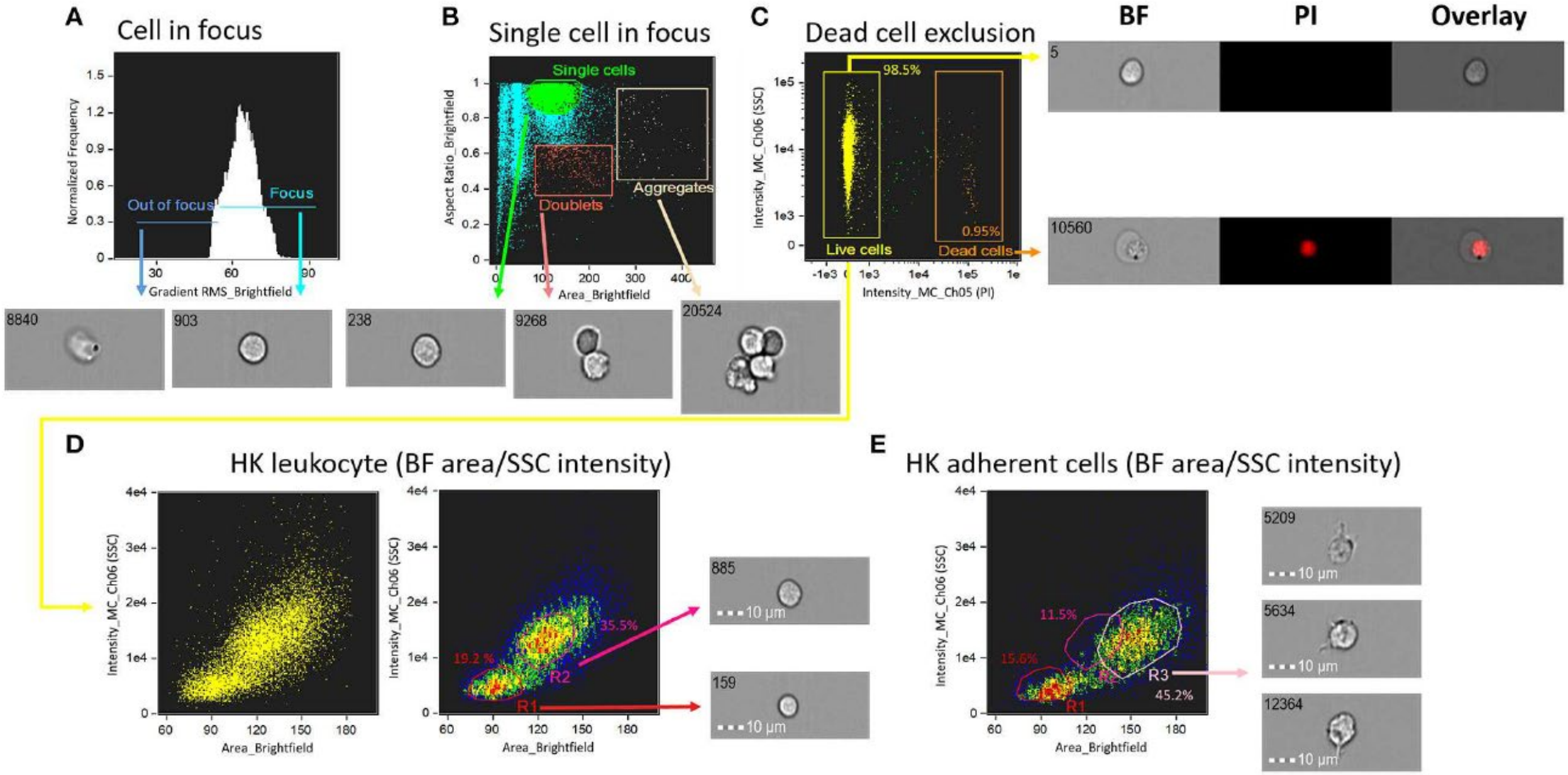
Available phagocytosis techniques

- The most common instrument used today is the flow cytometer, in which fluorescently labeled prey and phagocytes can be quantified in a high-throughput manner.
- Fluorescence microscopy can be used for qualitative confirmation.
- Imaging flow cytometry combines the high-throughput benefits of flow cytometry with the ability to do single-cell image analysis and provides an option for detailed analysis of phagocytosis.

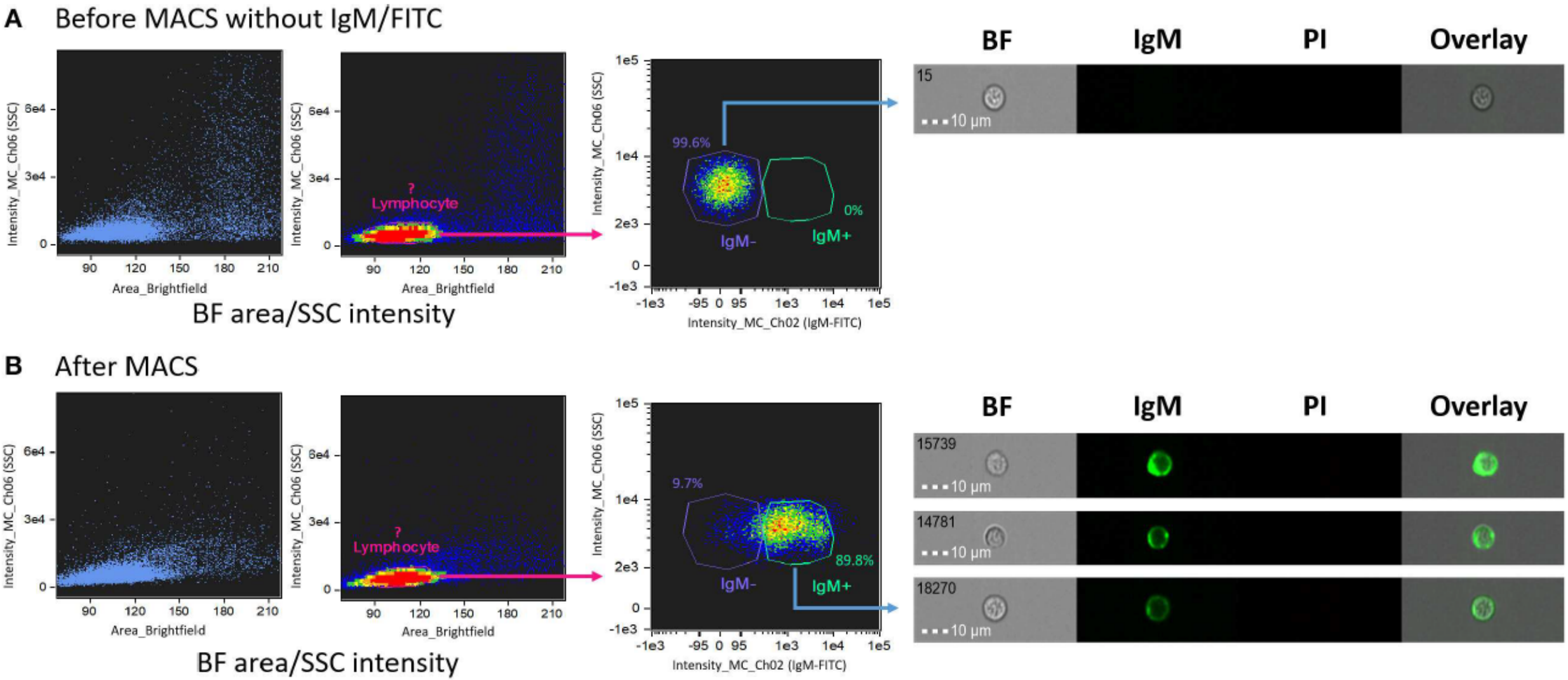
Aim of the study

- Live/dead cell assays and identification of cell types;
- Phagocytosis of degradable and non-degradable particles by Atlantic salmon head kidney cells;
- The effect of incubation temperature on phagocytosis of degradable particles in three aquatic animals–Atlantic salmon, Nile tilapia, and blue mussel;

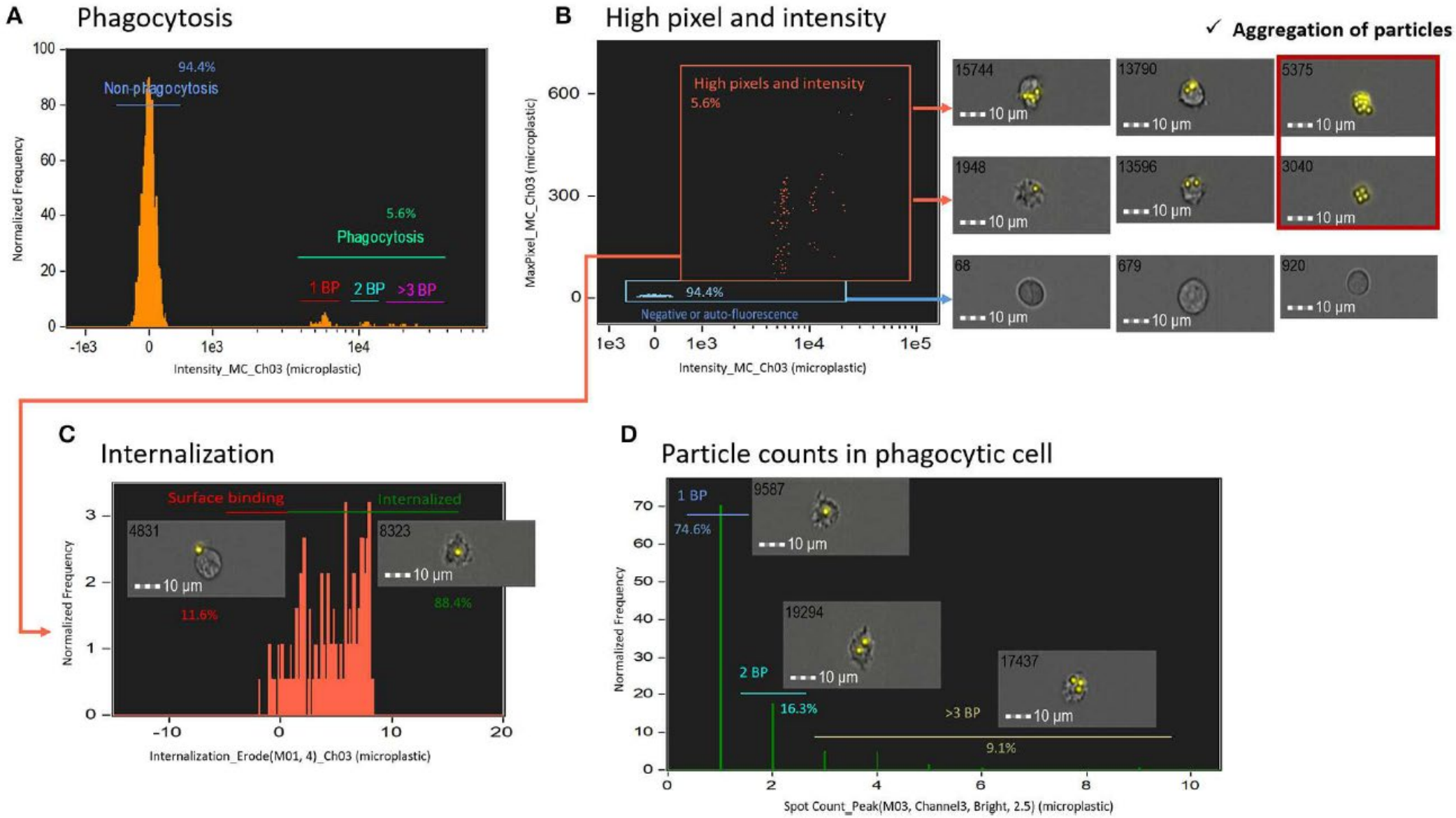
Identification of cell types



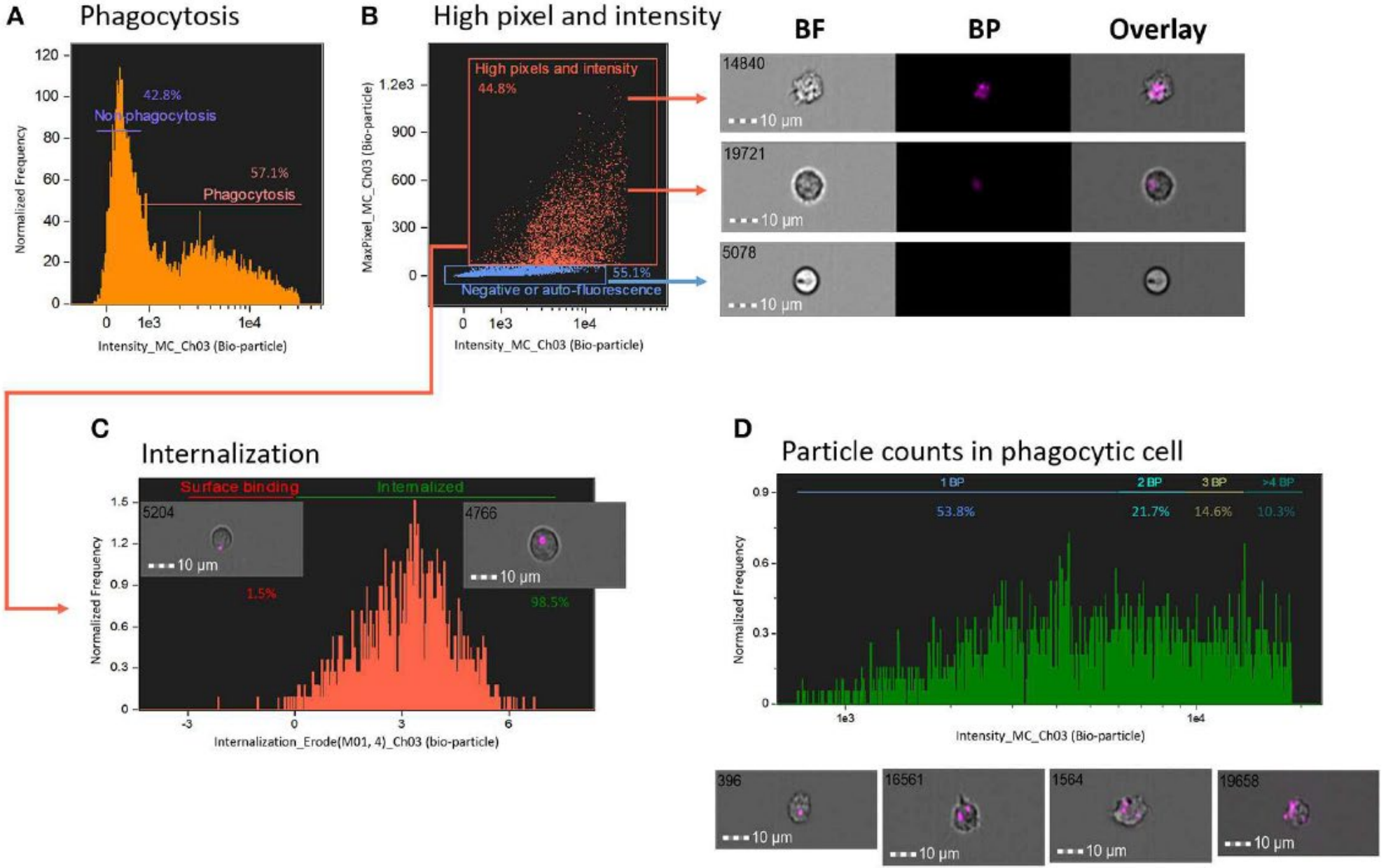
Identification of cell types



Phagocytosis using non-degradable fluorescent particles (microplastics)



Phagocytosis using degradable fluorescent particles (pHrodoTM)



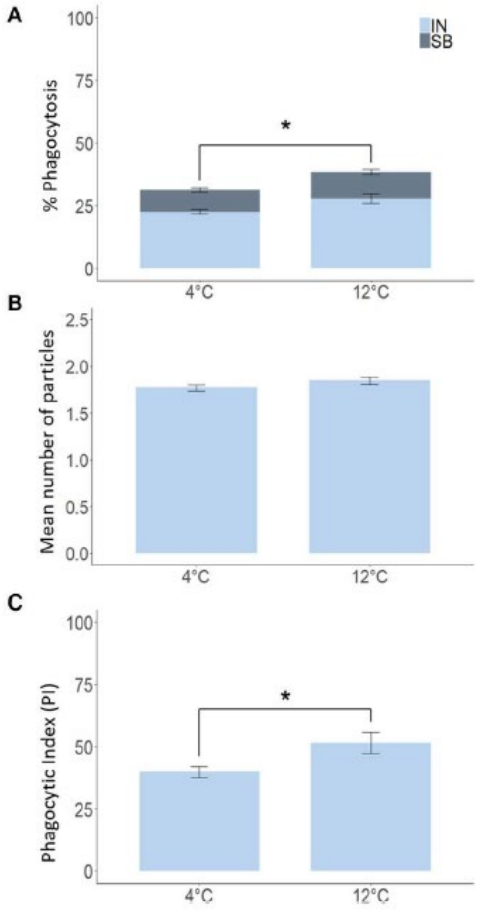
Phagocytosis of Atlantic salmon head kidney macrophages incubated at different temperatures

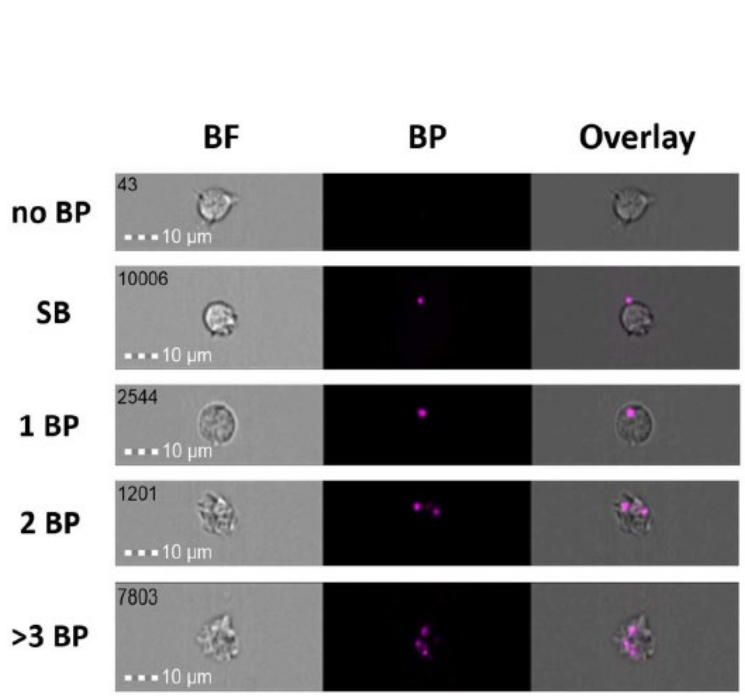
Phagocytic index (PI)=

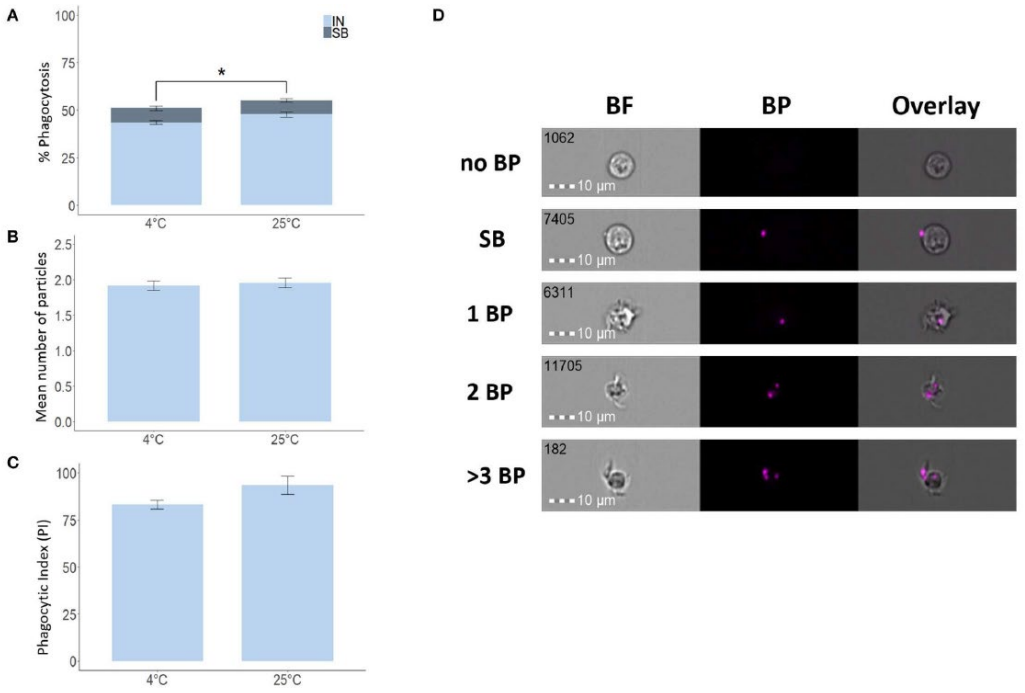
[%phagocytic cells containing at least one particle]

×

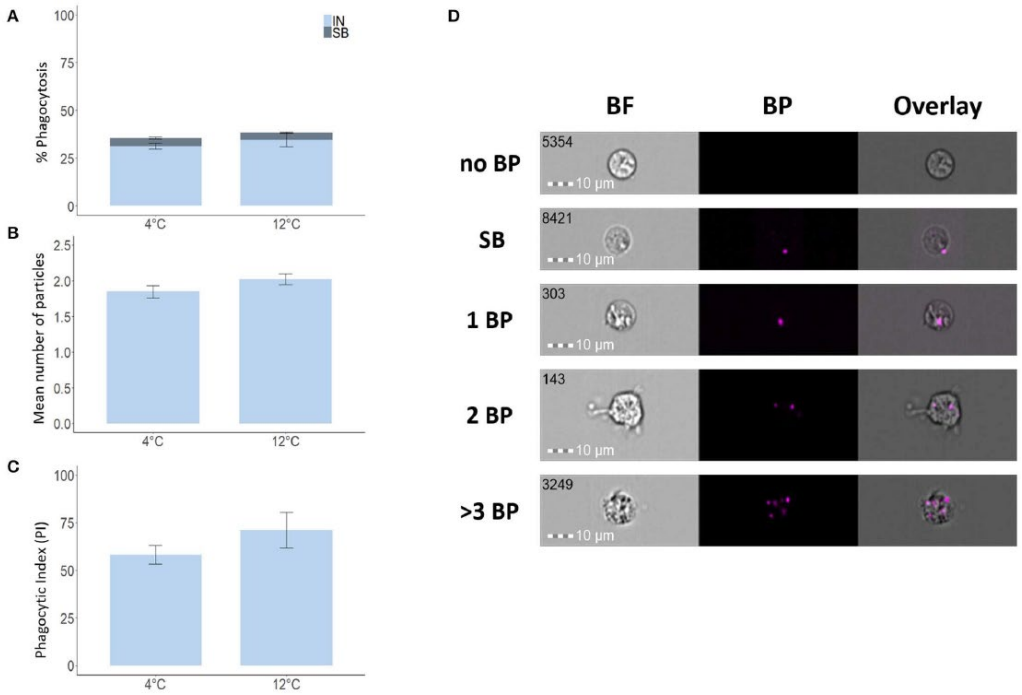
[mean particle count per phagocytic cell]







Nile tilapia head kidney macrophages



blue mussel hemocytes

Summary

- Imaging flow cytometry combines the power of fluorescent microscopy and flow cytometry.
- Qualitatively and quantitatively analyze phagocytosis.

frontiers in Immunology

METHODS
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Evaluation of Canonical Inflammasome Activation in Human Monocytes by Imaging Flow Cytometry

Silvia Lucena Lage[1]*, *Venina Marcela Dominical*[2]*, *Chun-Shu Wong*[1] and *Irini Sereti*[1]

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The Journal of Immunology

Inflammasome and Caspase-1 Activity Characterization and Evaluation: An Imaging Flow Cytometer–Based Detection and Assessment of Inflammasome Specks and Caspase-1 Activation

Abhinit Nagar,* Richard A. DeMarco,† and Jonathan A. Harton*

The Journal of Immunology

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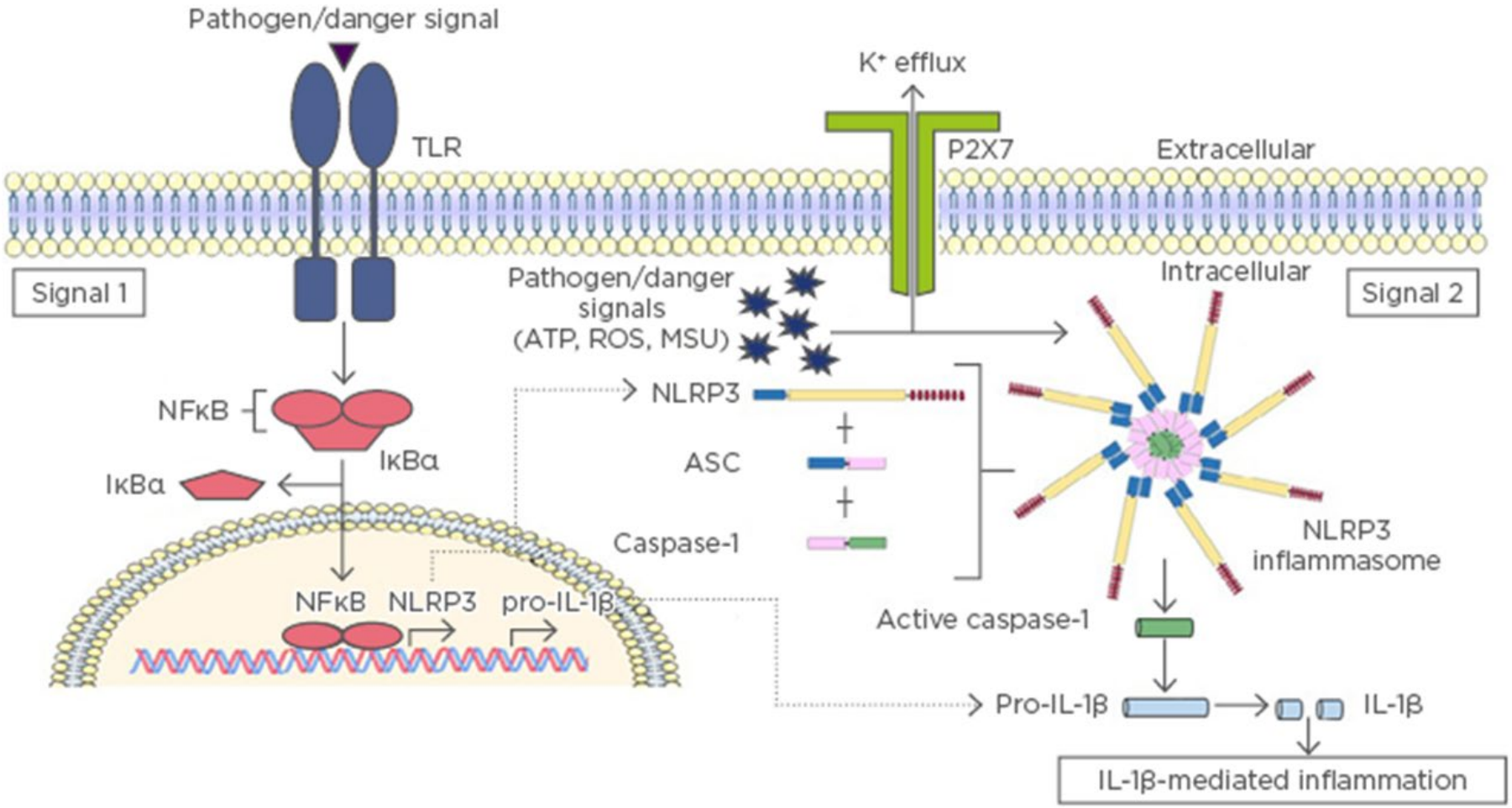
Inflammasome: Cytosolic multiprotein oligomers of the innate immune system responsible for the activation of inflammatory responses.



EMJ Allergy Immunol. 2018;3[1]:106-113.

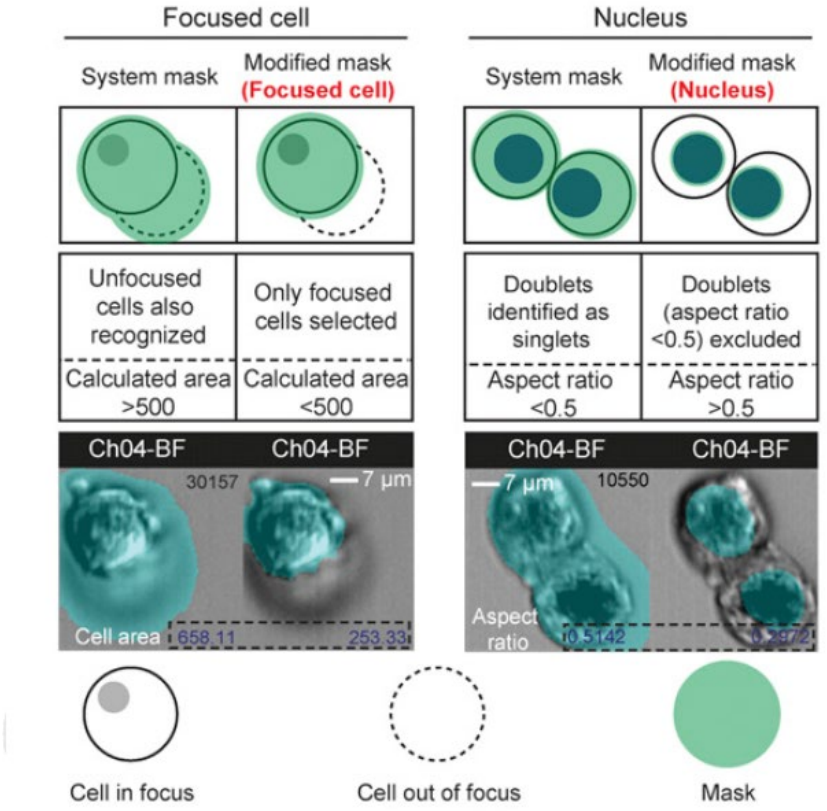
Structure of inflammasome

- Inflammasome assembly is accompanied by the formation of a typically singular, perinuclear structure called a “speck.”
- Speck has a toroidal appearance with an apparent diameter of ~1 um.
- Speck formation is a rapid, all-or-none event that coincides with activation of caspase-1, it is frequently used as complementary readout for inflammasome activation.

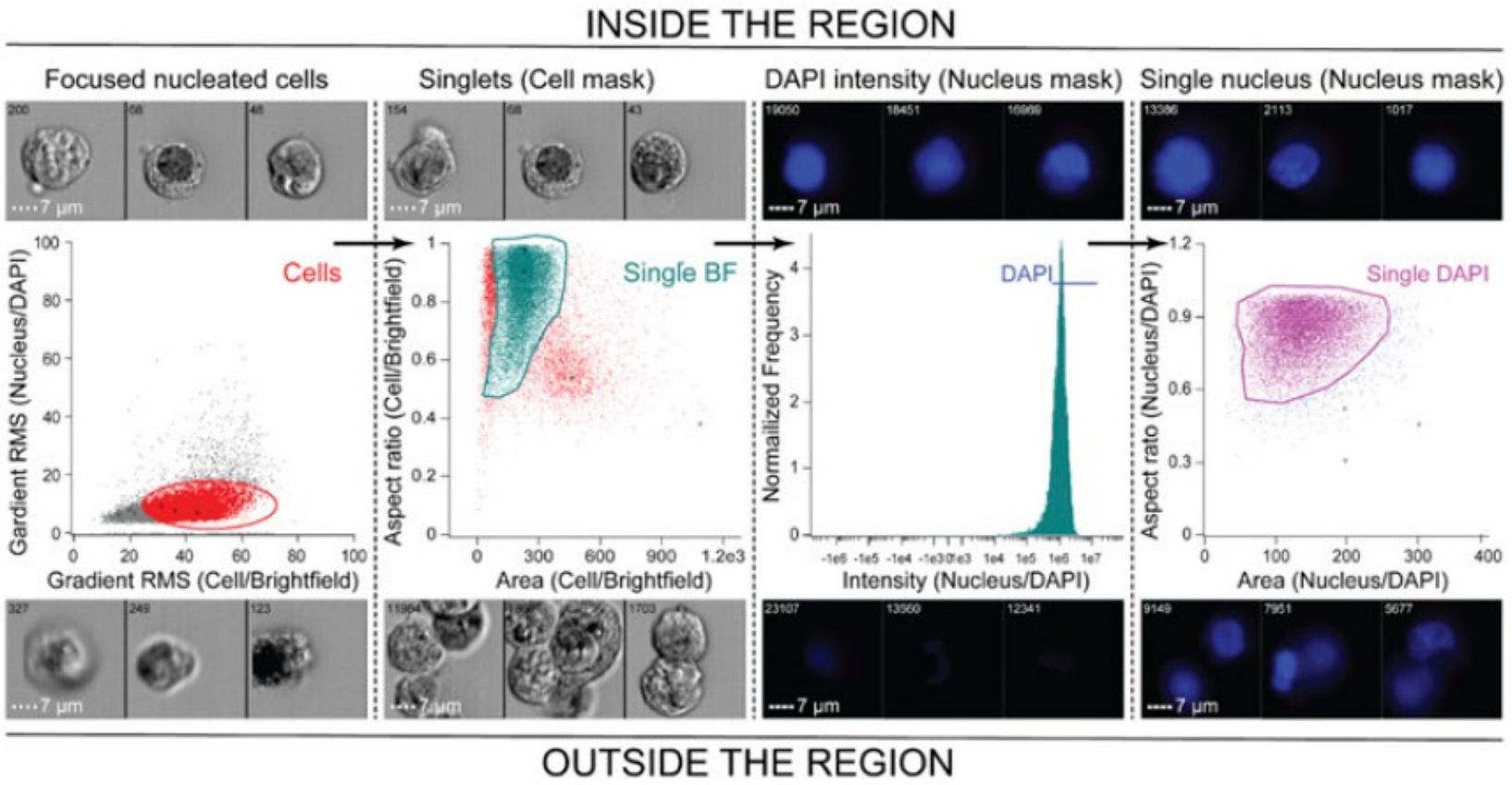
ICCE: inflammasome and caspase-1 activity characterization and evaluation

- Imaging flow cytometry
- Computational quantitative image analysis
- Single-cell analysis: simultaneous assessment of caspase-1 activity, including its distribution and localization in ASC-expressing cells.
- Quantifies ASC speck-containing cells and evaluates speck size
- Eliminates nonspeck-like aggregates of ASC (false positive artifacts)

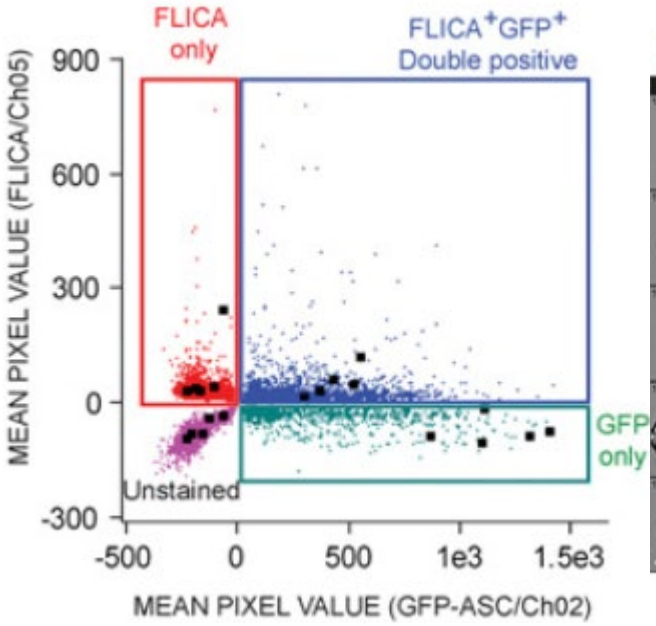
Gating and masking strategy:
Identifying focused cells & single nuclei

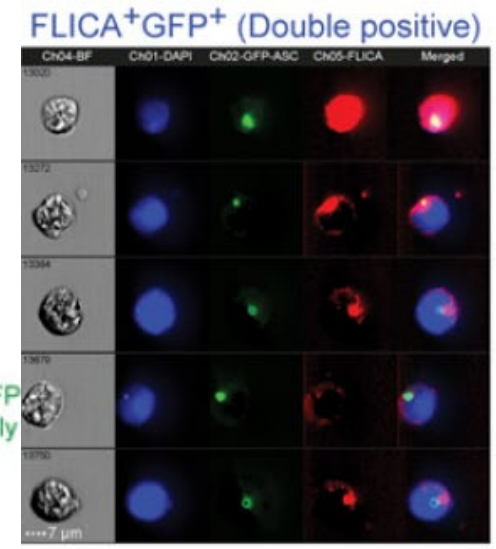


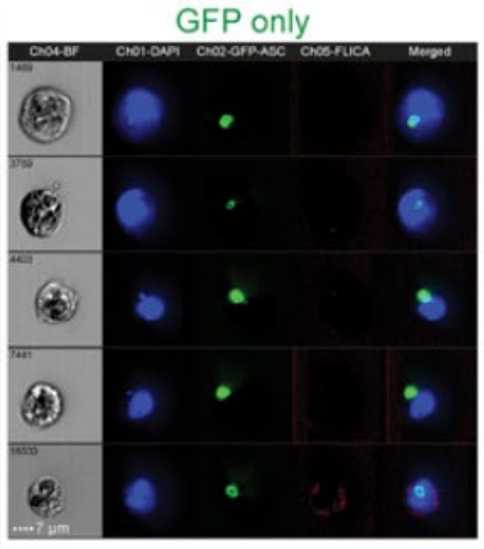
Gating and masking strategy:
Identifying single cells

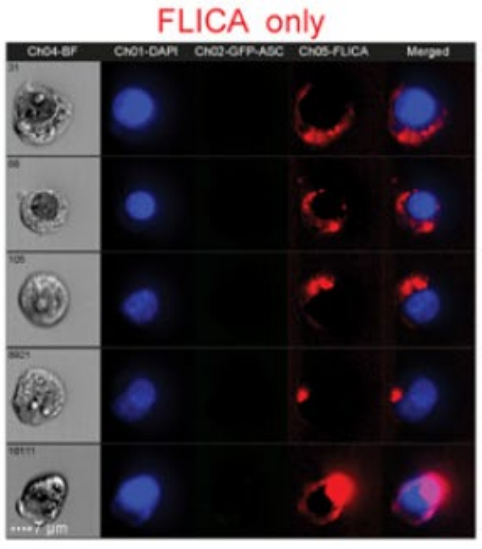


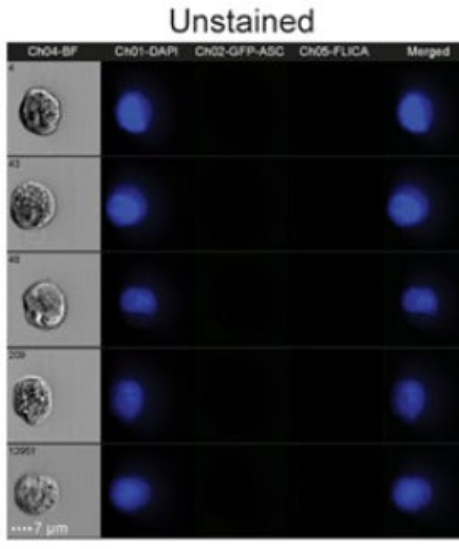
Gating and masking strategy:
identify ASC (GFP) and active caspase-1 (FLICA) double-positive cells







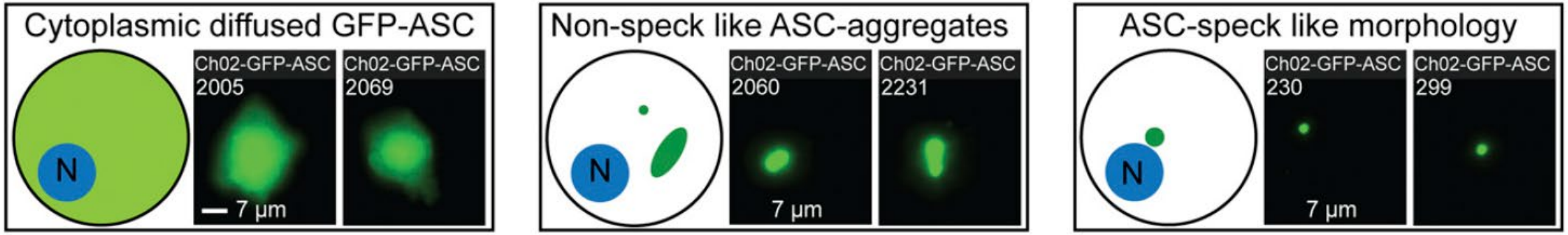




ASC was cloned into the pEGFP-C3 expression vector backbone using HindIII and KpnI digestion to generate GFP-ASC and transfected into HEK293T cells.

Validating masking strategy for ASC specks

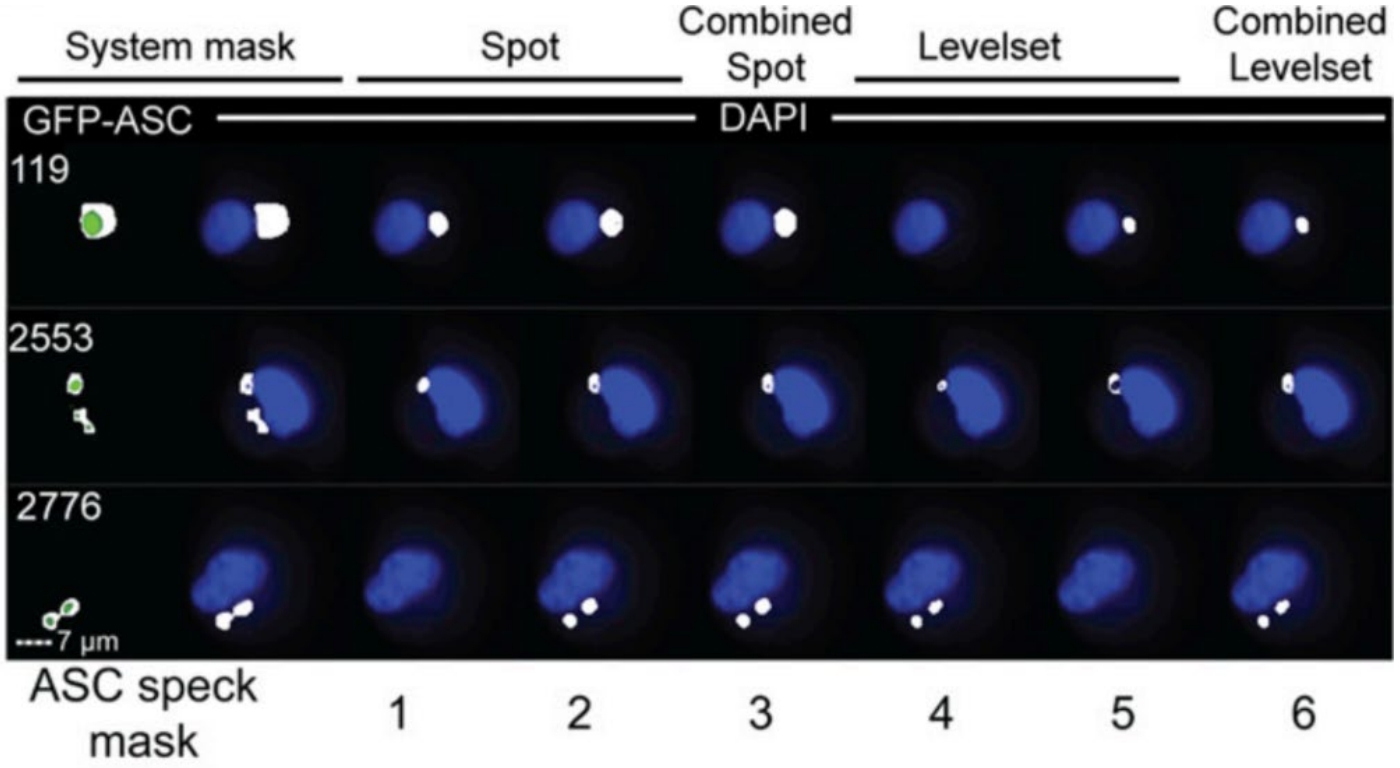
Automate detection of bona fide ASC specks and eliminate nonspeck-like aggregates



- GFP has a weak tendency to oligomerize, which could result in false positives.

Mask Name	Reconstituted ASC Specks; HEK293T (ASC-GFP)	Native ASC Specks	
		THP-1	Primary Human Monocytes
ASC mask 1	Spot (M02, Ch02-GFP-ASC, bright, 2, 10, 3)	Spot (M02, Ch02-AF488, bright, 3, 5, 1)	Spot (M05, Ch05-AF594, bright, 3, 5, 1)
ASC mask 2	Spot (M02, Ch02-GFP-ASC, bright, 1, 20, 3)	Spot (M02, Ch02-AF488, bright, 3, 3, 1)	Spot (M05, Ch05-AF594, bright, 3, 3, 1)
ASC mask 3	ASC mask 1 Or ASC mask 2	ASC mask 1 Or ASC mask 2	ASC mask 1 Or ASC mask 2
ASC mask 4	Levelset (ASC mask 3, Ch02-GFP-ASC, middle, 5)	Levelset (ASC mask 3, Ch02-AF488, middle, 5)	Levelset (ASC mask 3, Ch05-AF594, middle, 5)
ASC mask 5	Levelset (ASC mask 3, Ch02-GFP-ASC, bright, 5)	Levelset (ASC mask 3, Ch02-AF488, bright, 5)	Levelset (ASC mask 3, Ch05-AF594, bright, 5)
ASC mask 6	ASC mask 4 Or ASC mask 5	ASC mask 4 Or ASC mask 5	ASC mask 4 Or ASC mask 5
ASC mask 7	Fill (ASC mask 6)	Fill (ASC mask 6)	Fill (ASC mask 6)
ASC mask 8	Range (ASC mask 7, 15–500, 0.4–1)	Range (ASC mask 7, 0–200, 0.3–1)	Range (ASC mask 7, 0–200, 0.5–1)
ASC mask 9	Intensity (ASC mask 8, Ch02-GFP-ASC, 750–4095)	Intensity (ASC mask 8, Ch02-AF488, 300–4095)	Intensity (ASC mask 8 Ch05-AF594, 750–4095)
ASC mask 10	Dilate (ASC mask 9, 1)	Dilate (ASC mask 9, 1)	Dilate (ASC mask 9, 1)
ASC mask 11	Fill (ASC mask 10)	Fill (ASC mask 10)	Fill (ASC mask 10)
ASC mask 12	Threshold (ASC mask 11, Ch02-GFP-ASC, 80)	Threshold (ASC mask 11, Ch02-AF488, 52)	Threshold (ASC mask 11, Ch05-AF594, 52)
ASC mask 13	Range (ASC mask 12, 15–450, 0.6–1)	Range (ASC mask 12, 2–18, 0.5–1)	Range (ASC mask 12, 5–20, 0.6–1)
ASC mask 14	Component (1, area, ASC mask 13, descending)	Component (1, area, ASC mask 13, descending)	Component (1, area, ASC mask 13, descending)

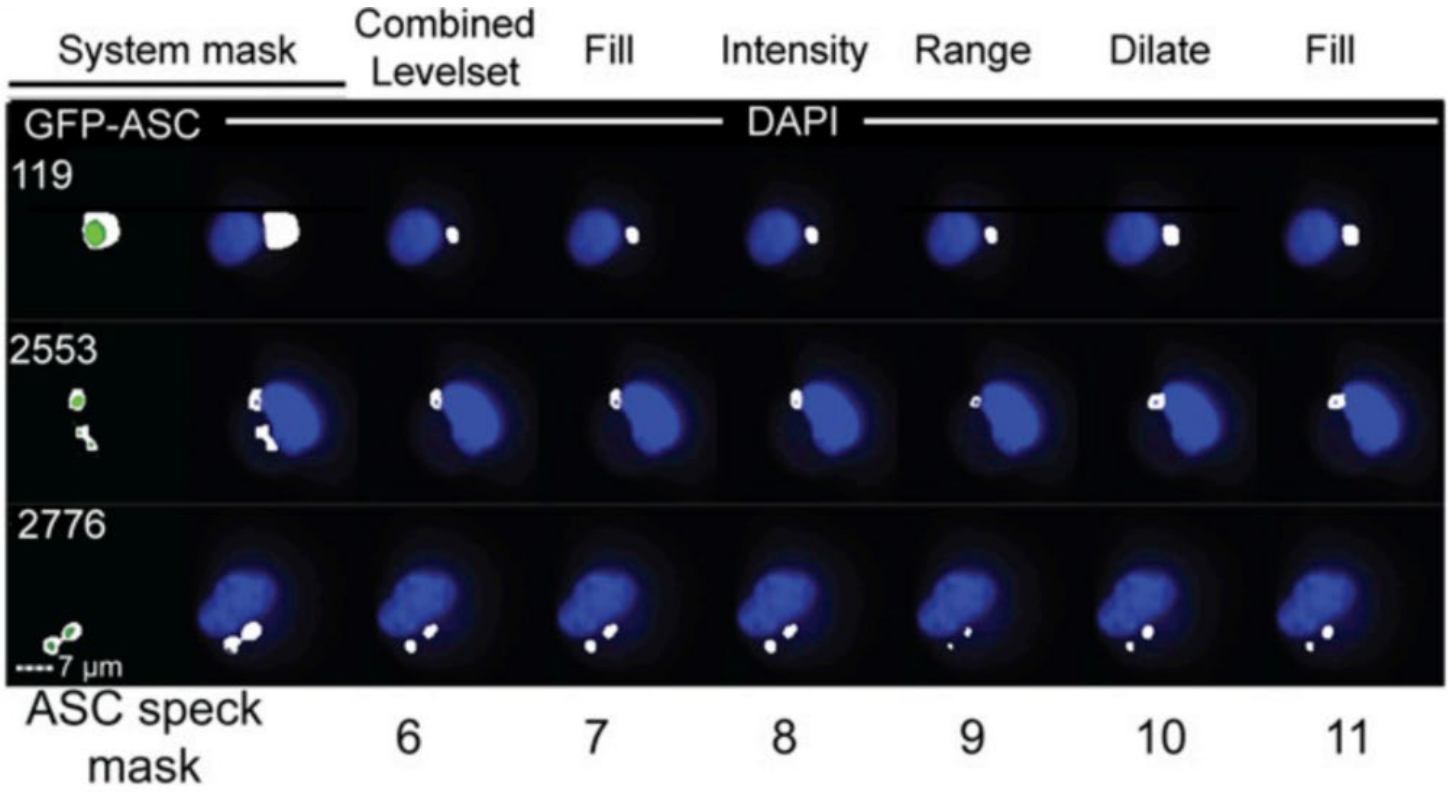
Strategy step 1: separation of punctate from diffuse signal



Identifying bright, punctate staining.

"ASC speck mask" : the default system mask on the GFP channel alone, with this mask superimposed on the DAPI channel;
ASC mask 1: recognize single punctate staining.
ASC mask 2: Identify multiple puncta.
ASC mask 3: Combined spot mask 1 and 2.
ASC mask 4: Levelset masks based on GFP signal threshold: detect dimmer signals.
ASC mask 5: Detect brighter signals.
ASC mask 6: Combined levelset masks 4 and 5.

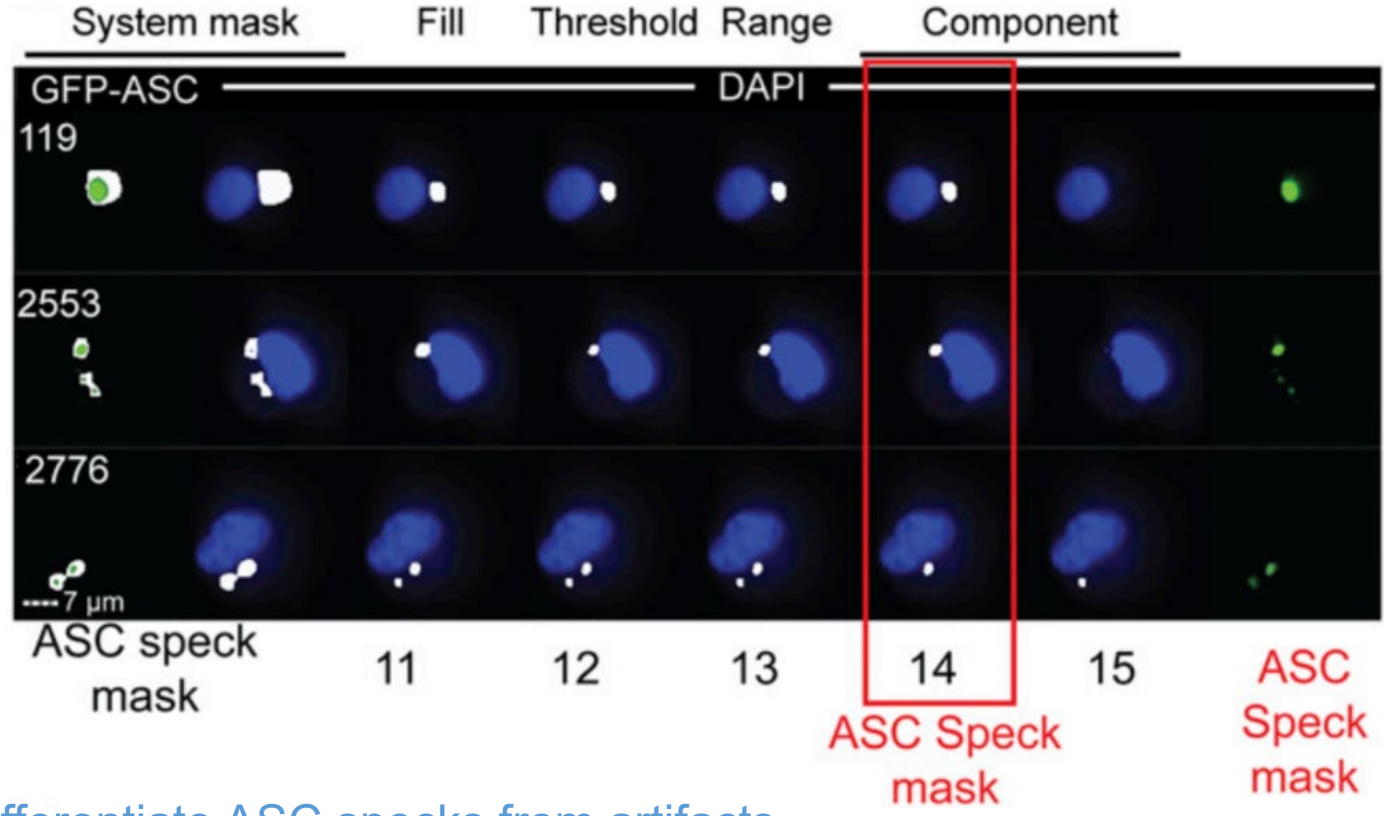
Strategy step 2: discrimination of circular from irregular structures



Differentiating GFP aggregates from ASC specks.

ASC mask 7: A fill mask was applied to make ASC mask 6 uniform.
ASC mask 8: An intensity mask to select pixels above a selected intensity.
ASC mask 9: A range mask was applied to define minimum diameter and near circularity criteria.
ASC mask 10: A dilate mask was used to refine the size of ASC mask 9.
ASC mask 11: Another fill mask for uniformity.

Strategy step 3: selection based on area, circularity, and number



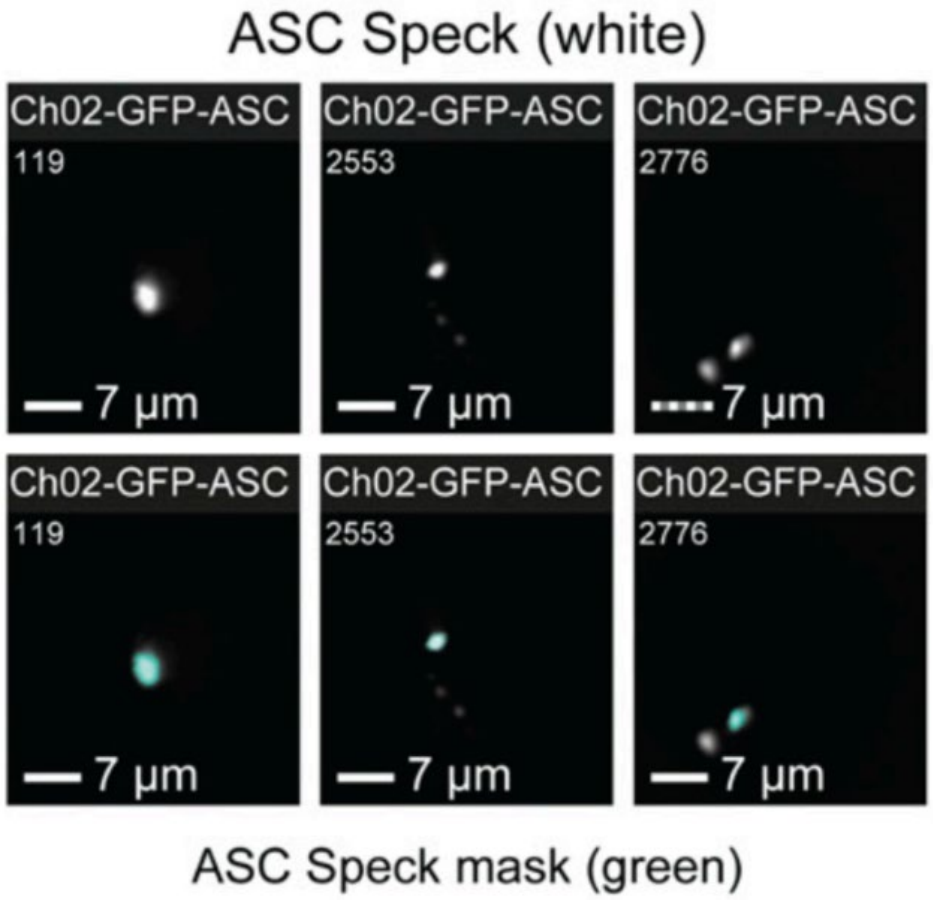
Final refinements to differentiate ASC specks from artifacts.

ASC mask 12: a threshold mask based on the intensity of visually inspected specks was used to exclude dimmer objects (GFP aggregates).

ASC mask 13: by a refined range mask to more stringently select for circularity (aspect ratio 0.6) and further constrain the signal area to within ~30 um2.

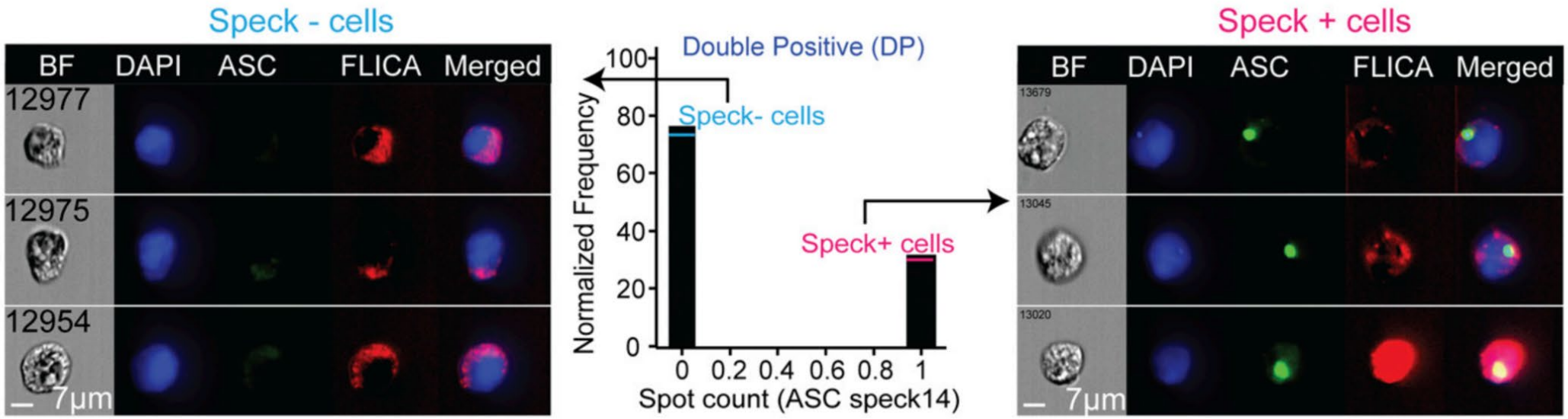
ASC mask 14: to ensure that cells with multiple ASC specks are not counted multiple times, which would inflate the frequency of speck-containing cells, the mask with the highest area (highlighted by red box) is referred to as ASC speck mask.

ASC speck mask accurately reflects speck morphology



- GFP has a weak tendency to oligomerize, which could result in false positives.
- GFP aggregates are expected to be random in shape with less signal intensity, whereas ASC specks are more organized and circular in appearance
- → To select for circular puncta with high signal intensity and eliminate non-speck, aggregate artifacts.

Determining ASC speck frequency



spot count of 0 = speck negative, 1 = speck positive [+]

Characterizing distribution and area of active caspase-1

- Active inflammasomes are characterized by the presence of active capsase-1, which can be visualized with FLICA, a fluorescent inhibitor that binds irreversibly to the caspase-1 active site.

- Use ImageStreamX to construct an appropriate FLICA mask to differentiate between diffuse and localized caspase-1 activity.

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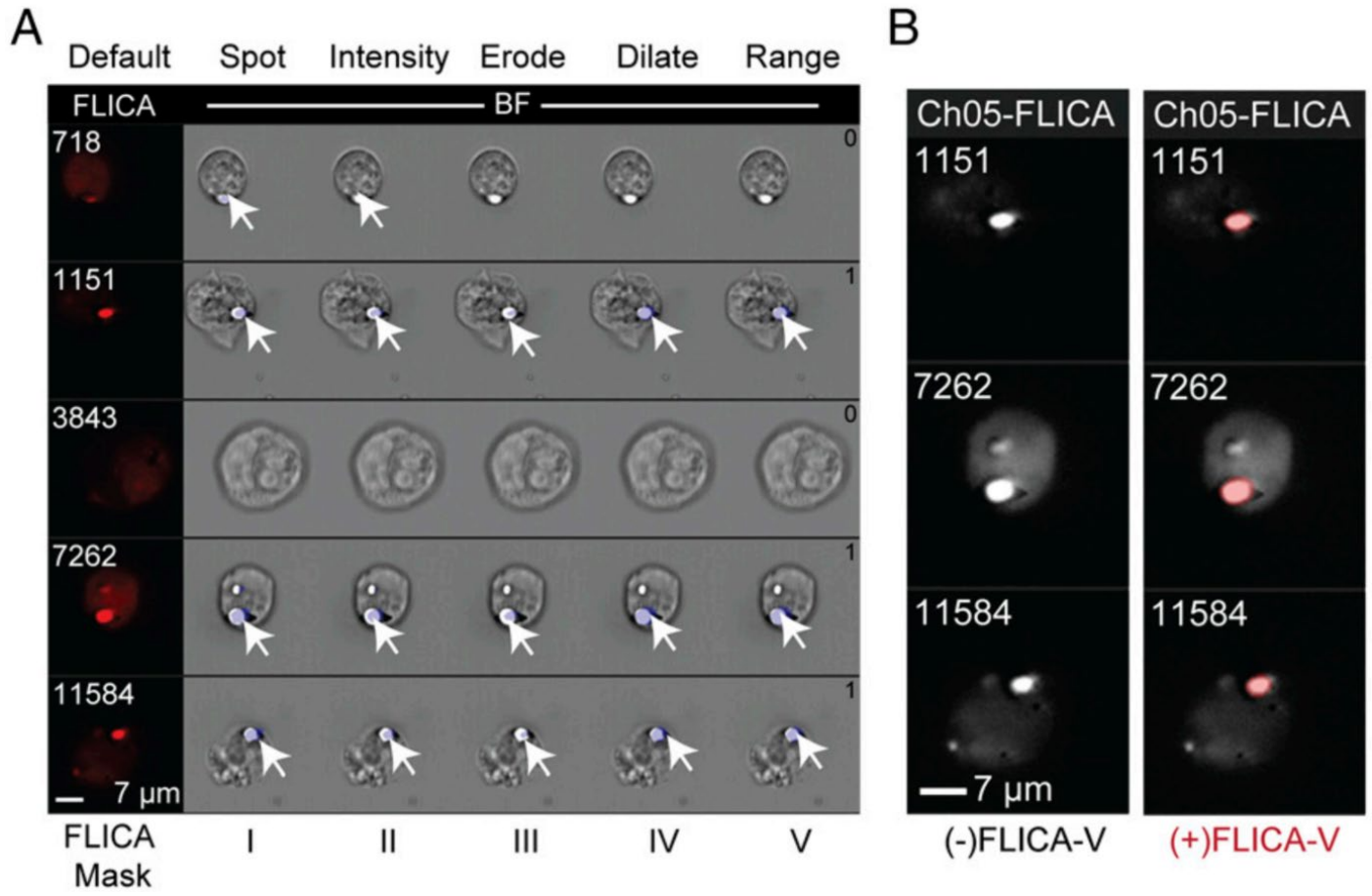
Table III. Masking strategy for FLICA aggregates

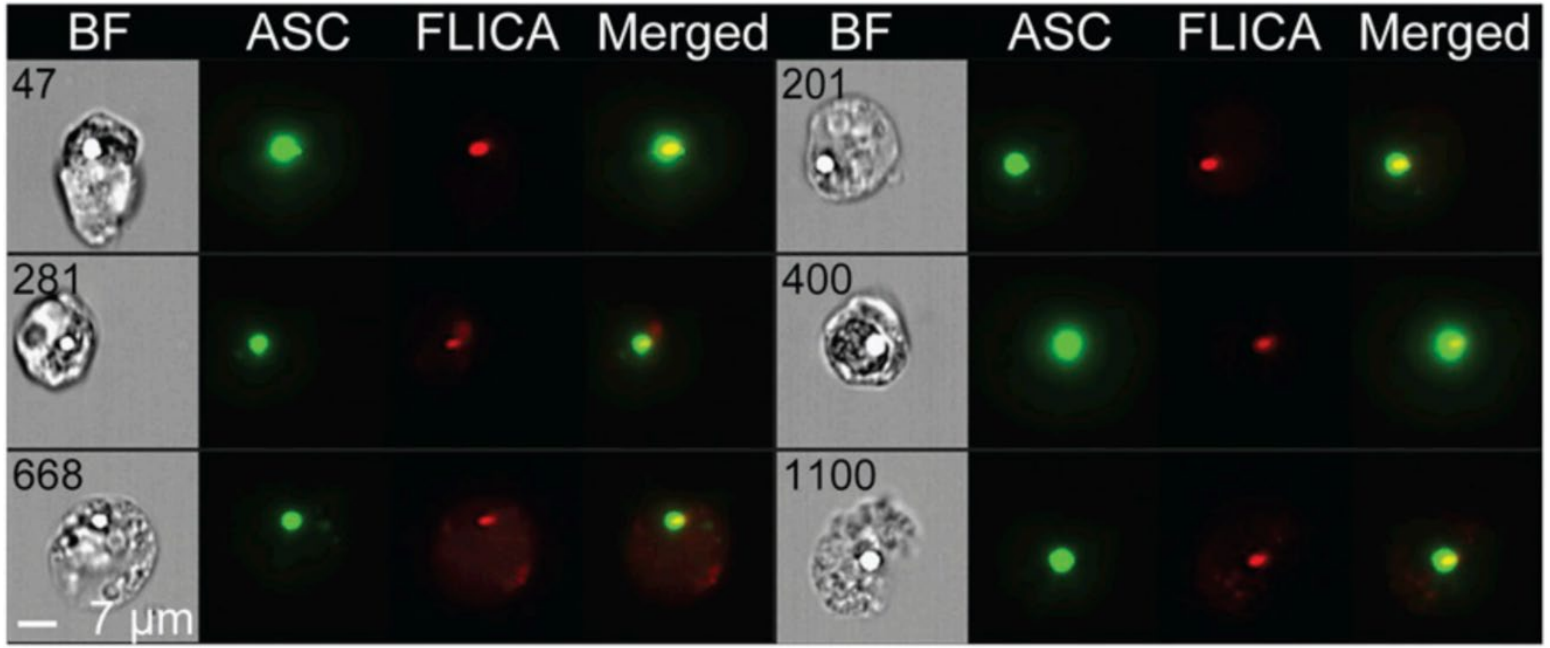
Mask Name	Function	Setting
FLICA-I	Spot	Spot (M05, Ch05-FLICA, bright, 5, 5, 2)
FLICA-II	Intensity	Intensity (FLICA-I, Ch05-FLICA, 150–4095)
FLICA spot-III	Erode	Erode (FLICA-II, 1)
FLICA-IV	Dilate	Dilate (FLICA-III, 3)
FLICA-V	Range	Range (FLICA-IV, 20–1000, 0.6–1)

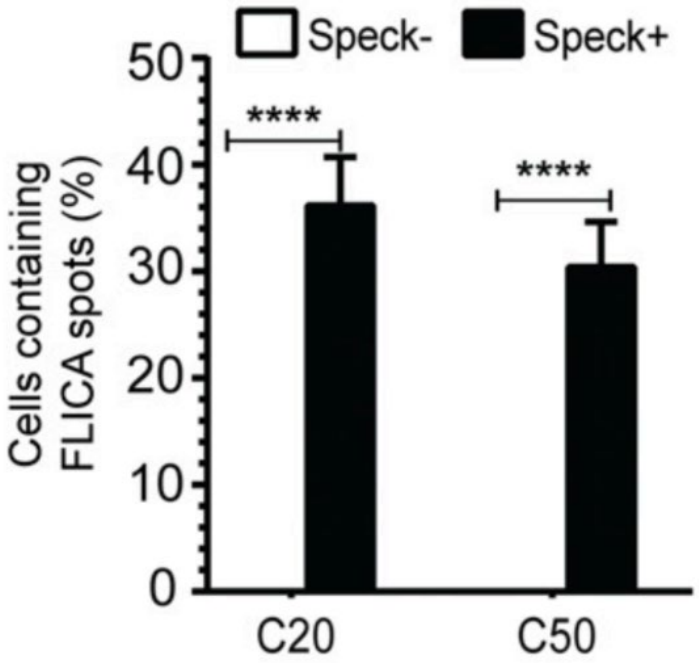
Mask name denotes the name given to individual mask. Function defines the type of mask used. Setting defines the parameters set for individual masks to achieve FLICA spot mask. FLICA-V mask identified all the FLICA aggregates in a cell. Spot count on FLICA-V was used to define diffused FLICA staining (spot count 0).



This masking strategy differentiates punctate FLICA staining from diffuse staining and more precisely defines clusters of FLICA signal.
Superimposing FLICA-V on FLICA spot images confirmed that this mask accurately reflects FLICA spot morphology.

ASC speck-positive cells showing covisualization of ASC speck and FLICA spots.





Summary

- Microscopy-based techniques permit visualization and analysis of inflammasome specks in single cells quantitatively and qualitatively.

→Time consuming

→Small sample size

→Require subjective selection of cells followed by image analysis, inherently subject to human error and bias

Summary

- Flow cytometry assay large numbers of cells.

→Unable to detect colocalized enzymatic activity

→Cant detect morphological features of the speck structure together with quantitative fluorescence analysis at the single-cell level

Summary

- Imaging flow cytometry avoids the shortcomings from both sides.
- The default masks supplied with the IDEAS software fail to eliminate background and decrease signal to noise ratios that can lead to inaccurate feature calculations.
- The masking/gating strategy allows accurate measurements by excluding false positive events and can be further customized to accommodate other cell-types.

FACS Canto II 2L - Irchel 1
FACS Canto II 3L - Irchel 2
LSR II Fortessa 4L - USZ 1
LSR II Fortessa 4L with HTS - USZ 2
LSR II Fortessa 4L - Schlieren
Sony SP6800 3L - Schlieren
FACSymphony 5L - Irchel
Cytek Aurora 5L - Irchel 1
Cytek Aurora 5L - Irchel 2
Image Stream X - Irchel
NanoFCM NanoAnalyzer - Irchel

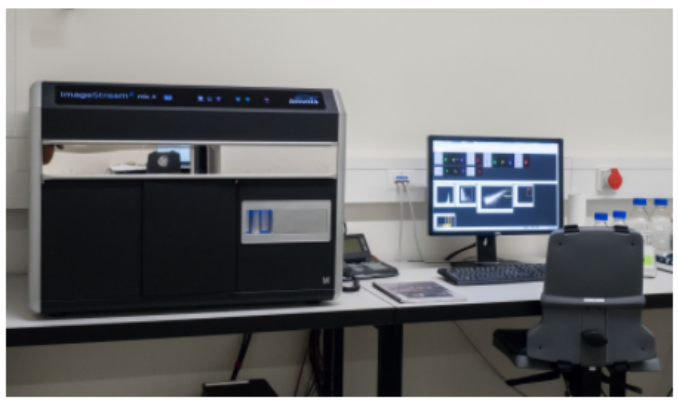
Image Stream X - Irchel

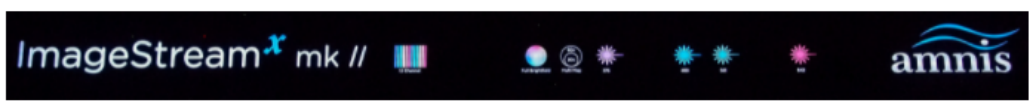
The Image Stream X Mark II imaging flow cytometer combines the speed, sensitivity, and phenotyping abilities of flow cytometry with the imaging capability and functional insights of microscopy.

The Image Stream can generate multiple images of every cell in flow, including brightfield, darkfield and up to 10 fluorescent markers (↓ Filter Guide (PDF, 149 KB)).

Potential applications include among many others the study of cell-cell interactions, phagocytosis, the characterization of circulating tumor cells, apoptosis and autophagy.

Telephone Image Stream Room: 044 63 55 319





The instrument is located in room Y44-G-3c

More detailed information about the image stream can be found ↗ here

Please contact → Claudia Dumrese for further discussion.

Optical configuration

The ISX has 5 Lasers 375nm, 405, 488nm, 561nm and 647nm for excitation

↓ Download optical configuration ISX (PDF, 149 KB)

SOPs

The standard operating procedures can be downloaded from here.

↓ SOP Imagestream X Mark II (PDF, 104 KB)
↓ Support Computer - SOP (PDF, 170 KB)

Operators for the Image Stream

An operator is available for project discussion, operator based image acquisition and data analysis. Please send your request to info@cytometry.uzh.ch.

Overview of the objectives used for imaging

The multimag option is incorporated

↓ Multimagnification (JPG, 45 KB)

- Thank you for your attention!
- Questions?