

ImageStream^X Cytometry:

影像化流式細胞儀-高速細胞影像定量之應用

Eric Chen

Cold Spring Biotech Corporation

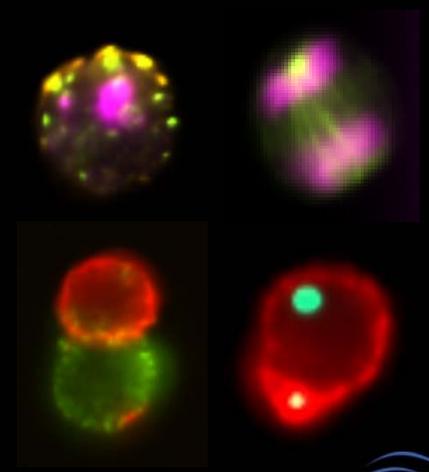
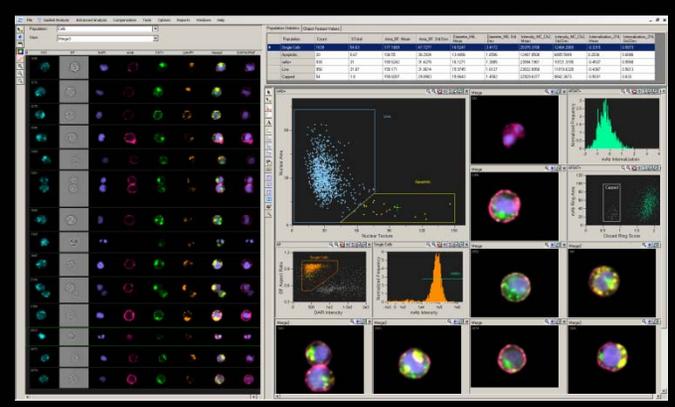


冷泉港生物科技股份有限公司



Amnis ImageStream[®] System

- **ImageStream^x Imaging Flow Cytometer**
Brightfield, darkfield, and several fluorescent images at high speed
- **IDEAS[®] Statistical Image Analysis Software**
Quantitative cellular image analysis and population statistics
- **Novel Applications**
Translocation, co-localization, cell classification, cell cycle, apoptosis, etc.



ImageStream^x combines FACS and Microscopy



ImageStream

Fast and quantitative
with high sensitivity
and spatial
resolution...

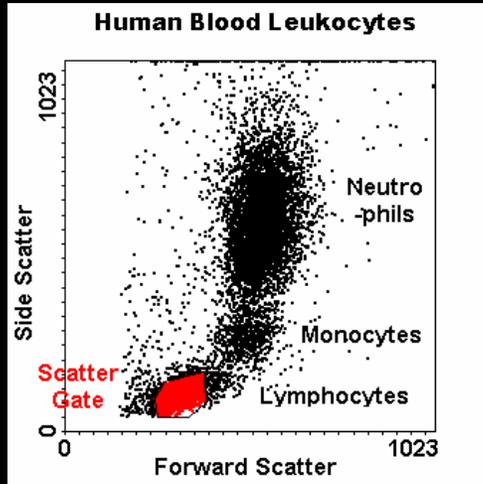
FACS

Fast, sensitive,
quantitative, but
lacks spatial
resolution...

Microscopy

High resolution,
less quantitative
features and
lower
throughput...

Flow Cytometry – *Well-established, powerful technology*



- provides quantitative data
- for multiple probes
- on a per cell basis
- for a large numbers of events/sample
- look at rare events
- determine subtle differences in populations



Lacks morphology – no ability to:

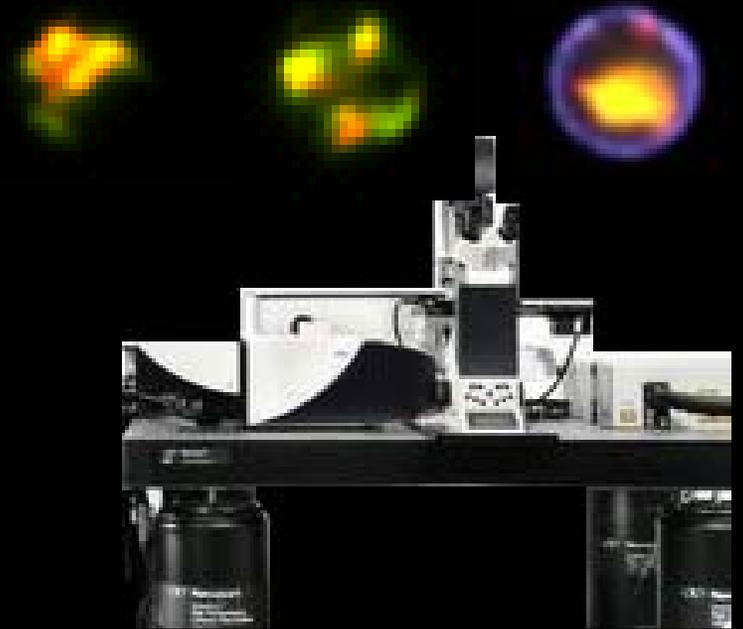
- *determine spatial distribution of signal*
- *determine morphological changes*
- *determine co-localizing events*
- *investigate trafficking, polarization, etc.*

Fluorescence Microscopy –

Well-established, powerful technology



- extremely high resolution images
- on a per cell basis
- for multiple probes
- on a per cell basis
- very high content information



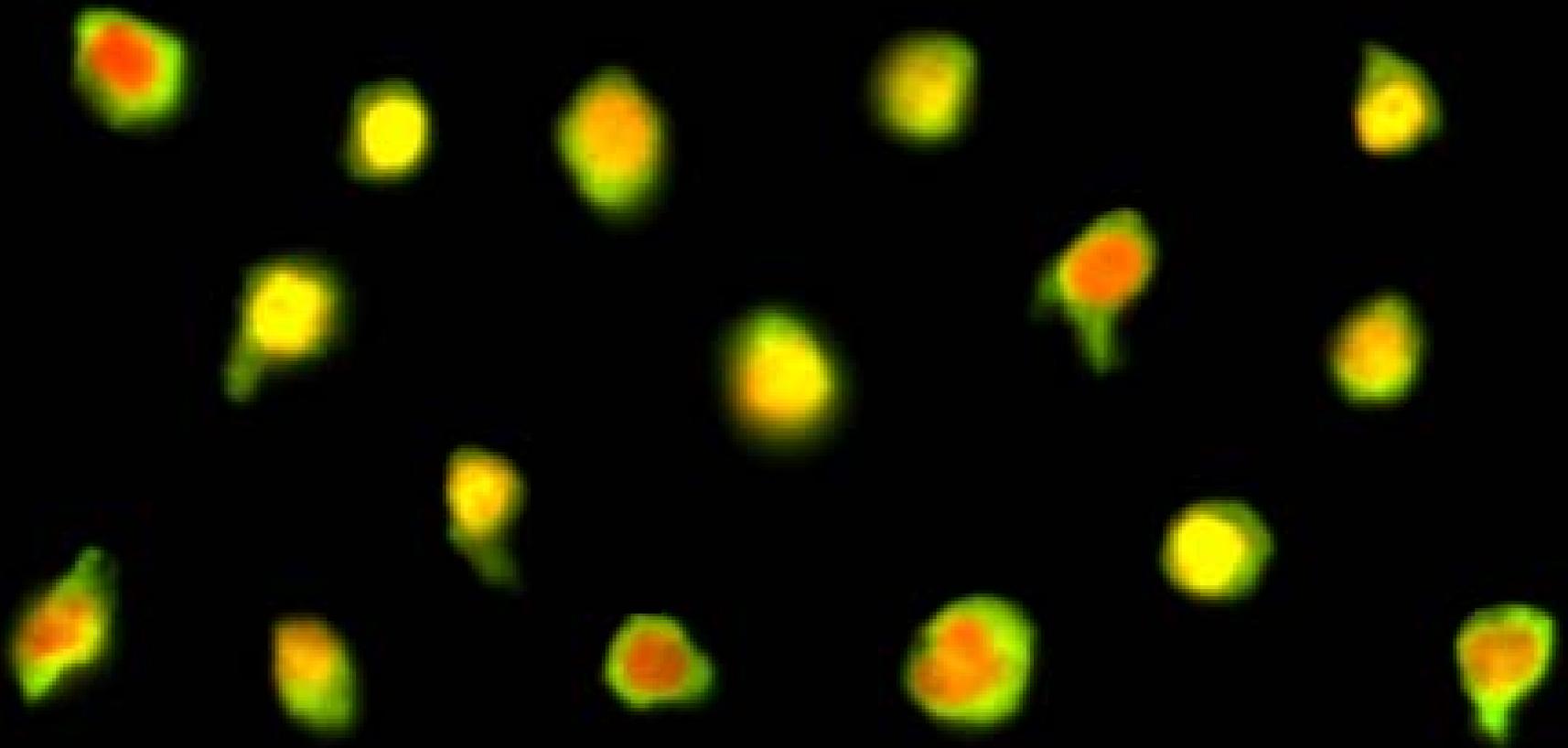
Microscopy limited by two problems:

- *difficult to get statistically large sampling size per sample*
 - *images are laborious and time consuming to take*
- *difficult to quantify images in an objective manner*
 - *despite the fact that there is a tremendous amount of information present in each image*

ImageStream Quantitation: How do I measure what I see?

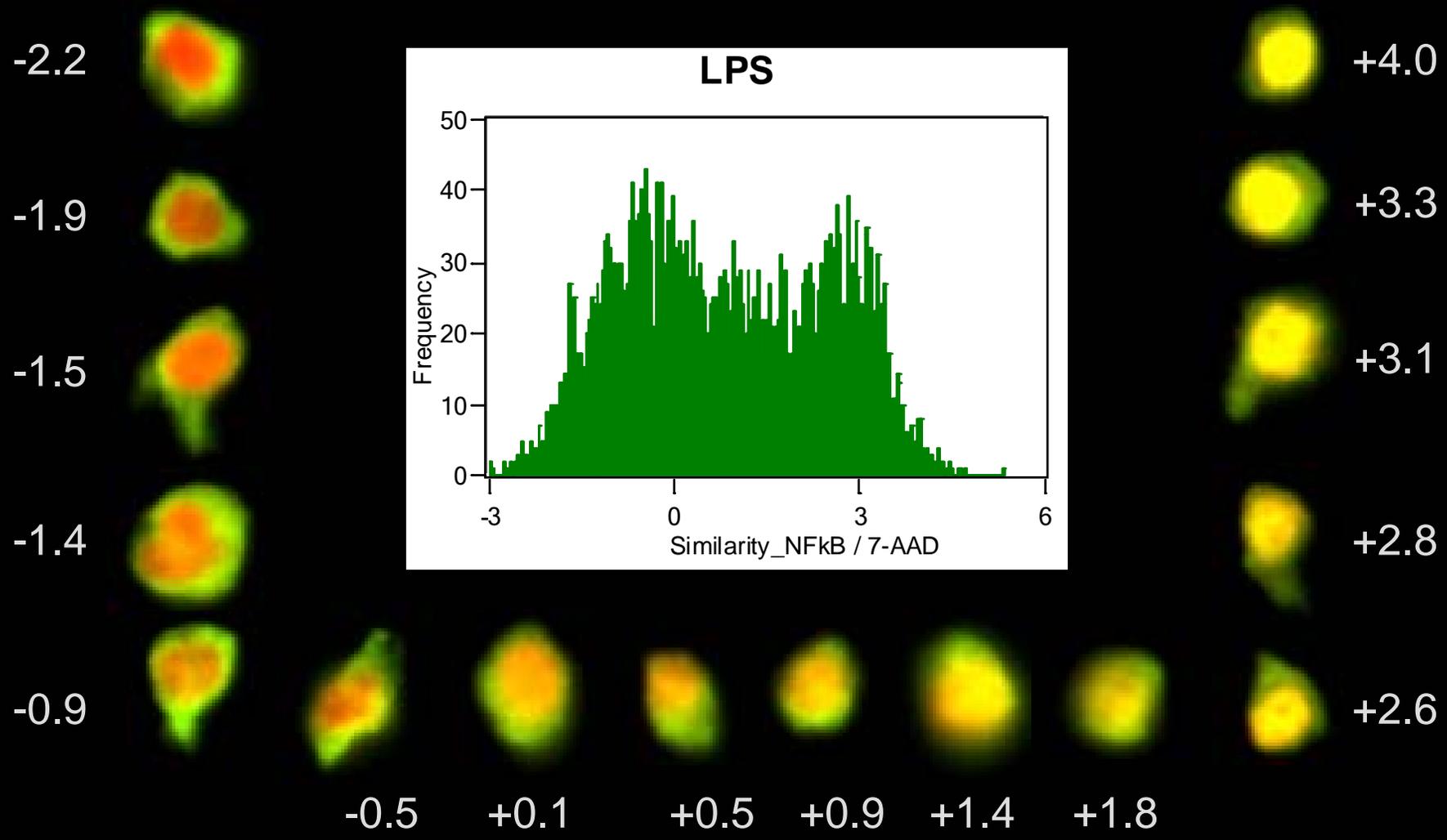


Incomplete NF- κ B translocation in LPS-stimulated monocytes



ImageStream Quantitation:

How do I measure what I see?



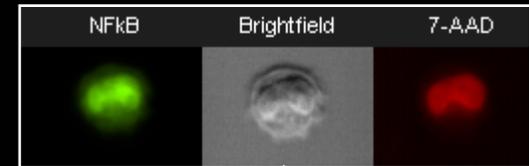
Quantitative Image Analysis

Example: NF-kB translocation assay in monocytes

LPS stimulated cell, low translocation



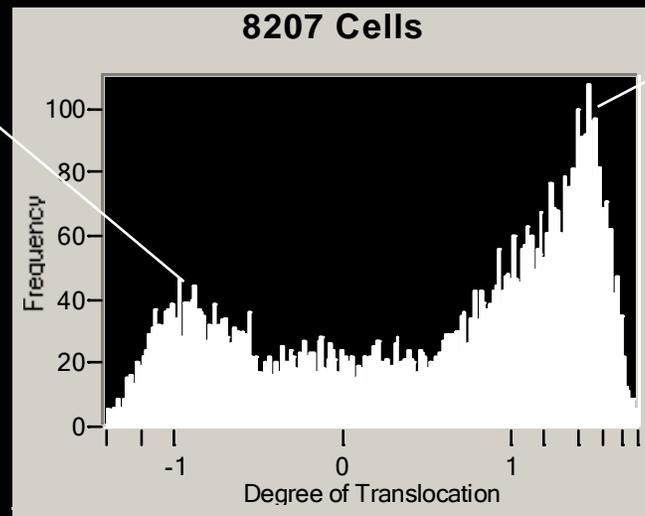
LPS stimulated cell, high translocation



With 50 images its impossible to say if there was a response to treatment.

The same is true even at 100 events

With 250 images a pattern begins to emerge.



With 500 images we see a pattern but lack statistical rigor.

With ~10,000 images its possible to see a bimodal distribution of responders and non responders.

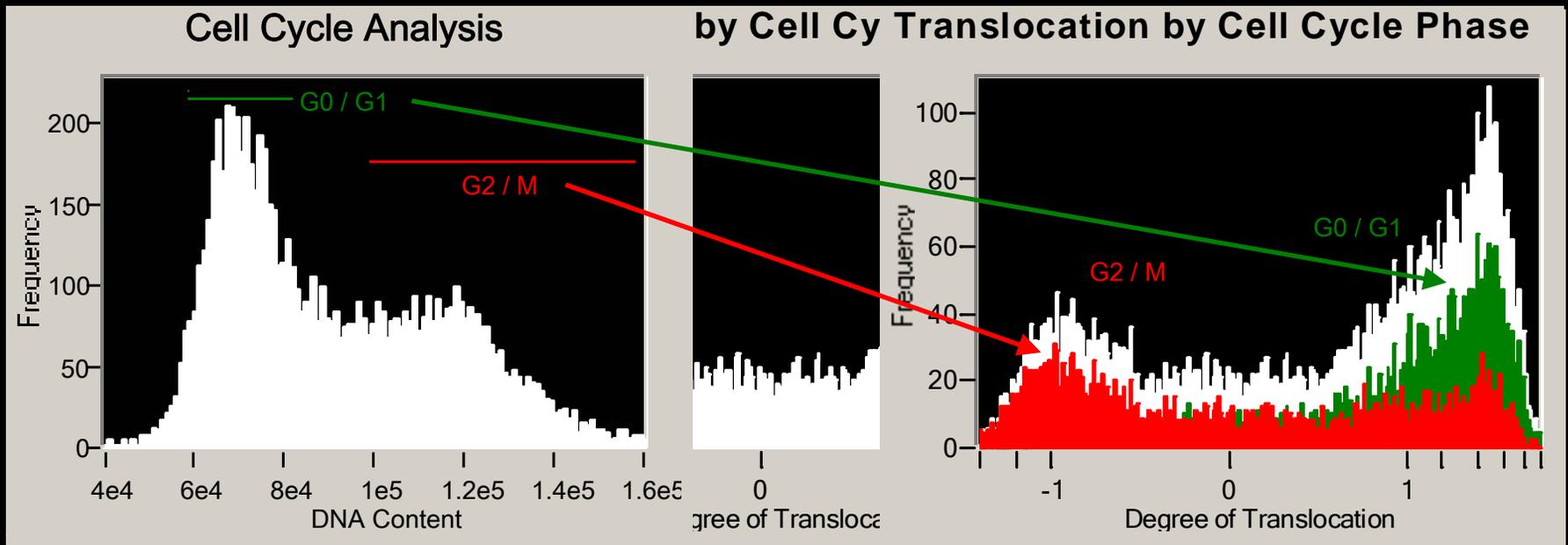
Conclusion:

Robust image-based assays can require the analysis of thousands of cells.

Using Images to Multiplex Experiments

Example:

Incomplete NF- κ B translocation in LPS-stimulated monocytes



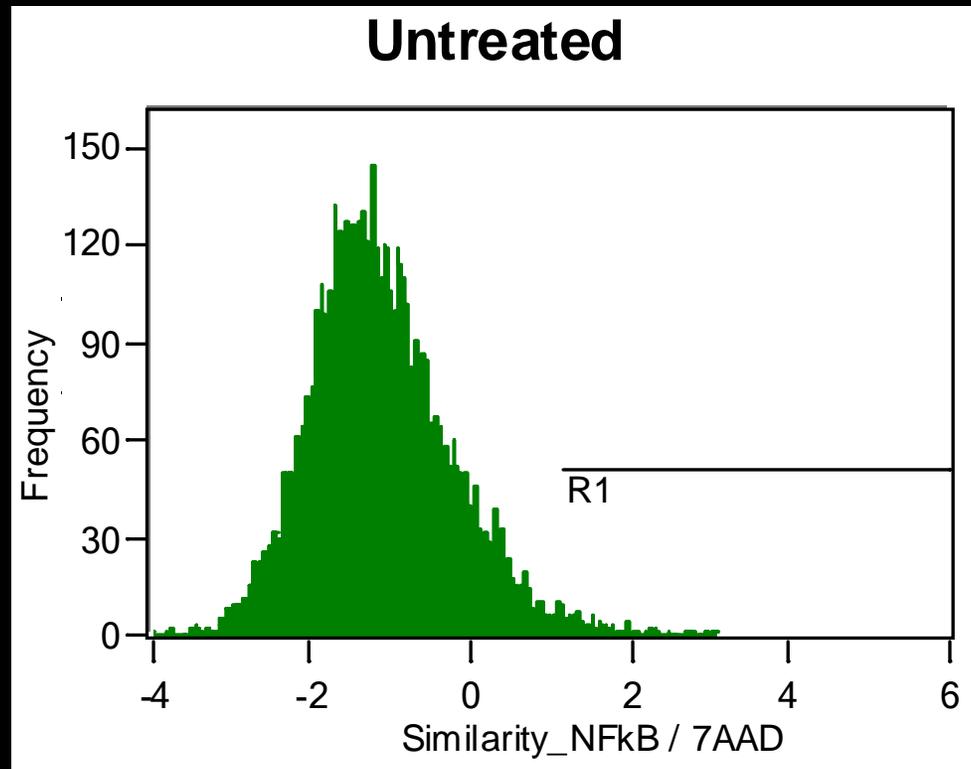
Conclusion:

Simultaneous measurement of translocation and DNA content can lead to better understanding...

ImageStream Quantitation: How do I measure what I see?



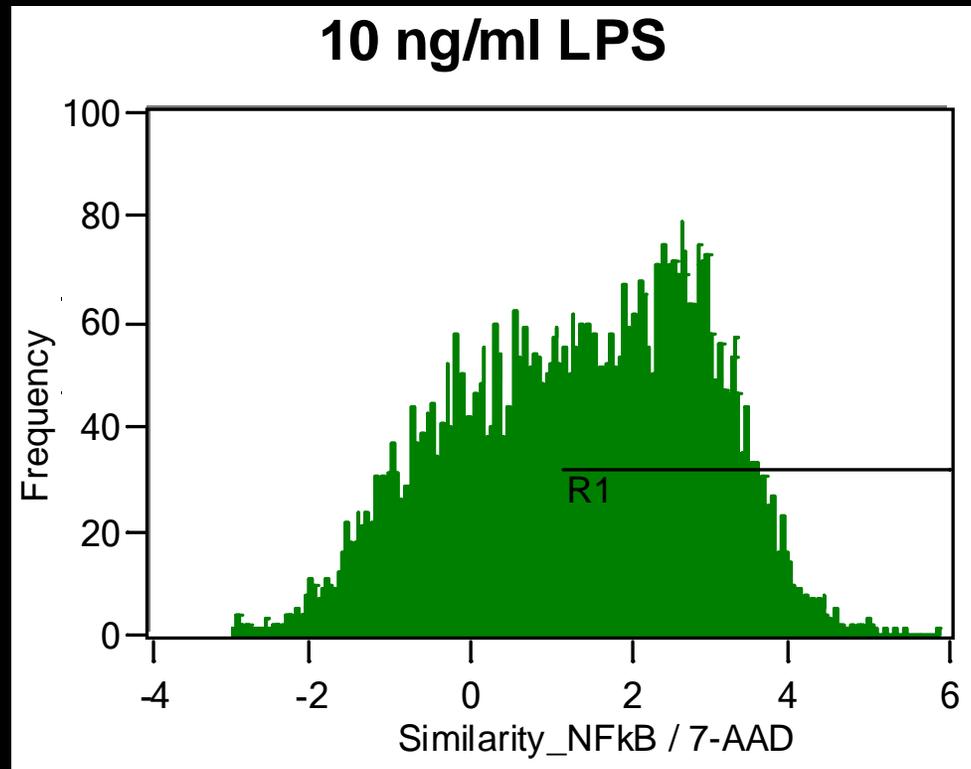
Dose response and time course: LPS-stimulated monocytes



ImageStream Quantitation: How do I measure what I see?



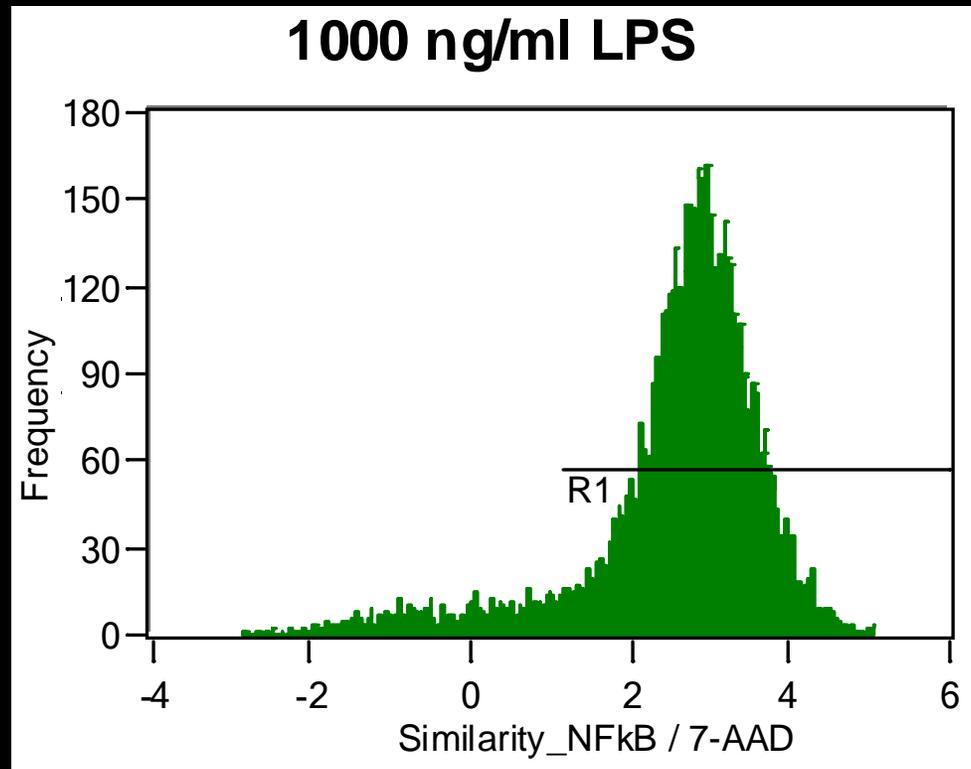
Dose response and time course: LPS-stimulated monocytes



ImageStream Quantitation: How do I measure what I see?



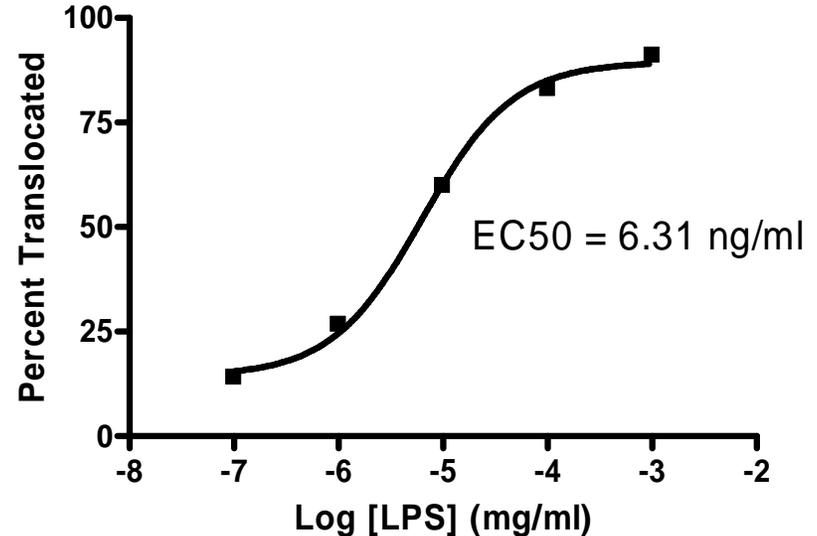
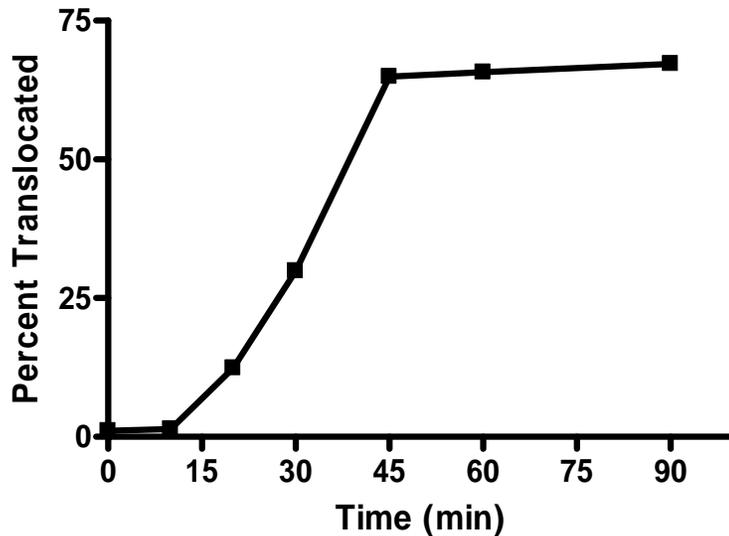
Dose response and time course: LPS-stimulated monocytes



ImageStream Quantitation: How do I measure what I see?



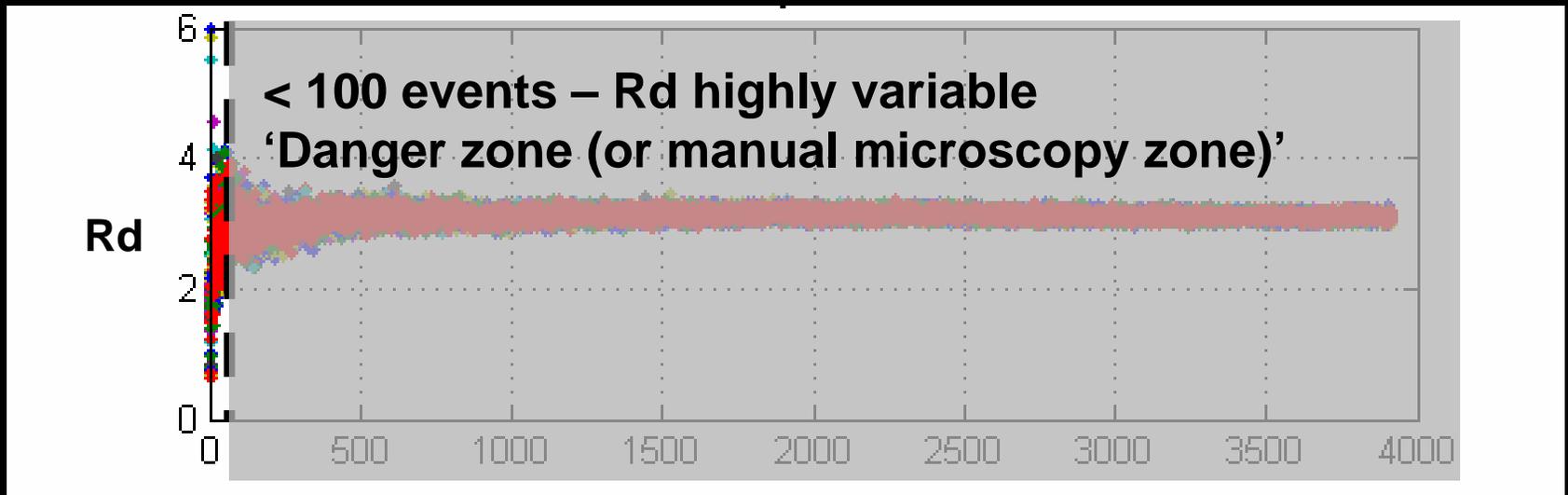
Dose response and time course: LPS-stimulated monocytes



ImageStream Quantitation: How many cells do I need?



LPS-induced nuclear translocation



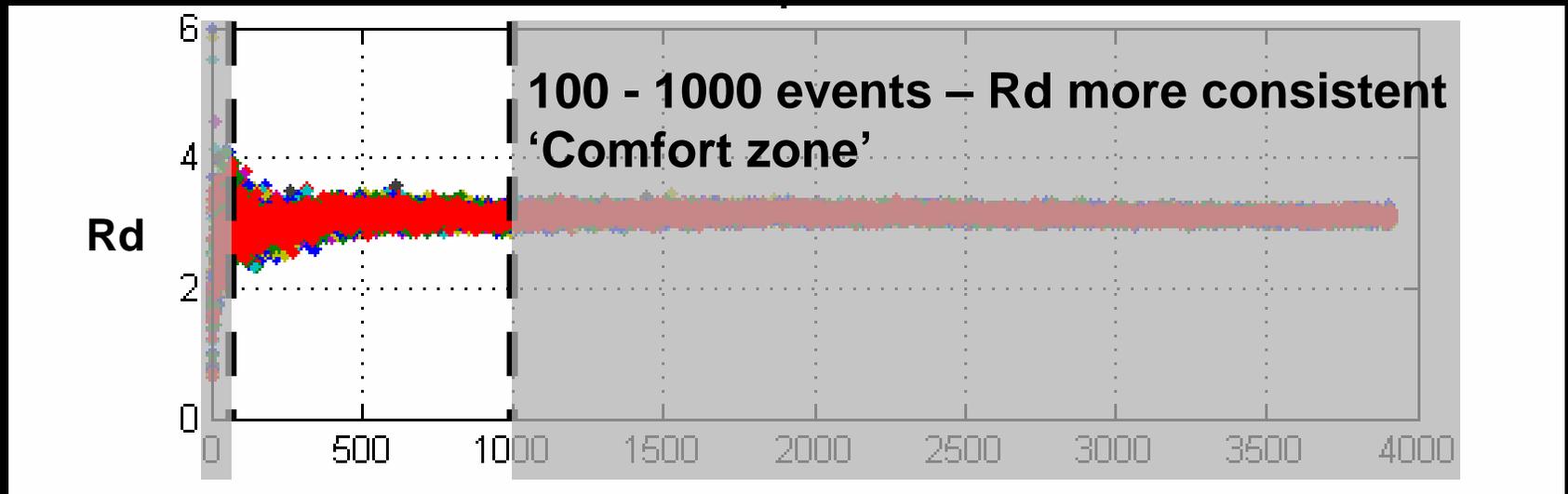
- Change between untreated/treated cells measured using Rd analysis (y-axis) for the indicated number of randomly sampled cells (x-axis)

Rd = statistical separation between positive and negative controls

ImageStream Quantitation: How many cells do I need?



LPS-induced nuclear translocation

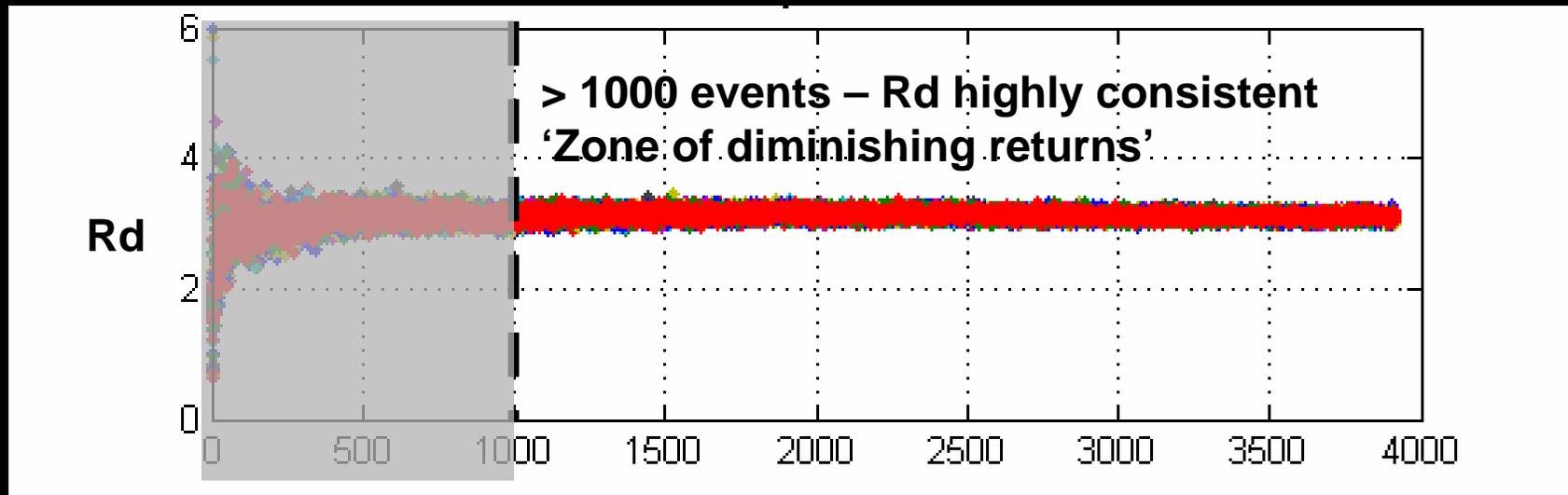


- Change between untreated/treated cells measured using Rd analysis (y-axis) for the indicated number of randomly sampled cells (x-axis)

ImageStream Quantitation: How many cells do I need?



LPS-induced nuclear translocation

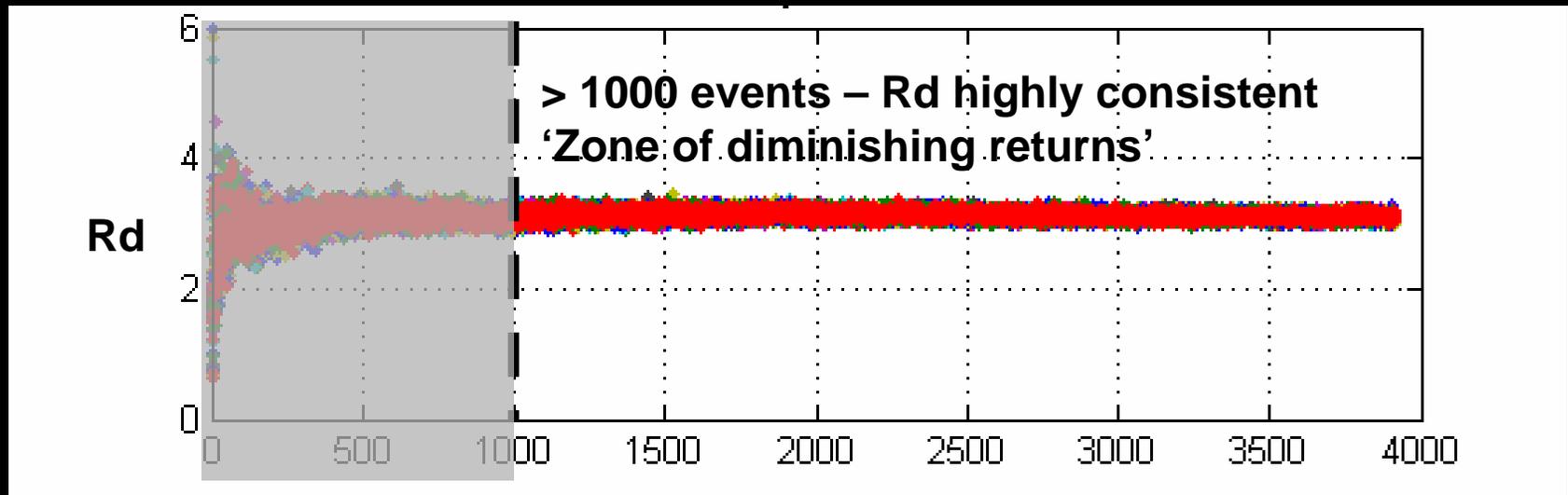


- Change between untreated/treated cells measured using Rd analysis (y-axis) for the indicated number of randomly sampled cells (x-axis)
- *Therefore collect at least 100 and ideally at least 1000 target events per sample*

ImageStream Quantitation: How many cells do I need?

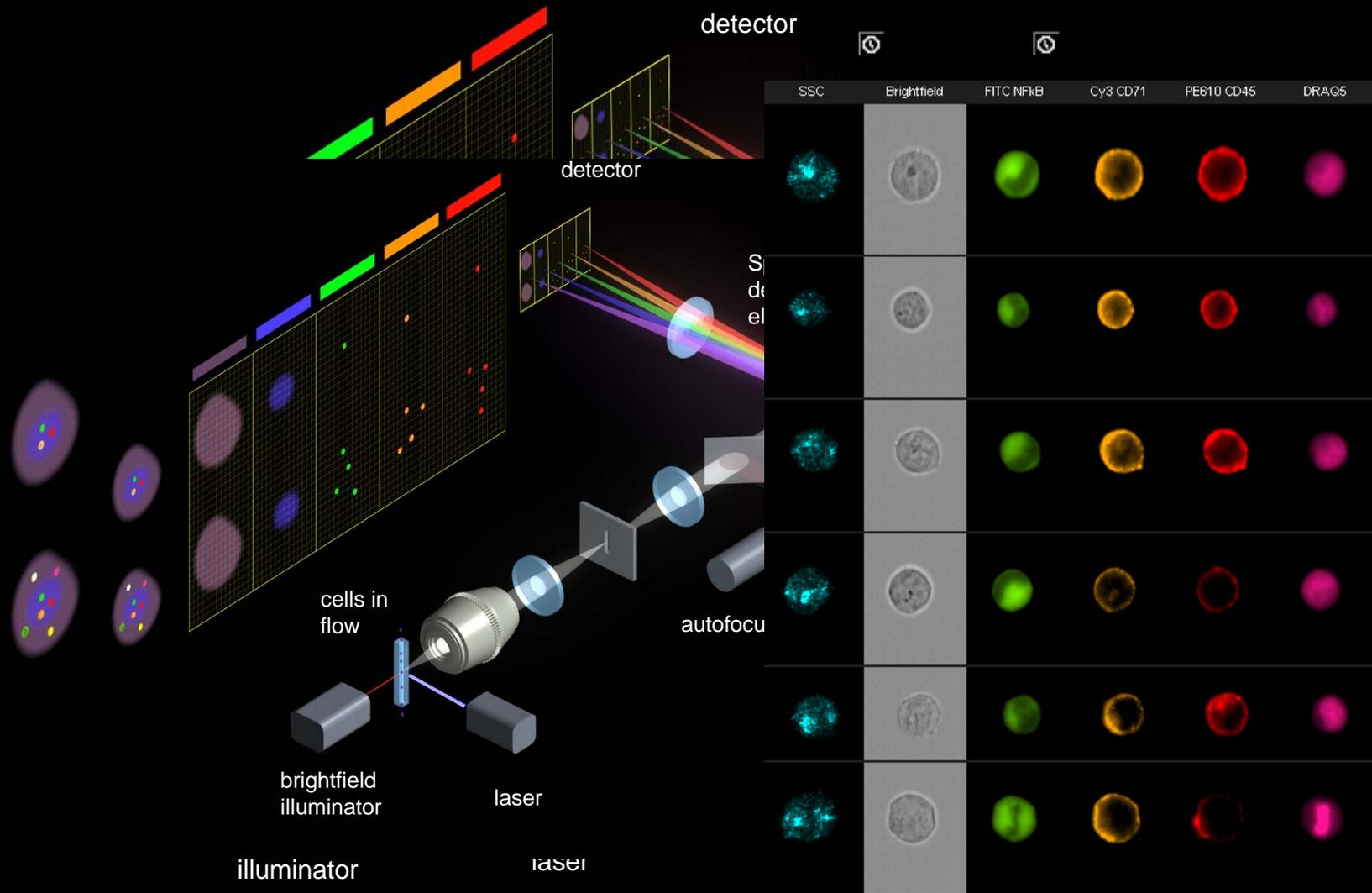


LPS-induced nuclear translocation



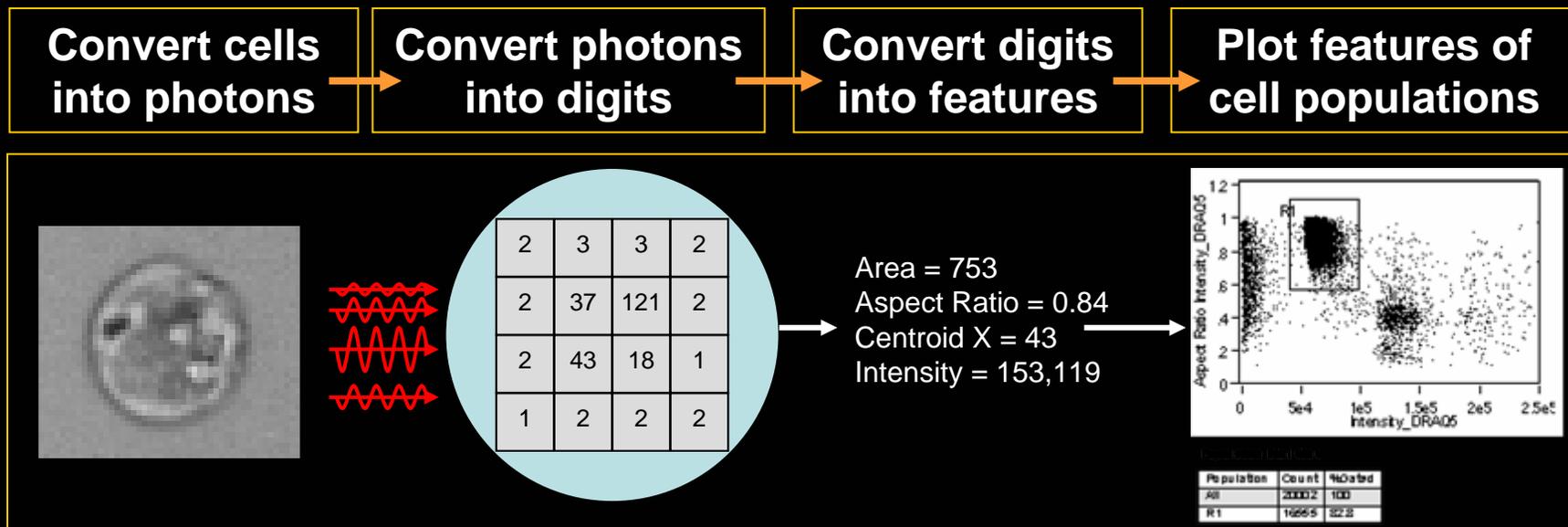
- Change between untreated/treated cells measured using Rd analysis (y-axis) for the indicated number of randomly sampled cells (x-axis)
- *Therefore collect at least 100 and ideally at least 1000 target events per sample*

ImageStream Layout

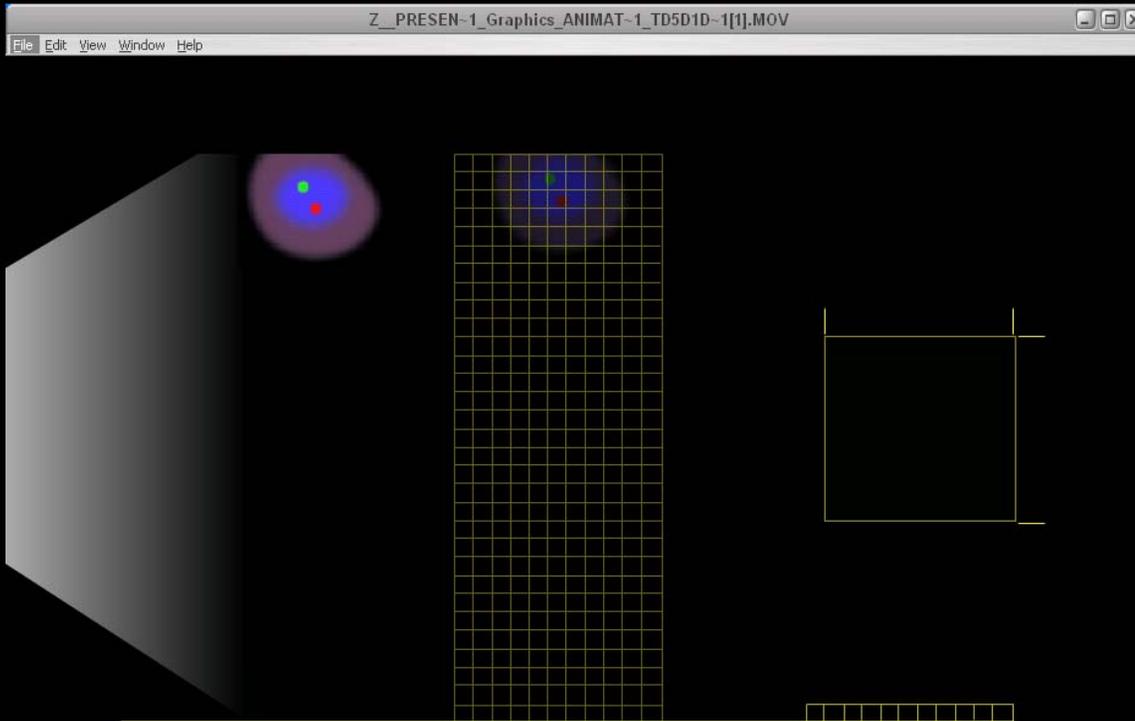


Pathway to quantitative image analysis

- Numerical measurement of morphology associated with an image; performed on many images per sample
- To achieve this:



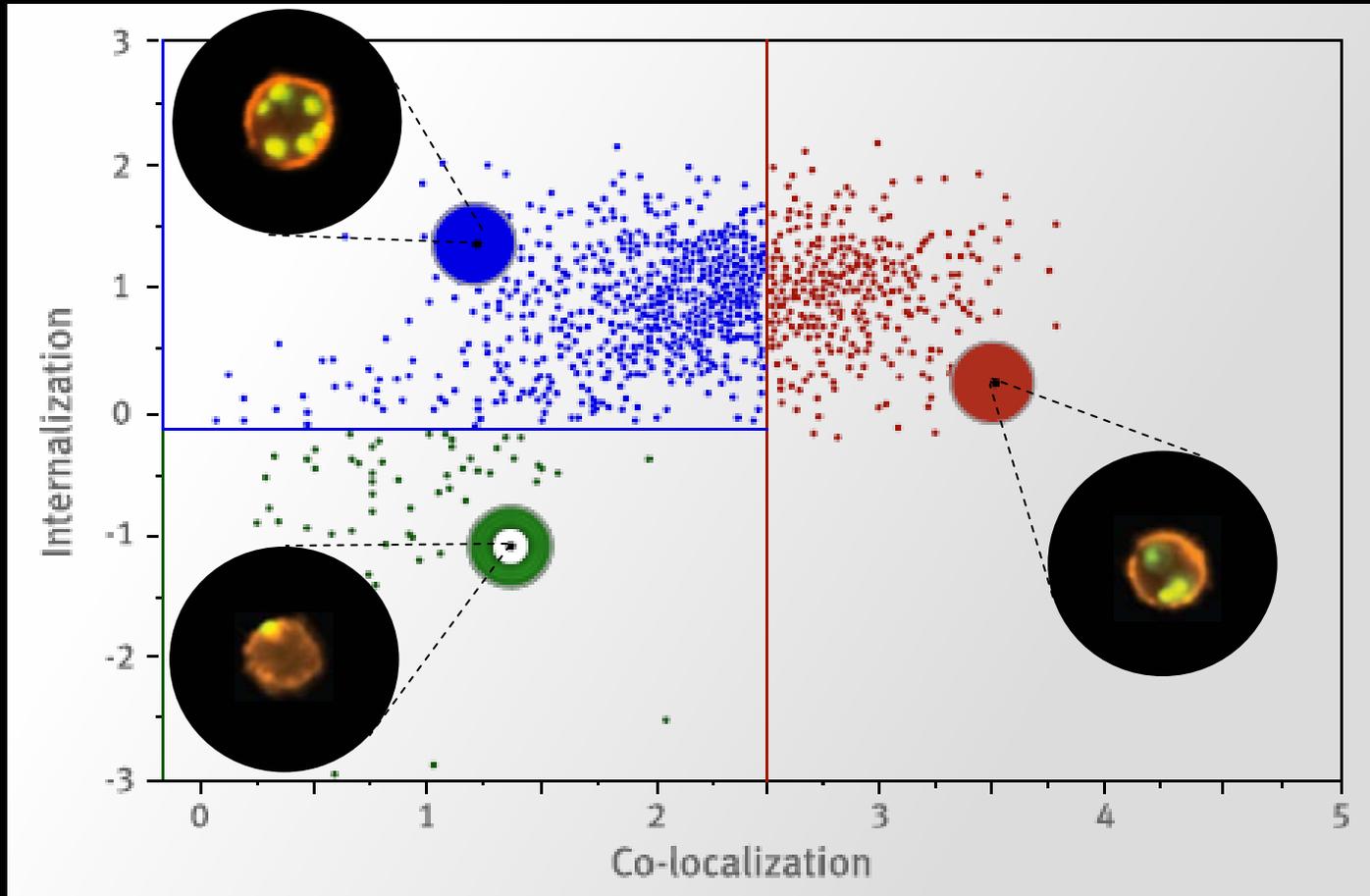
Time Delay Integration



TDI CCD

- Excite fluorescence over the entire height of the detector
- Light is detected in the first pixel row and transferred to the pixel below in exact synchrony with the velocity of the cell as it goes streaming by.
- Light is integrated over the entire height of the detector to achieve high photonic sensitivity
- Images don't streak or blur and maintain 0.5 μ m per pixel resolution.

Digital Imaging in Flowcytometer



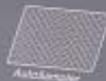
One dot represents one cell image



ImageStream X

- 1,000 cells per second
- 12 image channels per cell: darkfield, brightfield, fluorescent
- Up to 5 lasers (488, 405, 561, 592, 658 nm)
- 430-800 nm imaging bandwidth
- Multiple magnifications (60X/.9NA, 40X/.75NA, 20X/.5NA)
- AutoSampler for 96 well plates
- Extended depth of field optics (EDF)

ImageStream *x*



IDEAS[®] Analysis Software



- High content morphometric analysis of tens of thousands of images
- Application wizards for validated protocols
- “Building block” analysis quickly finds the features you need
- 85 parameters per channel, 14 masks, and user defined features for advanced image-based discrimination of cells

Welcome to IDEAS[®] ...
Version 4.0.249.0

To begin using IDEAS, select an application below or choose the "Open File" option to perform manual analysis or view previously processed data.

	Open File	Open ImageStream data files
	Apoptosis	Identify apoptotic events based on brightfield and nuclear morphology
	Cell Cycle - Mitosis	Distinguish mitotic and apoptotic events
	Co-localization	Measure the co-localization of two probes on, in, or between cells
	Internalization	Measure the internalization of a probe
	Nuclear Localization	Measure the nuclear localization of a probe
	Shape Change	Measure circular morphology

IDEAS[®] Software



The screenshot displays the IDEAS software interface with the following components:

- Population Statistics Table:**

Feature	Value	Definition
1_Area	3445	Area M1 0
1_Aspect Ratio	0.7613	Aspect Ratio M1 0
1_CentroidX	39.5376	CentroidX M1 0
1_CentroidY	55.1402	CentroidY M1 0
1_Frequency	3.9042	Frequency M1 0
1_Major Axis	35.5175	Major Axis M1 0
1_Mean Intensity	7.683	Mean Intensity M1 0
1_Minor Axis	30.9907	Minor Axis M1 0

- Scatter Plot:** A plot of 488 Scatter Frequency vs. Brightfield - 7AAD Area. The x-axis ranges from -500 to 1.5e3, and the y-axis ranges from 0 to 25. Data points are colored by population.
- Histogram:** A histogram of HLA-PE Total Intensity. The x-axis is on a log scale from 10e2 to 10e5. A peak is labeled 'Necrotic by HLA'.
- Table:**

Population	Count	% Total	% Gated
Double Positive	1526	35.3	100
Necrotic by HL	1046	24.2	68.5

Image Gallery

see every cell
flexible viewing
enhance & color
tag populations
virtual cell sort

Tabular Data

100's of params/cell
population statistics
object values

Workspace

uni + bivariate
flexible gating
click dot to view cell
custom parameters



Features 85 per channel, up to 12 channels

Size features are in microns:

Area; Diameter; Major Axis; Minor Axis; Major Axis Intensity; Minor Axis Intensity; Perimeter; Thickness Max and Min; Spot Area Min; Width; Height; Length

Shape features define the mask shape, units vary with the feature:

Aspect Ratio; Aspect Ratio Intensity; Circularity; Compactness; Elongatedness; Lobe Count; Shape Ratio; Symmetry 2,3,4

Location features are in X,Y pixel coordinates:

Angle; Angle Intensity; Centroid X; Centroid Y; Centroid X Intensity; Centroid Y Intensity; Delta Centroid X; Delta Centroid Y; Delta Centroid XY; Max Contour position; Spot Distance Min; Valley X and Valley Y

Texture features determine local intensity variations in images:

Bright Detail Intensity R3; Bright Detail Intensity R7; Contrast; Gradient Max; Gradient RMS; Modulation; Spot Count; Std Dev; and the Haralick (H) texture features H-Contrast; H-Correlation; H-Energy; H-Entropy; H-Homogeneity; and H-Variance

Signal Strength features are measured in counts:

Bkgd Mean; Bkgd StdDev; Intensity; Raw Intensity; Raw Max Pixel; Raw Min Pixel; Raw Mean Pixel; Raw Median Pixel; Max Pixel; Min Pixel; Mean Pixel; Median Pixel; Saturation Count; Saturation Percent; Spot Intensity Min

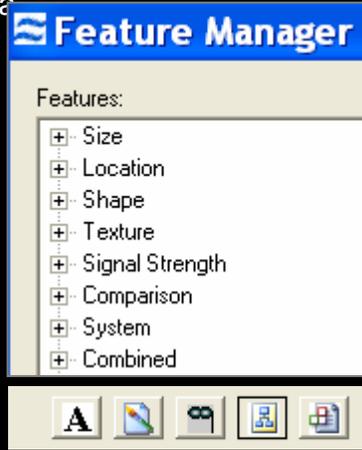
Comparison features quantify intensity differences between masks or pixels:

Intensity Concentration Ratio; Internalization; Similarity; Bright Detail Similarity R3

System features do not require a mask:

Camera Line Number; Camera Timer; Flow Speed; Object Number; Objects per second; Objects per ml and Time

Combined features are created by using Boolean Logic



Alphabetical
Image
Mask
Category
Base



ImageStream applications



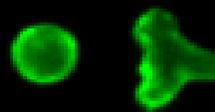
Cell signaling



Cell death & autophagy



Internalization & phagocytosis



Shape change & chemotaxis



Surface and intracellular co-localization



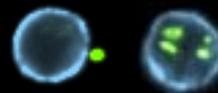
Stem cell biology



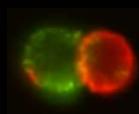
DNA damage and repair



Parasitology



Microbiology



Cell-cell interaction



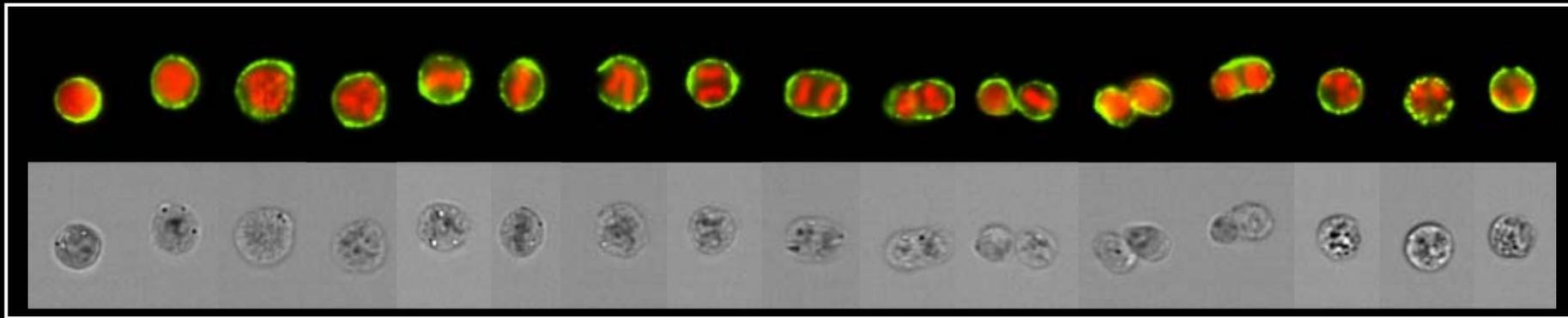
Cell cycle & mitosis



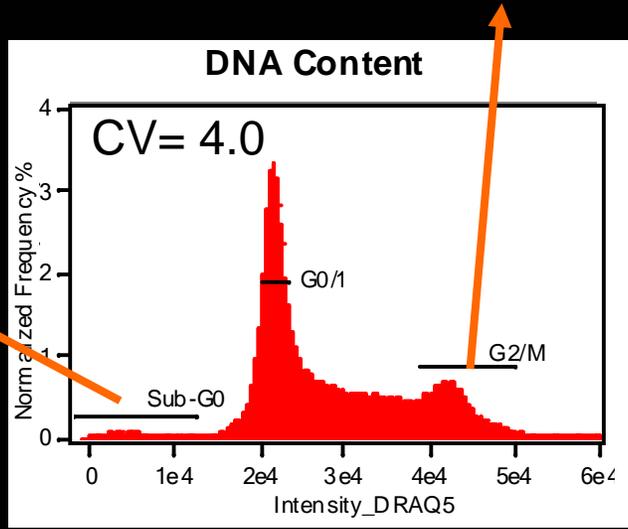
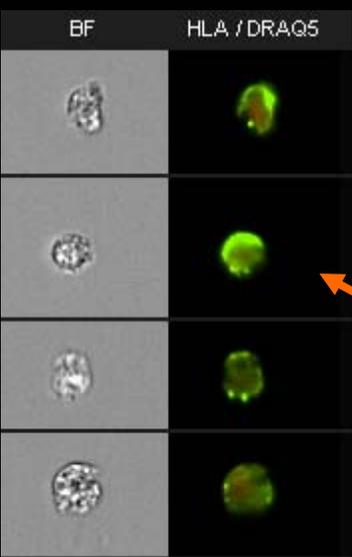
Oceanography



Cell Cycle / Mitosis / Apoptosis



Interphase Prophase Metaphase Anaphase Telophase Atypical Apoptotic



- HL60 cells were labeled with anti-HLA AF488 and DRAQ5
- DNA condensation pattern and Brightfield contrast help distinguish the phases of mitosis from apoptotic and interphase nuclei

NF- κ B translocation in whole blood subsets



Incubate whole blood with LPS



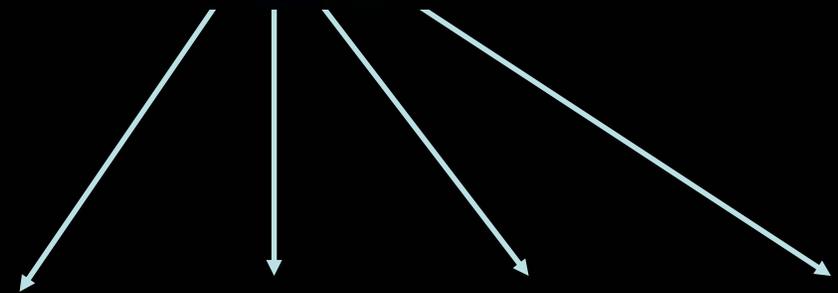
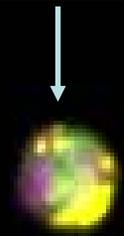
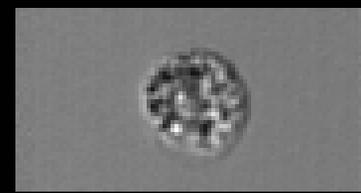
4-color immunophenotype for leucocytes and stain for NF- κ B and DAPI



Collect 20,000 event image files at 60X on the ImageStream^x



Quantify: nuclear localization in whole blood subsets

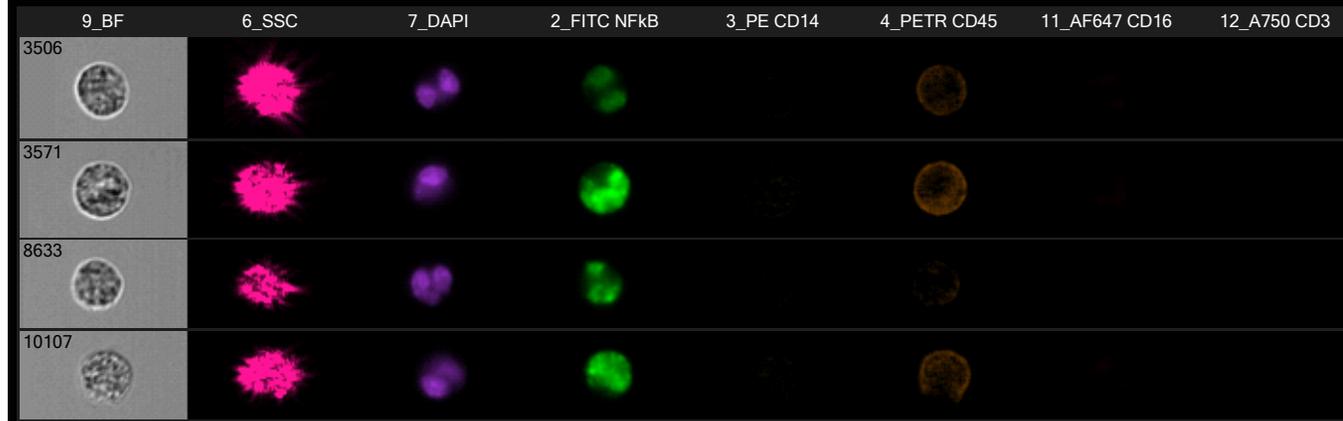
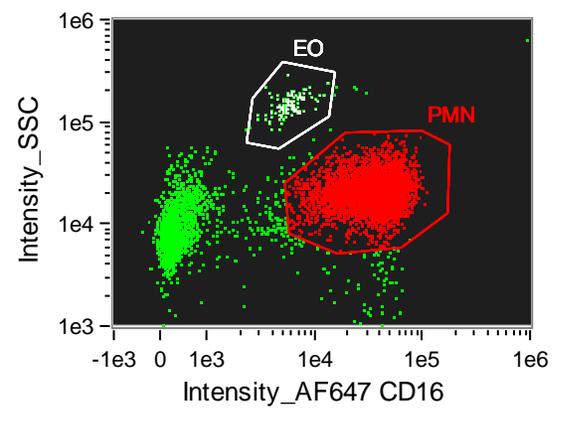


	9_BF	6_SSC	7_DAPI	2_FITC Nf κ B	3_PE CD14	4_PETR CD45	11_AF647 CD16	12_A750 CD3
9095								
969								
1142								

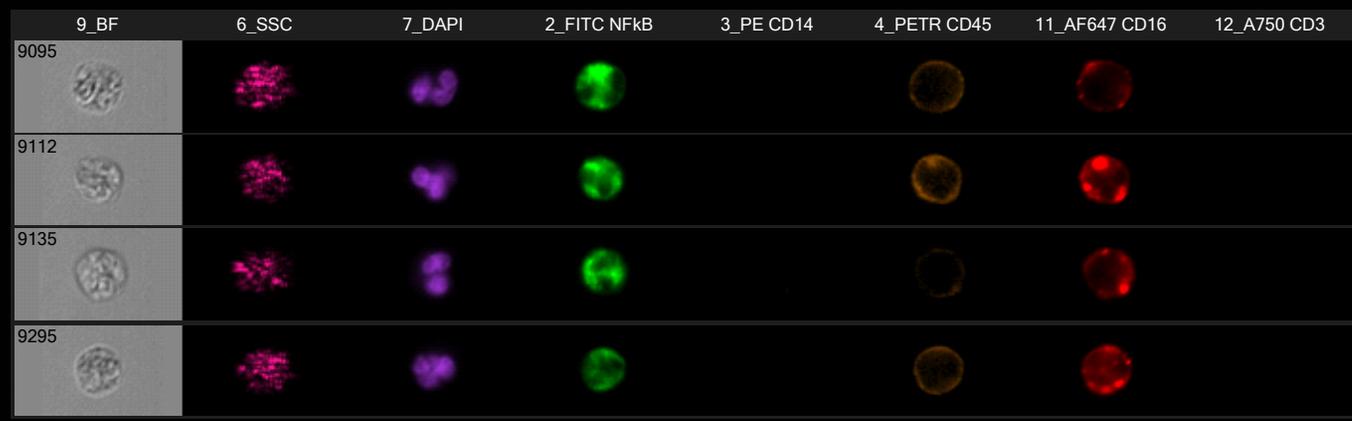
NF- κ B translocation in whole blood subsets



Eosinophils



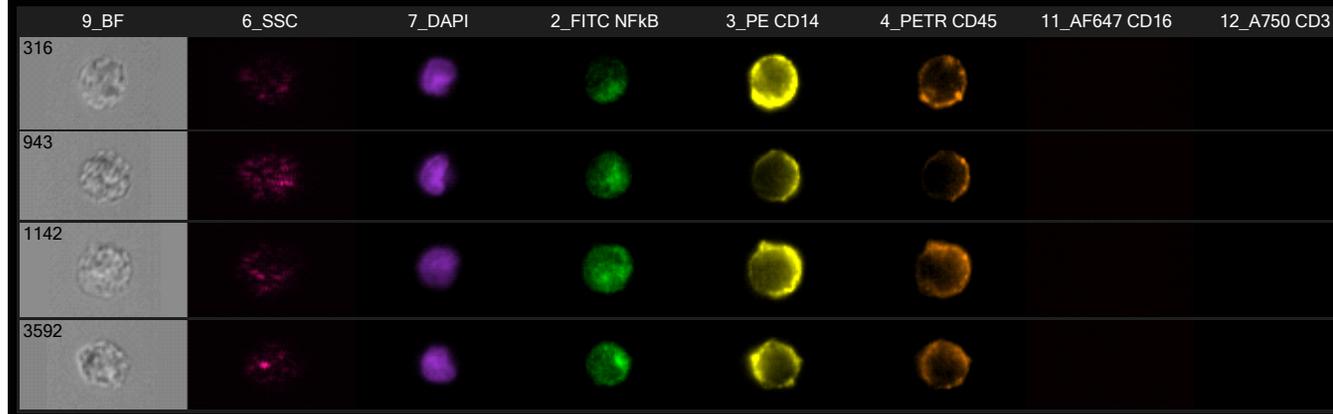
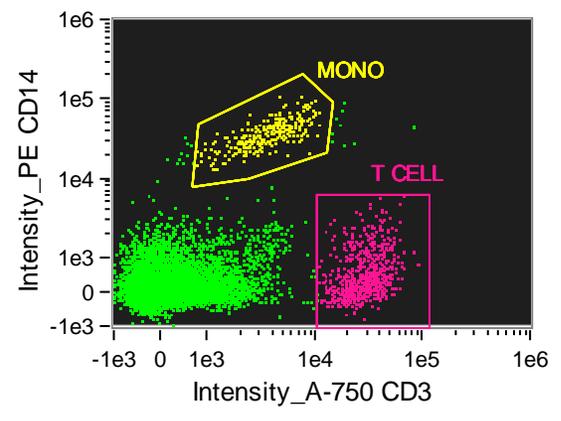
Neutrophils



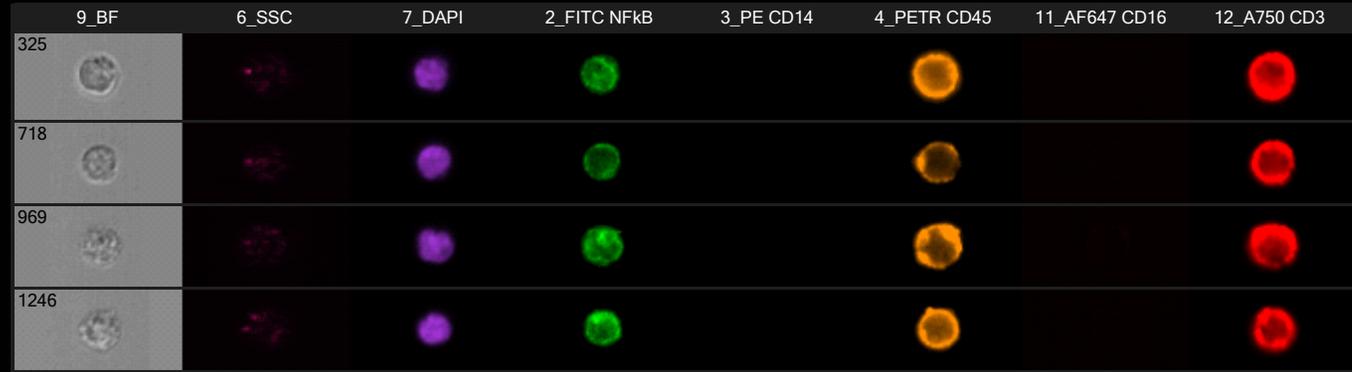
NF- κ B translocation in whole blood subsets



Monocytes



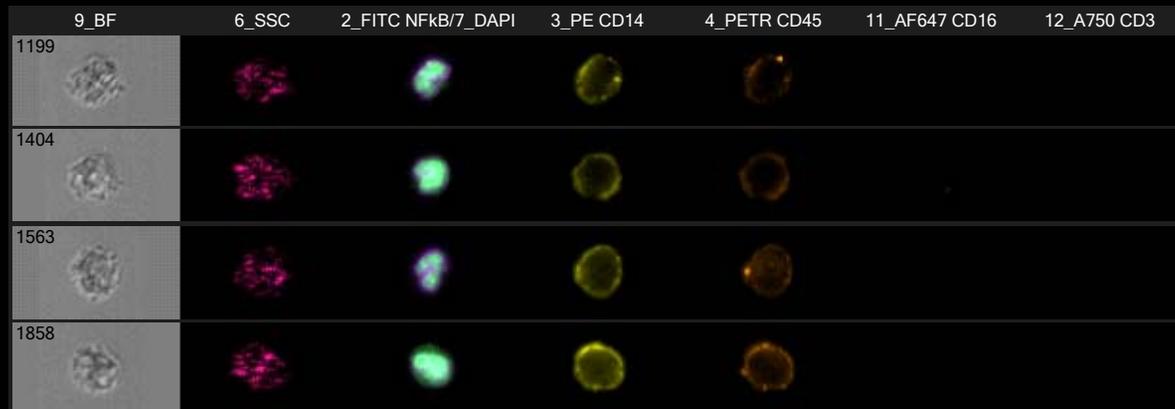
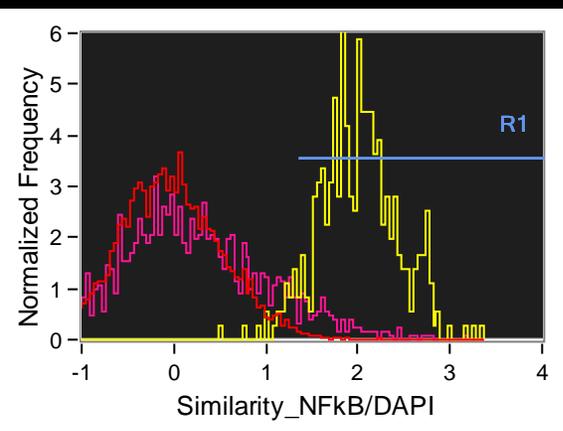
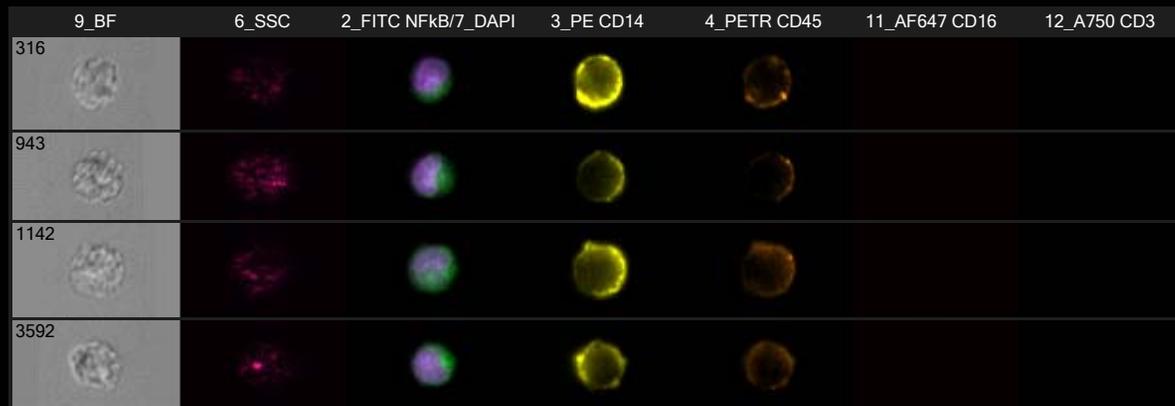
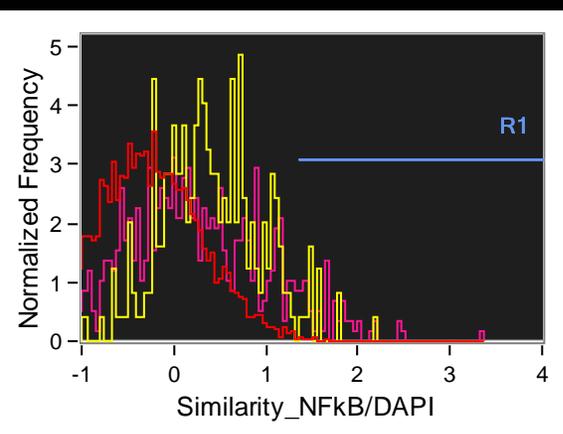
T cells



LPS induces specific NF-kB translocation in monocytes

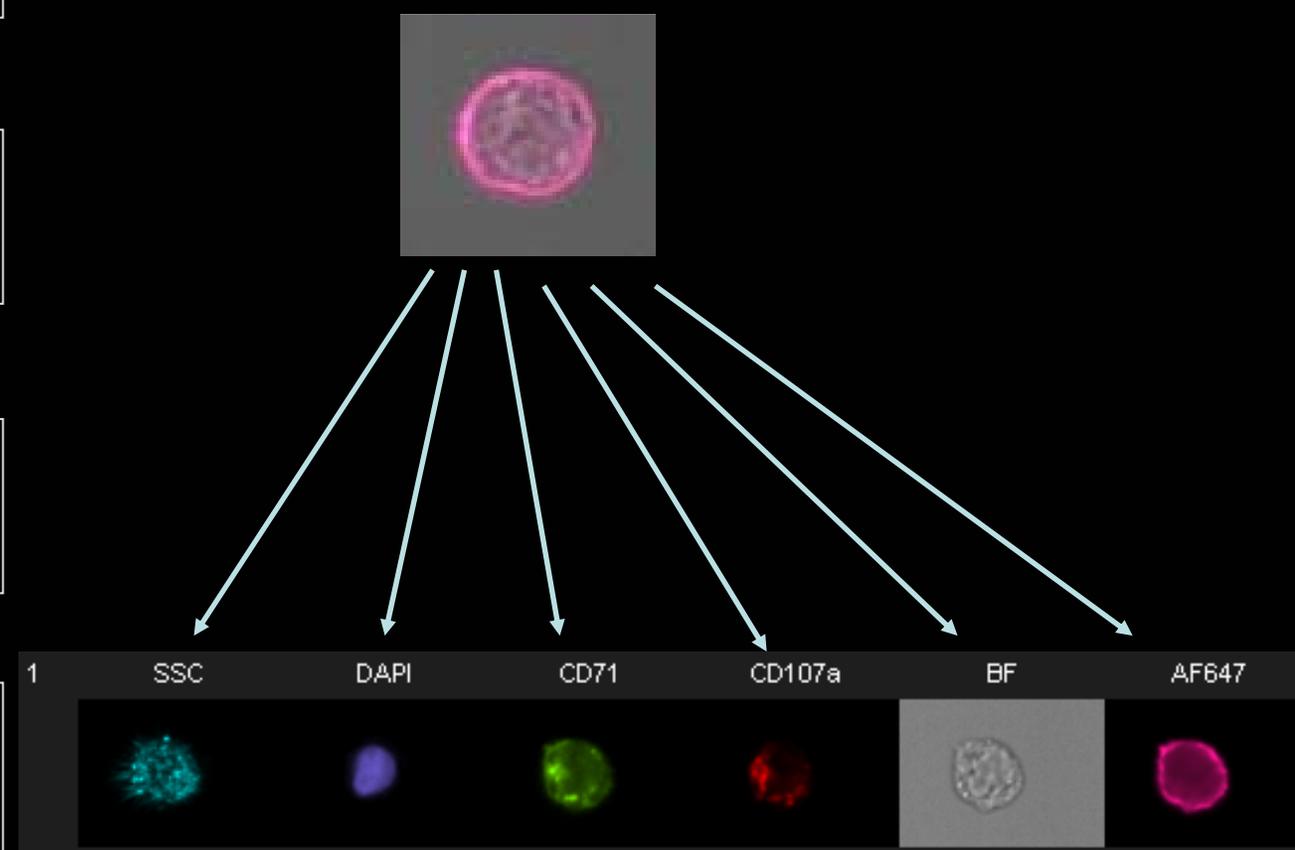
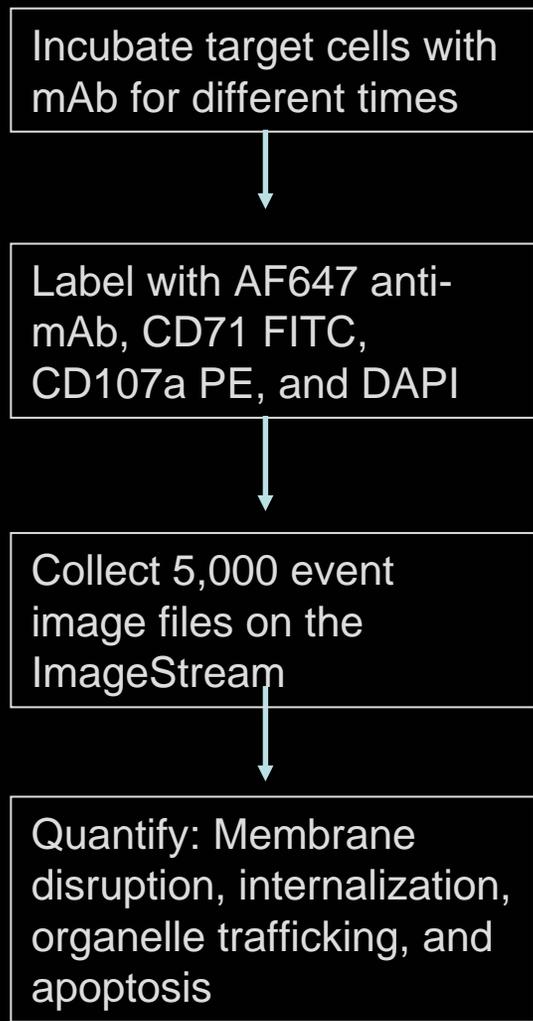


Mono, PMN, T cell



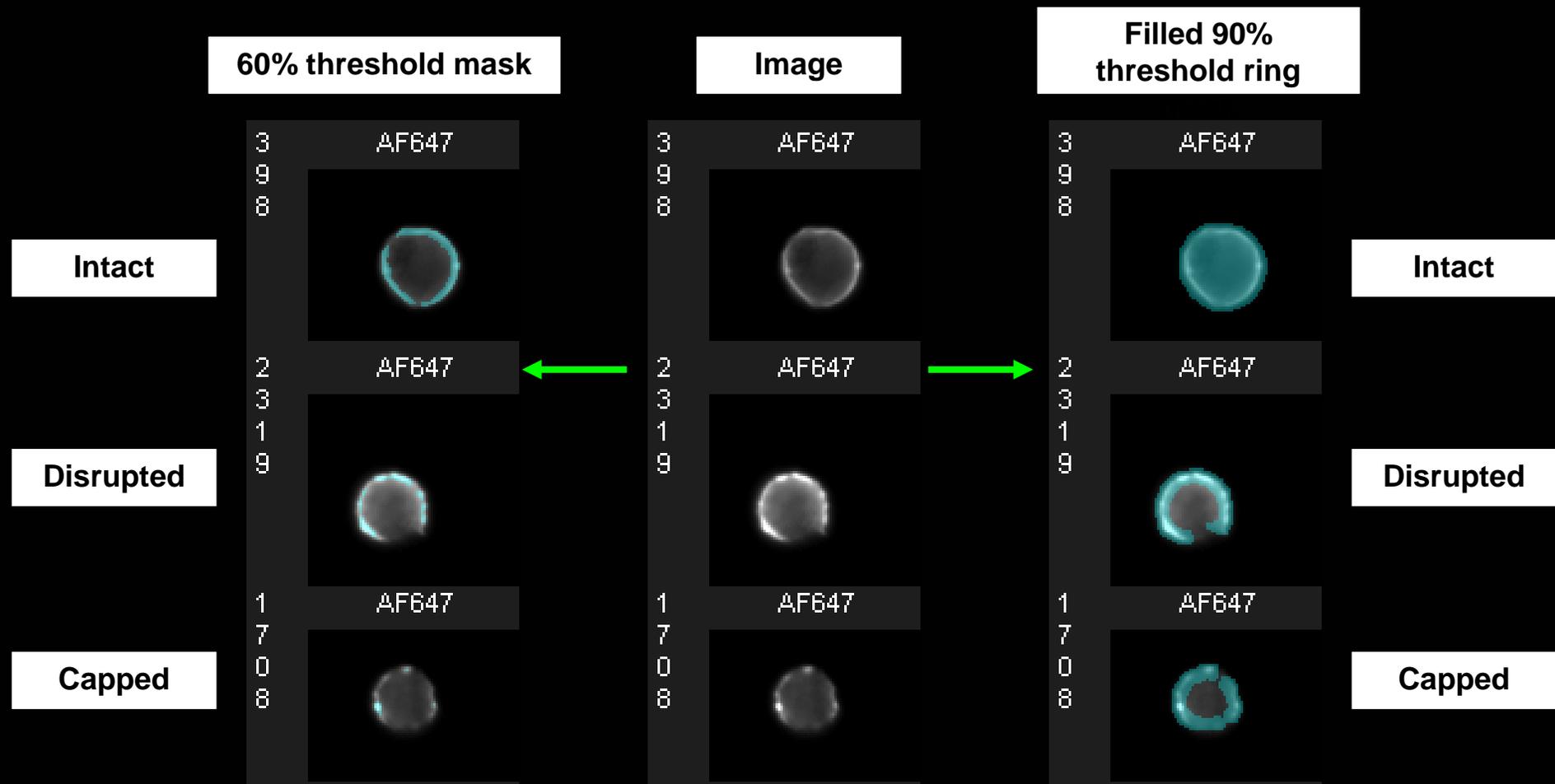


Mechanism of Action – Killer mAb





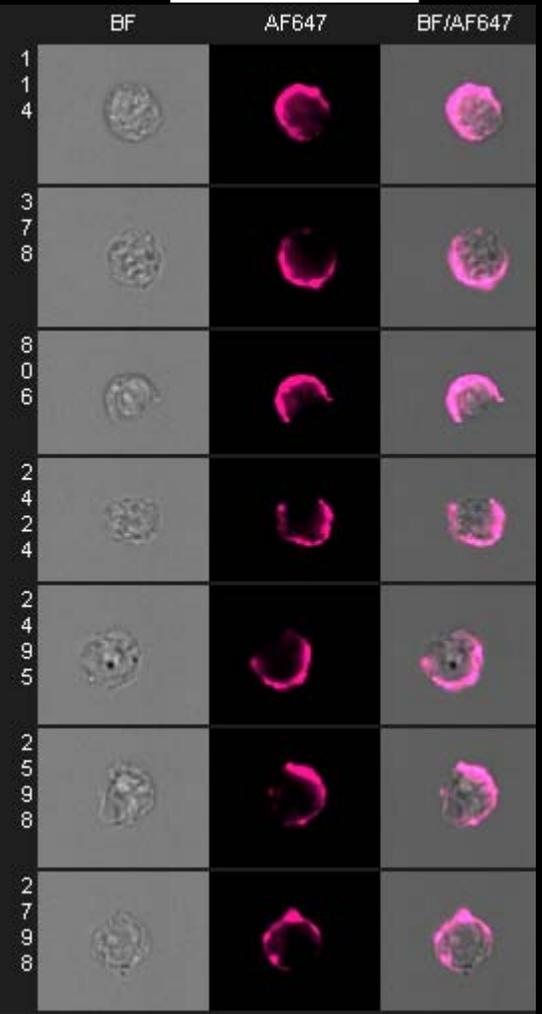
Mechanism of Action – Killer mAb



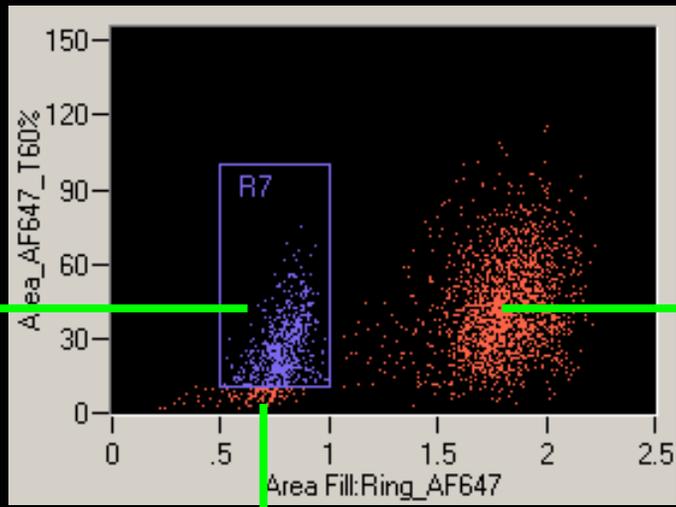
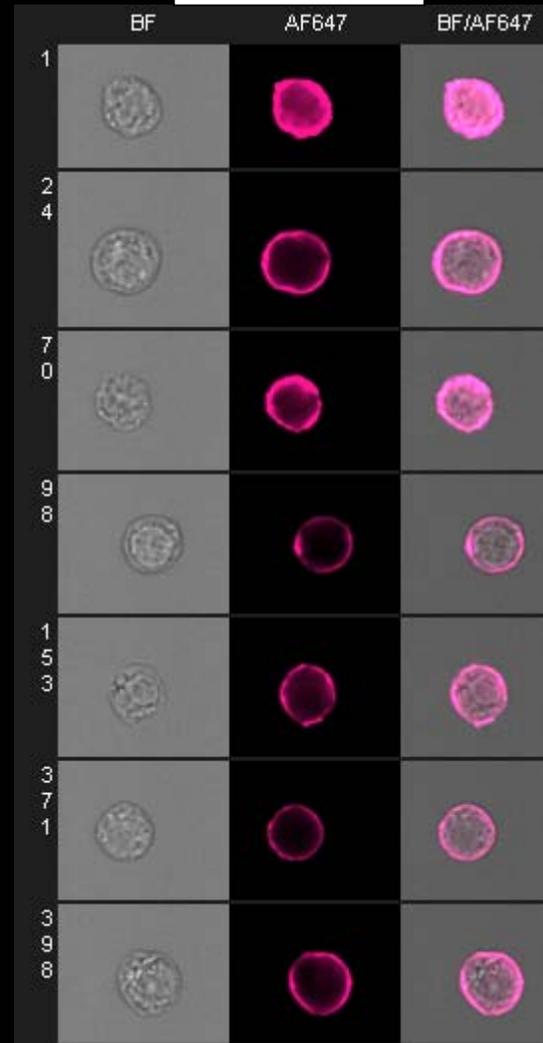


Mechanism of Action – Killer mAb

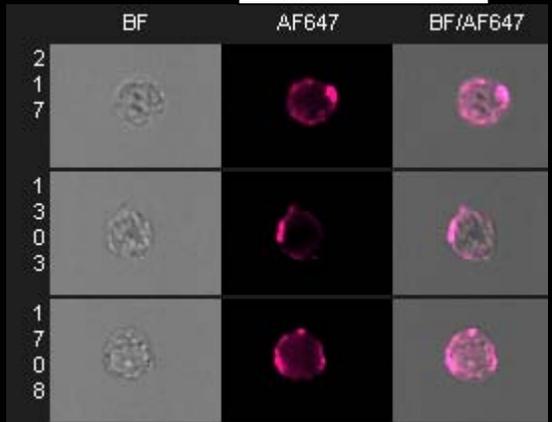
Disrupted



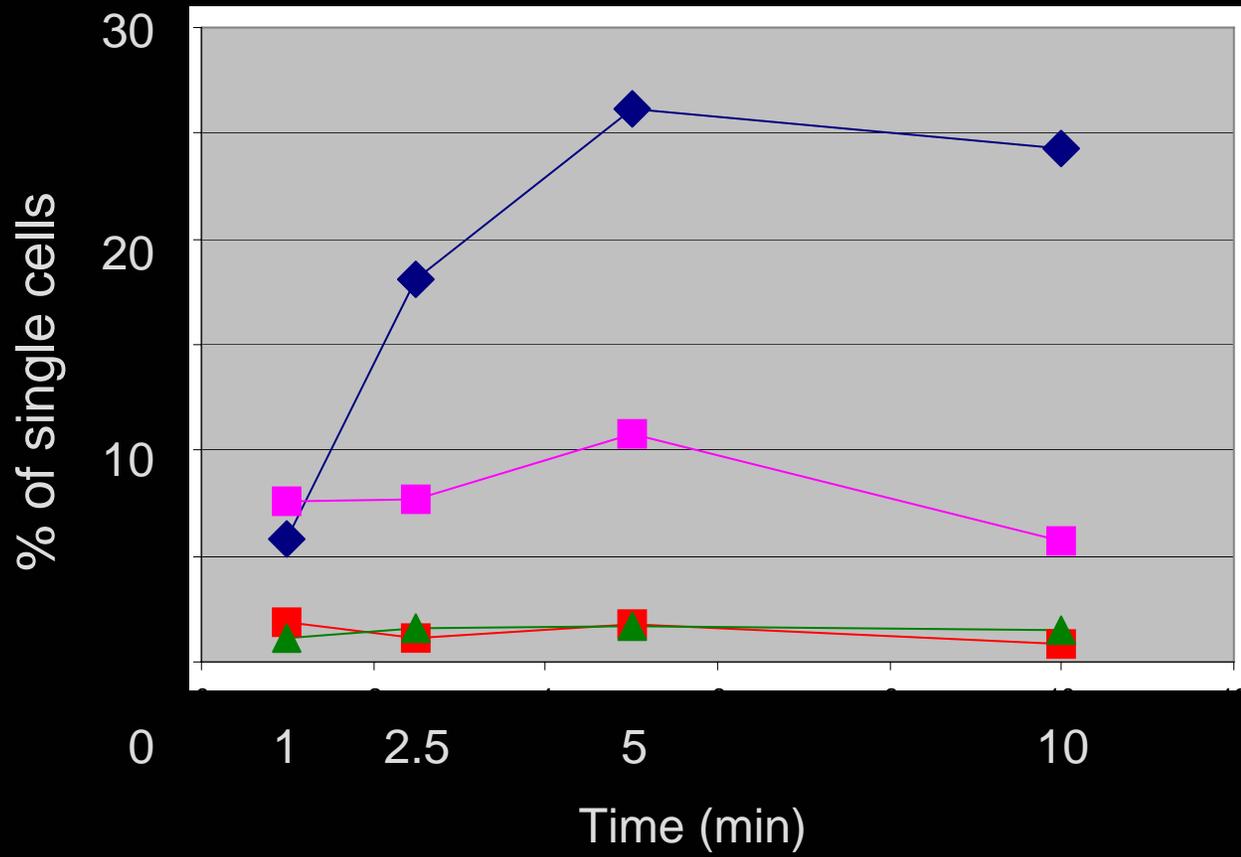
Intact



Capped



Mechanism of Action – Killer mAb



- ◆ Disruptions
- Internalization
- Apoptosis
- ▲ Endo Localization



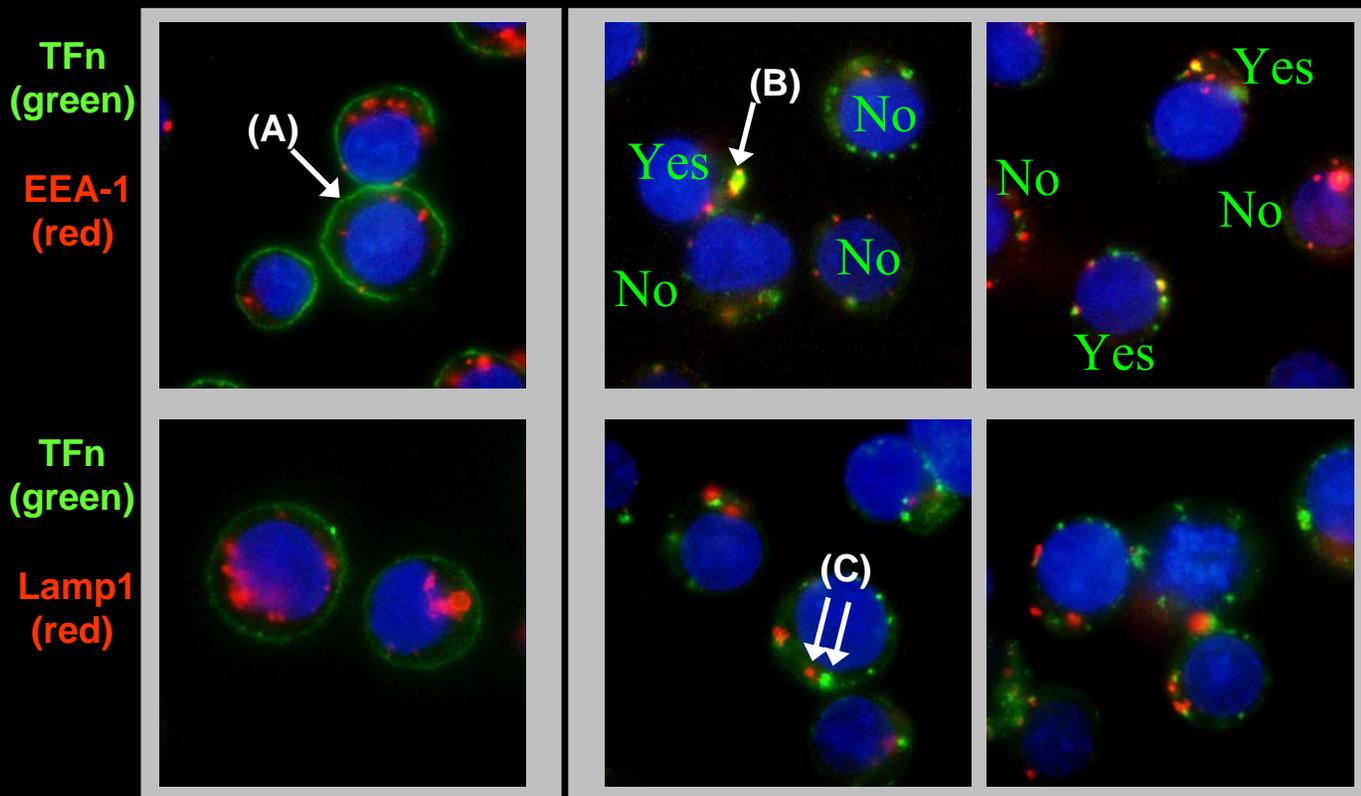
Internalization and Trafficking of Human Transferrin with confocal microscopy...

- In a well characterized system, the transferrin (TFn) receptor is distributed on the cell surface (A).

- At 60min TFn localizes to the endosome (B) but not the lysosome (C).

- This is a model system for internalization and intracellular trafficking of novel ADCs and can be quantified.

How effective was the endosomal colocalization?



How effective was the endosomal colocalization in the top panel?
3 cells in 8 is only 38%

Internalization / trafficking in primary cells

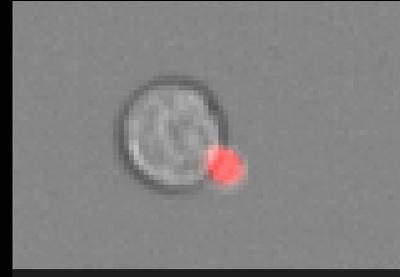


Incubate PBMC with
CpGB

Label pDC with BDCA PE,
CD71 or CD107a FITC

Collect 20,000 event
image files on the
ImageStream

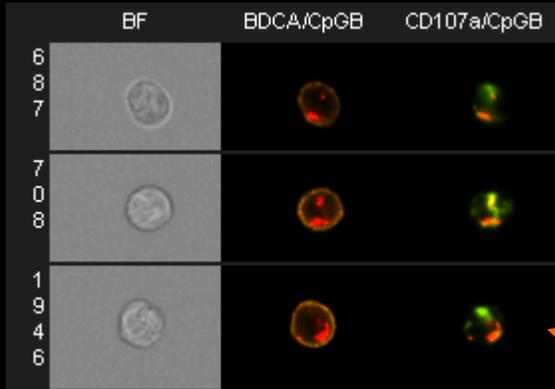
Quantify: Internalization
and colocalization of
CpGB to endosomes and
lysosomes



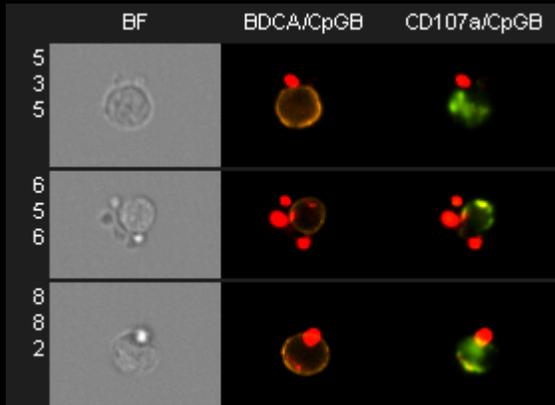
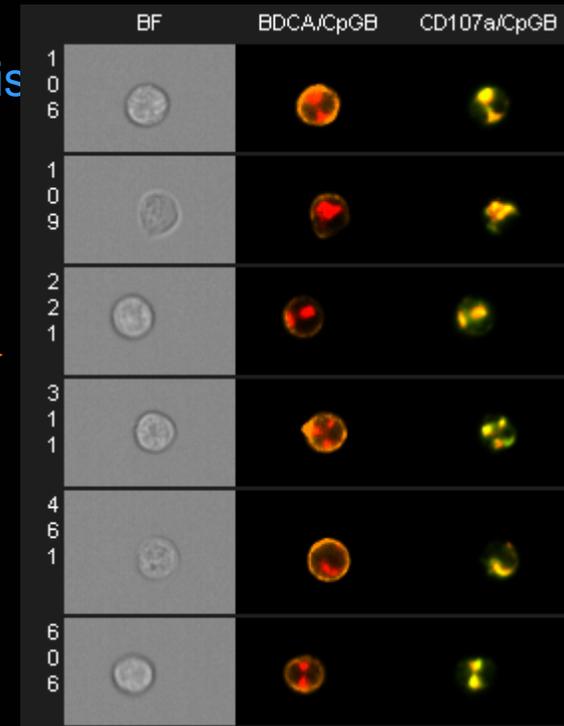
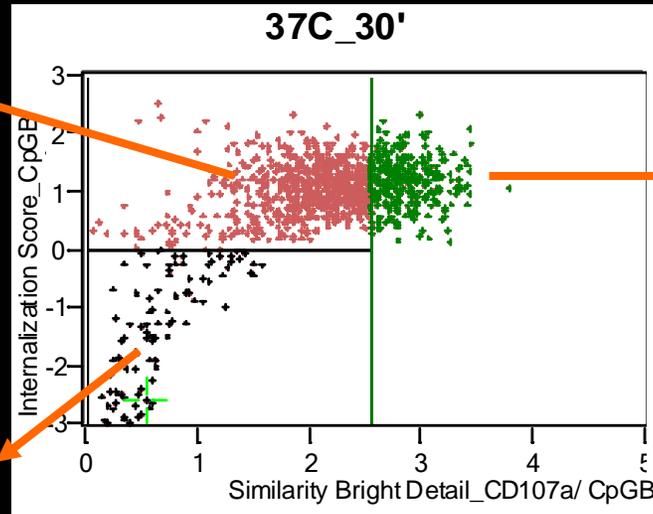
Chongwei Cui
Dr. Pat Fitzgerald-Bocarsly
UMDNJ



Internalization / trafficking in primary cells



Cy5-CpGB Internalization on y-axis

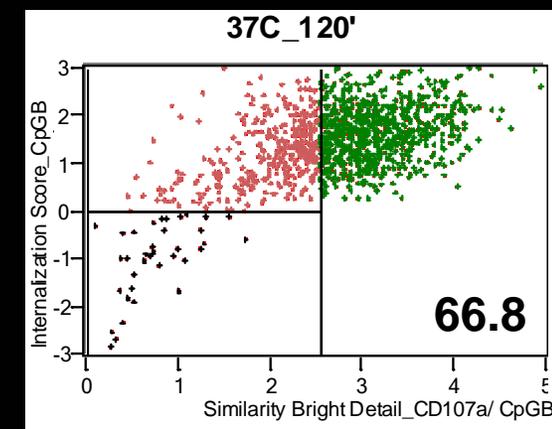
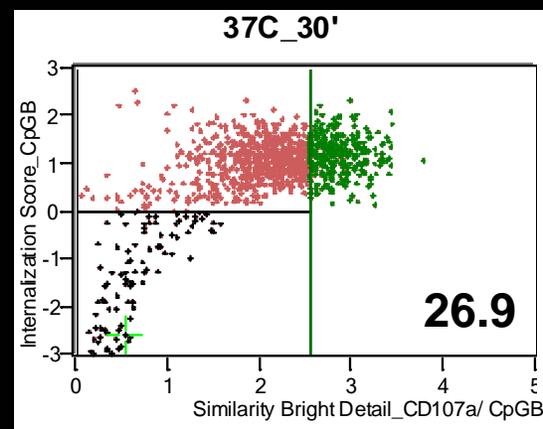
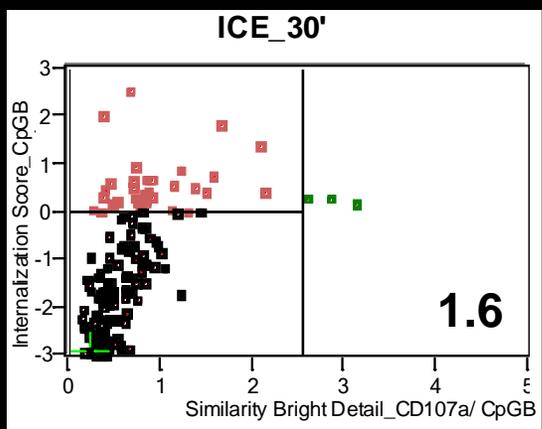
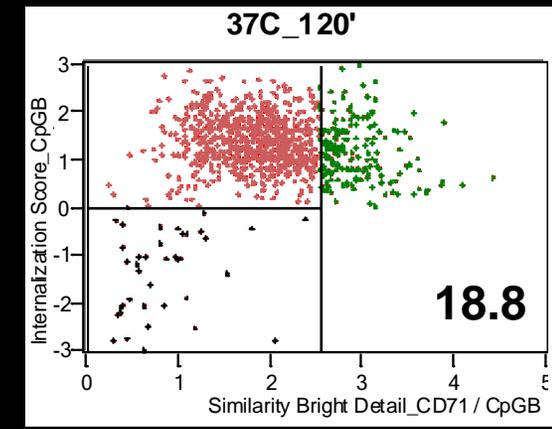
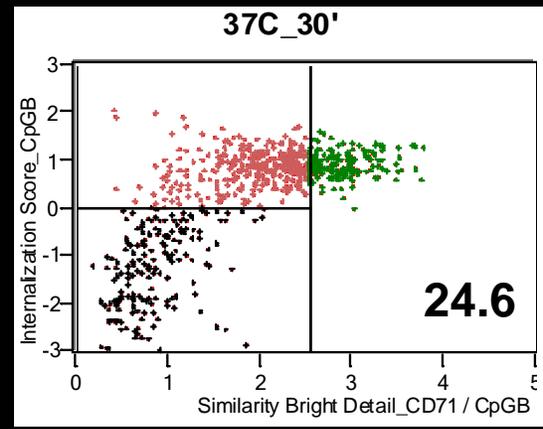
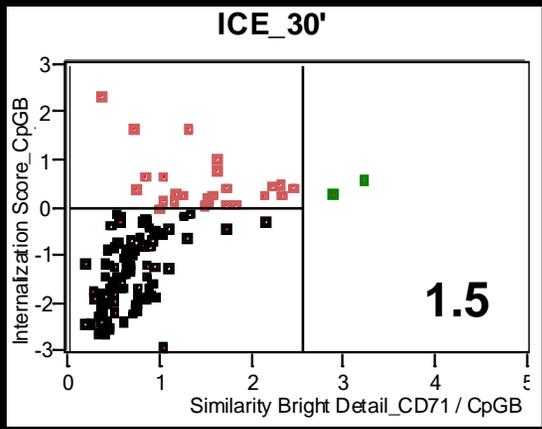


Similarity Bright Detail (x-axis) used to measure organelle colocalization



Internalization / trafficking in primary cells

CD71 (endosomes)

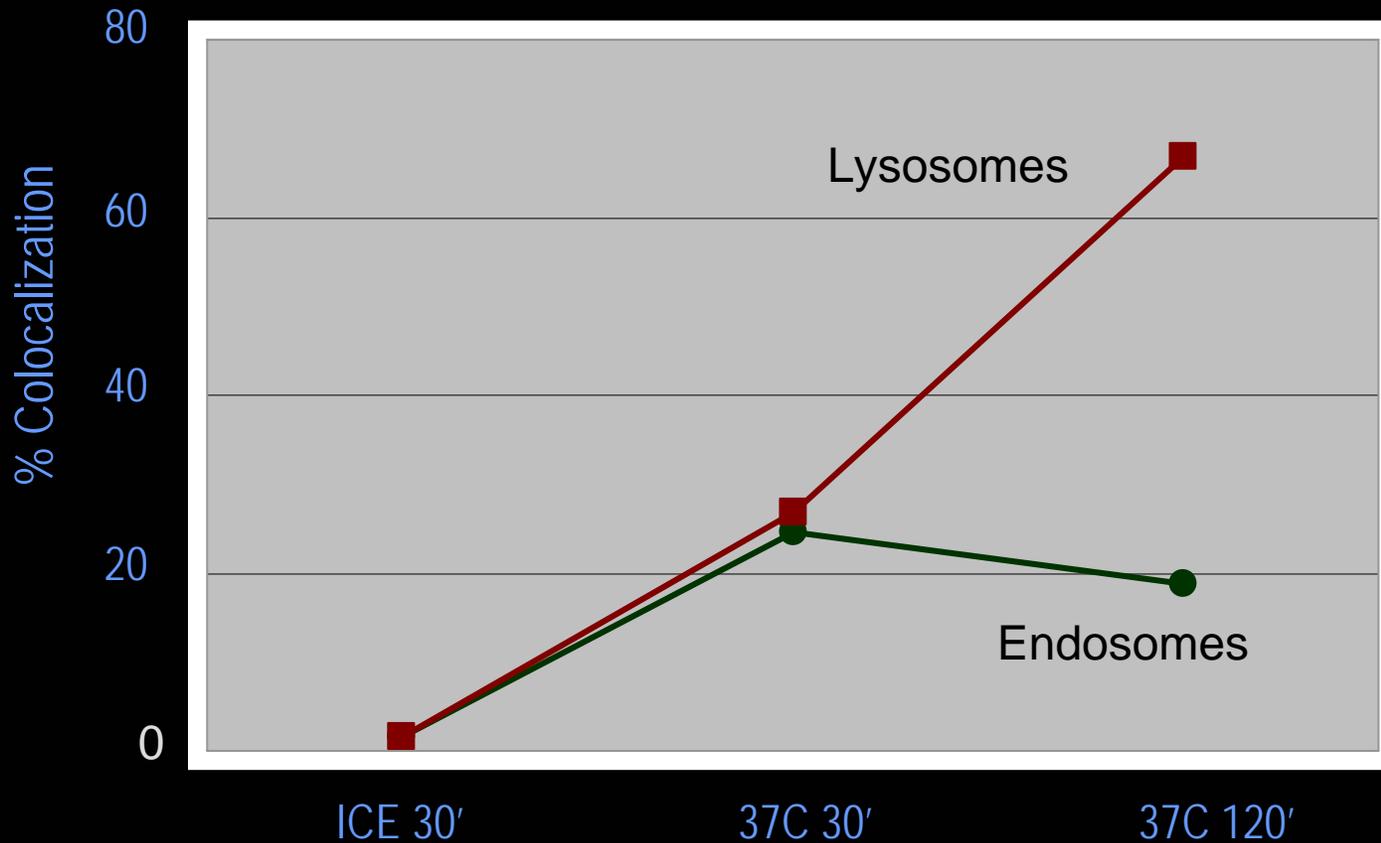


CD107a (lysosomes)

Internalization / trafficking in primary cells

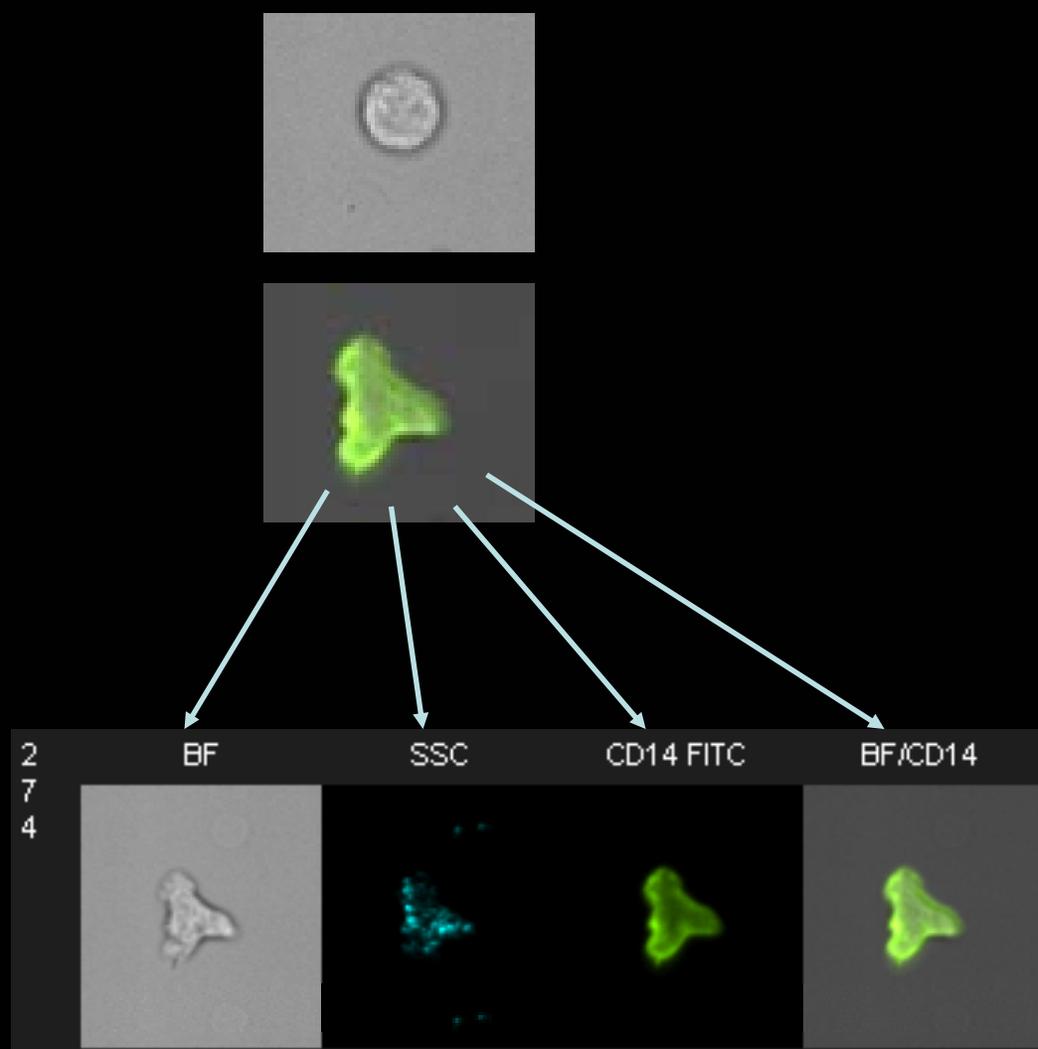
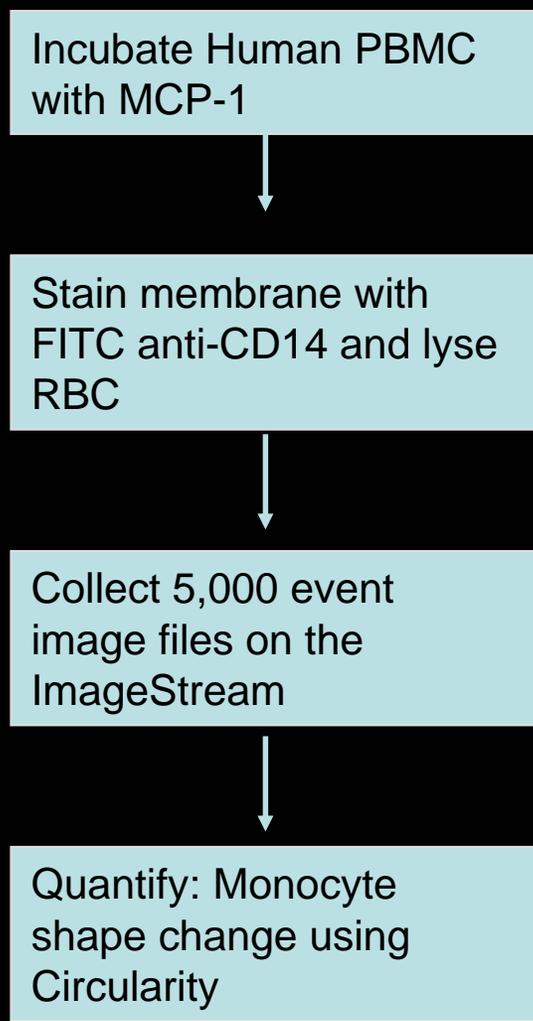


Cy5-CpGB: organelle trafficking





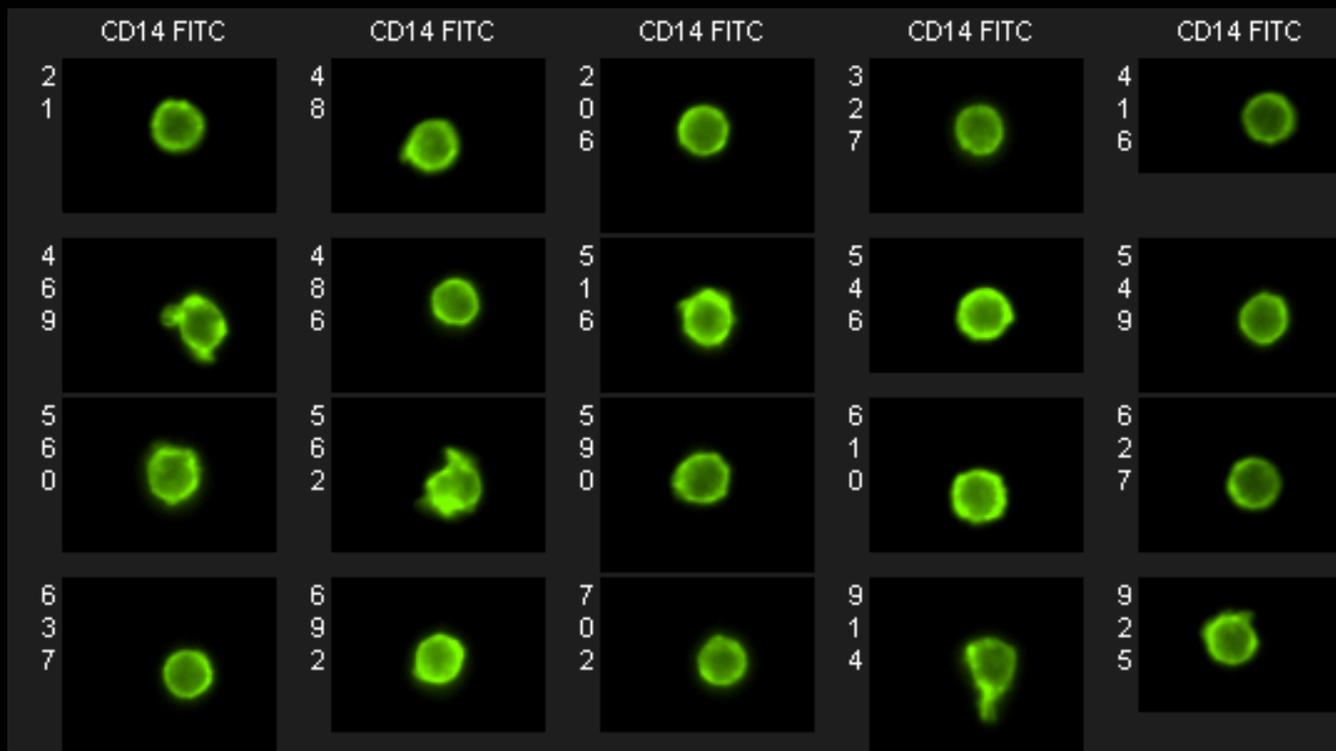
Shape Change, Primary Monocytes





Shape Change, Primary Monocytes

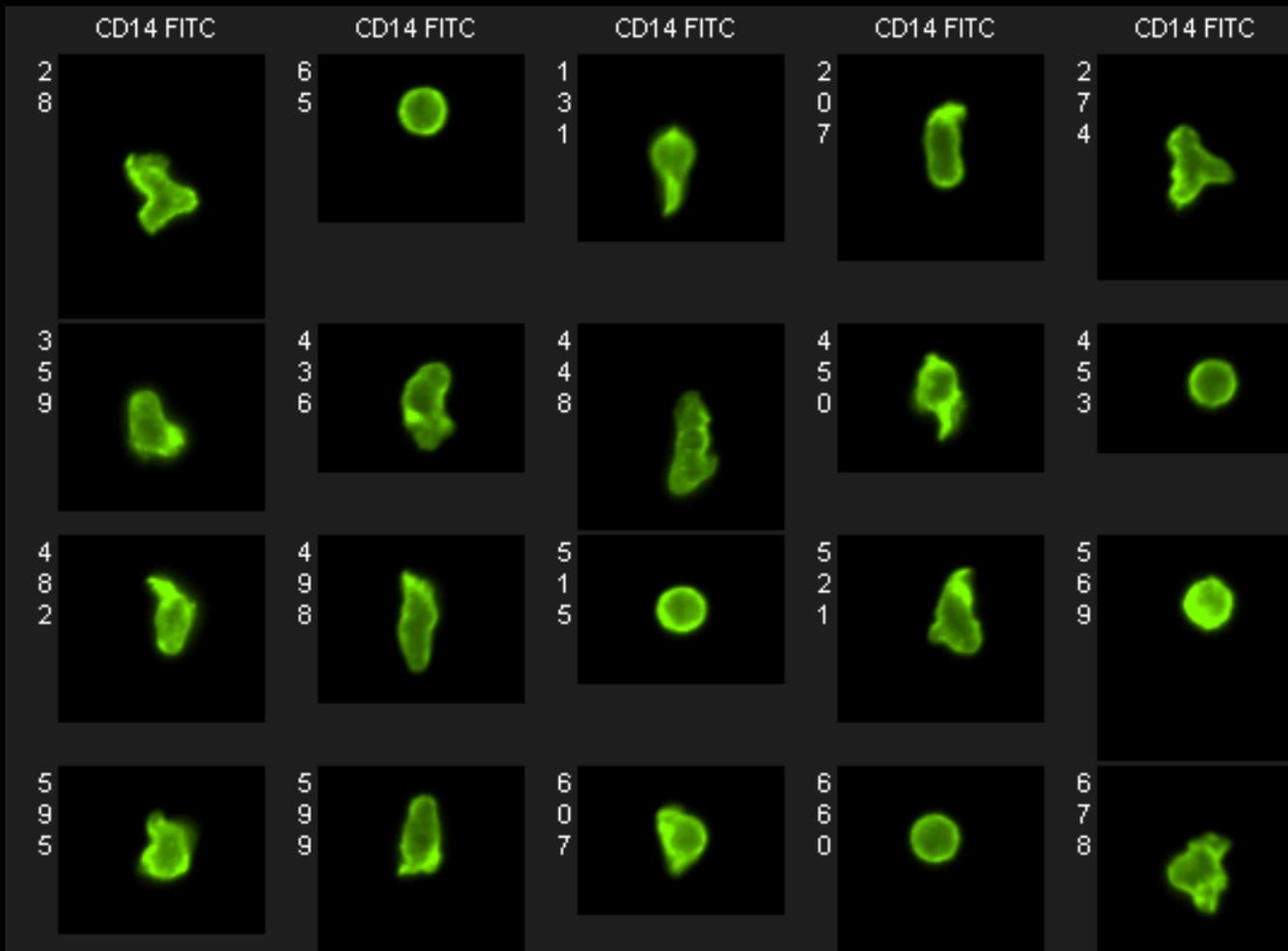
Untreated





Shape Change, Primary Monocytes

MCP-1

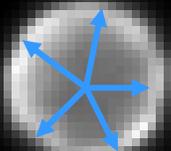




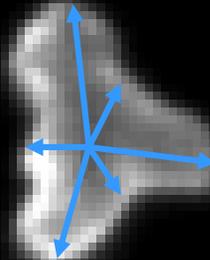
Shape Change, Primary Monocytes

Circularity = average radius divided by radial variance

Untreated



MCP-1



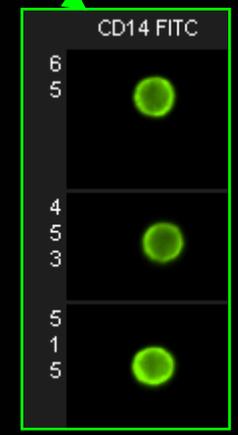
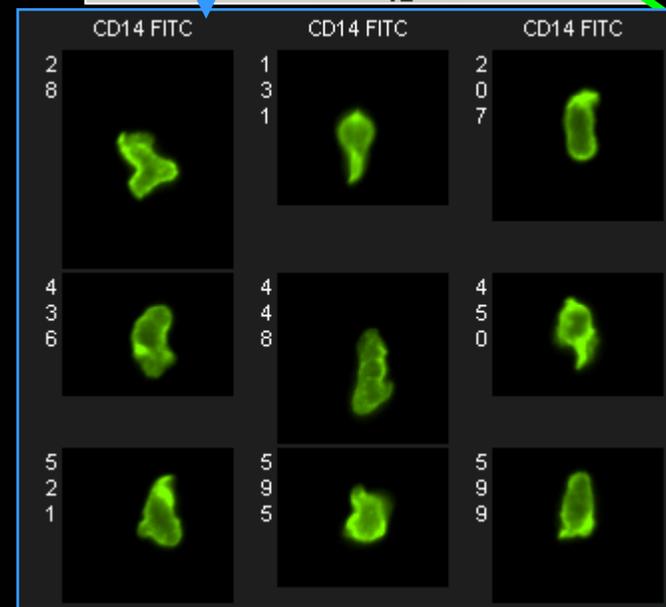
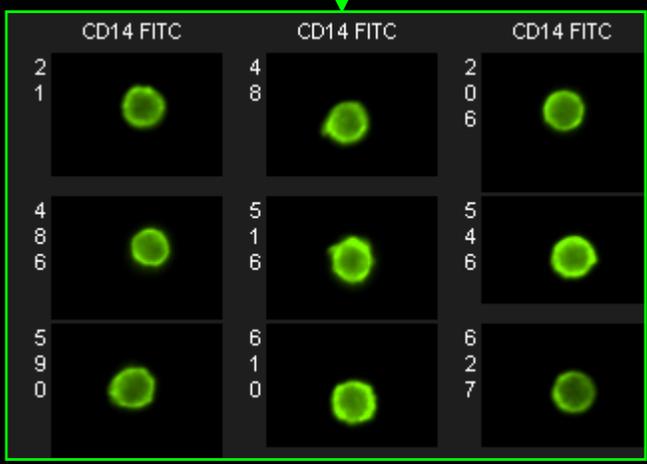
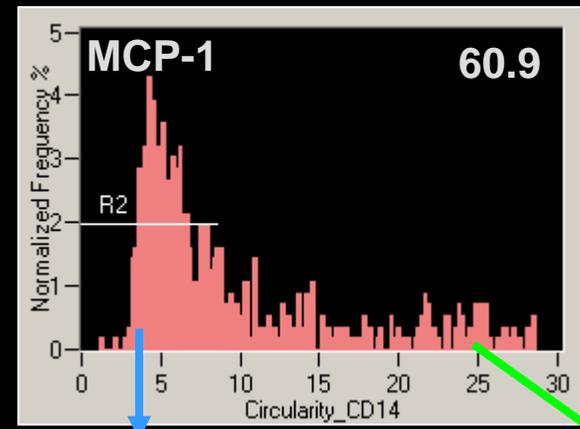
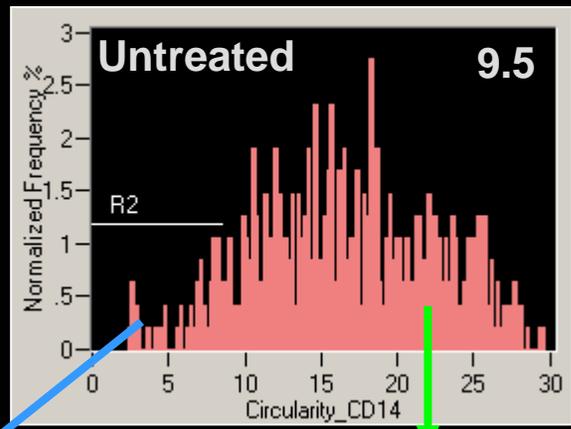
- Radius is the distance from the center of the cell to the edge, at a given angle.

- Radial variance is the average squared deviation from the radial mean, and is a measure of a non-uniform radius.



Shape Change, Primary Monocytes

MCP-1 causes a 'loss in circularity' in the majority of the monocytes





ImageStream applications



Cell signaling



Cell death & autophagy



Internalization & phagocytosis



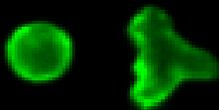
DNA damage and repair



Surface and intracellular co-localization



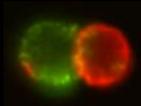
Stem cell biology



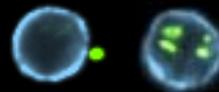
Shape change & chemotaxis



Oceanography



Cell-cell interaction



Microbiology



Cell cycle & mitosis



Parasitology

Multiple Excitation Lasers



405nm 100mw Laser
(DAPI, Hoechst, Pacific Blue...)

488nm 100/500mw Laser
(FITC, PE, PI, ECD, PerCP...)

560nm 200mw Laser
(PE, Spectrum Orange...)

592nm 300mw Laser
(Texas Red, AF594, DS Red...)

658nm 120mw Laser
(AF647, Cy5, APC, APCCy7...)

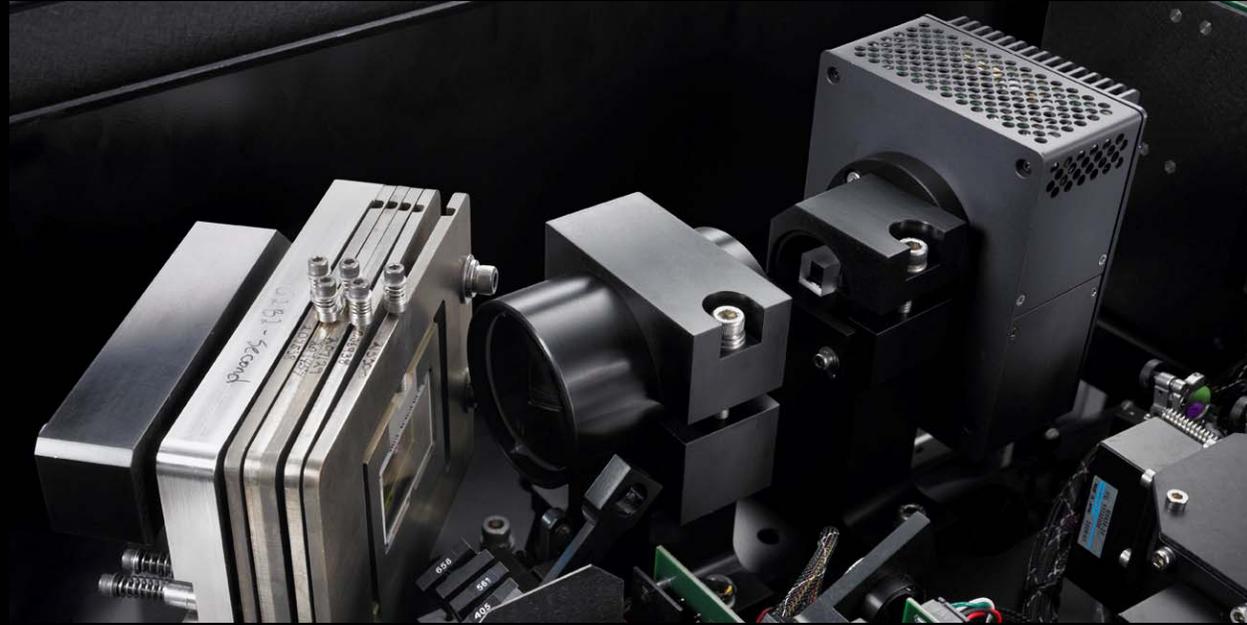


More lasers for greater flexibility in choosing fluorochrome, and collecting
More channels of data...

12 Channel 2 Camera System



- 12 channel data, two brightfield, SSC and up to 10 colors of fluorescence.



Camera 1, ex.488, 560, 785

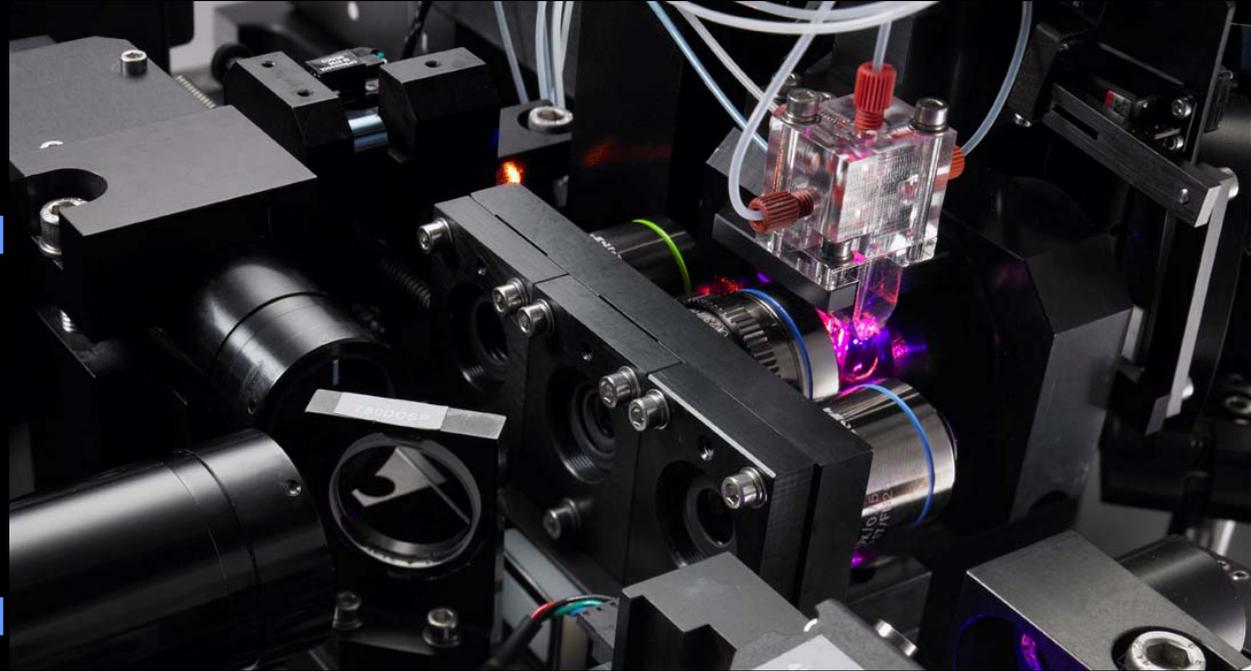
Camera 2, ex 405, 592, 658

Channel 1	Channel 2	Channel 3	Channel 4	Channel 5	Channel 6	Channel 7	Channel 8	Channel 9	Channel 10	Channel 11	Channel 12
430-480	480-560	560-595	595-660	660-745	745-800	430-505	505-575	575-595	595-660	660-745	745-800
Brightfield	FITC	PE	ECD	PerCP	SSC	DAPI	PacOrng	Brightfield	TxR	AF647	APC Cy7
	AF488	Cy-3	PE-TxR	Draq5	PE-Cy7	PacBlu	AF430		AF594	AF660	APC AF750
	GFP	AF555	PI	PerCp5	PE-750	MarBlu			AF568	Cy5	
	YFP	DS-Red	7AAD	PE-647	SSC	Hoechst			AF610	APC	
	Syto		PE-610	PE-Cy5						APC-Cy5.5	
	SpecGrn			PE-680							

Multiple Magnifications



- 40x objective
60um field of view
0.5um per pixel
4um eff depth of field
0.75 NA
- 20x objective
120um field of view
1um per pixel
8um eff depth of field
0.5NA
- 60x objective
40um field of view
0.33um per pixel
2.5um eff depth of field
0.9NA



AutoSampler for Unattended Operation



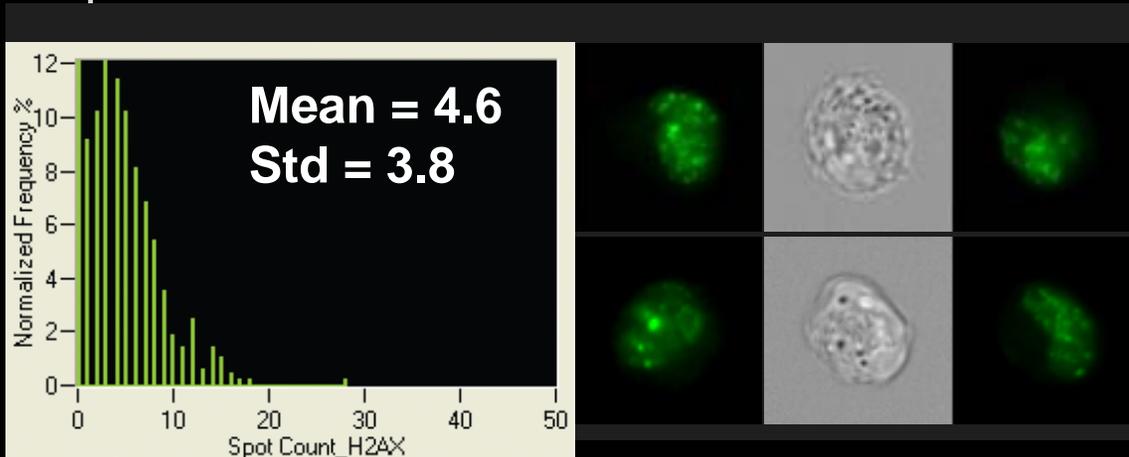
- 96 well plate format
- Software to facilitate multiple experiments per plate
- Failure warning and sample recovery
- Robotics compatible
- Auto-mixing with the uptake probe.
- Membrane piercing probe.



Extended Depth of Field (EDF) – Spot count analysis

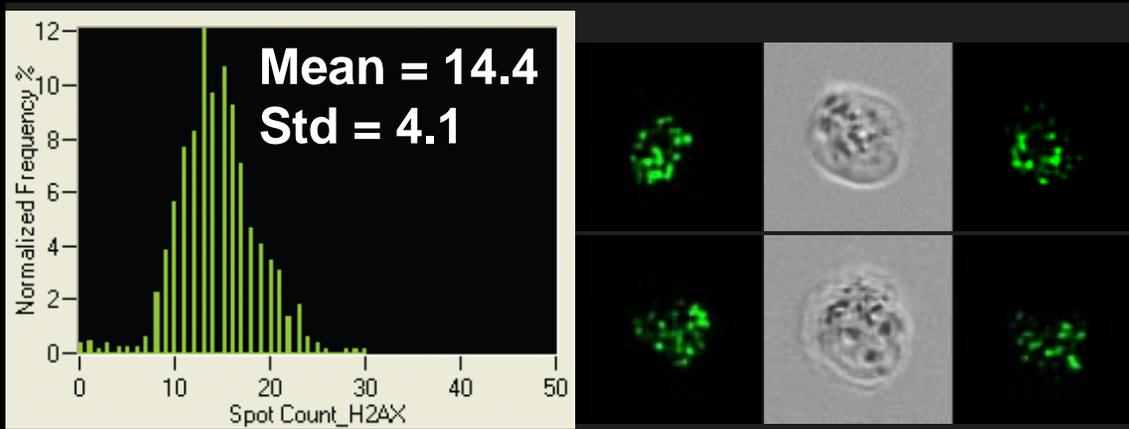


Tox assay - Lung carcinoma cells probed for γ -H2AX (FITC) expression



Standard Imaging

- Some spots in focus
- Defocused spots contribute to high background
- Reduced Spot count values



EDF Imaging

- All spots in focus
- Background reduced

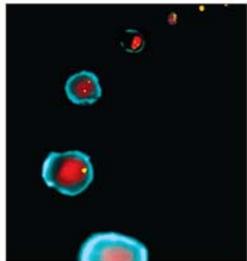
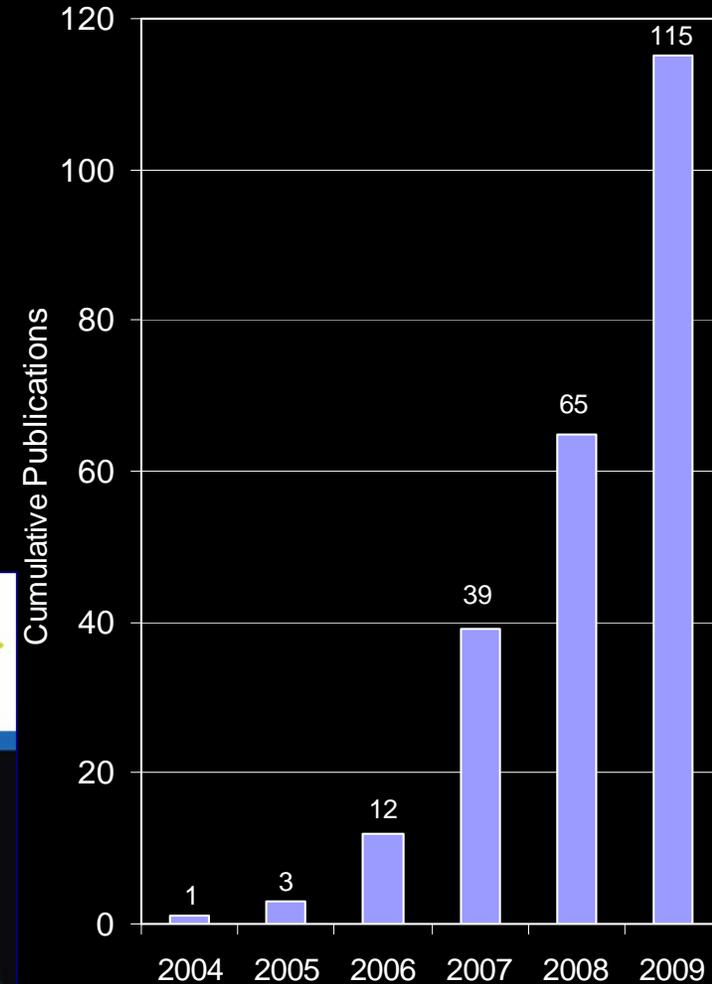
Brightfield

H2AX

Validated with Scientific Publications



- Peer reviewed scientific publications are essential for broad adoption
- >125 Scientific publications from customer base
- Publications are high profile and demonstrate unique capabilities



Journal of
Virology



MAY 2007, VOLUME 81, NUMBER 10



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Cytometry

Volume 71A

Number 4

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ImageStream Sites



Summary



The ImageStreamX delivers:

- Localization of biomarkers on, in, or between cells
- Cell classification with quantitative morphology
- Characterization of rare sub-populations in complex samples
- Image-based applications with flow cytometry protocols



Clarity from Complexity



- ***Thank you for your attention!!***

