# Harnessing imaging and spectral analysis potential in flow cytometry with Attune flow cytometers


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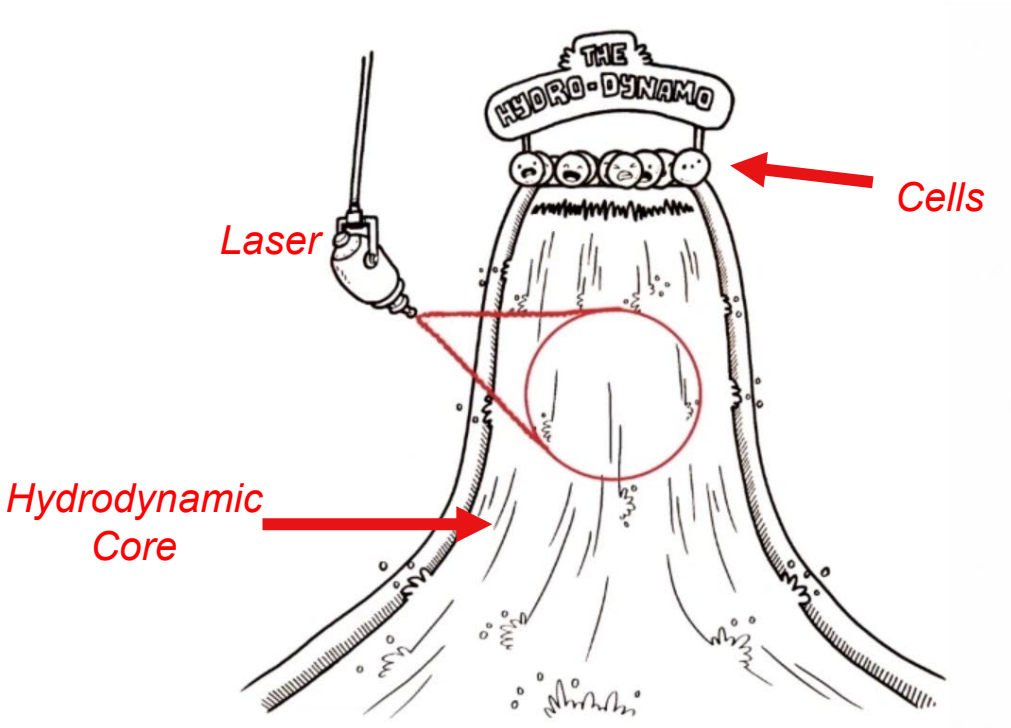
**Australasian Cytometry Society – 2024, Hobart.**

The world leader in serving science

# Acoustic Focusing

*Hydrodynamic Focusing*




***High Flow Rates**

# Why acoustic focusing?

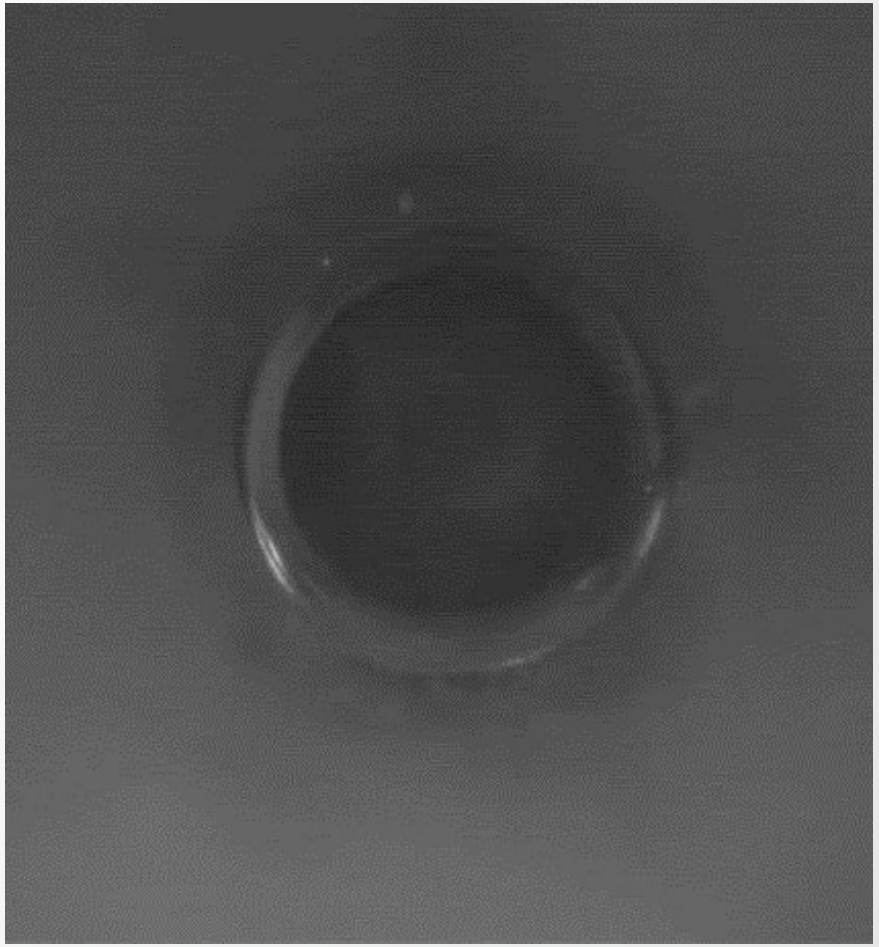
- Precise alignment of cells with laser
  - **Consistent and reproducible data** with lower CVs
  - Enables **higher flow rates** without sacrificing **data quality**

- **Reduced time to statistically significant data**
  - Attune CytPix Flow Cytometer offers flow rates of 12.5 µL/ minute – 1000 µL/minute

- Specified volumetric delivery
  - **Clog resistant**, even for difficult sample types
  - Consistent concentration results

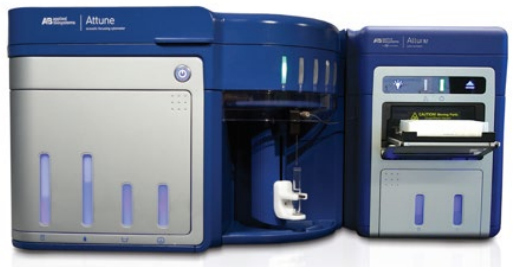
| Instrument/flow rate | Time to 1 million granulocytes | Relative rate |
|---|---|---|
| Hydrodynamic focusing high flow rate | 63 min 33 sec | – |
| Acoustic focusing at 200 µL/min | 13 min 20 sec | ~5x faster |
| Acoustic focusing at 500 µL/min | 5 min 47 sec | >10x faster |

Data from AACR scientific poster, “Acoustic Cytometry for Rare Event Detection of PNH Cells”



Fluorescent beads in capillary tube showing particle distribution with and without acoustic focusing

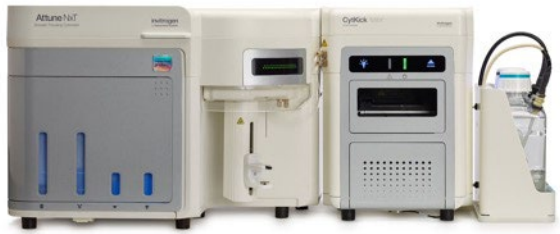
# Evolution of Attune Flow Cytometers



## Invitrogen™ Attune™ Acoustic Focusing Cytometer

**2010** First acoustic flow cytometer released to the market

**2012** Invitrogen™ Attune™ Autosampler released, bringing standard and deep-well plates to customers



## Invitrogen™ Attune™ NxT Flow Cytometer

**2014** Attune NxT Flow Cytometer introduced to the market

**2016** Green laser introduced

**2017** Attune NxT Flow Cytometer Violet 6-channel option released

**2018** Integration of Thermo Scientific™ Orbitor™ RS2 Microplate Mover

**2020** 21 CFR Part 11 compliance; release of Invitrogen™ CytKick™ Autosampler and CytKick™ Max Autosampler with Microsoft™ Windows™ 10 software compatibility

## Invitrogen™ Attune™ CytPix™ Flow Cytometer

**2021** **Attune CytPix Flow Cytometer introduced**

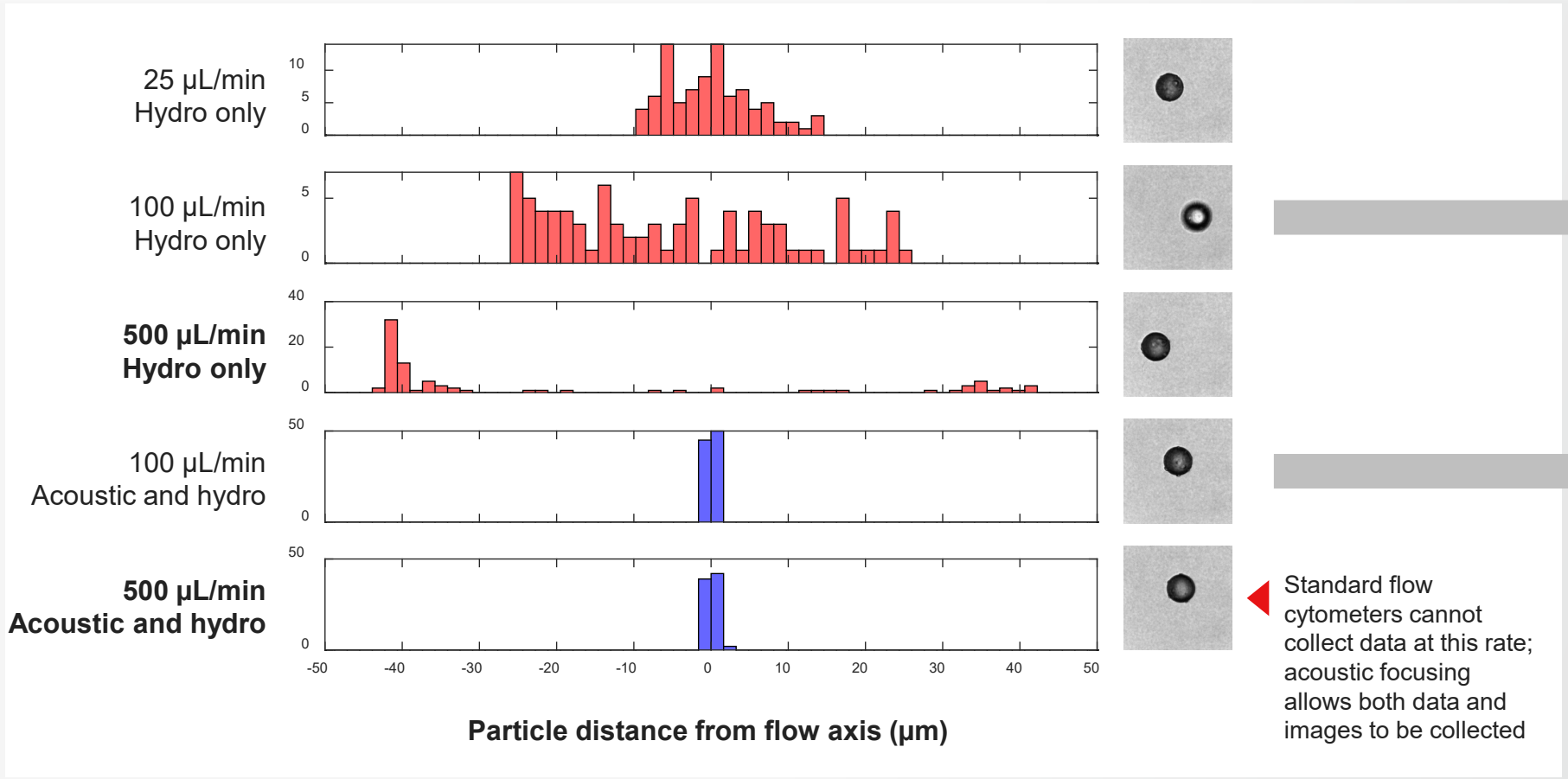
**2023** **Major software upgrade — *Automated Image Analysis* (AIA) feature**

# Benefits of acoustic focusing for imaging

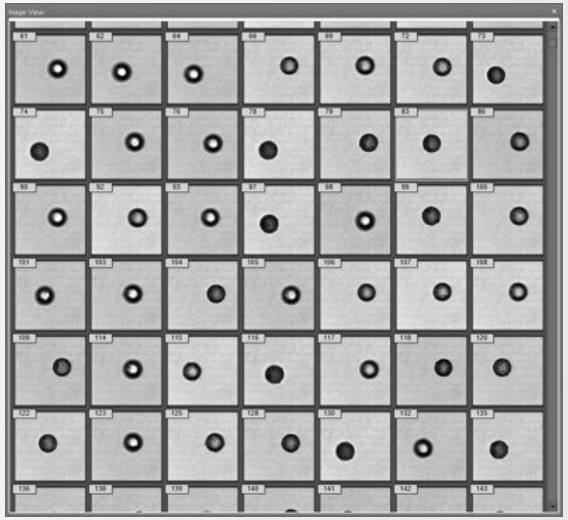
**Particles fall outside microscope's depth of field (DOF) when using hydrodynamic focusing alone (~6 µm DOF)**

- Reason why imaging cytometers cannot run at standard flow rates

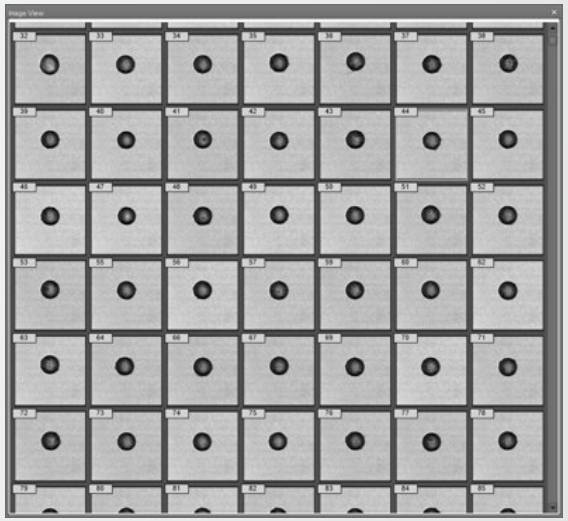
**Acoustics-assisted hydrodynamic focusing places particles within DOF, producing in-focus images at standard cytometry rates**




Particles in and out of DOF



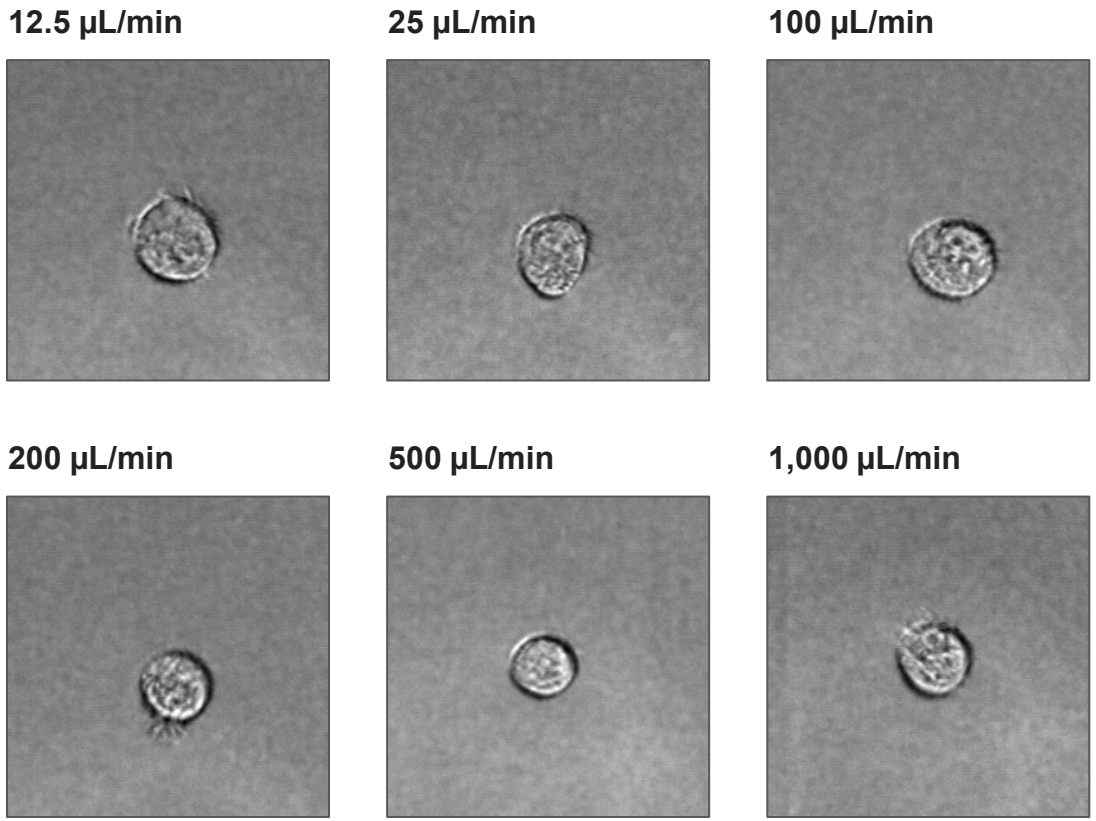
Particles contained within DOF



Note: Corner case: Number of out-of-focus images increases for small particles (<1 µm).

# An imaging cytometer with high-throughput capacity

## Image cells at every flow rate



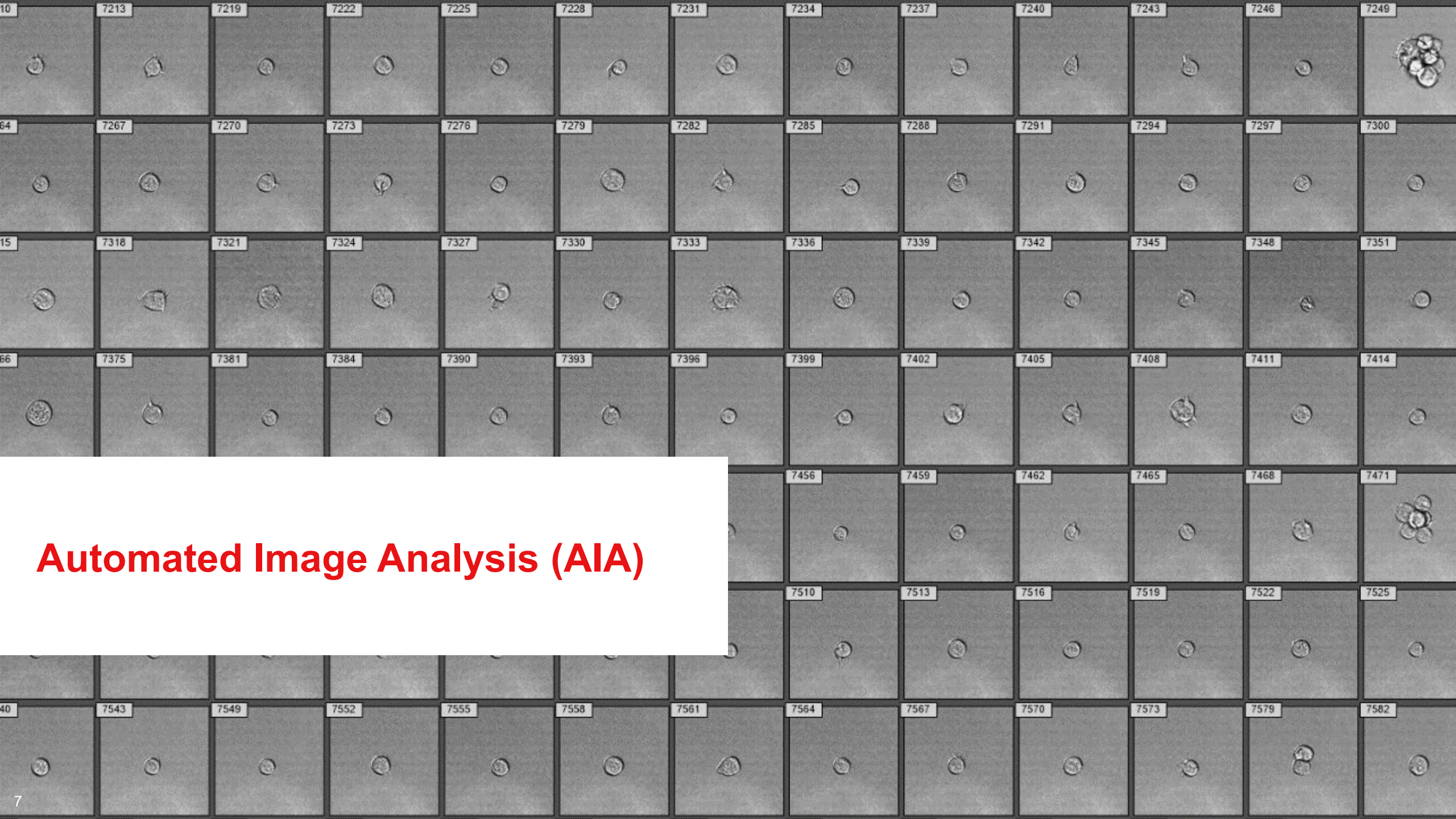

**Acoustic focusing combined with a high-speed camera enables consistent image quality at each flow rate**

- No changes to sample rate required for imaging capabilities

**CAR T cells imaged at each flow rate**

- Single sample (~1 x $10^6$ cells/mL)
- User-friendly focus setting adjustments
  - Easily adjust camera settings to match experimental requirements

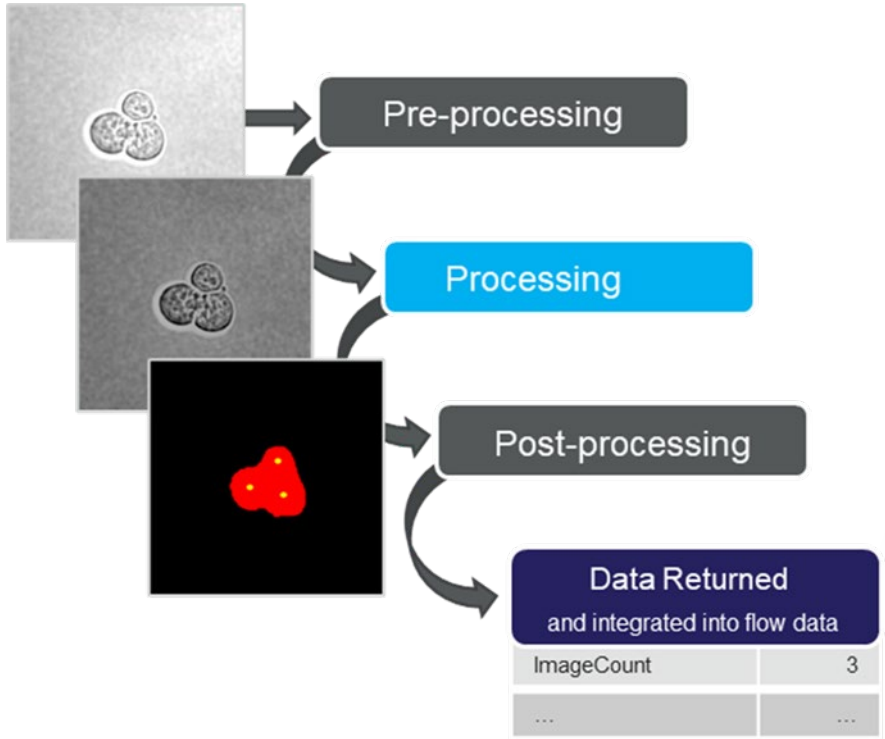
Late log phase CAR T cells from a single sample imaged at each flow rate. CAR T cells: proliferating human CART cells targeting the human CD19 antigen, supplied by Thermo Fisher (similar to Juno JCAR019), ~1 x $10^6$ cells/mL.



# Automated Image Analysis (AIA)

# Events identified and image data extracted in software

## Software removes the most resource intensive image analysis step: Manual annotations




- Software identifies events in the image
  - Events between 5-20 um
  - Error rate below 10%
  - Validated on leukocytes
- 26 image parameters are calculated based on the software's annotation
- These parameters are integrated into the normal workspace or can be exported with traditional FCS data files

# Automated image analysis

List of available image parameters for processing

| System features |
| --- |
| On border |
| Confidence score |
| Processed |
| Processable |

| Object features |
| --- |
| Particle/cell count |

| Pixel features |
| --- |
| Pixel count |

| Shape features |
| --- |
| Area ($\mu m^2$) |
| Perimeter area (µm) |
| Circularity (%) |
| Pseudo diameter (µm) |
| Major axis (µm) |
| Minor axis (µm) |
| Minor to major axis ratio (%) |
| Eccentricity (%) |

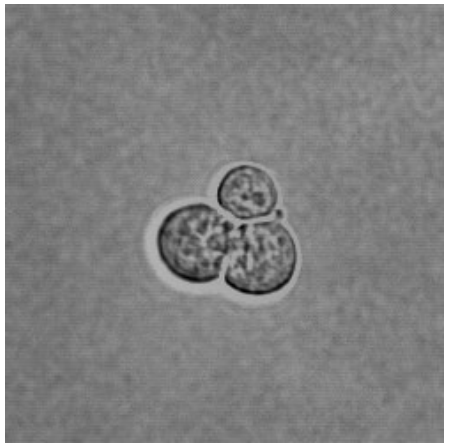
| Intensity features |
| --- |
| Maximum intensity |
| Minimum intensity |
| Total intensity |
| Average intensity |
| Intensity standard deviation |
| Intensity %CV |
| Average normalized intensity |
| Normalized intensity SD |
| Normalized intensity %CV |
| Intensity skewness |
| Intensity kurtosis |
| Intensity entropy |

# Image parameters—examples of use

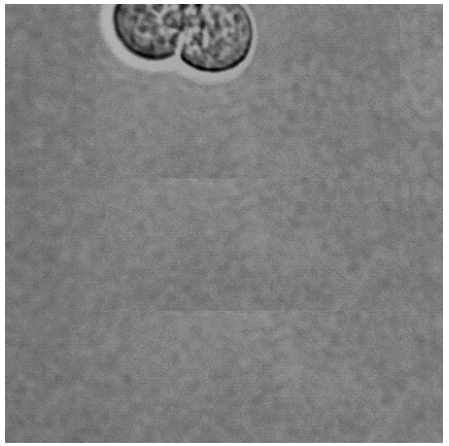
Improve accuracy for label-free analysis

## System features

**IsOnBorder = excludes events on the borders**



**Included**



**Excluded**

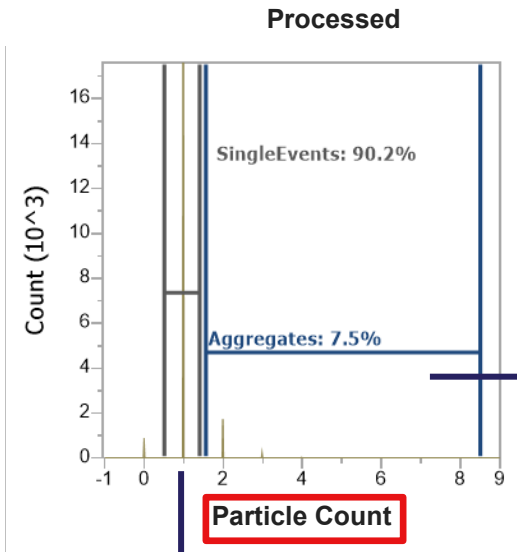
# Image parameters—examples of use

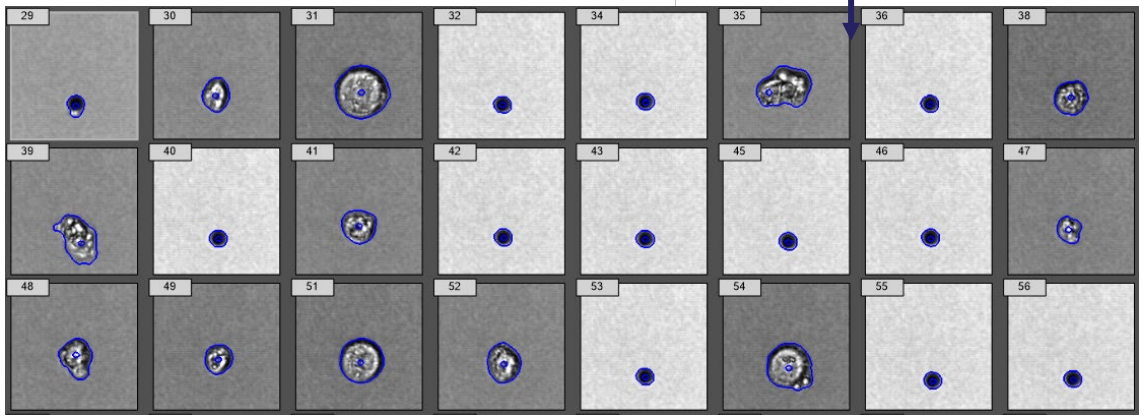
Improve accuracy to exclude aggregates, or unwanted events

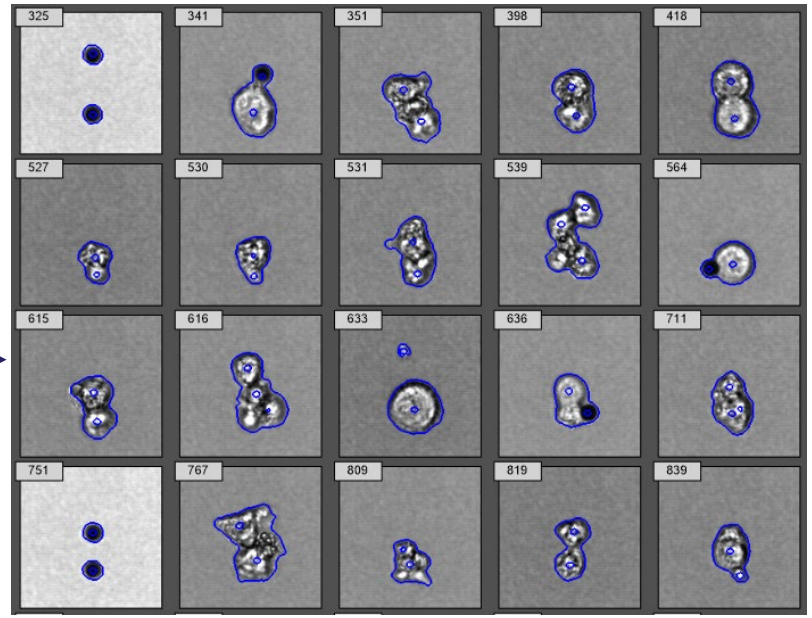
## Object features

### Particle count

**Exclude**
non-singlets



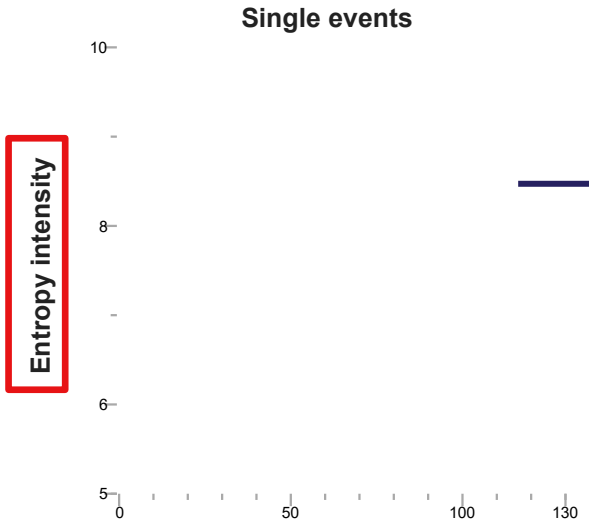


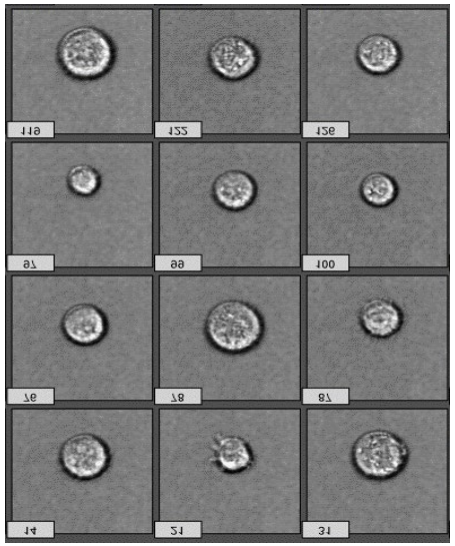
# Image parameters—examples of use

Distinguish surface complexity, uniformity, and granularity using light and dark spots

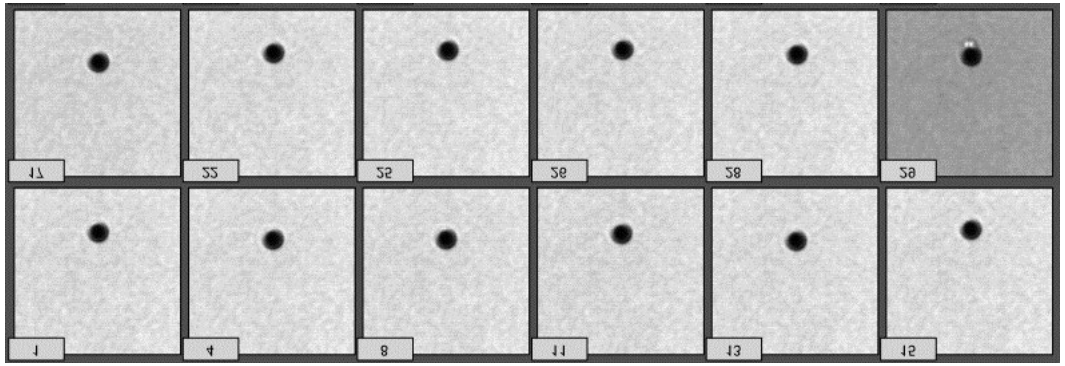
## Intensity features

### Entropy Intensity



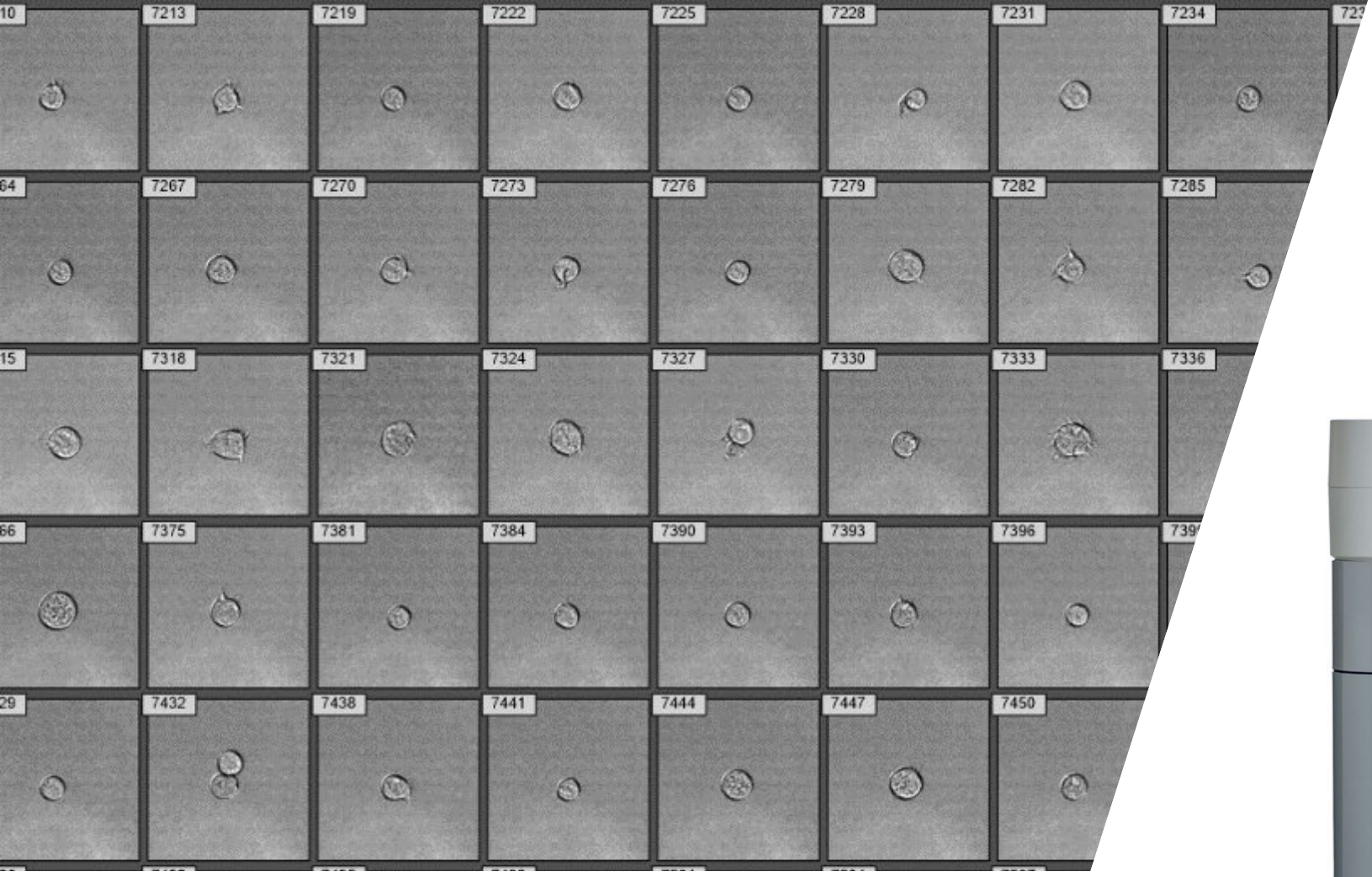



High entropy



Low entropy

- Cells have higher complexity compared to beads

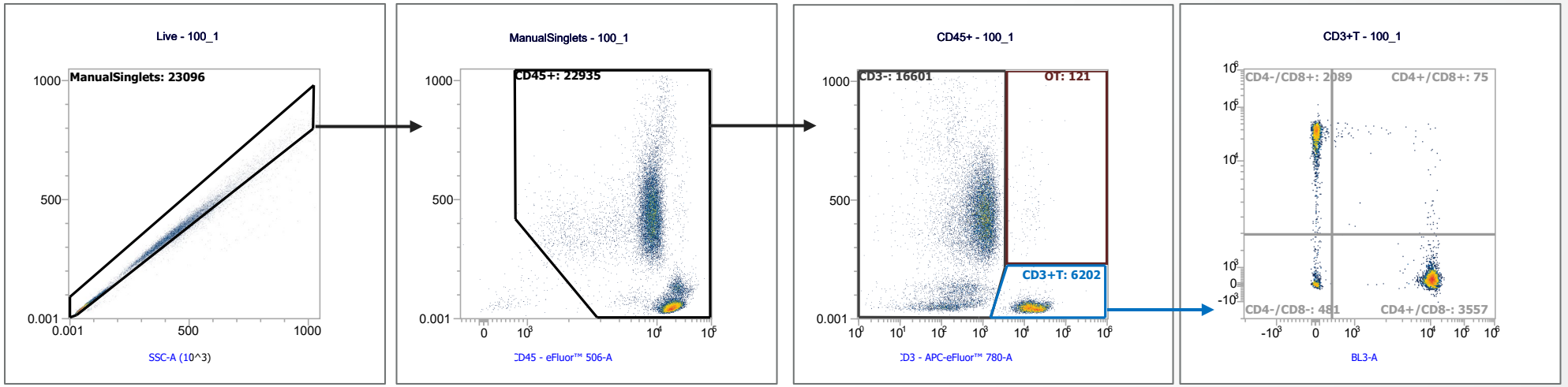



# *Attune Cytpix™*

## More then just pretty pictures

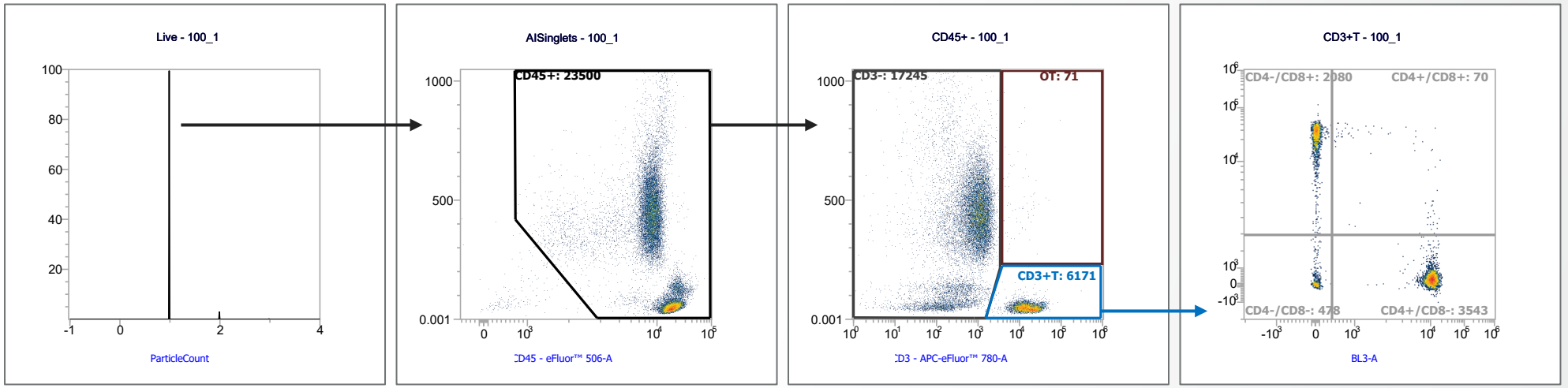
# Combining fluorescent detection and imaging data

## Lysed blood leukocytes – gating strategies to double positive T cells




### Without image data

Singlet gating performed manually




### With image data

Small change in workspace (here, singlet gating) causes a 7% shift in ultimate downstream gate (CD4+/CD8+ T cells)



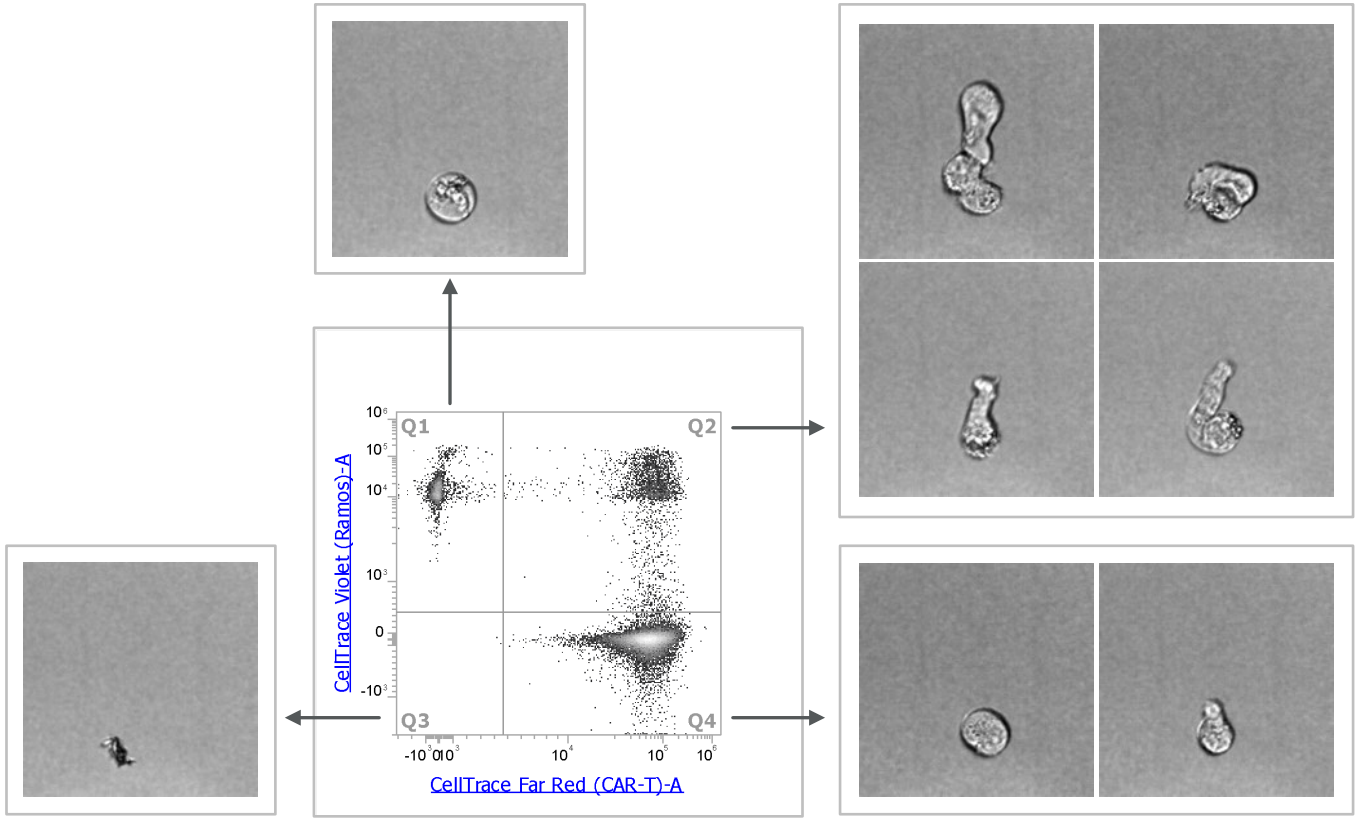

Values shown are count of events in each gate. See previous slides for sample preparation and upstream gating.

# Gain visual clarity of difficult sample types

## Evidence for cell-cell interaction now possible

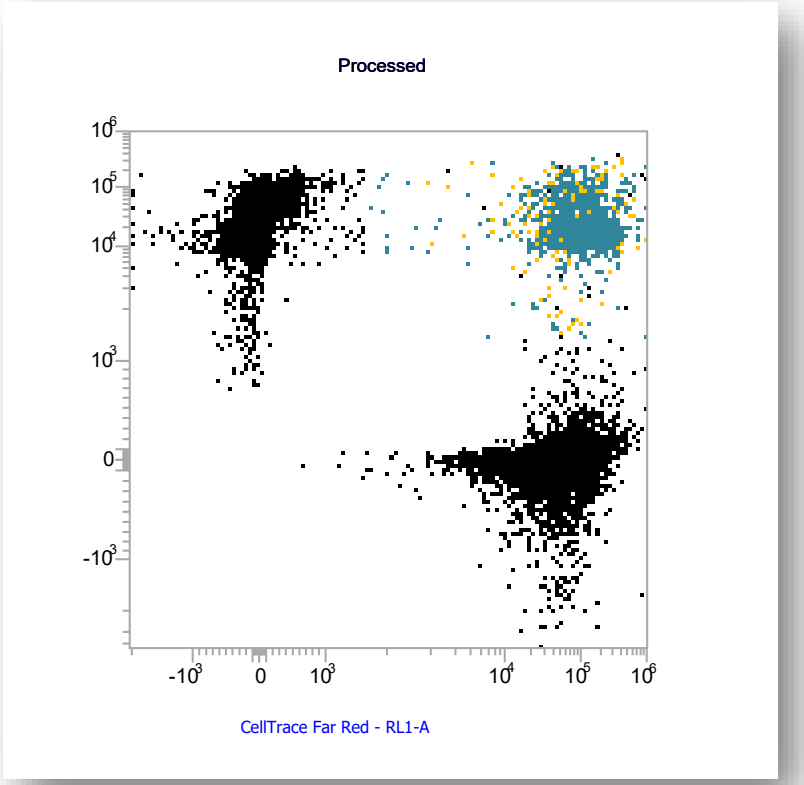
**Visualize lymphoma targeting by CAR T cells after 1 hour of co-incubation**

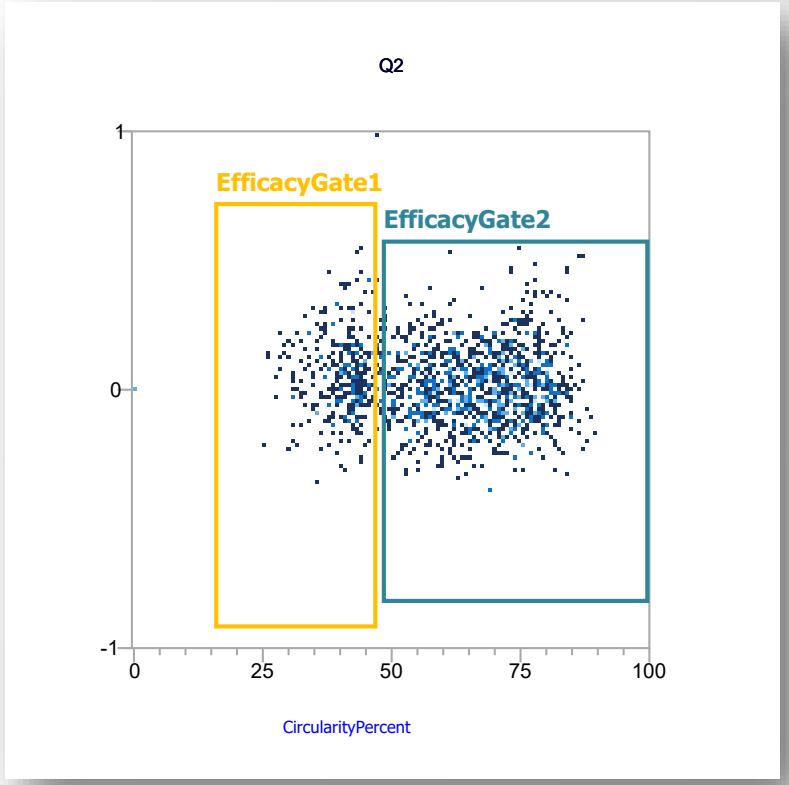
- **Q1** = Ramos cells without interaction with CAR T cells
- **Q2** = Potential immune synapse between the two cell phenotypes
- **Q3** = Debris/double negative events
- **Q4** = CAR T cells without interaction with Ramos cells




**Freshly labeled CAR T cells (Invitrogen™ CellTrace™ Far Red kit) and Ramos cells (Invitrogen™ CellTrace™ Violet kit) incubated at 1:1 ratio for 1 hr at 37°C. CAR T cells: single-chAIn variable fragment CD19 supplied by Thermo Fisher (similar to Juno JCAR019). All samples unfiltered prior to analysis. Samples acquired at 200 µL/min, >8 x $10^5$ cells/mL.**

# Conventional Flow Cytometry displays events only



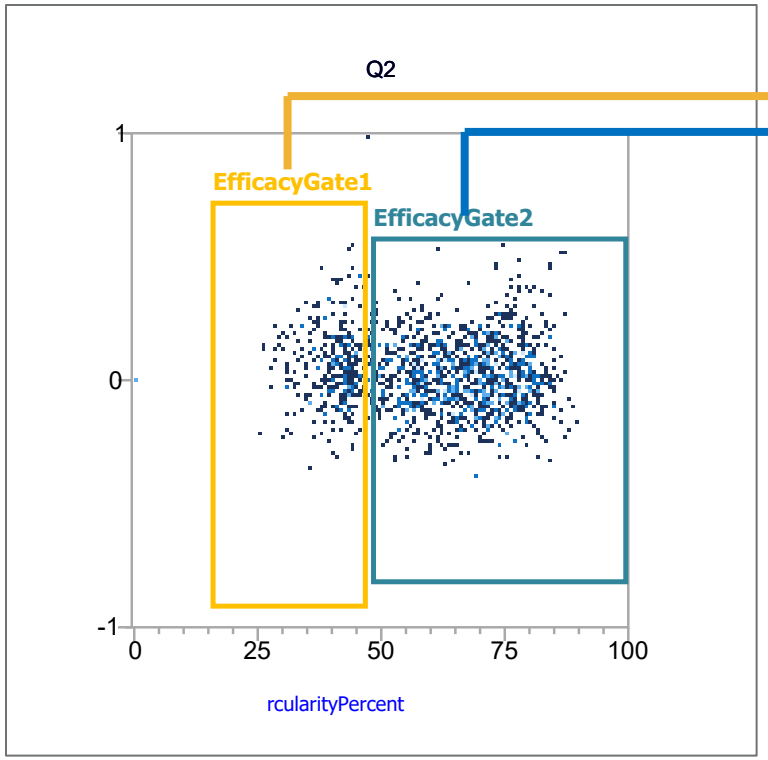




**With images and automated image analysis, new discovery is enabled with a click**

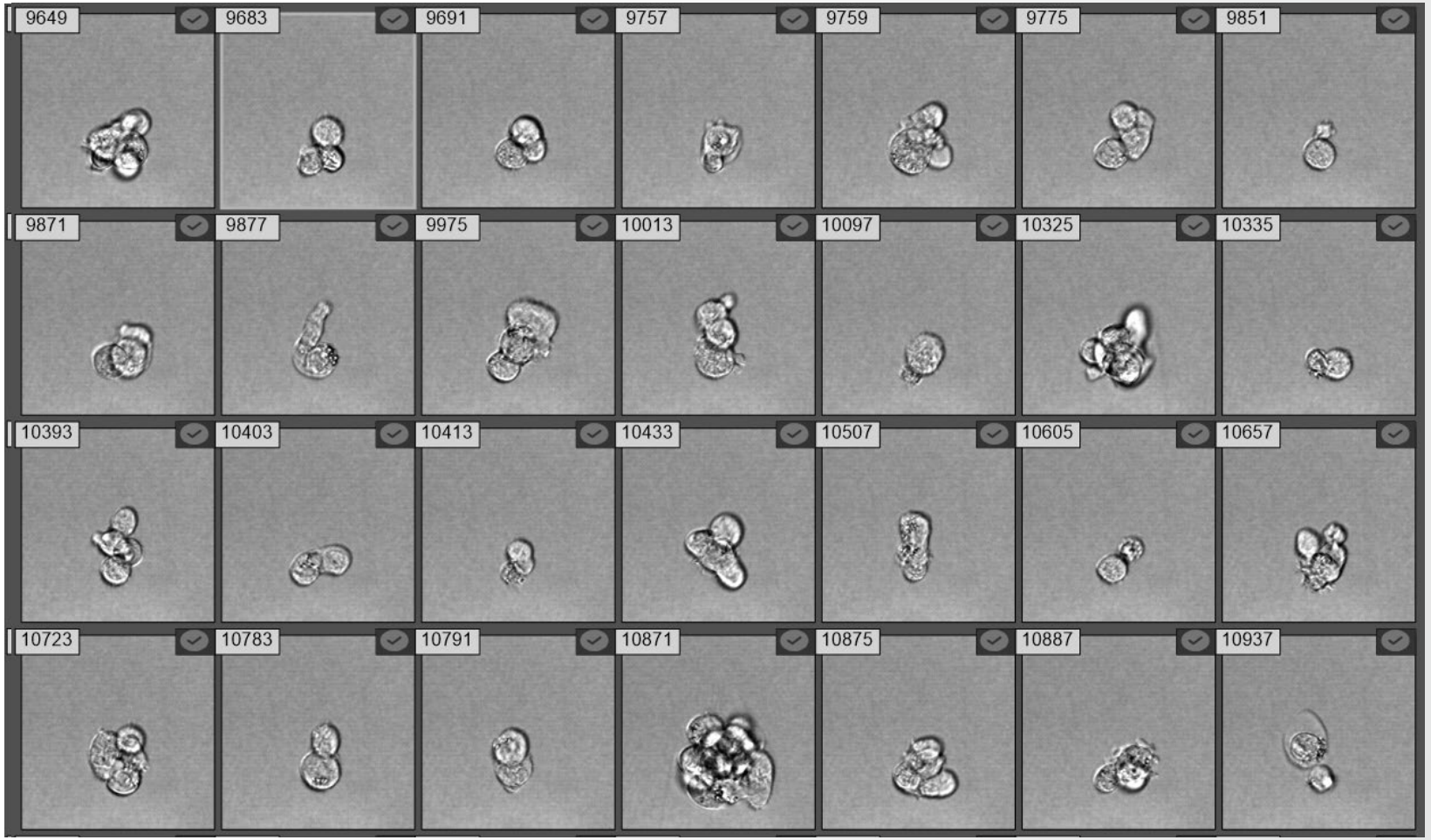
CART-Tcells incubated with Ramos Cells for 1 hour.

# Let the images guide the way

## Use images and feature parameters to uncover heterogeneity



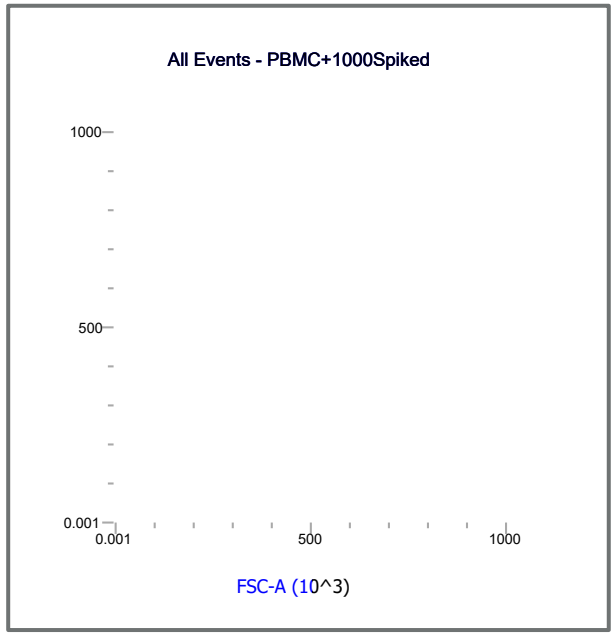

**EfficacyGate1 – Predominately untargeted/detached**
**EfficacyGate2 – Predominately targeted/attached**




See previous slides for upstream gating strategy and sample preparation.

Cytpix + AIA was able to define population within population

# Rare event analysis requires accuracy

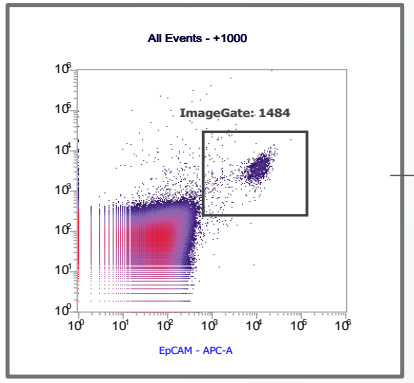


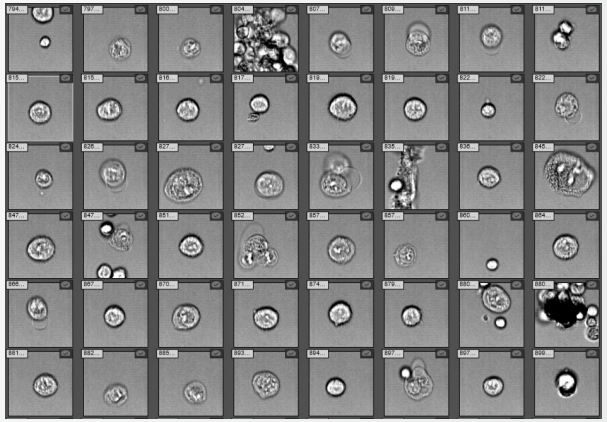

**Very rare event analysis**

Researcher collected >4E6 events for 1000 spiked-in EGFR/EpCAM+ cells

**Inaccurate recovery count before:**

**148%**

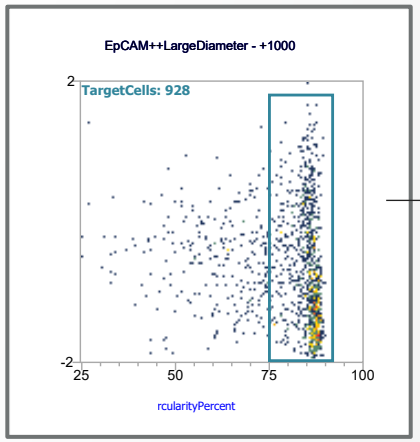


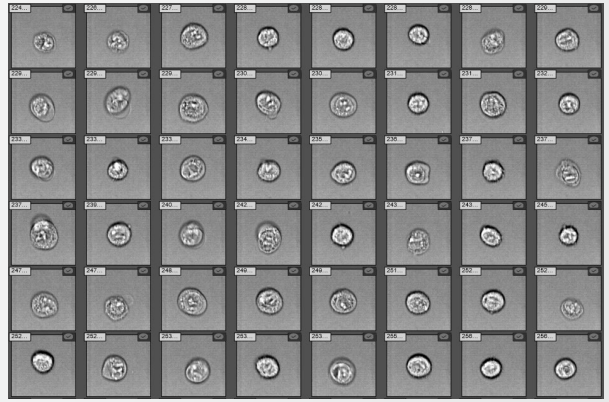



Images show many of these double positive events are debris/aggregates of unexpected morphology.

**Improved recovery count after:**

**92.8%**



Images show parameters improved quantitation of the EpCAM+EGFR+ cells

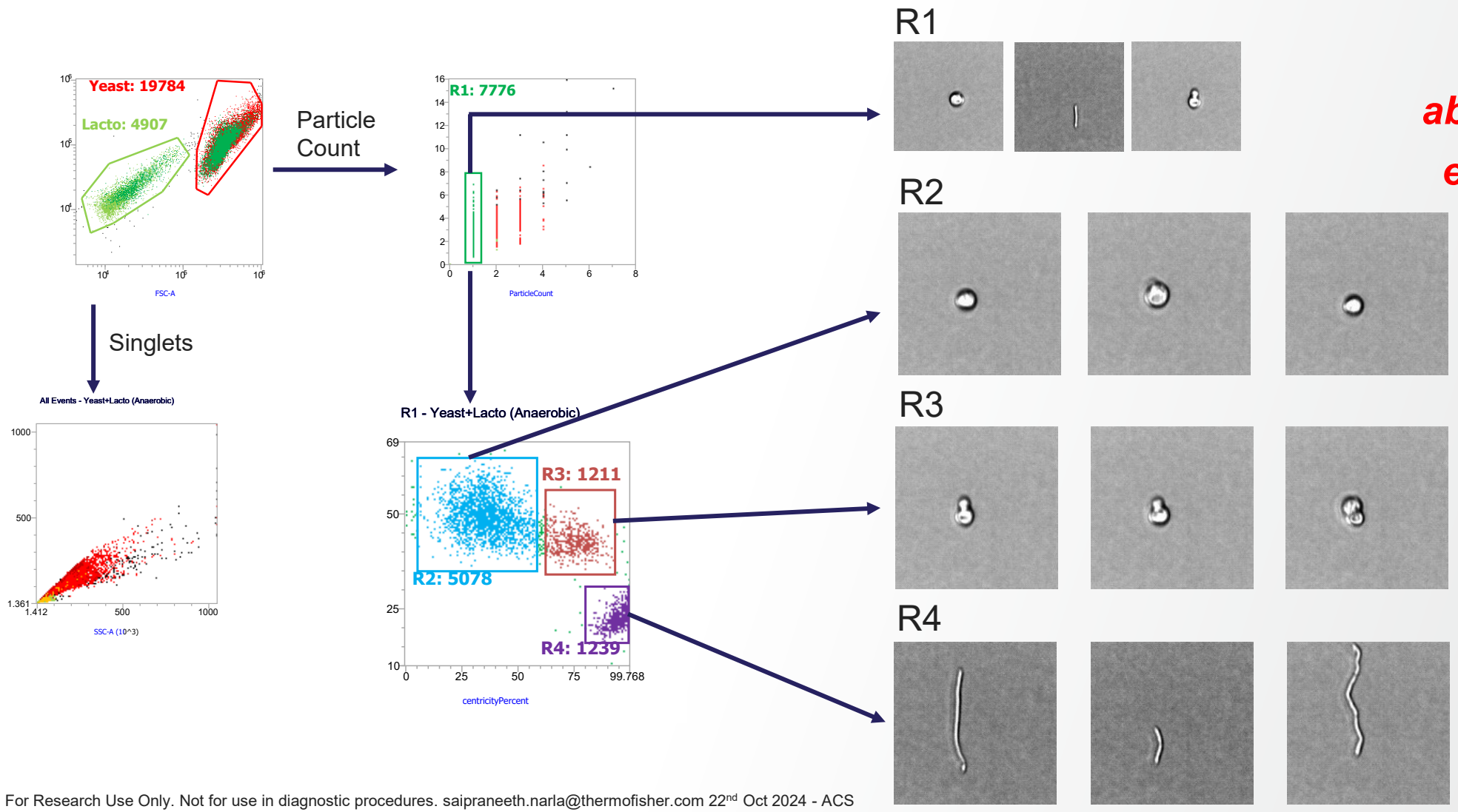
**Problem solved:**

**Image parameters reduce error and enable accurate quantitation than using fluorescence alone**

Data generated by and used with permission from Prof. M. van Egmond, Amsterdam UMC.

# Cytpix AIA software was able to identify different populations in yeast based on cell sizes

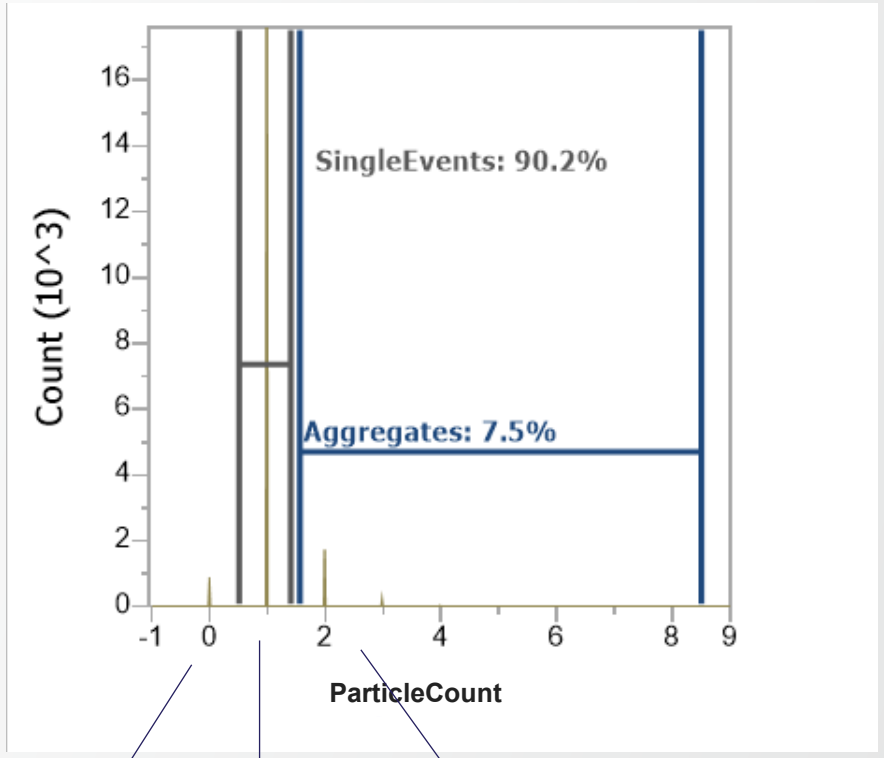
Cytpix can identify and separate bacteria with yeast. AIA can further characterize them into separate populations.

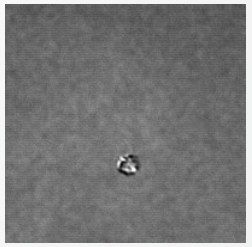



***Cytpix and AIA is able to optimize your experiments better***

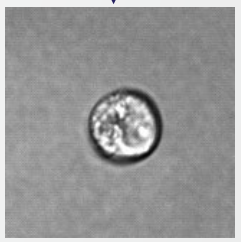
# Why does Attune™ CytPix and Automated Imaging Analysis (AIA) Matter?

- Two Datasets. One step. Zero doubt!
- Further define populations based on morphology ( label-free)
- Reduce error and improve accuracy
- Enable new insights and applications
- Improve gating strategy

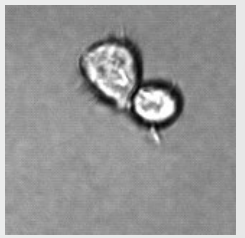


Debris



1 cell



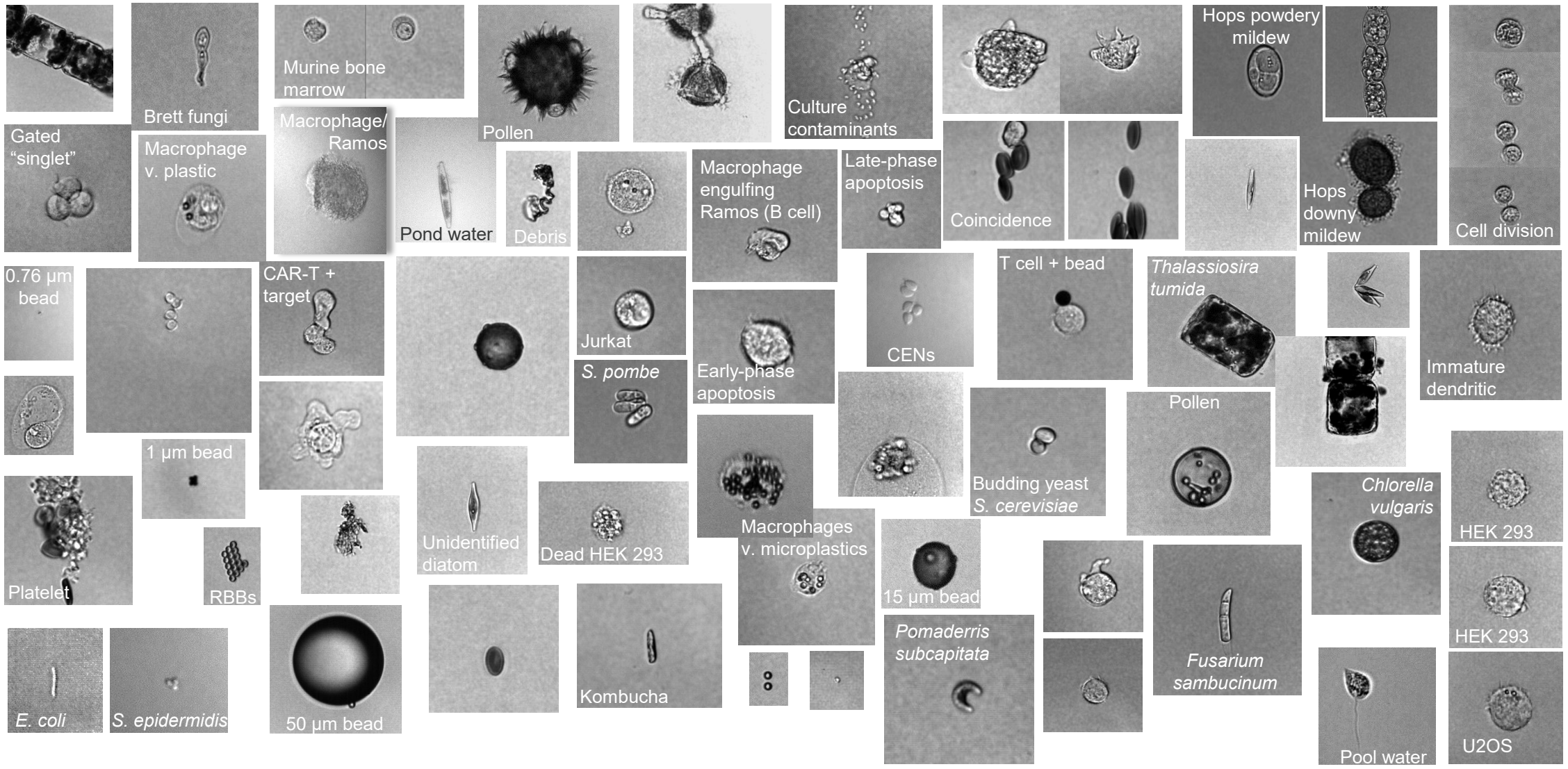
2 cells

# What’s in my flow sample?

“Phagosaurus Rex”

A grand contribution to science!

# What's in your flow sample?



# Growing needs of the flow cytometrist

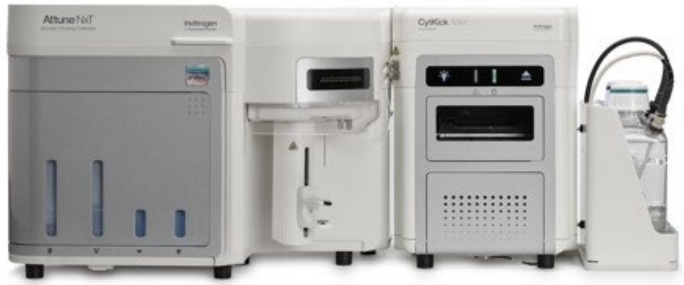
ThermoFisher SCIENTIFIC

**2015** → **2021** → **Present**

**Reliable workhorse instrumentation**

**More information needed per cell**

**Increasing panel size, rarer populations**



**Invitrogen™ Attune™ NxT Flow Cytometer**

**Efficiency, speed, accuracy**

**Invitrogen™ Attune™ CytPix™ Flow Cytometer**

**Two data sets, one step, zero doubt**

**Now announcing…**

**Understand your cells on a whole new level**

# Invitrogen™ Attune™ Xenith™ Flow Cytometer

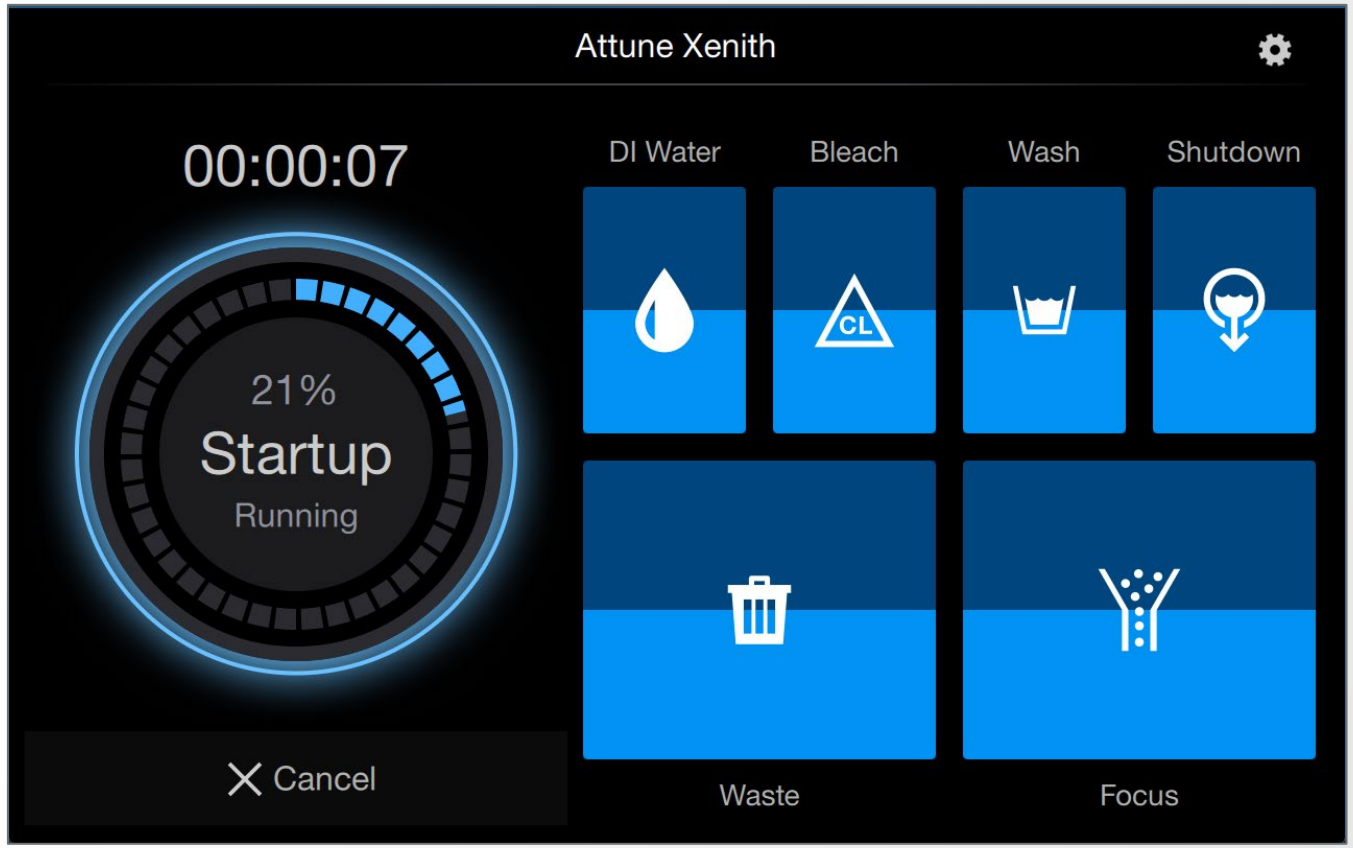
# Attune Xenith Flow Cytometer

## Discover the most advanced acoustic focusing flow cytometer

- Proven **acoustic focusing** core technology designed for **efficiency, speed and accuracy**
- Enables **high resolution spectral unmixing** and **conventional compensation** workflows
- Exceptional **automation** with the Invitrogen™ CytKick™ Max Autosampler ;
- Coming soon in **2025**

# Simplified touchscreen maintenance

- **Continuous level sensing** of onboard fluidics
  - Color-coded fluid level indicators visible across the lab
- One touch **startup and shutdown** independent of PC
- Maintenance instructions and acquisition progression



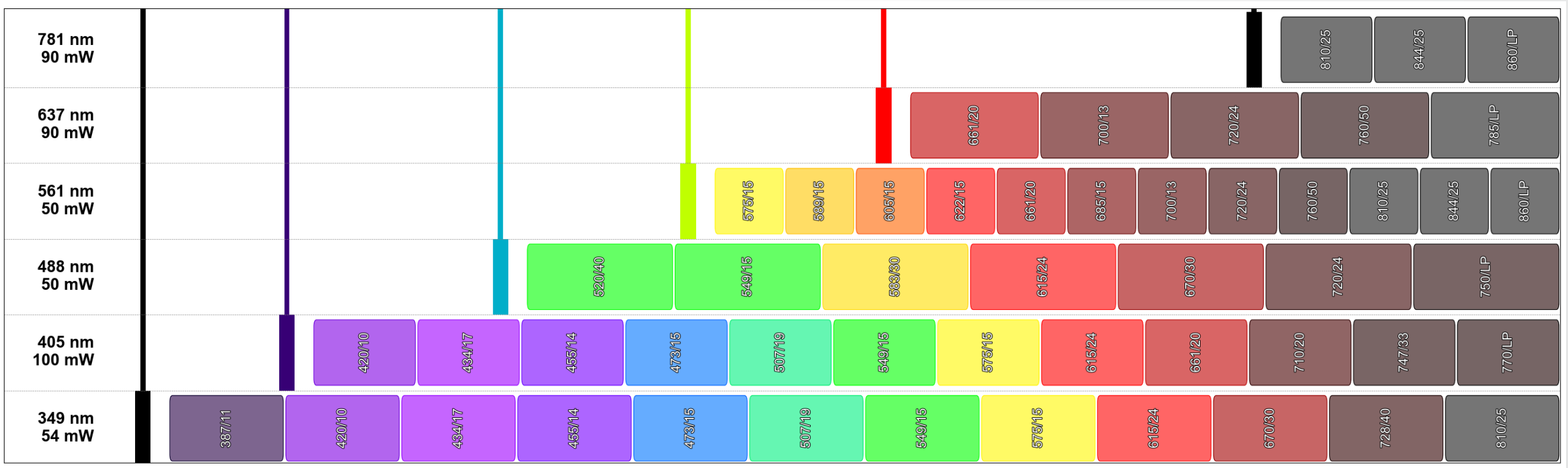

# Expanded optical detection for large panels

## Fluorescent detection: 51 channels

- **Six lasers:** 349, 405, 488, 561, 637 and 781 nm
- Customizable with user-changeable detection filters

## Scatter detection: 6 channels

- 488 nm standard FSC and SSC
- 405 nm FSC and SSC for small particle resolution
- Additional 488 nm FSC and SSC for expanded range/polarized detection



# Thank you