# Imaging Flow Cytometry: Theory and Application

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ISAC SRL Emerging Leader

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# The Flow Cytometry Core Facility (FCCF) @ Newcastle University


**International Centre for Life**

**Meeting the cytometry needs of over 200 users across several different disciplines/ institutes**

**Newcastle University Medical School**

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# Presentation Overview

- A brief reminder about what Cytometry is all about
- An introduction to Imaging (Flow) Cytometry
- Principles and theory of IFC:
  - Signal generation and image capture
  - Basics of pixel-based image analysis
- Challenges and benefits of Image-based cytometry data
- Two short examples:
  - Measuring $Ca^{2+}$ within organelles sites in activated T cells
  - Label-free analysis of the cell cycle by machine learning
- Conclusions

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# What is Cytometry?

# Cytometry

**Greek = “Kytos”**
**“hollow basket”**
**Relates to CELL**

**Greek = “Metria”**
**“Process of Measuring”**

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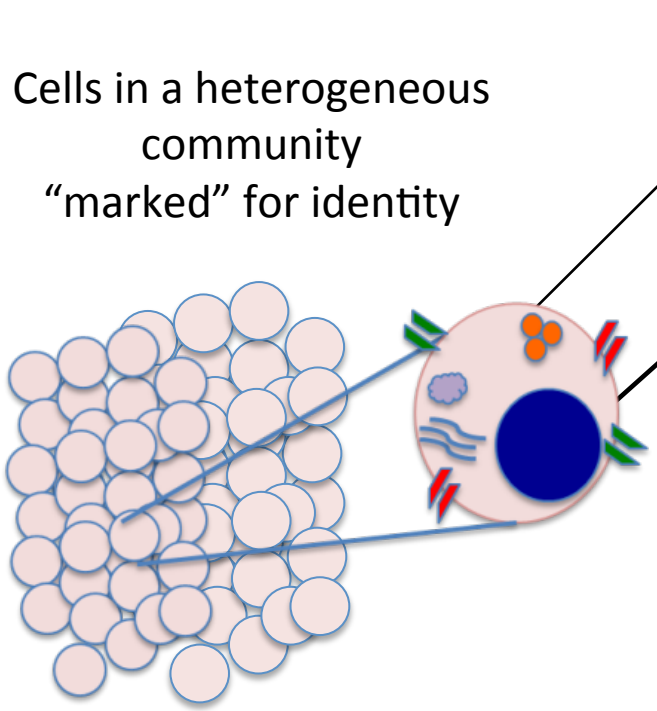
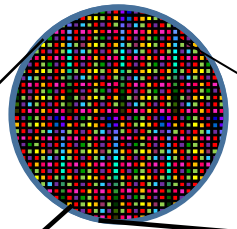
"**Cytome**try is the measurement of cell phenotype, frequency, form and function at the single cell level but conducted on a **population**-wide basis"

# High resolution single cell analysis should provide a high definition picture of the underlying biology

*n* parameters/cell

Cells in a heterogeneous community "marked" for identity





If we want our experiments to be like a piece of fine art, then surely we want the highest-possible level of resolution at the single cell level?

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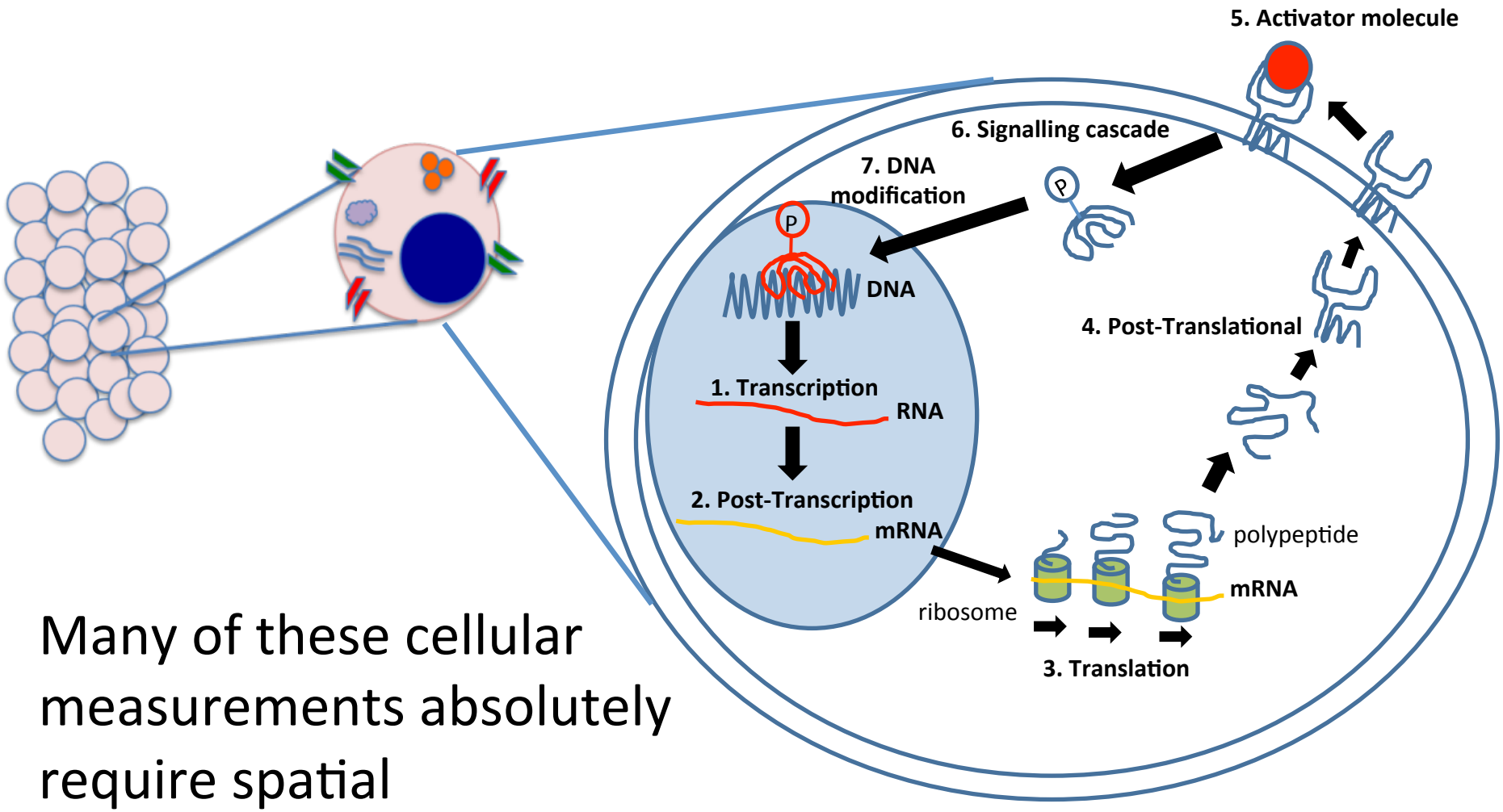
# To me, cytometry includes ALL possible single cell-based measurements




Many of these cellular measurements absolutely require spatial information (images)

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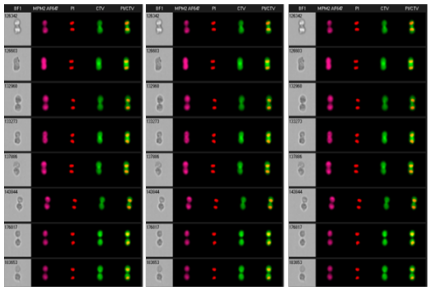
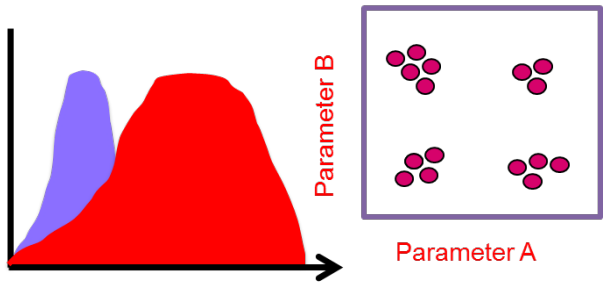
# Introduction to Imaging (Flow) Cytometry

**3. Apply to population**



**4. Analyse population distributions**

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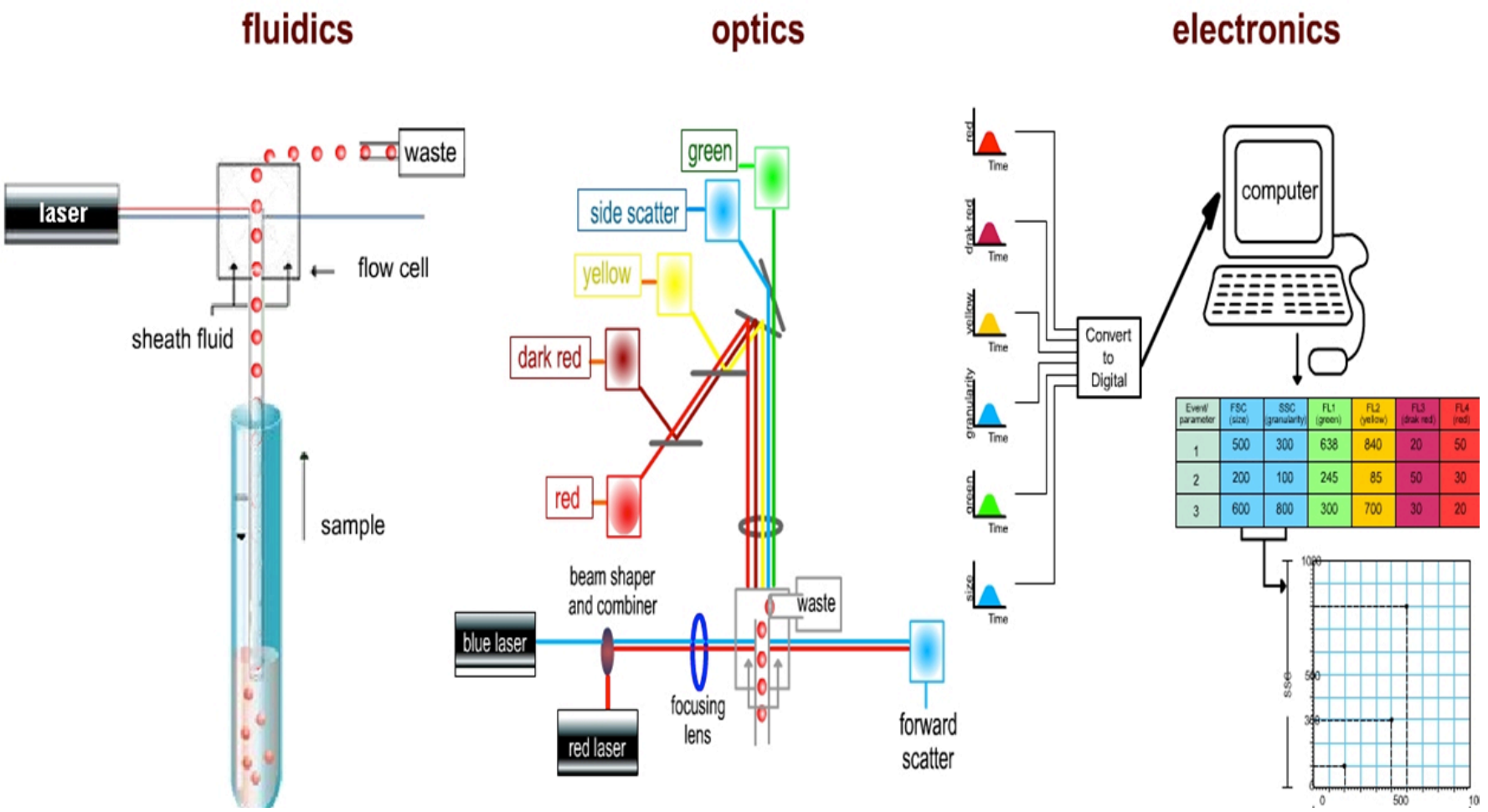
# How does a Flow Cytometer Work?

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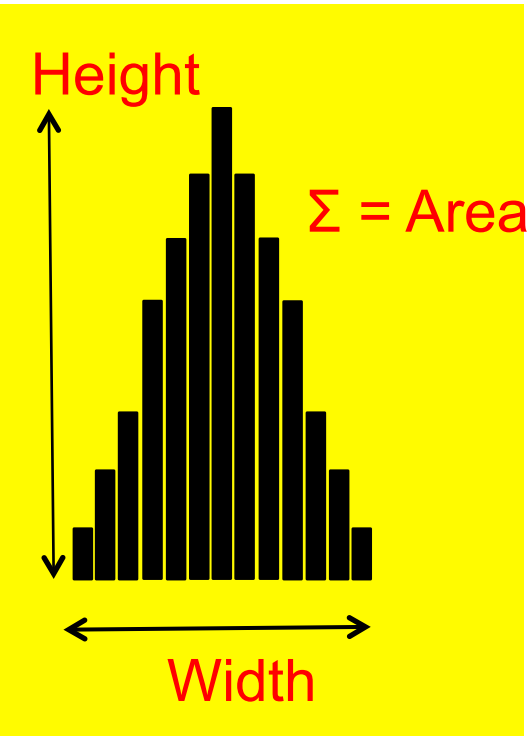
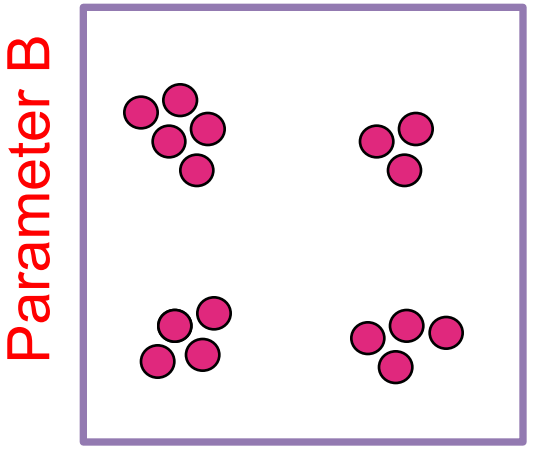
# We take all that amazing cellular structure and smash it into single numbers




| Event Number | FSC | SSC | Blue 530-30-A | Yellow 585-30-A | Violet 450-50-A | Red 670-30-A |
|---|---|---|---|---|---|---|
| 1 | 100 | 450 | 10 | 500 | 670 | 780 |
| 2 | 150 | 443 | 15 | 23 | 1000 | 456 |
| 3 | 180 | 250 | 2000 | 670 | 6000 | 3000 |
| 4 | 167 | 432 | 11 | 50 | 123 | 2500 |

**Numbers, numbers, numbers….**

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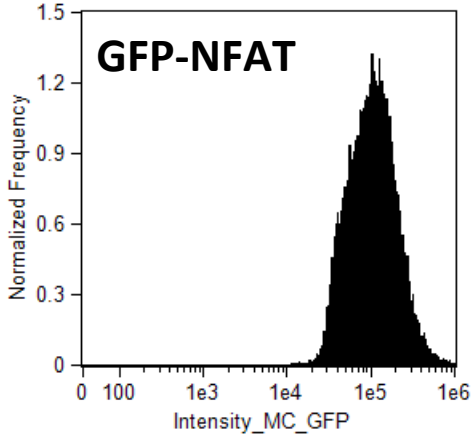
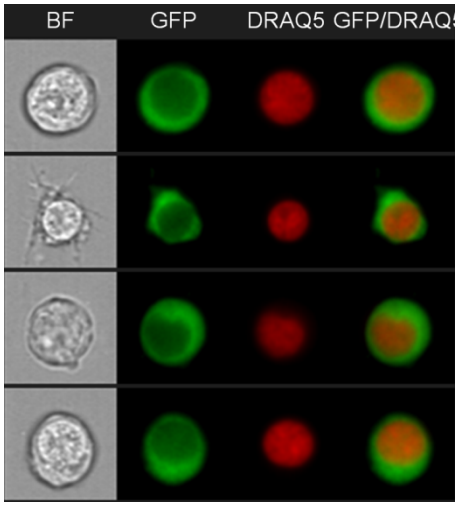
# But what are we (potentially) missing?

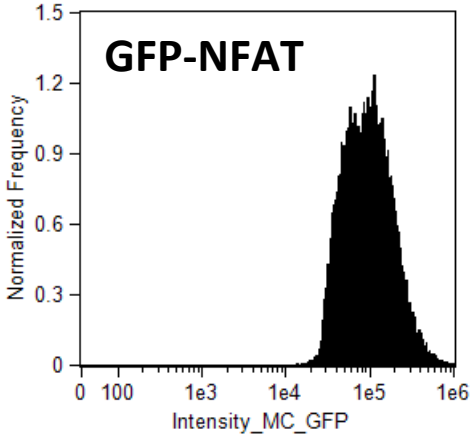
Un-stimulated



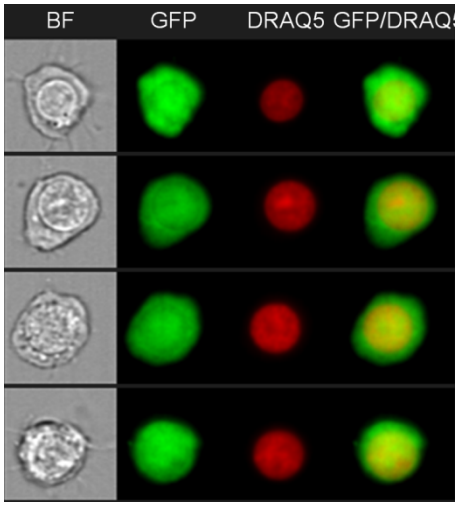

| ılation | Count | %Gated | Median |
|---|---|---|---|
| trans & gfp pos & R2 & R1 | 26632 | 100 | 103418.24 |




Stimulated




| tion | Count | %Gated | Median |
|---|---|---|---|
| cated & gfp pos & R2 & R1 | 39028 | 100 | 92669.62 |




**Location and spatial context may be ESSENTIAL to the biological question.**

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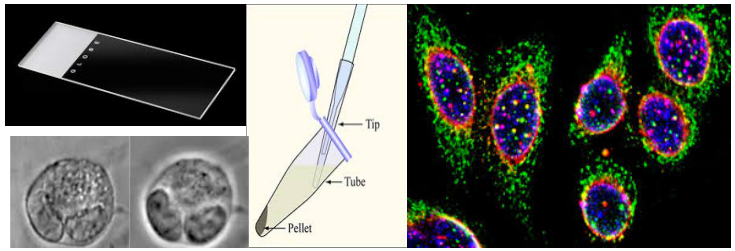
# What makes an Image Cytometer?

- Ability to:
  - Capture images in a spatially registered digitised format (pixels).
  - Capture images in a manner that allows for direct comparisons (relative quantitation) of pixel properties within and between cells.
  - Multi-parameter (highly desirable).
  - Capture images in a high-throughput manner (how many cells do we need for confidence in a measurement?).
- There is overlap with quantitative microscopy BUT we are dealing with population scale.
- IF calibrated correctly, a conventional microscope can be considered an “Image Cytometer”.

# Generic Image Cytometry workflow

## 1. Sample prep

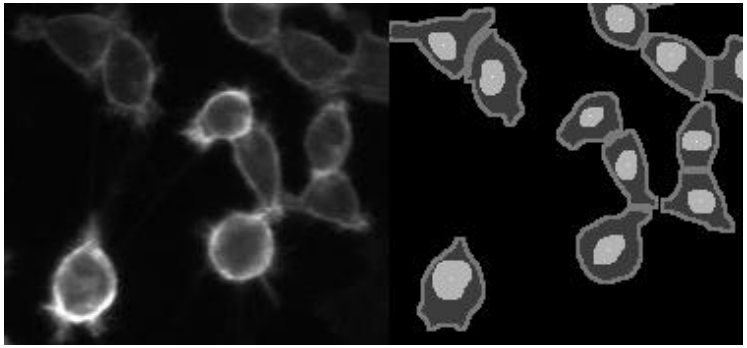



*Not always fluorescence

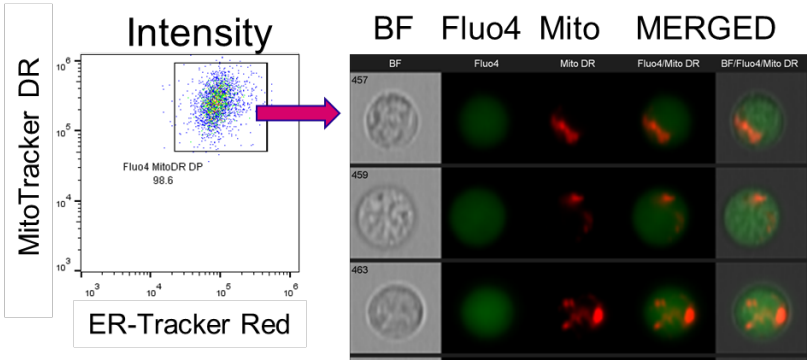
## 2. Controlled Image capture

Well Characterized detectors such as CMOS, CCD PMT

## 3.Identification/segmentation of single cells and cellular structures



## 4. Feature extraction and “flow-like "data analysis from images

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# Image Cytometry benefits from dedicated systems/platforms

## Imaging Cytometry in Flow


## Slide/plate-based Image Cytometry

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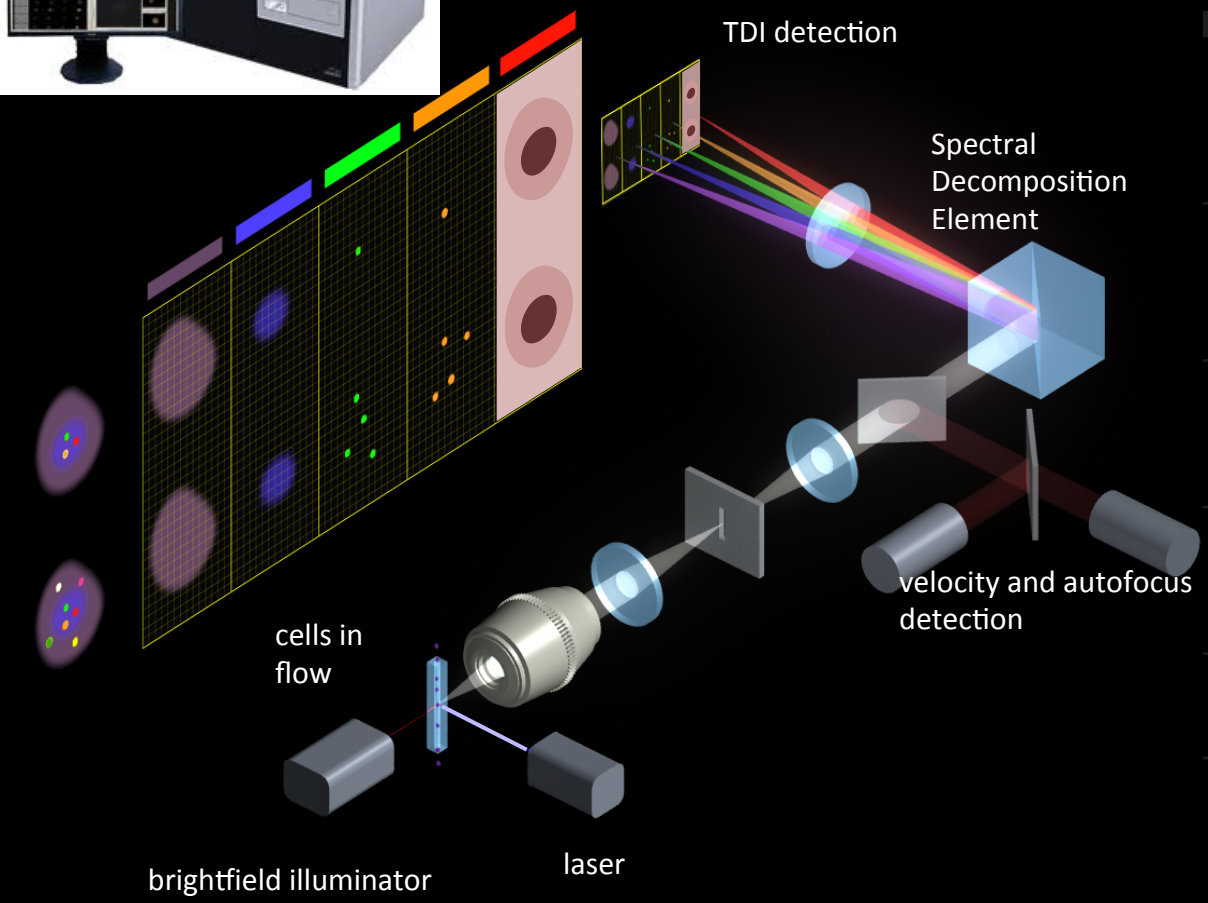
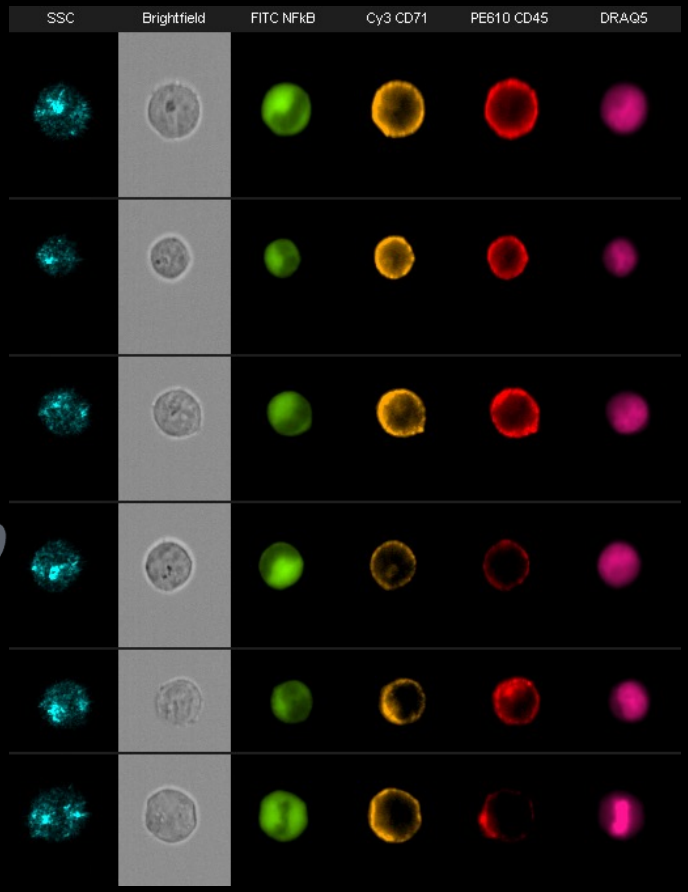
# ImageStream X Optical Configuration (single camera)




Multi-spectral / Multi-mode Imagery

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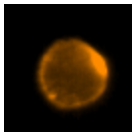
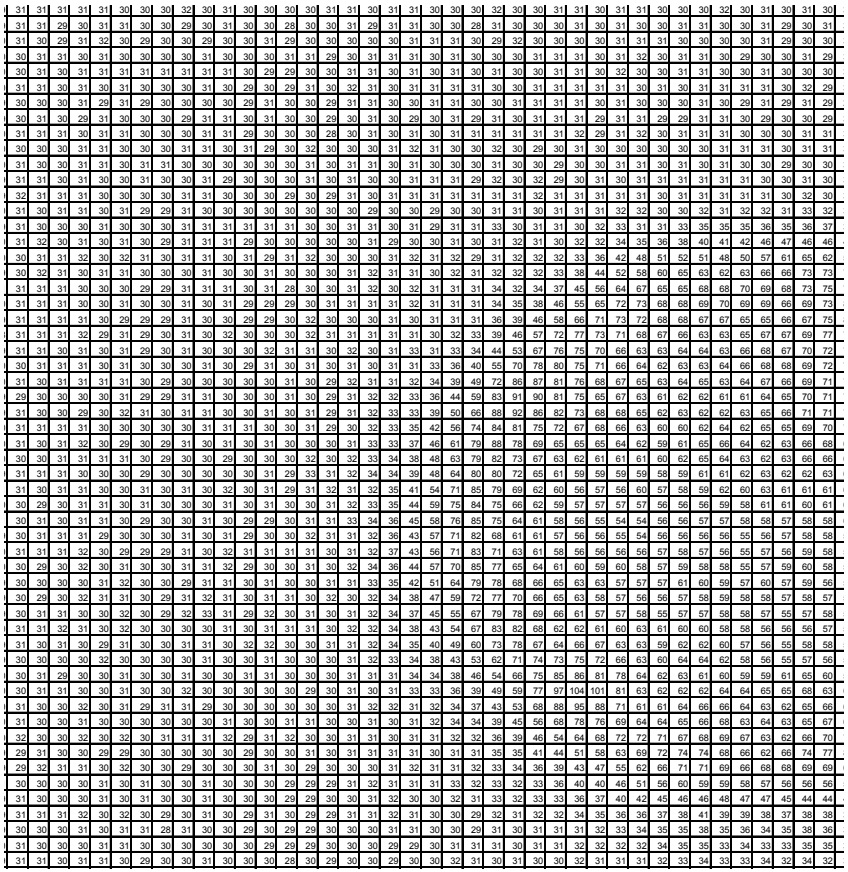
# It’s all about the pixel location and associated values





- 6 or 12 Channel Images are collected using a TDI CCD.
- Pixel values from a 12 bit detector range from 0 to 4096.
- Each image is a grey scale two dimensional image of the cell

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# It's all about the pixel location and associated values

| | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 31 | 32 | 31 | 30 | 32 | 31 | 32 | 31 | 30 | 32 | 31 | 32 | 31 | 30 | 32 | 31 | 32 | 31 | 30 | 32 |
| 32 | 32 | 32 | 32 | 32 | 32 | 32 | 32 | 450 | ### | 900 | 32 | 32 | 32 | 32 | 32 | 32 | 32 | 32 | 32 |
| 32 | 32 | 30 | 32 | 31 | 32 | 425 | 500 | ### | ### | ### | 800 | 700 | 450 | 31 | 32 | 32 | 30 | 32 | 31 |
| 32 | 32 | 32 | 31 | 32 | 780 | ### | ### | ### | 980 | ### | ### | ### | ### | 500 | 32 | 32 | 32 | 31 | 32 |
| 32 | 31 | 31 | 32 | 750 | ### | 690 | 31 | 32 | 30 | 32 | 31 | 31 | 500 | ### | 650 | 31 | 31 | 32 | 30 |
| 32 | 32 | 32 | 400 | ### | 700 | 32 | 32 | 32 | 32 | 32 | 32 | 32 | 32 | 450 | ### | ### | 500 | 32 | 32 |
| 31 | 30 | 32 | 800 | ### | ### | 30 | 32 | 32 | 32 | 31 | 30 | 32 | 32 | 32 | 500 | ### | 550 | 32 | 32 |
| 32 | 32 | 32 | ### | 700 | 32 | 32 | 32 | 32 | 31 | 32 | 32 | 32 | 32 | 31 | 32 | ### | 800 | 32 | 31 |
| 30 | 32 | 300 | ### | 700 | 30 | 32 | 30 | 30 | 32 | 30 | 32 | 30 | 30 | 32 | 30 | 700 | ### | 900 | 32 |
| 32 | 250 | ### | ### | ### | 32 | 31 | 31 | 32 | 30 | 32 | 31 | 31 | 32 | 30 | 32 | 600 | ### | ### | 30 |
| 32 | 400 | ### | ### | ### | 32 | 32 | 32 | 31 | 32 | 32 | 32 | 32 | 31 | 32 | 32 | 890 | ### | 600 | 32 |
| 31 | 32 | 320 | ### | 210 | 31 | 32 | 30 | 32 | 32 | 31 | 32 | 30 | 32 | 32 | 31 | ### | 30 | 32 | 32 |
| 32 | 32 | 230 | 310 | ### | 430 | 32 | 32 | 32 | 31 | 32 | 32 | 32 | 32 | 31 | 750 | ### | 600 | 32 | 31 |
| 32 | 32 | 31 | 420 | ### | 250 | 32 | 31 | 32 | 32 | 32 | 32 | 31 | 32 | 32 | ### | 780 | 31 | 32 | 32 |
| 32 | 31 | 32 | 30 | 320 | ### | 31 | 32 | 30 | 32 | 32 | 31 | 32 | 30 | 600 | ### | 31 | 32 | 30 | 32 |
| 32 | 32 | 32 | 32 | 200 | ### | ### | 32 | 32 | 32 | 32 | 32 | 32 | 32 | ### | 500 | 32 | 32 | 32 | 32 |
| 32 | 32 | 32 | 31 | 32 | 32 | ### | 600 | 800 | 700 | 32 | 450 | 300 | 250 | ### | 650 | 32 | 32 | 31 | 32 |
| 31 | 32 | 31 | 32 | 32 | 31 | 600 | ### | ### | ### | 900 | ### | ### | ### | 700 | 31 | 32 | 31 | 32 | 32 |
| 32 | 32 | 32 | 32 | 31 | 32 | 32 | 800 | ### | ### | ### | 32 | 32 | 32 | 31 | 32 | 32 | 32 | 32 | 31 |
| 30 | 32 | 30 | 32 | 31 | 30 | 32 | 30 | 32 | 31 | 30 | 32 | 30 | 32 | 31 | 30 | 32 | 30 | 32 | 31 |

Pixelated Imagery

- Each pixel has an X coordinate, a Y coordinate and an intensity value that corresponds to the amount of light captured at that location.
- The value of these pixels relate to the dynamic range of the detector (12-bit = 0 to 4096)

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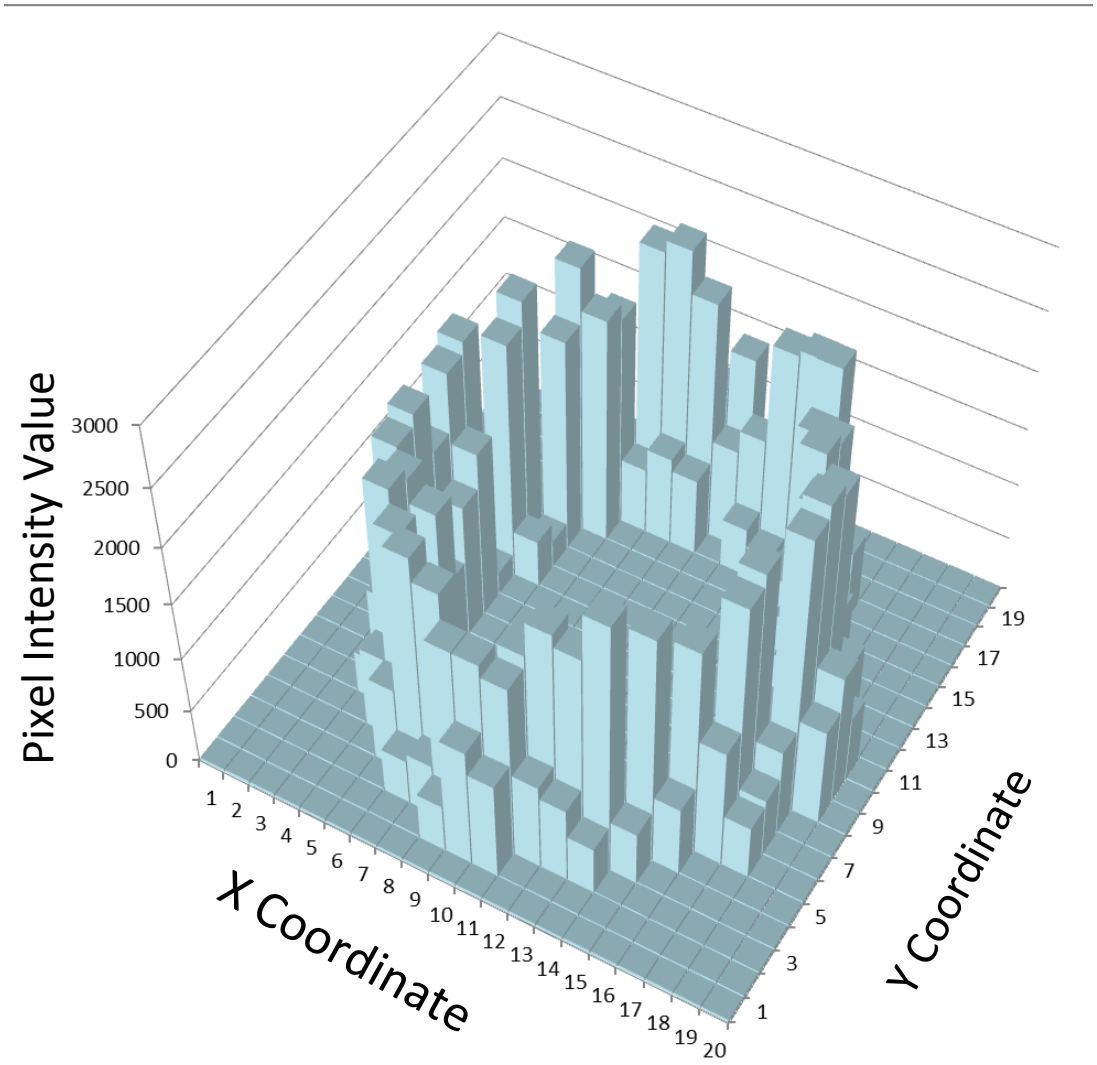
# It's all about the pixel location and associated values




Pixelated Imagery

- Each pixel has an X coordinate, a Y coordinate and an intensity value that corresponds to the amount of light captured at that location.
- The value of these pixels relate to the dynamic range of the detector (12-bit = 0 to 4096)

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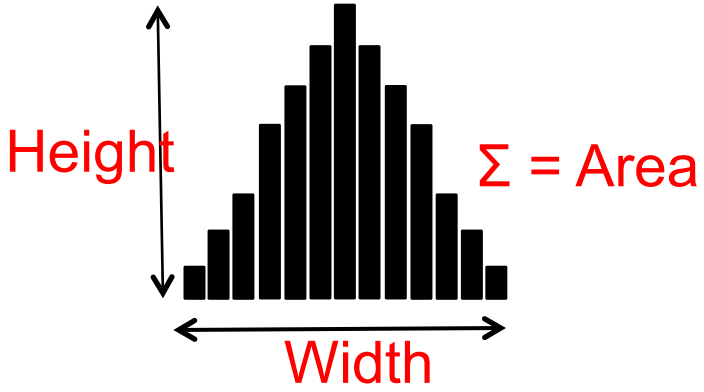
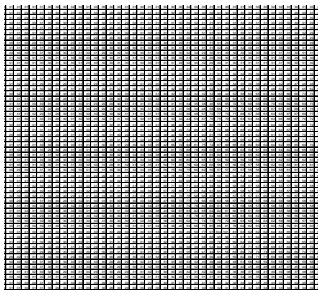
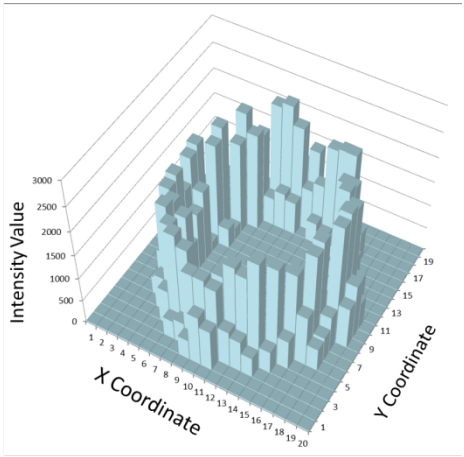
# Translating between Flow and Image Cytometry




Maximum Signal = **PULSE HEIGHT** **MAX PIXEL**

Total Fluorescence = **PULSE AREA** **SUM OF ALL PIXEL VALUES**

Doublet Discrim = **PULSE WIDTH** **AREA OF PIXELS**

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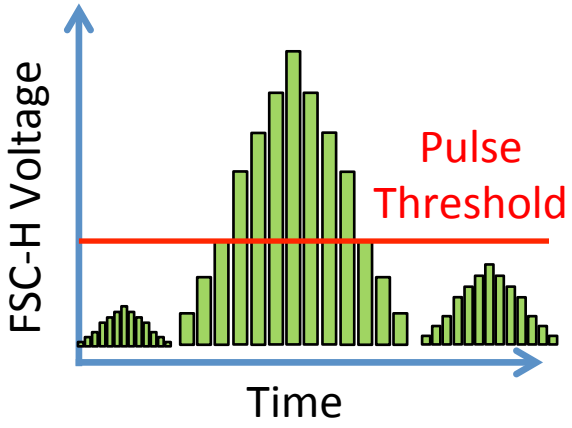
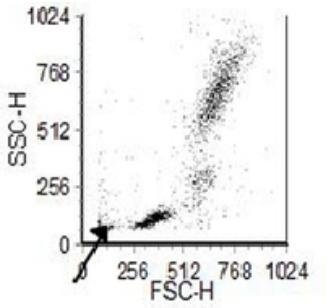
# Thresholding a pulse versus thresholding on an image

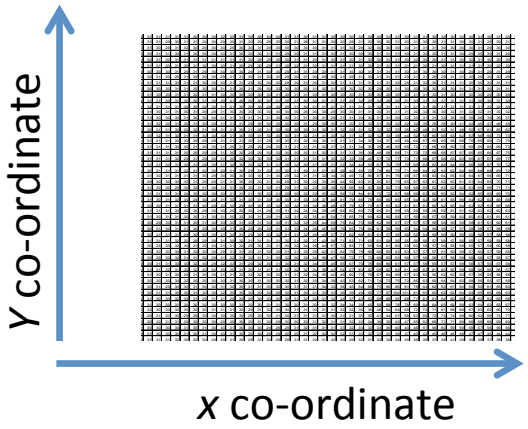
## Conventional Flow Cytometry



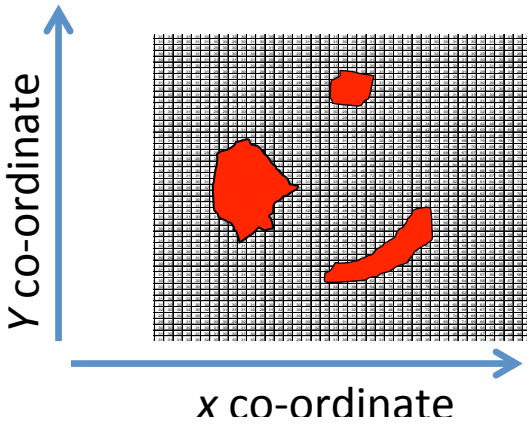



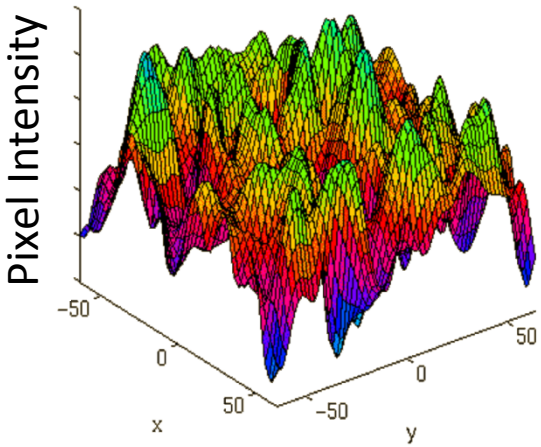

## Imaging (Flow) Cytometry

All pixels in frame considered




Brightest *x*% pixels only considered

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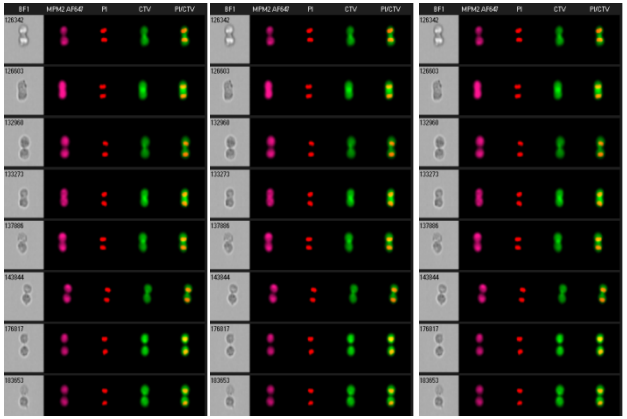
# Identifying pixels, measuring properties, plotting population-wide measurements

## 1. Identify (threshold) Pixels

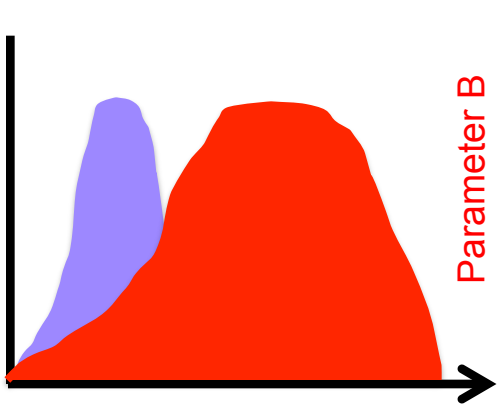

## 2. Measure Masked Pixels

- Intensity based
  - Sum Intensity
  - Max Pixel
- Texture Based
  - Modulation
  - Standard Dev
- Shape based
  - Circularity
  - Aspect ratio
- Locational
  - Internalisation
  - Translocation

## 3. Apply to population



## 4. Analyse population distributions

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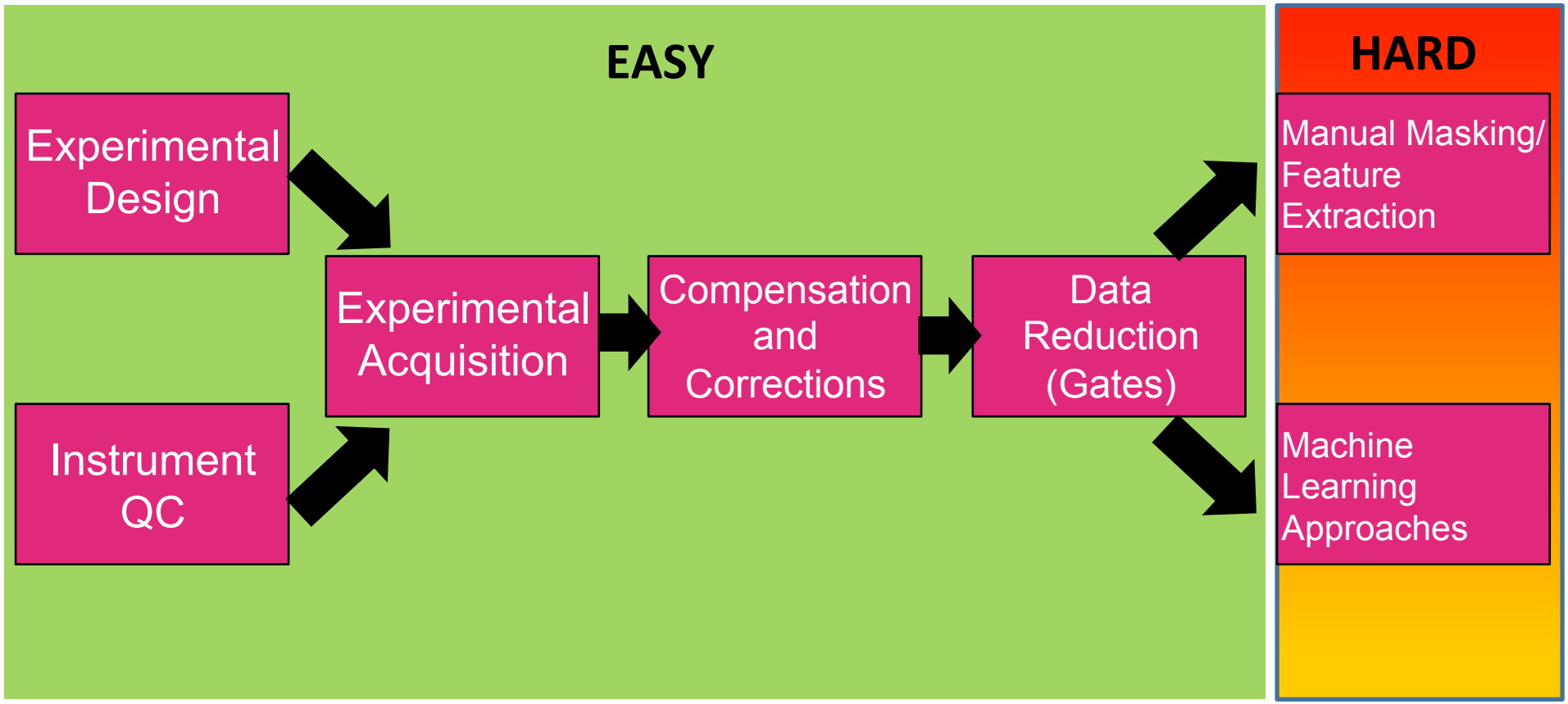
# Typical IFC Experimental Work Flow

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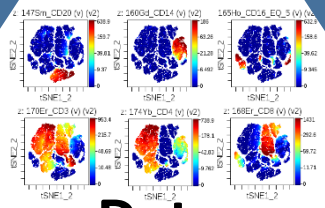
# The Cytometry "Triangle of Data Dimensionality"

**Image n = ∞**



**Data**

**"dimensionality"**

**"Conventional" Flow (n=~35)**

**MASS n = ~45**

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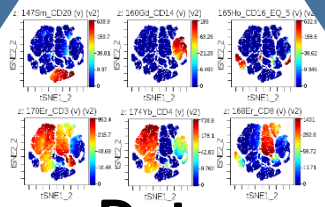
# The Cytometry "Triangle of Data Dimensionality"

**Image n = ∞**

**"highest"**



**Data**

**"dimensionality"**

**"high"** **"higher"**

**"Conventional" Flow (n=~35)**

**MASS n = ~45**

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# Why do humans find image analysis so hard?


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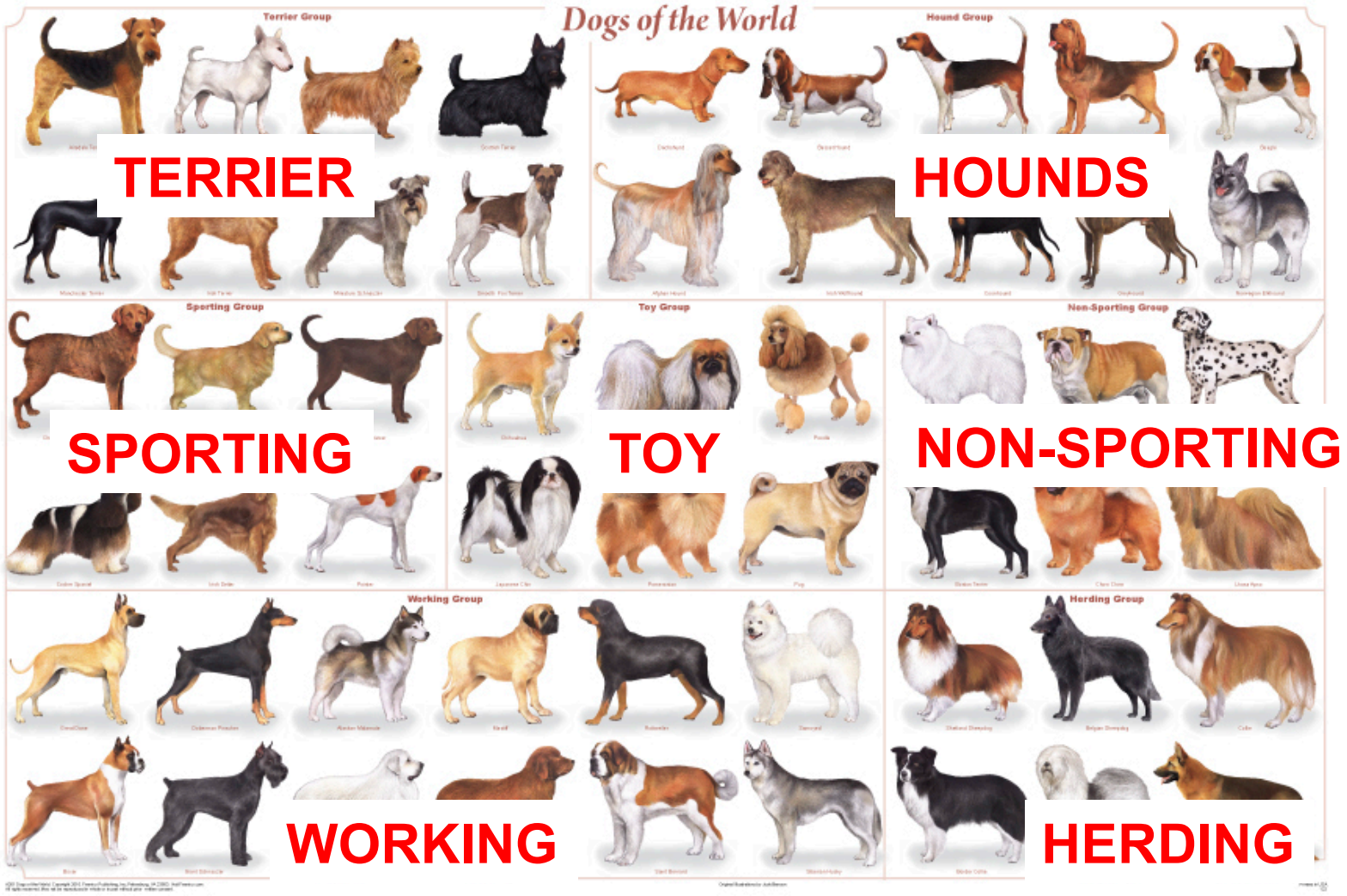
# Why do humans find image analysis so hard?

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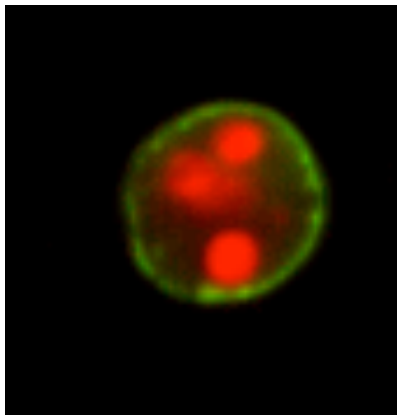
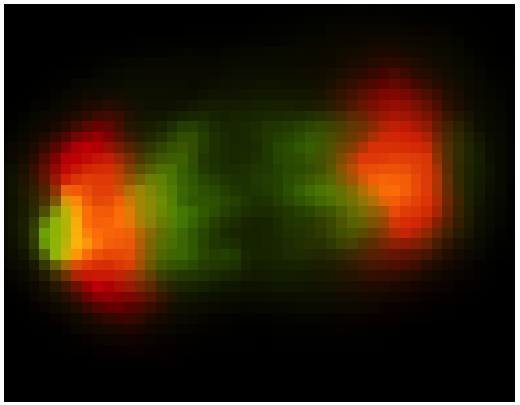
# What are we asking and how do we ask it?

A



B



How does cell A differ from cell B?
Don't describe it, enumerate it…

**Limitless combination of masks and measurements available.**

**Based on pixel information**

**Example of "Features"**

**Pixel Intensity: Total, Max, Min**
**Pixel Texture: SD, Modulation, RMS**
**Size: Area, Diameter, Height**
**Shape: Aspect Ratio, Circularity**
**Location: Delta Centroid, Angle**

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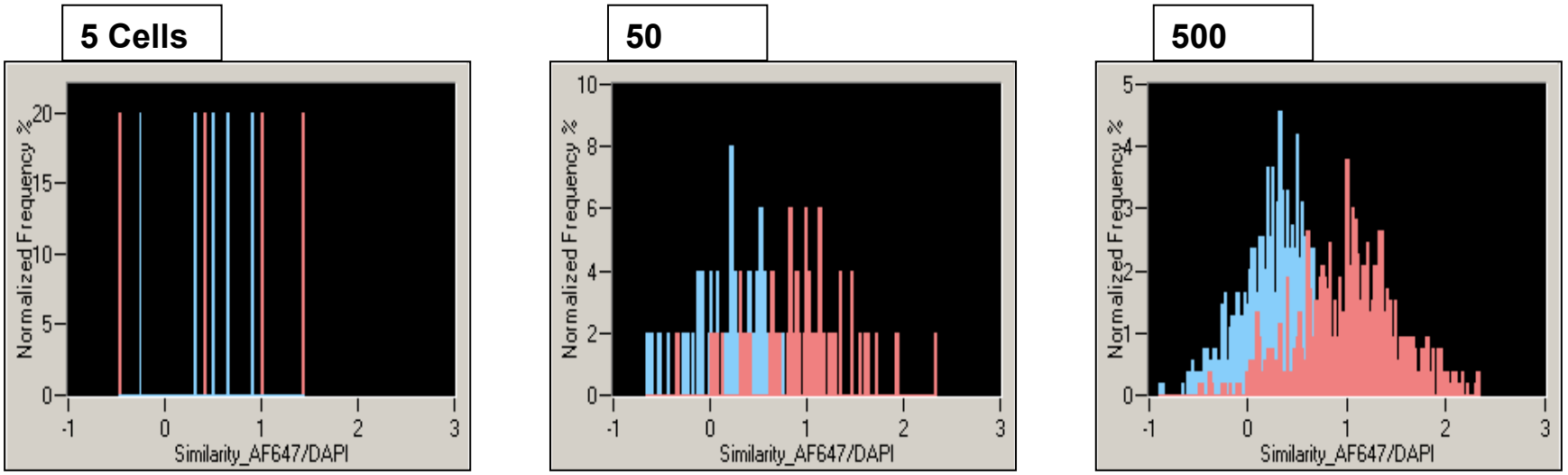
# Remember this point and you will not be disappointed by IFC

- Non-image based flow cytometry provides RELATIVE measurements of fluorescence
- Is a cell really GFP positive?
- Must compare to know GFP negative population

- IFC is no different, except measurement is derived from spatial/morphometric parameters.
- STILL RELATIVE measurements (image-based)
- MUST use fluorescent AND spatial controls to provide CONTEXT

# How many cells do I need to collect to be confident in my FINAL measurement?

**Spatial/Morphometric Measurement**




- In other words, how many cells are needed for me to feel confident that the measured difference is representative and not an artifact of only sampling FEW CELLS?

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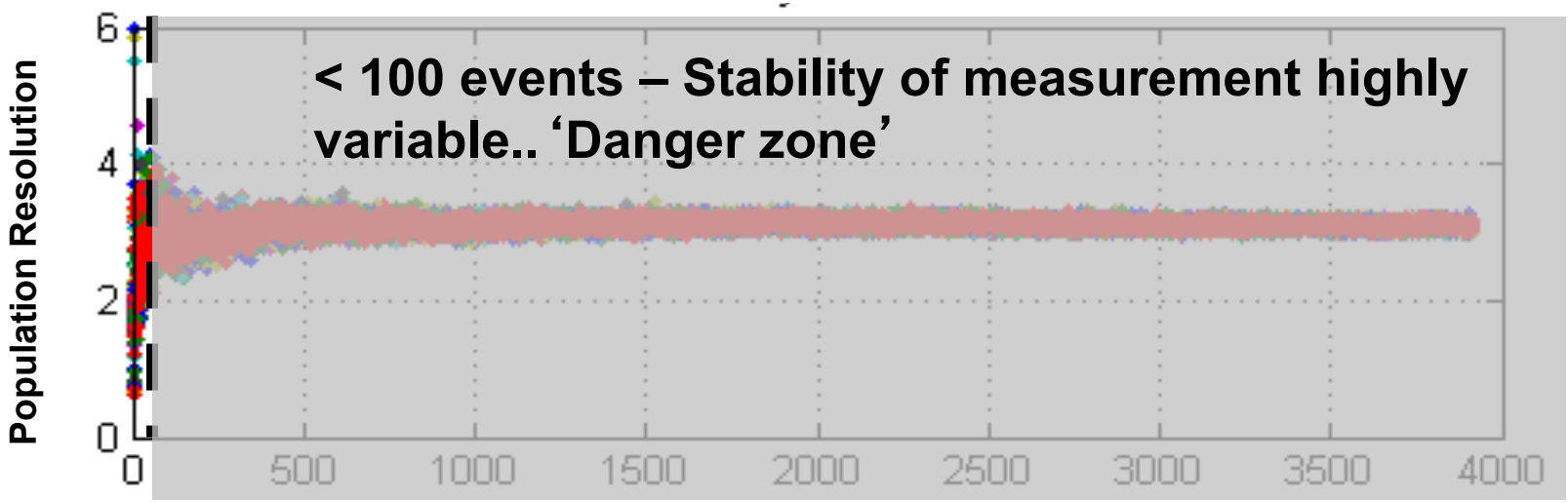
# How many cells do I need to collect to be confident in my FINAL measurement?

Repeated random sampling of increasing numbers of cell sets from a larger data set. How many do we need to measure to ensure they represent ENTIRE population?

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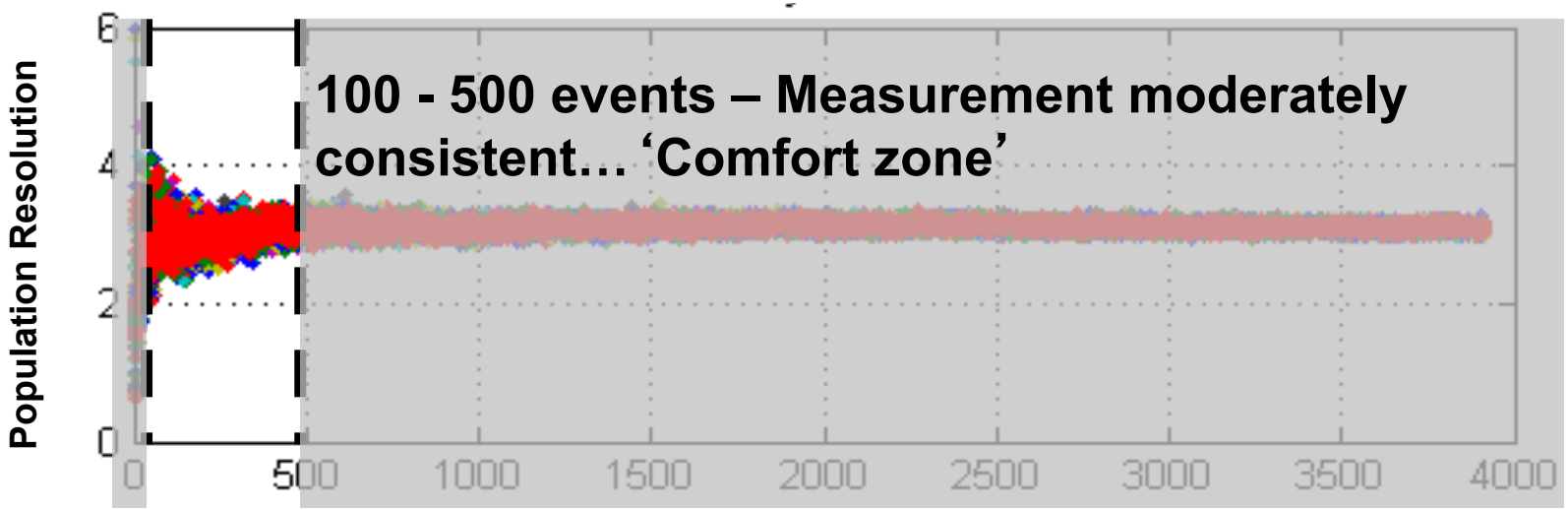


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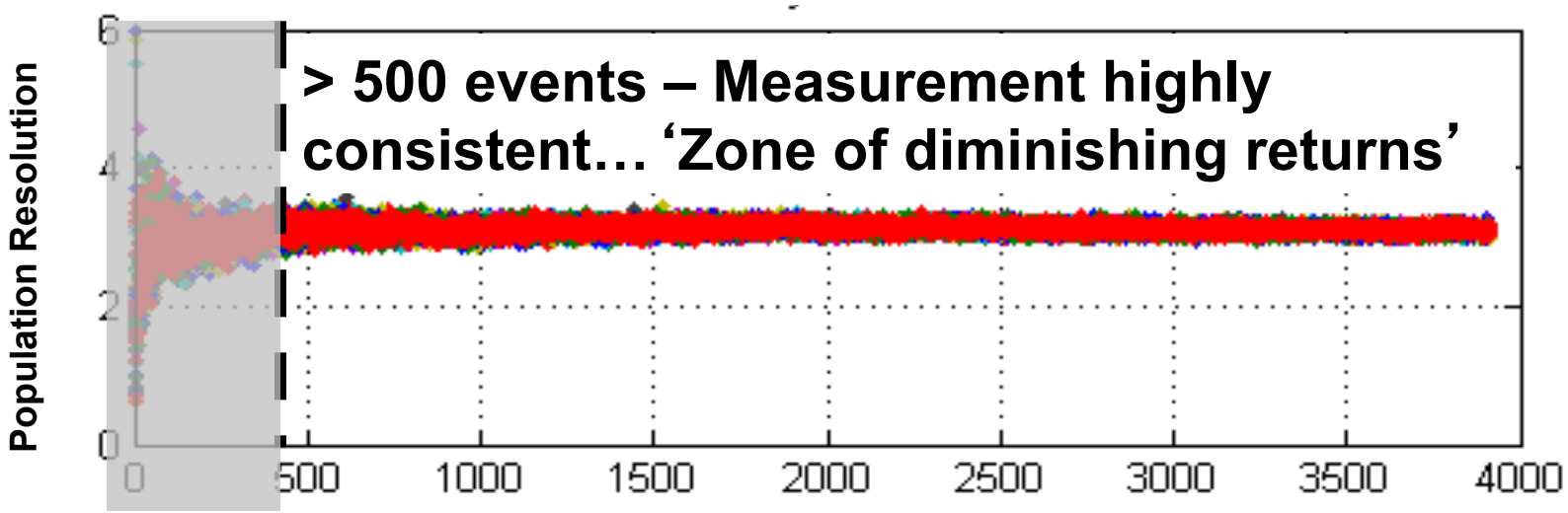


- *Therefore collect at least 100 and ideally at least 500* ***(FINAL) target*** *events per sample*

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# Summary slide

- Spatial/Morphometric biological questions require imagery
- An Imaging Cytometer MUST:
  - Allow for rapid image collection (100+ FINAL cell number)
  - Be multiparameter in nature to deal with heterogeneity.
  - Must acquire imagery in a fully calibrated, comparable manner
- Analysis must take into consideration:
  - Masking/ROI/Segmentation of single cells and structures
  - Extraction of pixel-based features to then plot as a population measurement.
  - Statistics that describe populations.

- IFC very much TICKS ALL BOXES.

- So what can be done with it?

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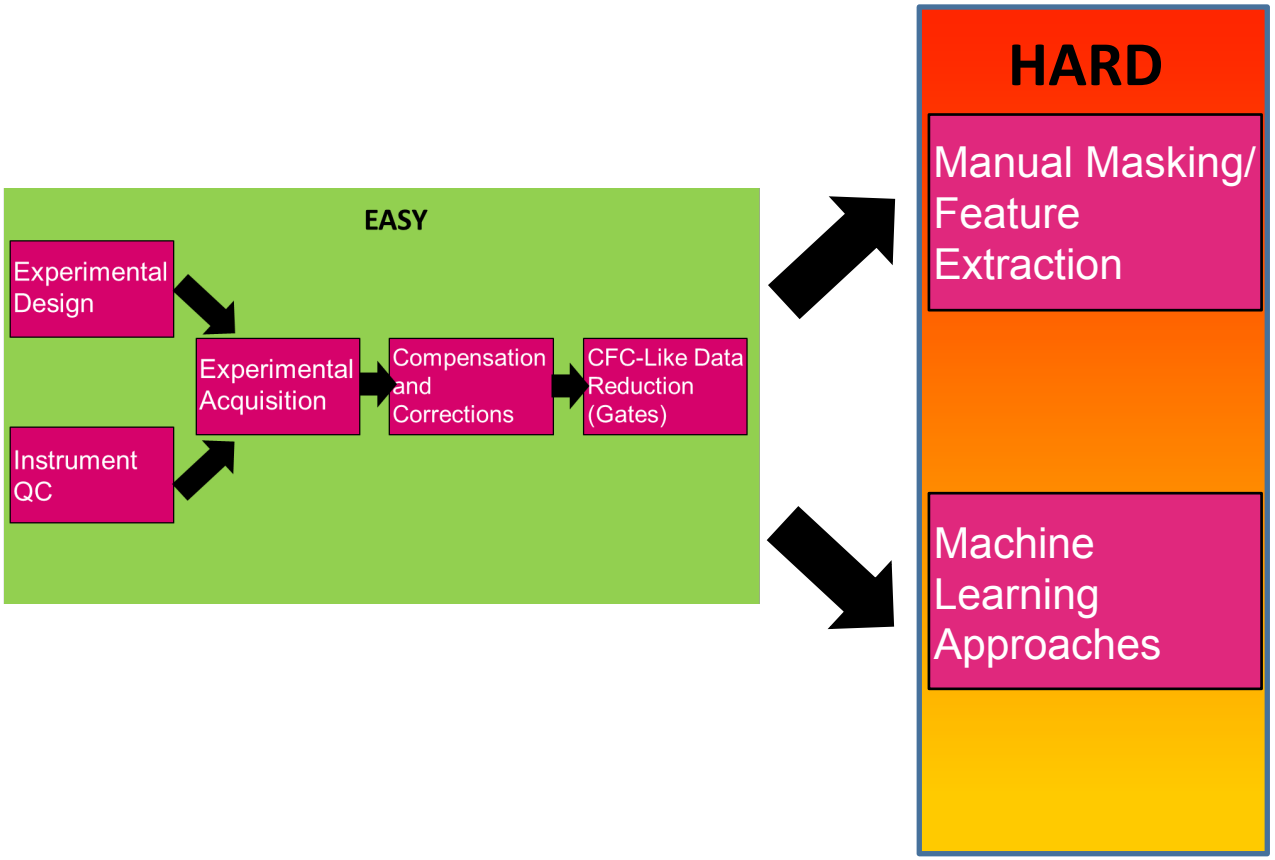
# Two examples of very different approaches to IC data analysis




Method 1:
Organelle $Ca^{2+}$ flux

Method 2:
Labe-free cell cycle

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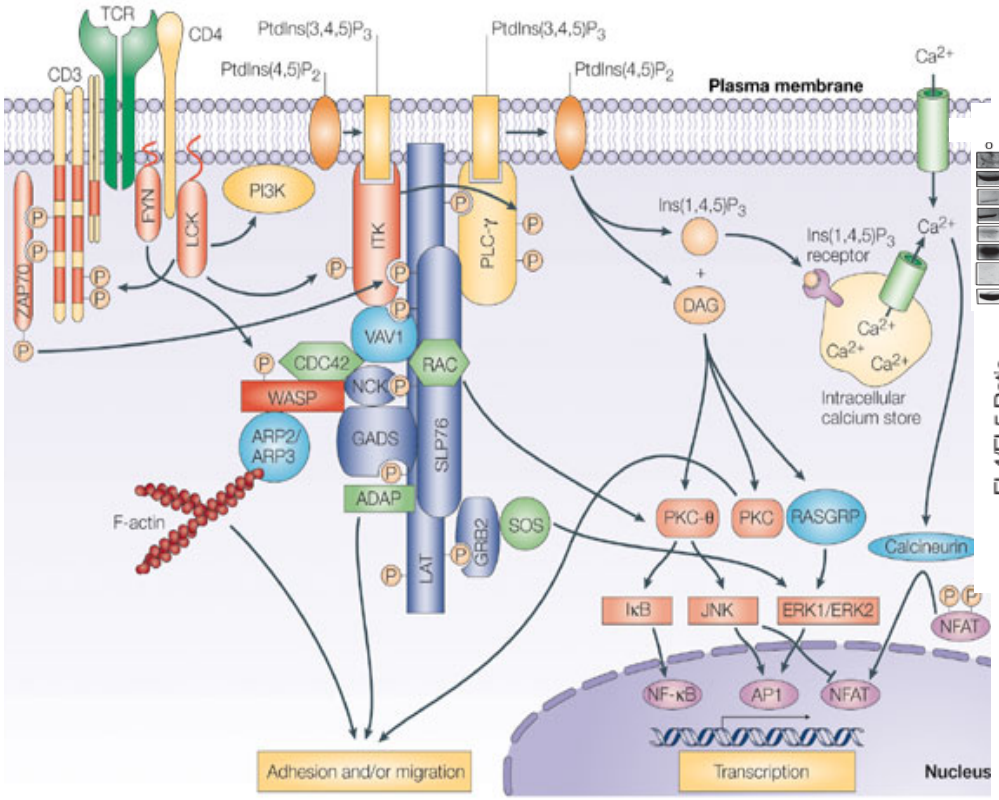
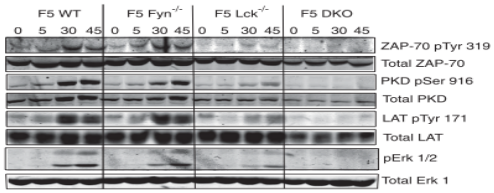
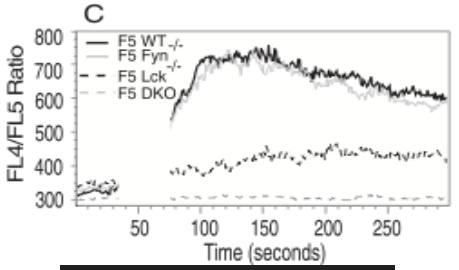
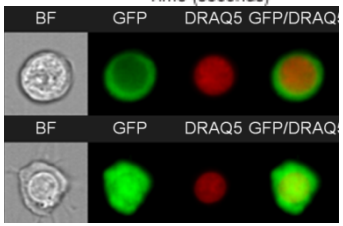
# Application 1: Spatiotemporal Calcium Signalling




## Cell Signalling





ELSEVIER

Journal of Immunological Methods

Volume 423, August 2015, Pages 120–130

Applications in Imaging Flow Cytometry


## An imaging flow cytometry-based approach to measuring the spatiotemporal calcium mobilisation in activated T cells


Joana Cerveira[b, 1], Julfa Begum[b, 1], Rafael Di Marco Barros[c], Annemarthe G. van der Veen[d], Andrew Filby[a, b]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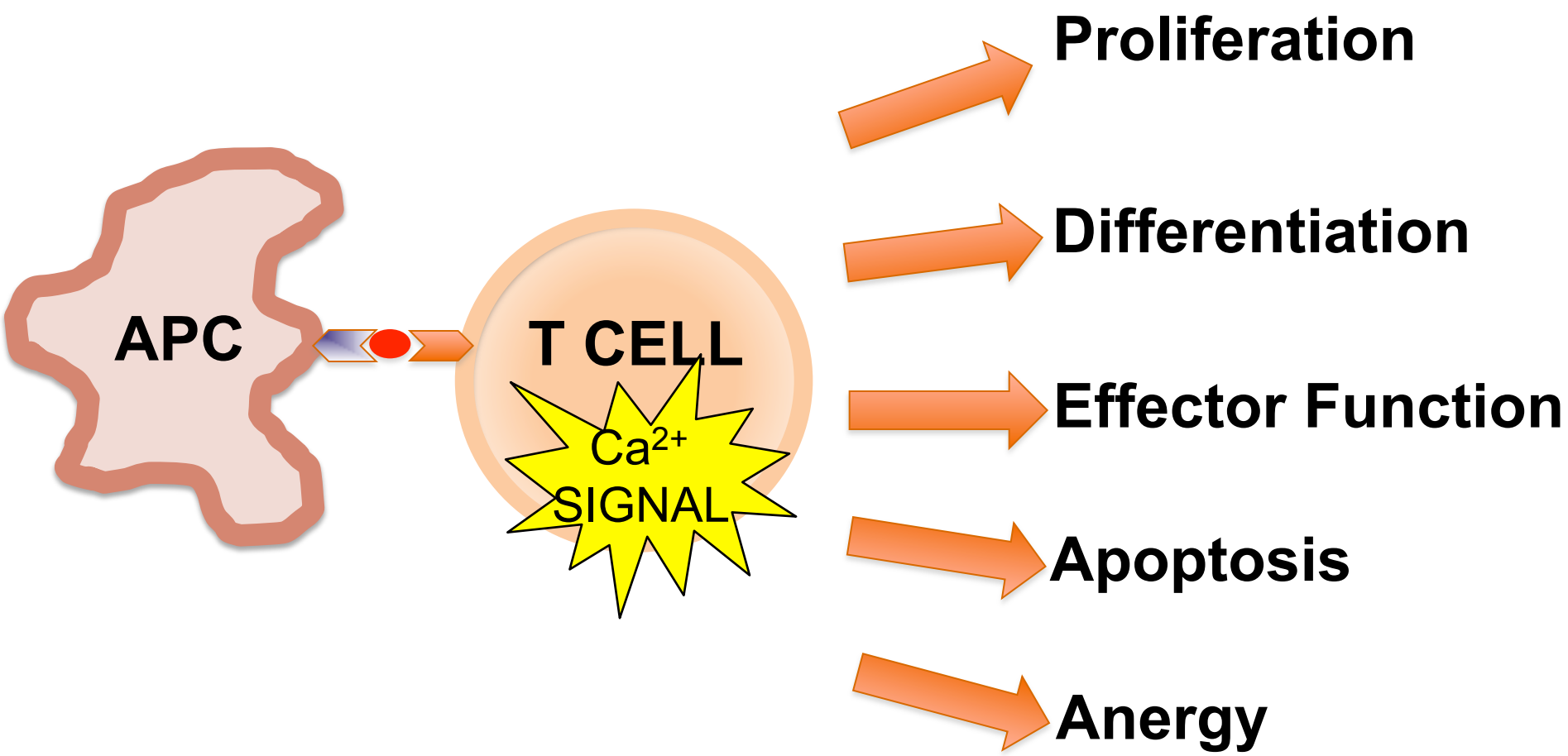
# TCR Engagement/signalling leads to diverse outcomes

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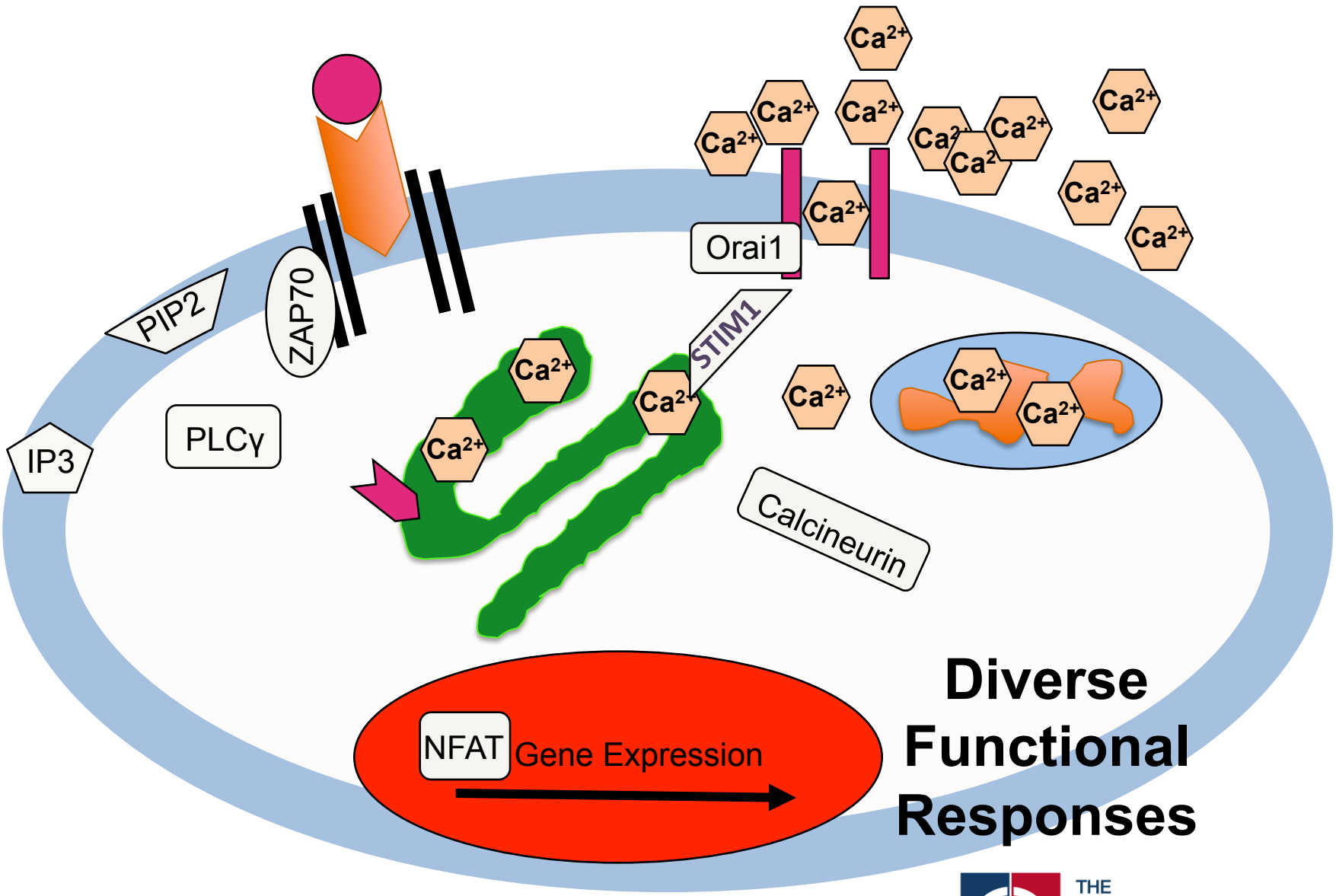
# Calcium signaling: Some of the Key Players

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# Approaches to Measure Calcium Mobilization in Immune Cells

- Conventional Flow Cytometry
  - Gives a temporal "snap shot" of calcium mobilization
  - High throughput, can analyse populations and subsets.
  - No spatial information

- Microscopy
  - Can provide live cell imaging data on single cells
  - Rapid measurement of calcium dynamics
  - Can use spatial information to localize the calcium signal within the cell
  - Lower throughput (generally)

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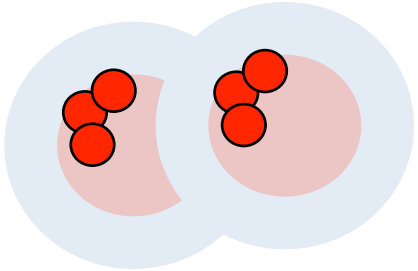
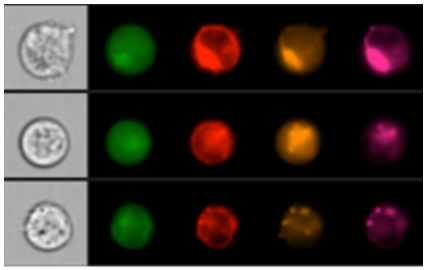
# IFC-protocol overview

1. Label T cells with vital dyes (Calcium/Organelles) spectrally compatible with IFC systems

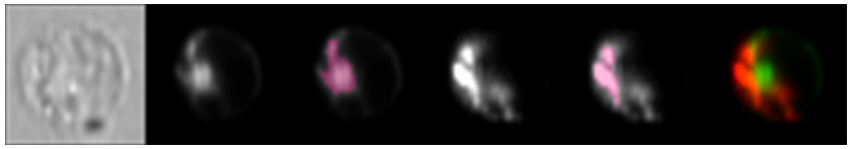


2. Activate with CD3 and acquire on IFC system

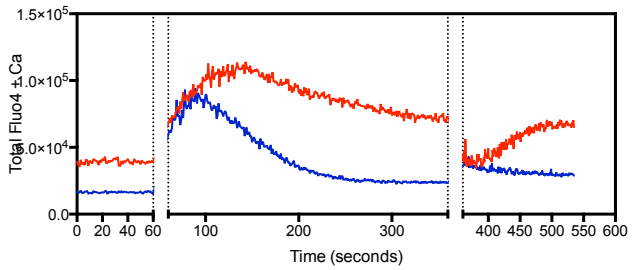
3. Perform compensation on multispectral data



4. Derive masks using organelle signals and measure calcium indicator fluorescence ONLY within that area



5. Plot organelle-directed intensity as a function of time

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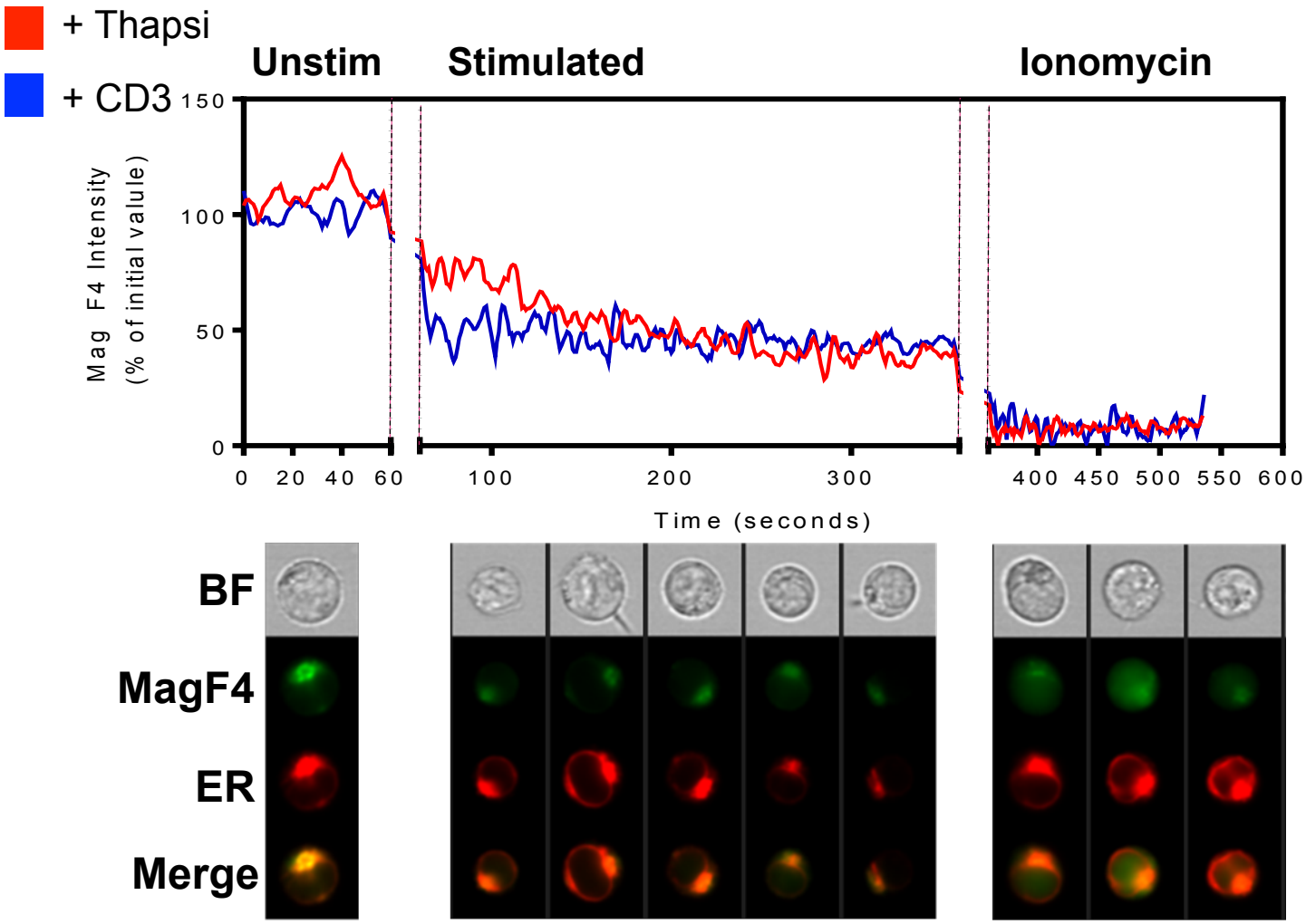
# Mag Flou-4 Reports ER store levels of Calcium and decreases with CD3/Thapsigargin Stimulation

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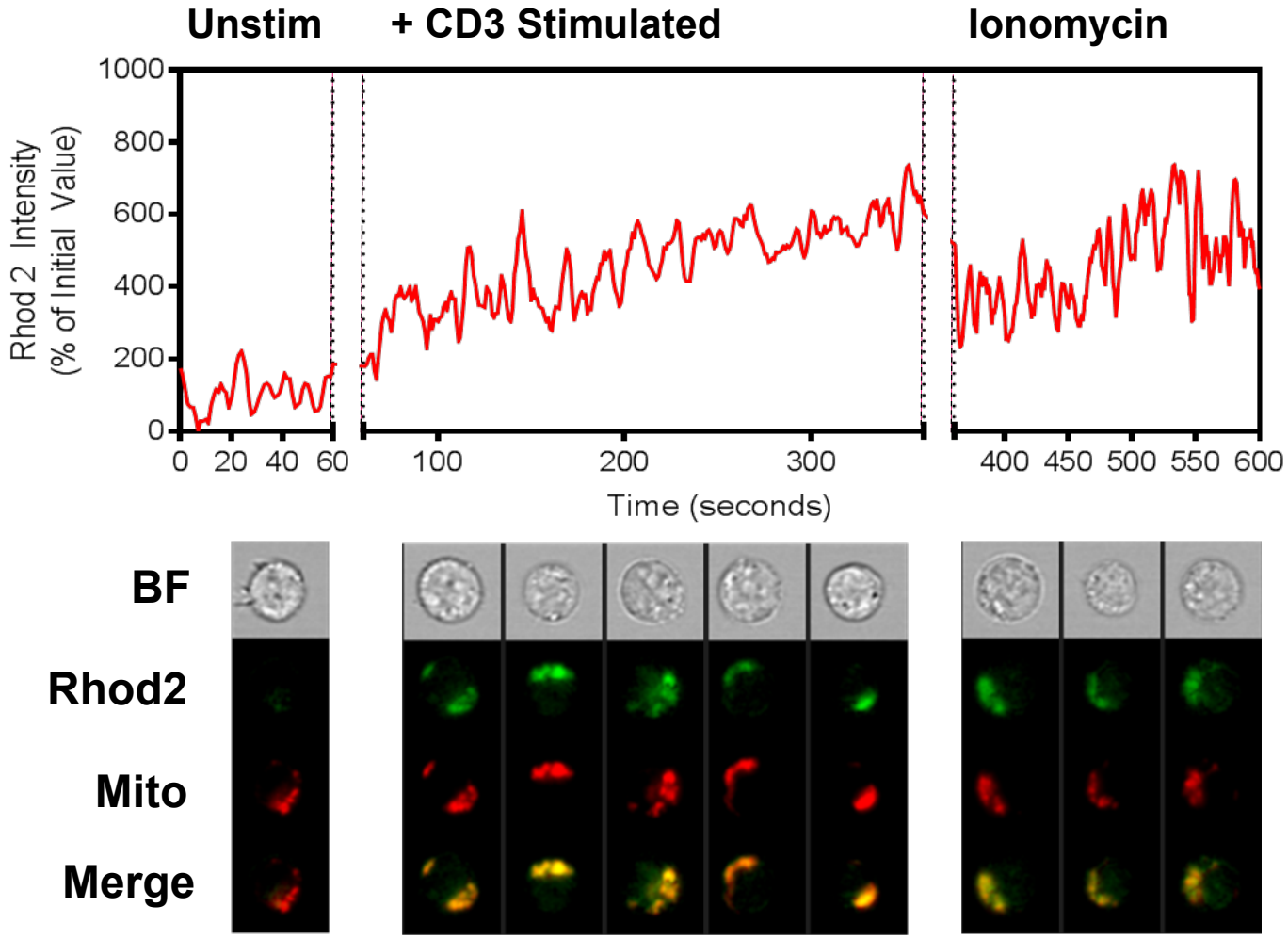
# Rhod 2 Reports Mitochondrial uptake of Calcium after CD3 Stimulation (even in Ca-free media)

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# A machine-learning based approach: Remove some of the human element

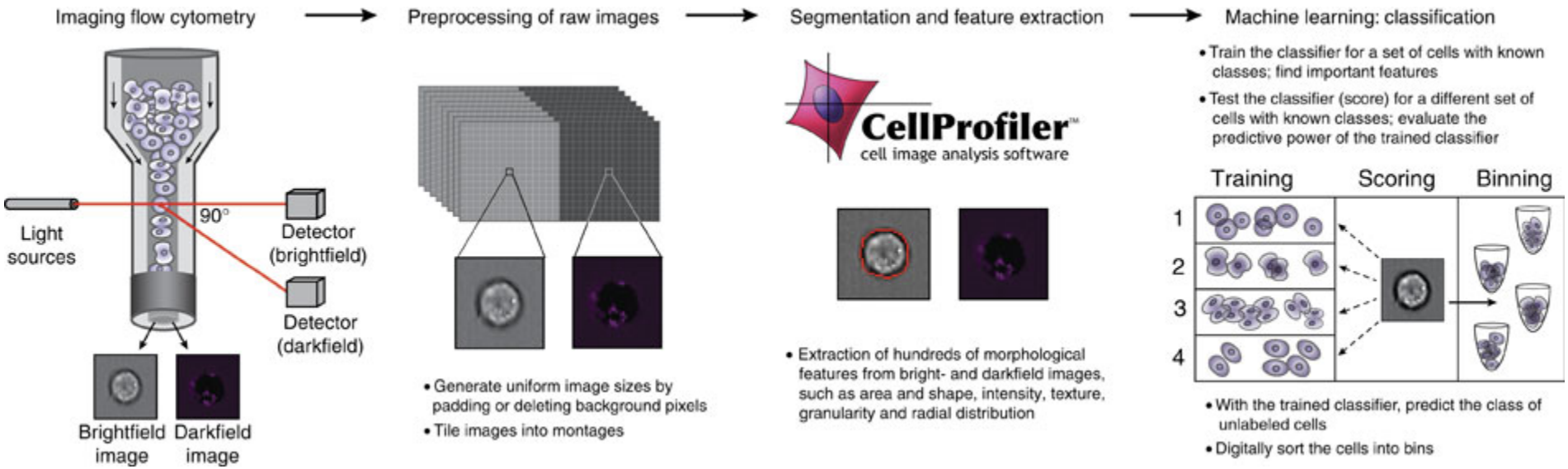
nature COMMUNICATIONS

ARTICLE

Received 1 Sep 2015 | Accepted 19 Nov 2015 | Published 7 Jan 2016 DOI: 10.1038/ncomms10256 OPEN

## Label-free cell cycle analysis for high-throughput imaging flow cytometry

Thomas Blasi[1,2,3], Holger Hennig[1], Huw D. Summers[4], Fabian J. Theis[2,3], Joana Cerveira[5], James O. Patterson[6], Derek Davies[5], Andrew Filby[7], Anne E. Carpenter[1] & Paul Rees[1,4]

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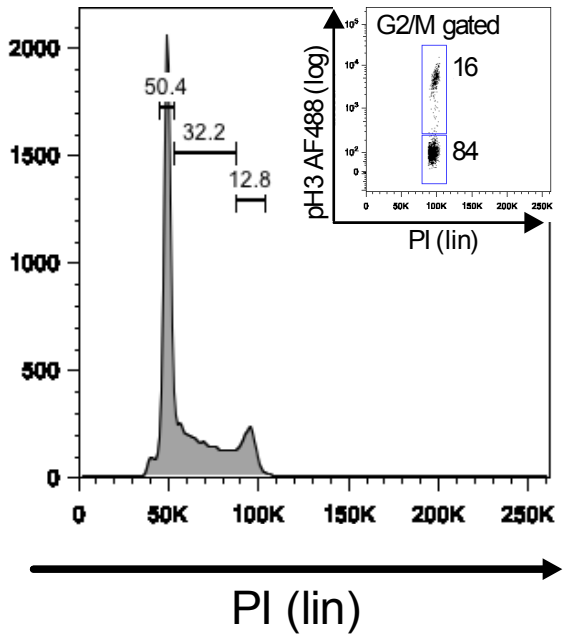
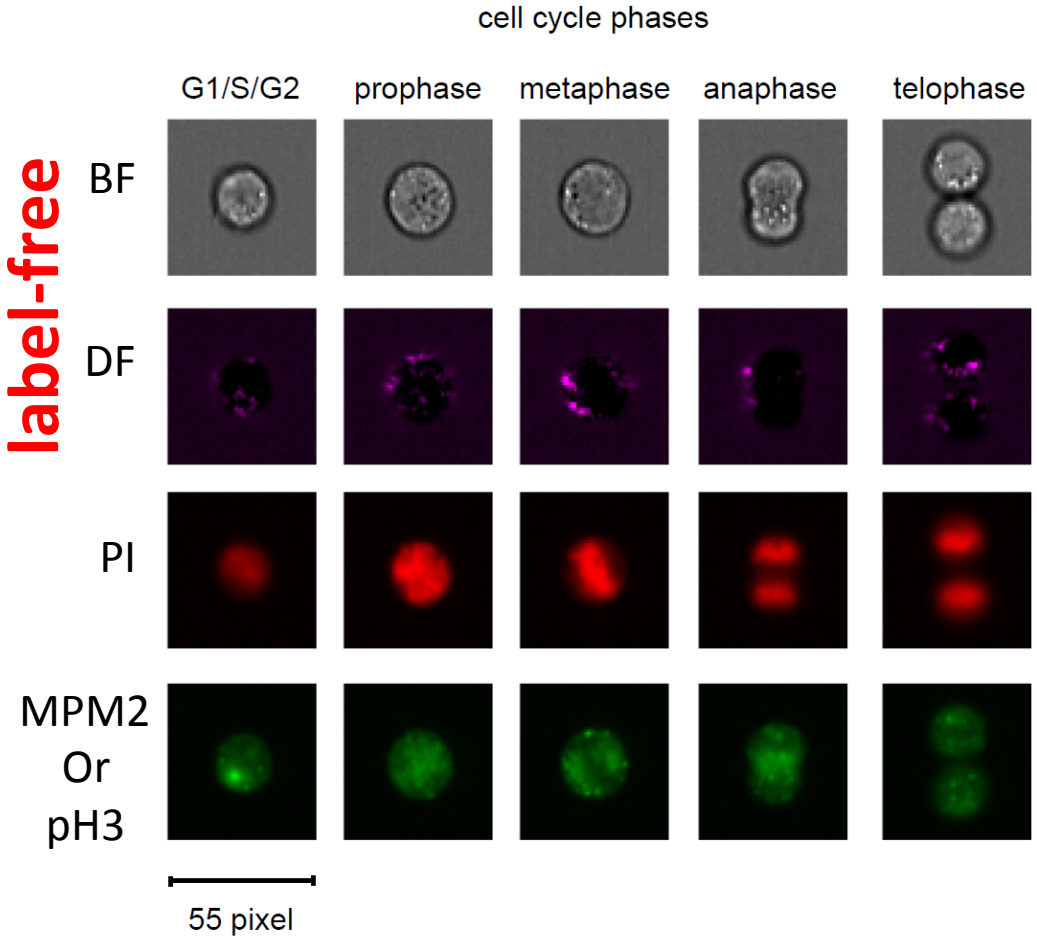
# A machine-learning based approach to cell cycle analysis

Traditional flow-based analysis




Imaging-flow based cell cycle analysis

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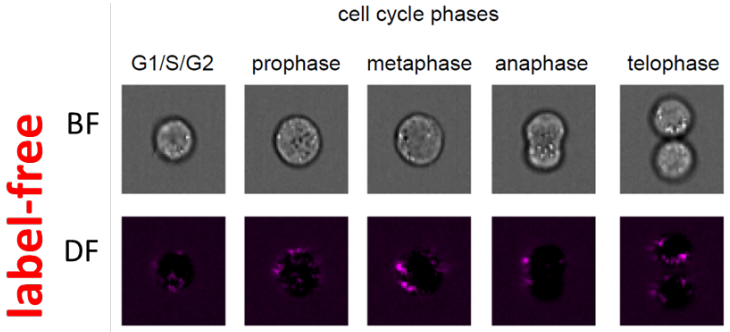
# We start with cell-cycle fluorescent stains then throw them away to be “label-free”

1. Collect lots of images




Throw away fluorescent labels

2. Ask computer to make 100s of pixel based measurements on BF and DF images and find the best correlates of cell cycle phase

| Feature class | Feature number | Feature name | Brightfield | Darkfield |
|---|---|---|---|---|
| Area and shape | 1 | AreaShape_Area | x | o |
| | 2 | AreaShape_Compactness | x | o |
| | 3 | AreaShape_Eccentricity | x | o |
| | 4 | AreaShape_Extent | x | o |
| | 5 | AreaShape_FormFactor | x | o |
| | 6 | AreaShape_MajorAxisLength | x | o |
| | 7 | AreaShape_MaxFeretDiameter | x | o |
| | 8 | AreaShape_MaximumRadius | x | o |
| | 9 | AreaShape_MeanRadius | x | o |
| | 10 | AreaShape_MedianRadius | x | o |
| | 11 | AreaShape_MinFeretDiameter | x | o |
| | 12 | AreaShape_MinorAxisLength | x | o |
| | 13 | AreaShape_Perimeter | x | o |
| Zernike polynomials | 14 | AreaShape_Zernike_0_0 | x | o |
| | ... | ... | x | o |
| | 43 | AreaShape_Zernike_9_9 | x | o |
| Granularity | 44 | Granularity_1 | x | x |
| | ... | ... | x | x |
| | 48 | Granularity_5 | x | x |
| Intensity | 49 | Intensity_IntegratedIntensityEdge | x | x |
| | 50 | Intensity_IntegratedIntensity | x | x |
| | 51 | Intensity_LowerQuartileIntensity | x | x |
| | 52 | Intensity_MADIntensity | x | x |
| | 53 | Intensity_MassDisplacement | x | x |
| | 54 | Intensity_MaxIntensityEdge | x | x |
| | 55 | Intensity_MaxIntensity | x | x |
| | 56 | Intensity_MeanIntensityEdge | x | x |
| | 57 | Intensity_MeanIntensity | x | x |
| | 58 | Intensity_MedianIntensity | x | x |
| | 59 | Intensity_MinIntensityEdge | x | x |
| | 60 | Intensity_MinIntensity | x | x |
| | 61 | Intensity_StdIntensityEdge | x | x |
| | 62 | Intensity_StdIntensity | x | x |
| | 63 | Intensity_UpperQuartileIntensity | x | x |
| Radial distribution | 64 | RadialDistribution_FracAtD_1 | x | x |
| | 65 | RadialDistribution_FracAtD_2 | x | x |
| | 66 | RadialDistribution_FracAtD_3 | x | x |
| | 67 | RadialDistribution_FracAtD_4 | x | x |
| | 68 | RadialDistribution_MeanFrac_1 | x | x |
| | 69 | RadialDistribution_MeanFrac_2 | x | x |
| | 70 | RadialDistribution_MeanFrac_3 | x | x |
| | 71 | RadialDistribution_MeanFrac_4 | x | x |
| | 72 | RadialDistribution_RadialCV_1 | x | x |
| | 73 | RadialDistribution_RadialCV_2 | x | x |
| | 74 | RadialDistribution_RadialCV_3 | x | x |
| | 75 | RadialDistribution_RadialCV_4 | x | x |
| | 76 | Texture_AngularSecondMoment_3_0 | x | x |

| Texture | | | | |
|---|---|---|---|---|
| | 77 | Texture_AngularSecondMoment_3_135 | x | x |
| | 78 | Texture_AngularSecondMoment_3_45 | x | x |
| | 79 | Texture_AngularSecondMoment_3_90 | x | x |
| | 80 | Texture_Contras_3_0 | x | x |
| | 81 | Texture_Contras_3_135 | x | x |
| | 82 | Texture_Contras_3_45 | x | x |
| | 83 | Texture_Contras_3_90 | x | x |
| | 84 | Texture_Correlation_3_0 | x | x |
| | 85 | Texture_Correlation_3_135 | x | x |
| | 86 | Texture_Correlation_3_45 | x | x |
| | 87 | Texture_Correlation_3_90 | x | x |
| | 88 | Texture_DifferenceEntropy_3_0 | x | x |
| | 89 | Texture_DifferenceEntropy_3_135 | x | x |
| | 90 | Texture_DifferenceEntropy_3_45 | x | x |
| | 91 | Texture_DifferenceEntropy_3_90 | x | x |
| | 92 | Texture_DifferenceVariance_3_0 | x | x |
| | 93 | Texture_DifferenceVariance_3_135 | x | x |
| | 94 | Texture_DifferenceVariance_3_45 | x | x |
| | 95 | Texture_DifferenceVariance_3_90 | x | x |
| | 96 | Texture_Entropy_3_0 | x | x |
| | 97 | Texture_Entropy_3_135 | x | x |
| | 98 | Texture_Entropy_3_45 | x | x |
| | 99 | Texture_Entropy_3_90 | x | x |
| | 100 | Texture_Gabor | x | x |
| | 101 | Texture_InfoMeas1_3_0 | x | x |
| | 102 | Texture_InfoMeas1_3_135 | x | x |
| | 103 | Texture_InfoMeas1_3_45 | x | x |
| | 104 | Texture_InfoMeas1_3_90 | x | x |
| | 105 | Texture_InfoMeas2_3_0 | x | x |
| | 106 | Texture_InfoMeas2_3_135 | x | x |
| | 107 | Texture_InfoMeas2_3_45 | x | x |
| | 108 | Texture_InfoMeas2_3_90 | x | x |
| | 109 | Texture_InverseDifferenceMoment_3_0 | x | x |
| | 110 | Texture_InverseDifferenceMoment_3_135 | x | x |
| | 111 | Texture_InverseDifferenceMoment_3_45 | x | x |
| | 112 | Texture_InverseDifferenceMoment_3_90 | x | x |
| | 113 | Texture_SumAverage_3_0 | x | x |
| | 114 | Texture_SumAverage_3_135 | x | x |
| | 115 | Texture_SumAverage_3_45 | x | x |
| | 116 | Texture_SumAverage_3_90 | x | x |
| | 117 | Texture_SumEntropy_3_0 | x | x |
| | 118 | Texture_SumEntropy_3_135 | x | x |
| | 119 | Texture_SumEntropy_3_45 | x | x |
| | 120 | Texture_SumEntropy_3_90 | x | x |
| | 121 | Texture_SumVariance_3_0 | x | x |
| | 122 | Texture_SumVariance_3_135 | x | x |
| | 123 | Texture_SumVariance_3_45 | x | x |
| | 124 | Texture_SumVariance_3_90 | x | x |
| | 125 | Texture_Variance_3_0 | x | x |
| | 126 | Texture_Variance_3_135 | x | x |
| | 127 | Texture_Variance_3_45 | x | x |
| | 128 | Texture_Variance_3_90 | x | x |

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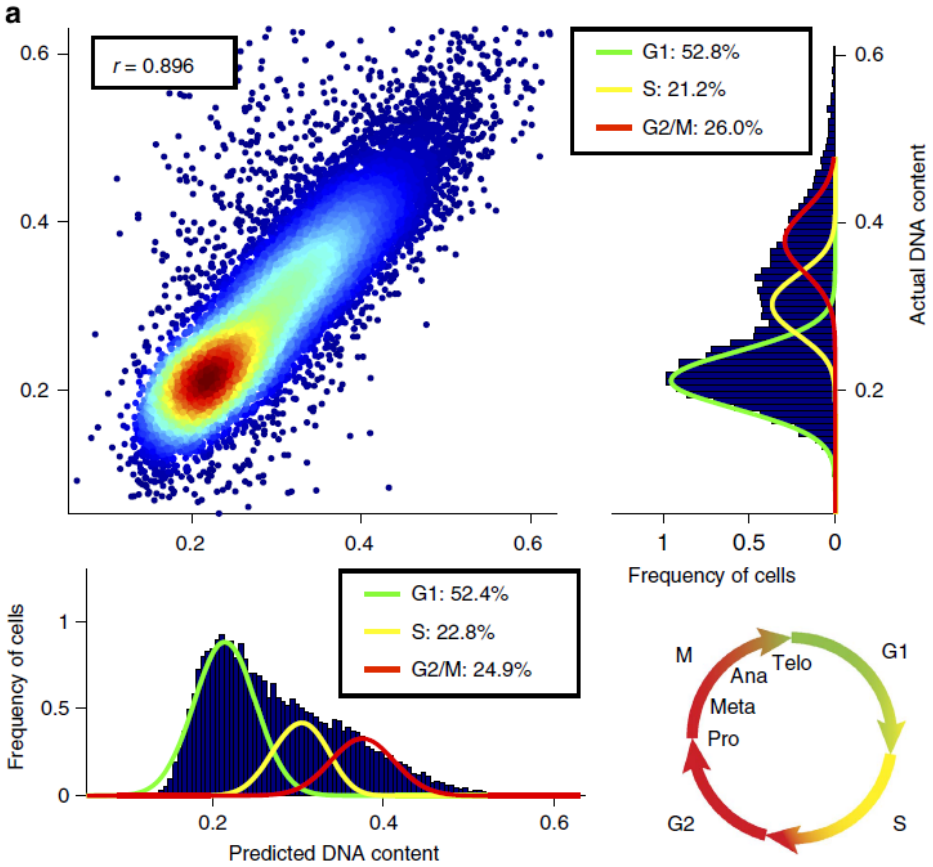
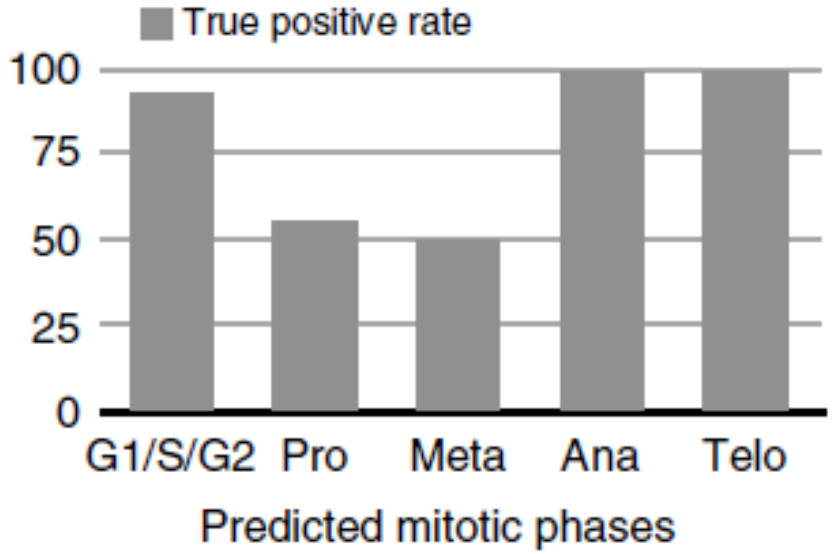
# A machine-learning based approach to cell cycle analysis




- Good label-free predictive rate for G1/S/G2
- Challenge with Pro/Meta (expected)
- Very accurate for Ana/Telophase

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# Summary and conclusions

- Preserves high-throughput capabilities of flow
- Provides spatial information to ask where the $Ca^{2+}$ mobilisation localises to in the T cell
- Provides a kinetic measurement on high numbers of cells.
- Number of cells analysed and quality of label free imagery (BF and SSC) allows for bold machine-learning approaches.

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# Acknowledgements


Andrew.filby@newcastle.ac.uk
http://www.ncl.ac.uk/fccf
Twitter: @NewcastleFCCF

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Sir Francis Crick Institute, UK
Derek Davies
Joana Cerveira
Julfa Begum

**Label-free Cell Cycle Story:**
Swansea University, UK
Paul Rees

Broad Institute, USA
Anne Carpenter
Holger Hennig

Helmholtz Zentrum, Germany
Fabian Theis
Thomas Blasi

bright red
Fighting blood cancer for a brighter future

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