Workshop Training Series

The Application of Amnis Image Flow Cytometry

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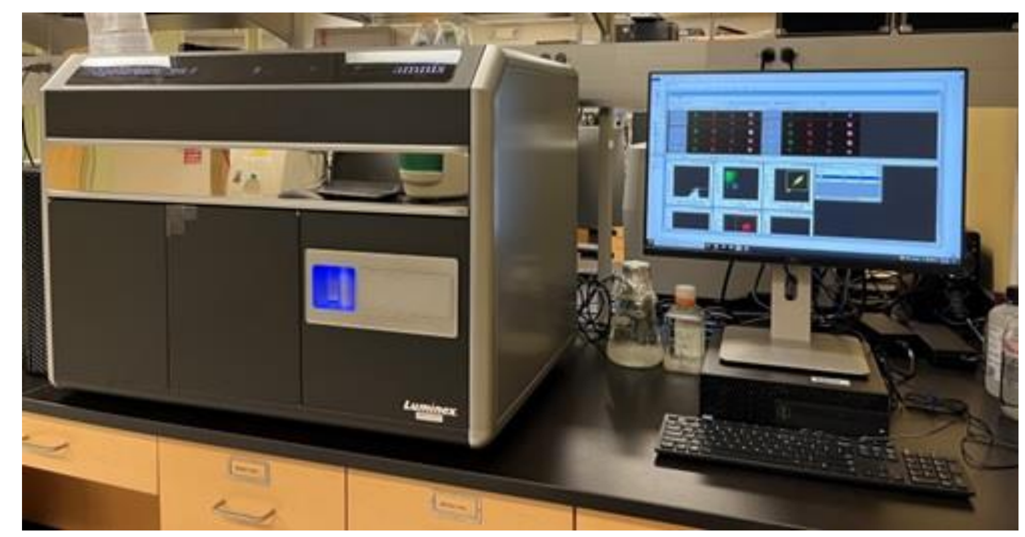
What's image flow cytometer?

- An image flow cytometer is an instrument that integrates high-speed fluorescent microscope system with a flow cytometer. It not only provides detailed cellular images and subcellular structure, but also offers cell phenotyping and counting function at the same time. It overcomes the limitations of both techniques and extends a range of novel application in biological research.

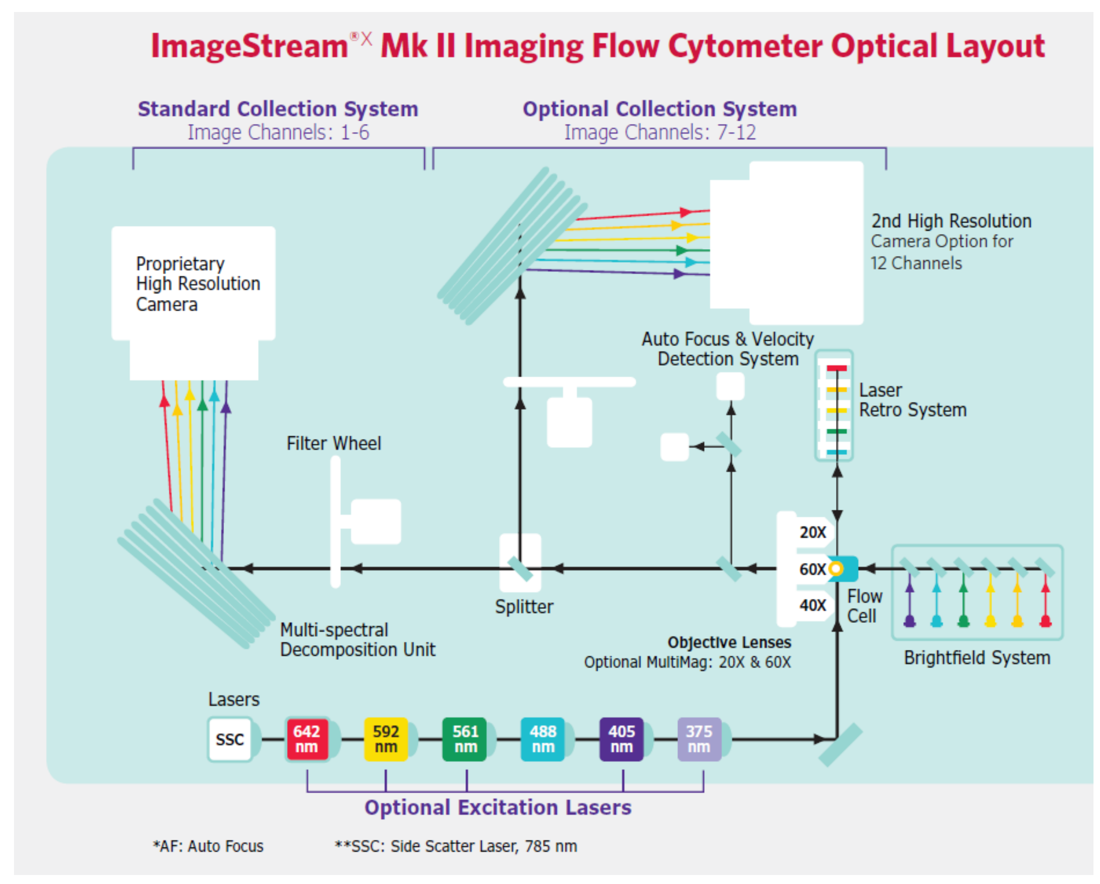


Amnis® ImageStream®X Mk II image flow cytometer

Comparison among Microscope, flow cytometry and Image flow Cytometry

	Microscope	Flow Cytometer	Image Flow Cytometer
Providing detailed information about cellular morphology and structure, sub-cellular localization	Yes	No	Yes
Phenotyping and counting cell population and sub population	No	Yes	Yes
User-friendly software to analyze data	No	N/A	Yes

The structure of Amnis ImageStream MK II image flow cytometer



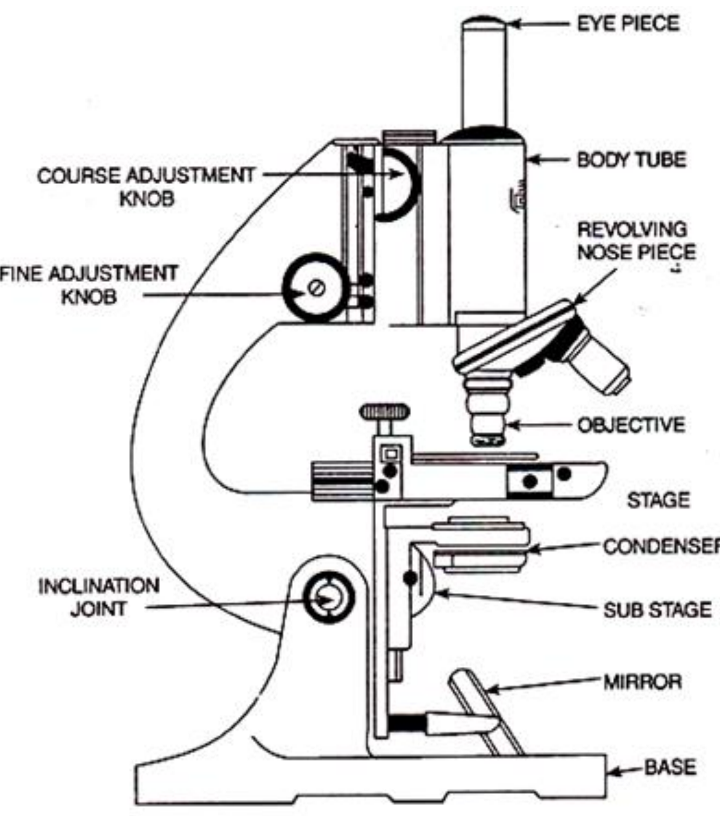
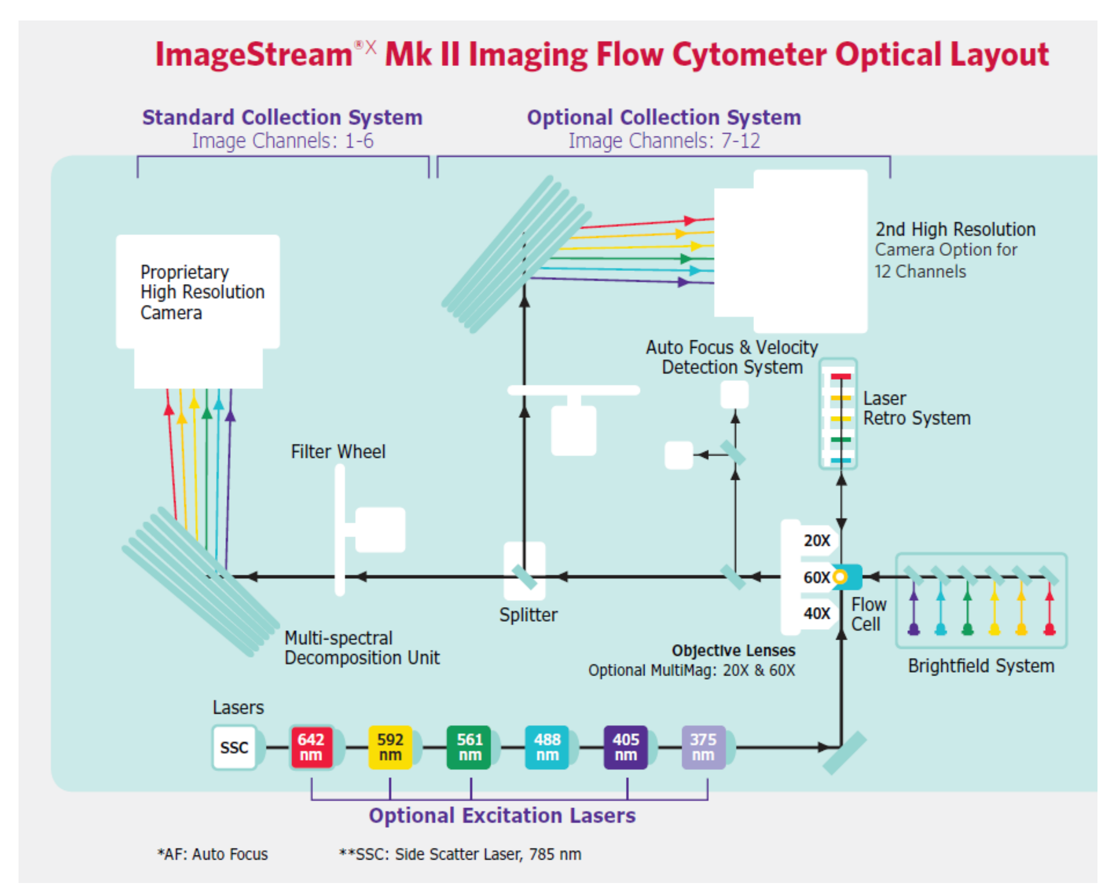


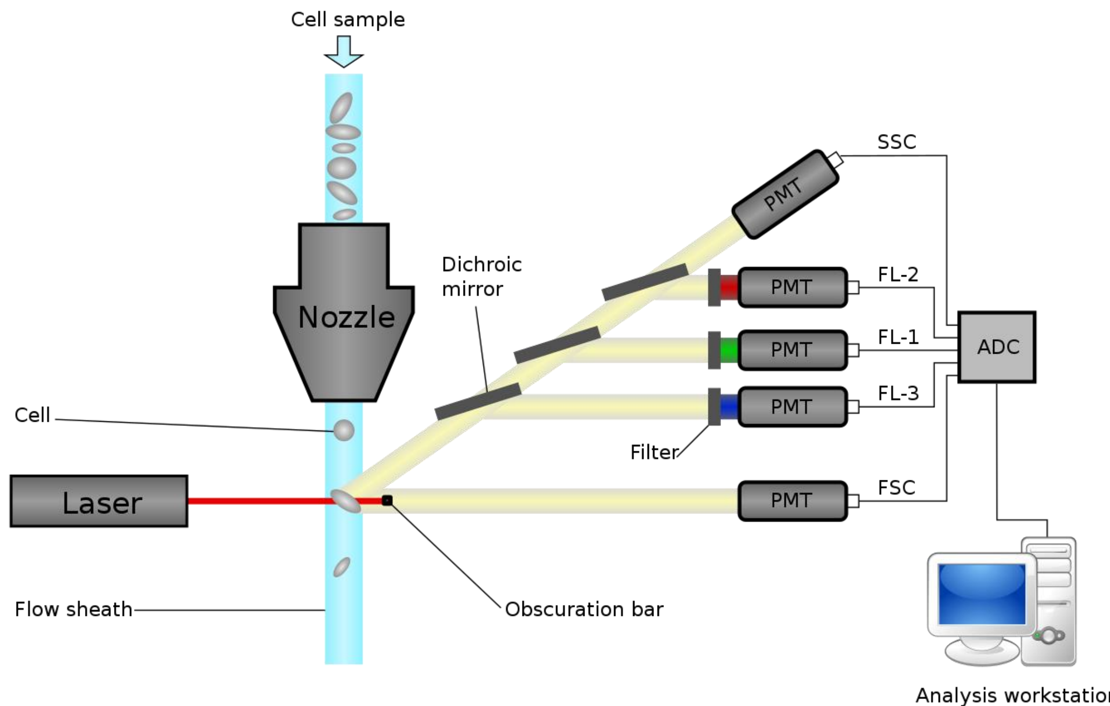
FIG. 15.1. The compound microscope showing its various parts.

Objectives, high gain and EDF

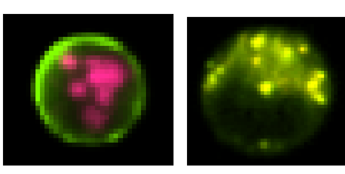
- The pixel size of the captured digital images are 0.1/0.25/1 μm^2 with 60X/40X/20X magnification.
- The EDF option can be considered as z-stack that provides additional depth information in each image, like in confocal imaging. It is recommended to use the EDF option for experiments where the images expected to be spotty or punctate. Using the EDF on samples where cellular staining is uniform can cause the images to become spotted and difficult to analyze.
- High Gain option allows users to collect information of Extracellular Vesicles (EVs) and other submicron-sized particles at 60x magnification.

The structure of Amnis ImageStream MK II image flow cytometer

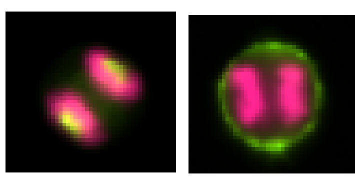




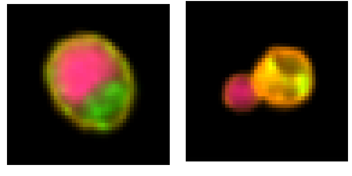
ImageStream applications-subcellular structure



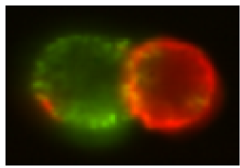
Apoptosis, nuclear fragmentation, LC3 clustering



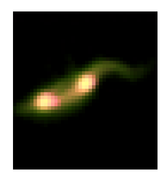
Morphological classification of mitosis



Eryithroid differentiation, hematopoiesis

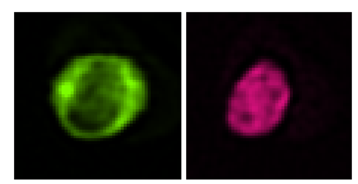


Immune synapse formation, T-cell APC conjugation

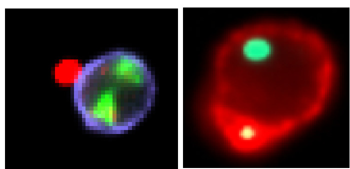


Babesia infection in RBCs, Trypanosomiasis

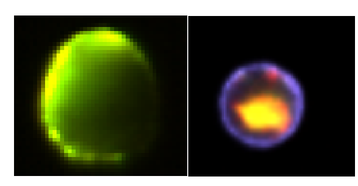
ImageStream applications-localization



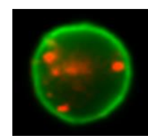
Protein localization and translocation,



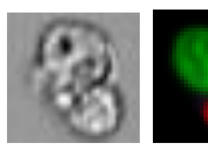
Internalization, phagocytosis of Bacteria by monocytes



Ligand colocalization to lysosomes

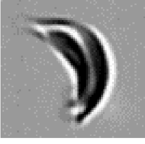


Exosome Internalization

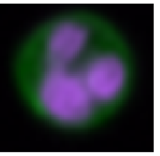


NK Cell Granzyme killing

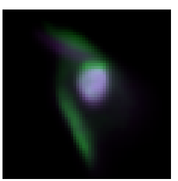
ImageStream applications - morphology



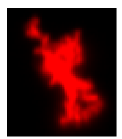
Sickle cell



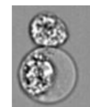
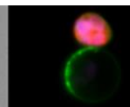
AML ALL classification



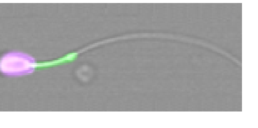
Clinical diagnostics: HPV



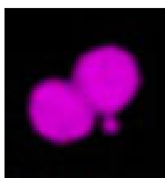
Protein Aggregation

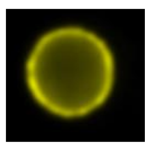
Netosis



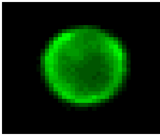
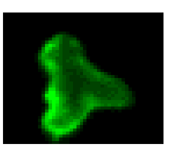
Sperm Analysis



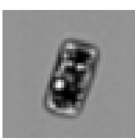
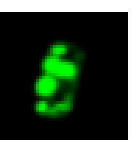
Micro-nuclei assay



Circulating tumor cell

Shape change
& chemotaxis

Water Quality Testing / Algae

ImageStream MKII workflow

1. Prepare samples
2. Collect data
3. Data analysis
4. Generate report

Sample preparation

1) Select fluorochromes appropriate for lasers
2) Prepare 10^6 cells per sample (Typically, 2 µm to 100 µm diameter), most of them have to be single cells.
3) Most FACS staining procedures should work.
4) Remember to label single color compensation controls along with multiple color experimental cells if needed.
5) label good positive biological controls to make sure the experiment works.
6) Filter out large cell aggregates to prevent clog before loading samples to the instrument (BORC provides filters)
7) The labeled cells are resuspended in 50ul running buffer in 1.5ml tubes

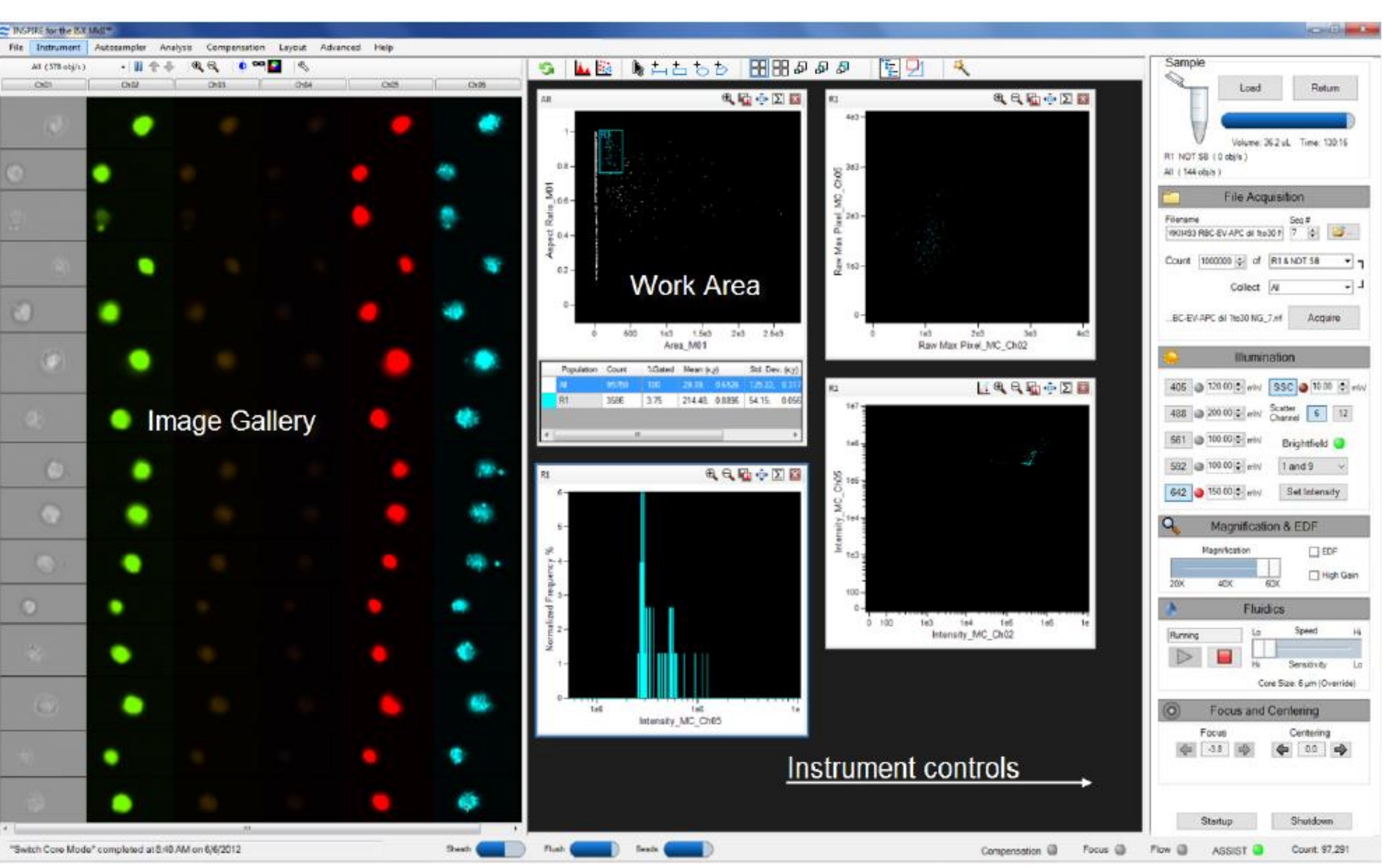
ImageStream channel system

Ch	Band (nm)	Excitation Laser (nm)						
		375	405	488	561	592	642	785
1	**435-505** (457/45)	***DAPI, BV421, AF350,** Hoechst, PacBlue, CascadeBlue,	***DAPI, BV421, AF405,** Hoechst, PacBlue, CascadeBlue, , eFluor450, DyLight405, CFP, LIVE/DEAD Violet					
2	**505-560** (533/55)	***BV510**, PacOrange, CascadeYellow, AF430, eFluor525, QD525,	***BV510**, PacOrange, CascadeYellow, AF430, eFluor525, QD525,	**FITC, AF488, GFP**, YFP, DyLight488, PKH67, Syto13, SpectrumGreen, LysoTrackerGreen, MitoTrackerGreen				
3	**560-595** (577/35)	*QD565, QD585, eFluor565	*QD565, QD585, eFluor565	**PE**, PKH26, DSRed, mOrange, CellMask/CellTracker/ SYTOX Orange, Cy3	**PE, AF546**, Cy3*, DyLight550, PKH26, DSRed, SpctmOrng, MTOrng			
4	**595-642** (610/30)	*QD625, eFluor625, BV605	*QD625, eFluor625, BV605	**PE-TexRed, ECD**, PE-AF610, RFP, QD625, eFluor625	**AF568**, Cy3*, PE-TexRed, ECD, TexRed, PE-AF610, RFP, mCherry, PI	**TexRed, AF594**, DyLight594, mCherry, SpectrumRed, PI,		
5	**642-745** (702/85)	*QD705, eFluor700, BV711	*QD705, eFluor700, BV711	**PE-Cy5, PE-AF647**, 7AAD, PI*, PerCP, PerCP-Cy5.5, DRAQ5 QD705, eFluor650, FuraRedlo, DRAQ5*, LDS 751	**PE-Cy5, PE-AF647**, DRAQ5*, 7AAD, LDS751	**AF647, AF660**, AF680, APC Cy5, DyLight649, PE-AF647, PE-Cy5, DRAQ5*	**APC, AF647, AF660, AF680, DRAQ5**, Cy5, DyLight649, DyLight680, PE-AF647, PE-Cy5, PerCP, PerCP-Cy5.5	
6	**745-785** (765/40)	*QD800, BV786	*QD800, BV786	**PE-Cy7, PE-AF750**, QD800	**PE-Cy7, PE-AF750**	**APC-Cy7, APC-AF750**, APC-H7, APC-eFluor750	**APC-Cy7, APC-AF750**, APC-H7 APC-eFluor750, Cy7, AF750, DyLight750, PE-Cy7, PE-AF750	**SSC**

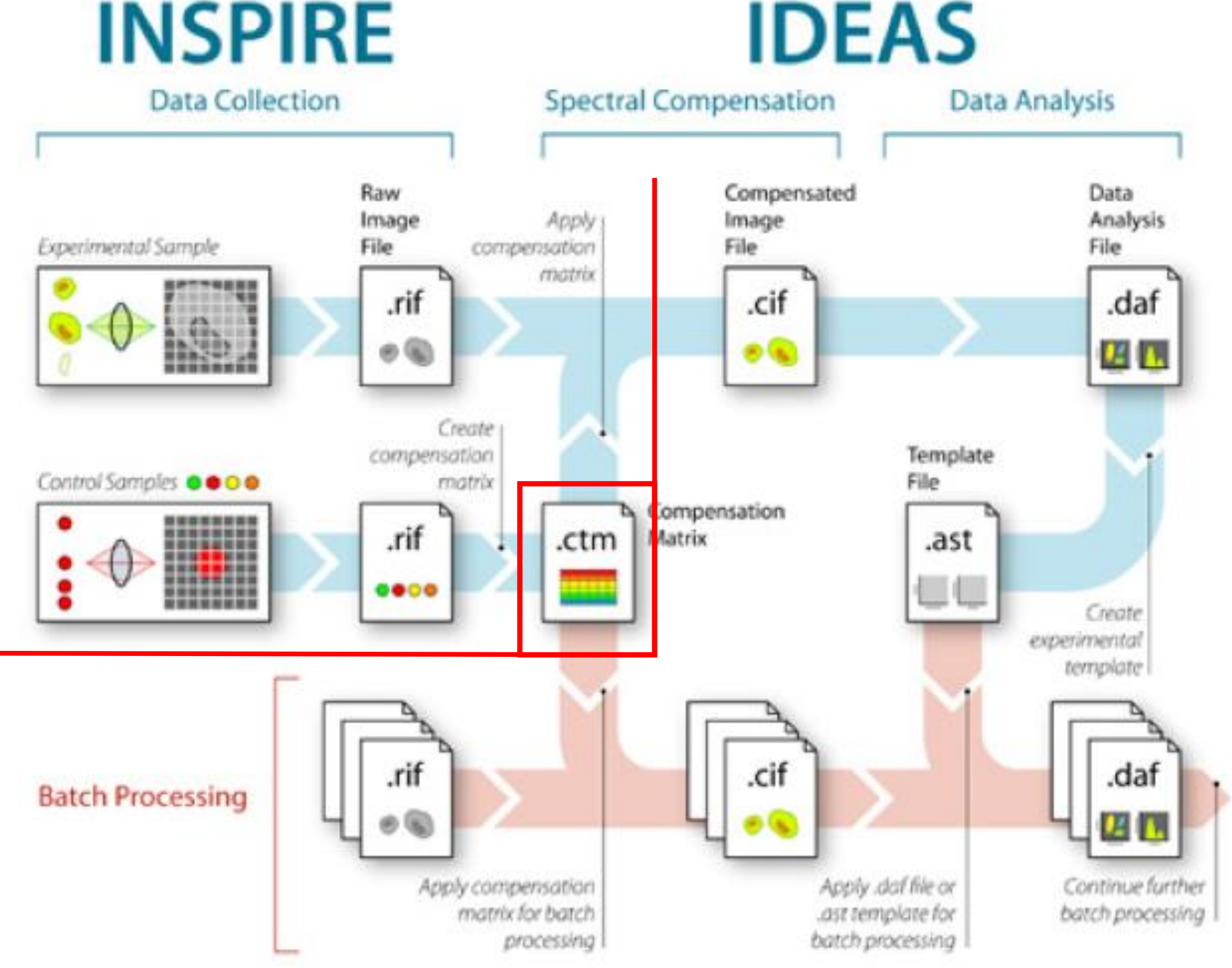
Data acquisition workflow

- Power on the system and log into the INSPIRE software
- Run ASSIST calibrations and tests
- Load sample
- Set **magnification** (choose with or without **EDF**)
- Set the appropriate **speed** (typically **Low**)
- Turn on and adjust each laser power
- Verify bright fluorescence and no **saturation** (Raw max pixel ~200 to 4000 counts)
- Collect experiment samples with consistent settings
- Collect compensation controls without BF and SSC

INSPIRE



Data Acquisition and Analysis



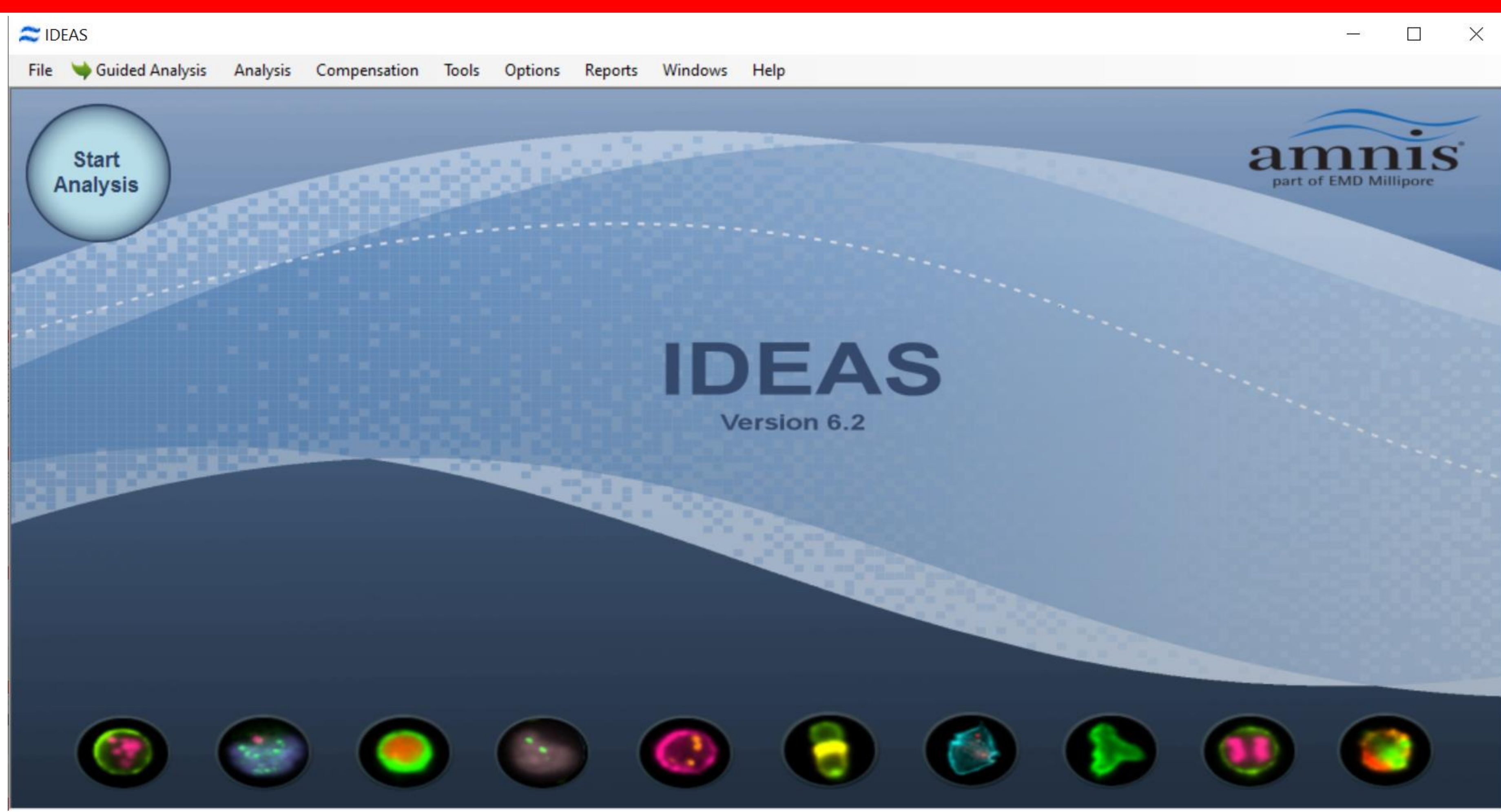
What's in the files

- **Raw Image File (.rif)**
  - Created in INSPIRE when collecting data.
  - Contains instrument setup data and uncompensated pixel intensity data.
- **Compensation Matrix File (.ctm)**
  - Created in INSPIRE during data acquisition or IDEAS in data analysis.
  - Created from multiple single color .rif files contains coefficients for .rif compensation
  - Applied to experimental raw image file to create compensated image file
- **Compensated Image File (.cif)**
  - Created in IDEAS when performing data analysis.
  - Contains image data that has been corrected and serve as a database of images used for feature-value calculations and imagery display.
- **Data Analysis File (.daf)**
  - Created in IDEAS when performing data analysis.
  - Contains calculated feature values, the graphs, and the statistics used for analysis.
  - The .daf file references the .cif and must be in the same folder as .cif files
- **Template files** (.ast) optional
  - Contains the structure for the analysis, used for batch processing.

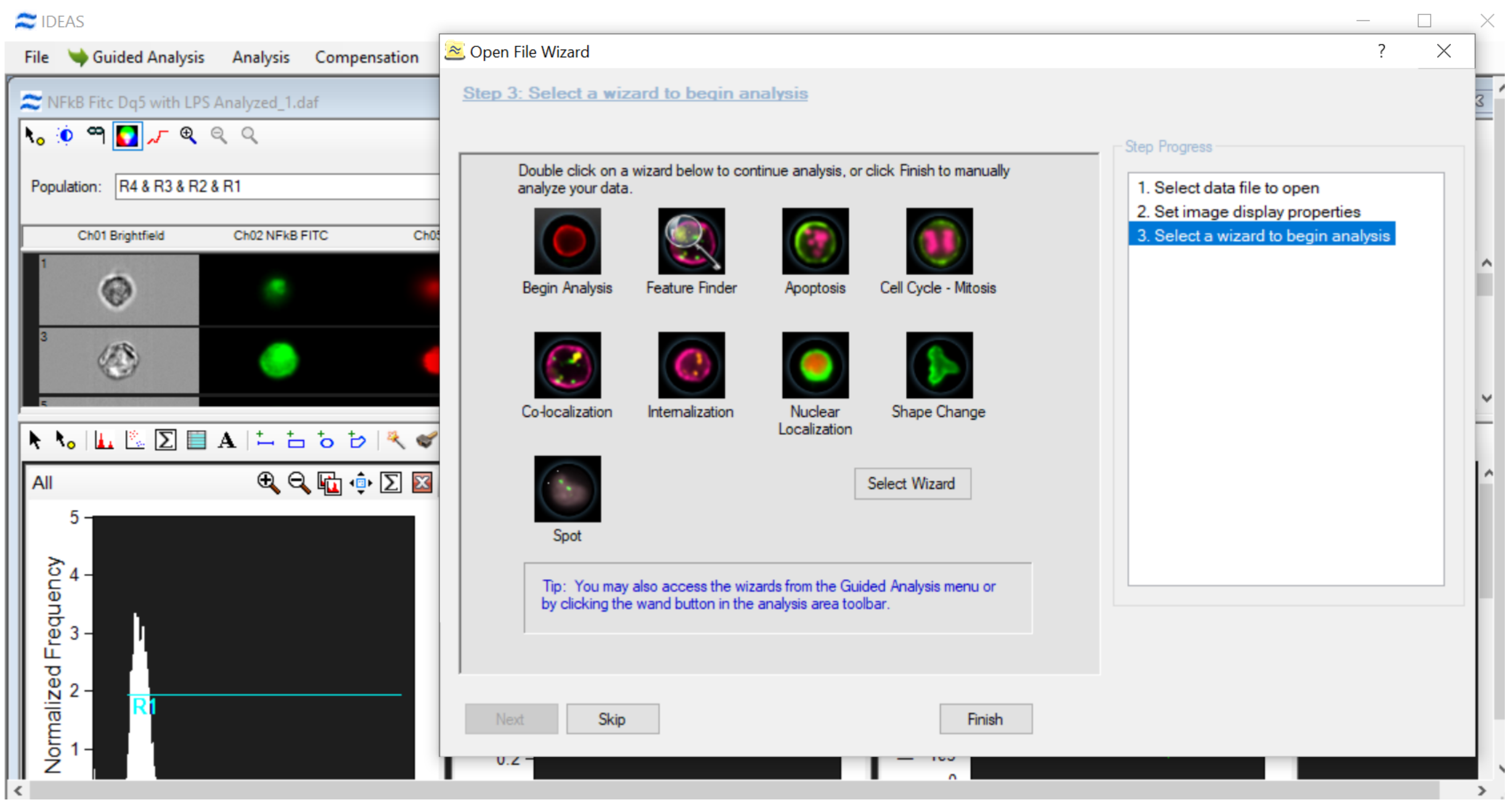
Data analysis outline

- Start IDEAS
- Open raw image files
- Create compensation file .ctm
- Apply compensation to raw image file to create compensated image files
- Graph histogram and scatter plot
- Gate target regions/populations
- Optimized image and set image display properties
- Create a template file
- Perform batch analysis on all data files in the experiment using the compensation matrix and analysis template.

IDEAS

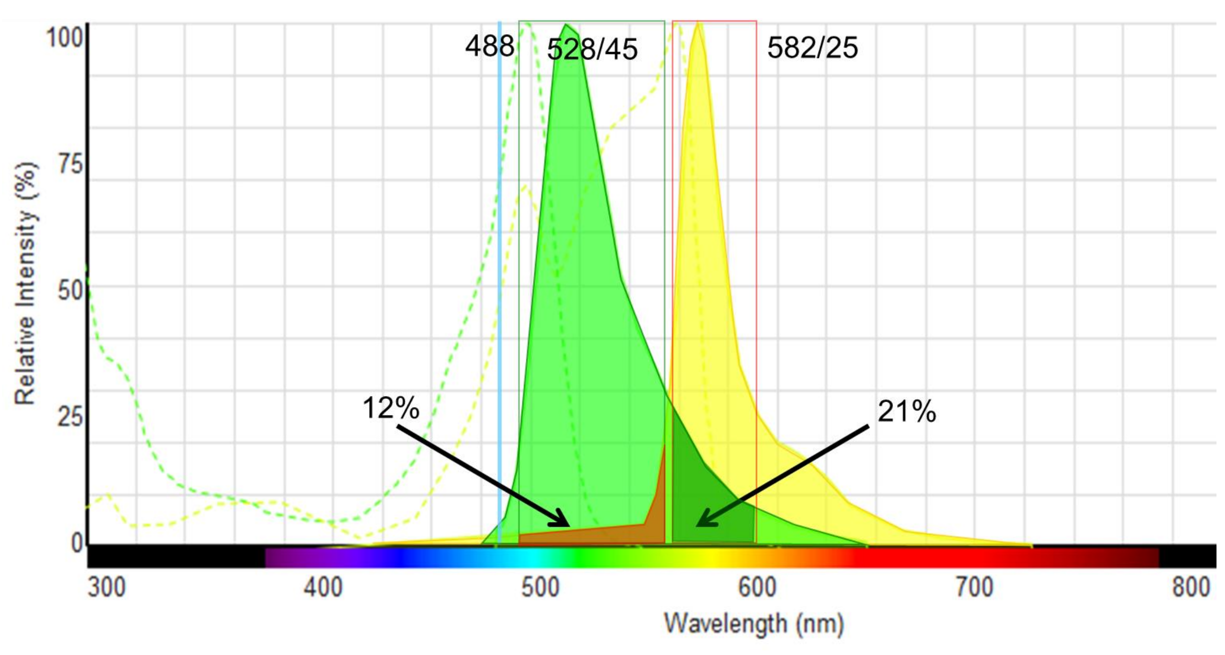


Built-in wizard for common analysis



What's compensation?

- All fluorochromes have broad emission spectra.
- The optical filters are not perfect.
- Signal in a given channel will come from multiple sources of fluorescence.
- Compensation is the removal of overlapping fluorescence from adjacent channels.

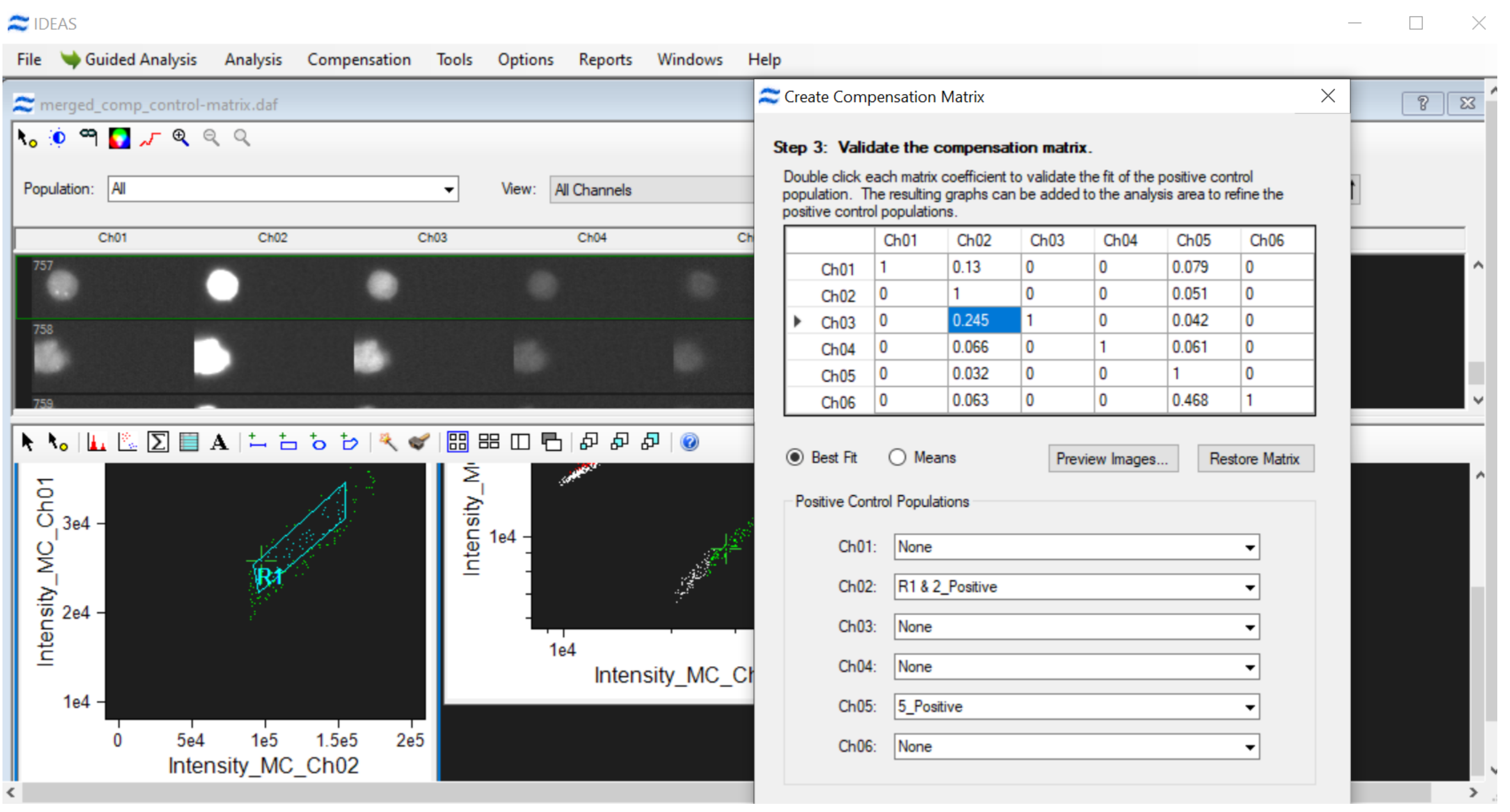


FITC overlaps the PE channel with about 21% crosstalk.
PE leaks back into FITC with about 12% crosstalk.

Check and correct compensation

- The software uses all the data from single color controls to create compensation matrix by default.
- The control samples may contain outliers or autofluorescence that will impact the accuracy of compensation.
- The biased compensation matrix can be corrected manually.

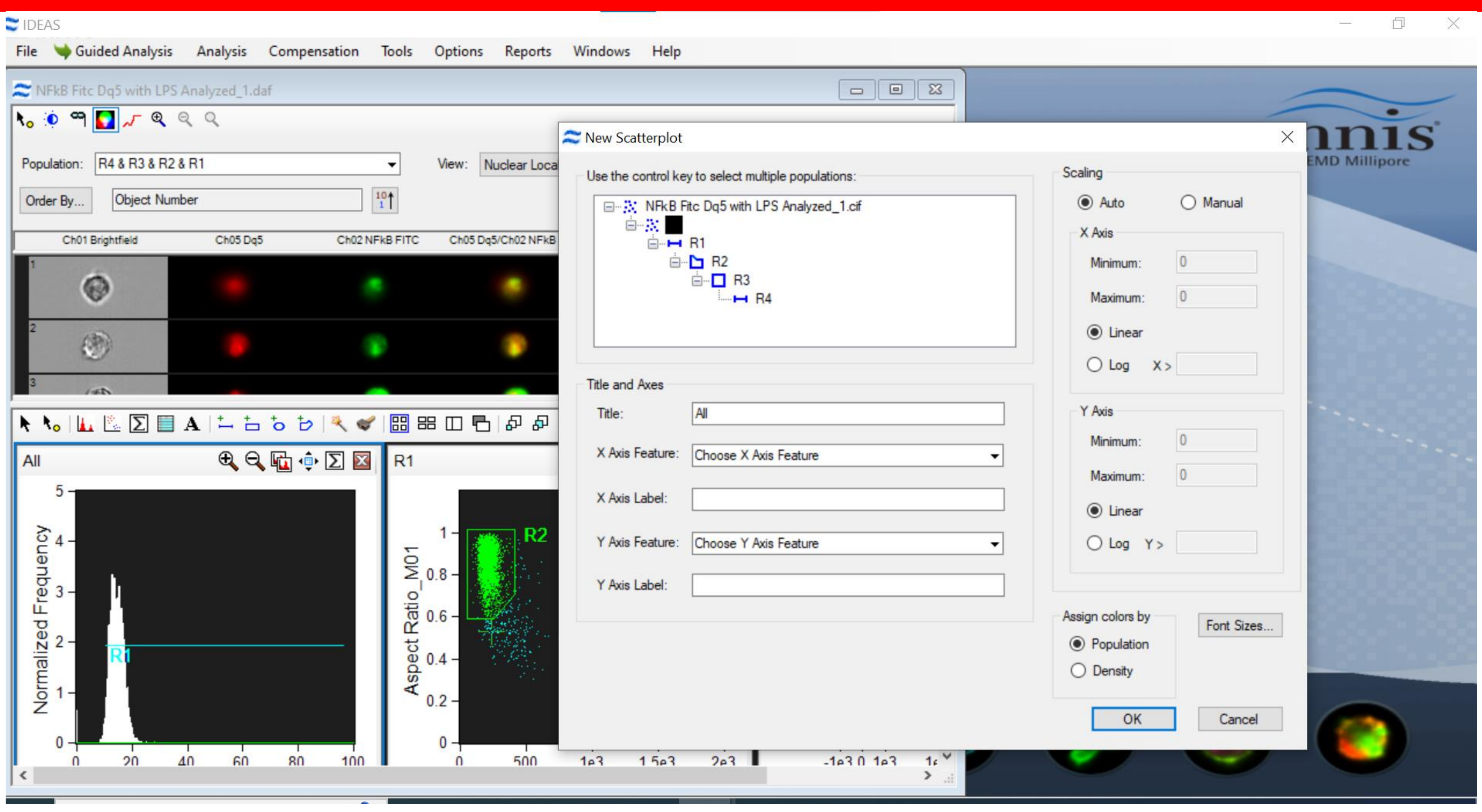
Check and correct compensation



Gate target populations

- **Gate cells in best focus**
  Gradient RMS histogram-The cells with better focus have higher Gradient RMS
- **Gate single cells**
  Single cells have an intermediate Area value and a high Aspect Ratio (scatter plot)
- **Gate sub-populations**
  Separate populations on scatter plot of channel intensity
- **Select target populations**
  apoptosis cells, internalization cells, etc.
- **Manage populations/regions**
  Resize and remove defined populations and regions

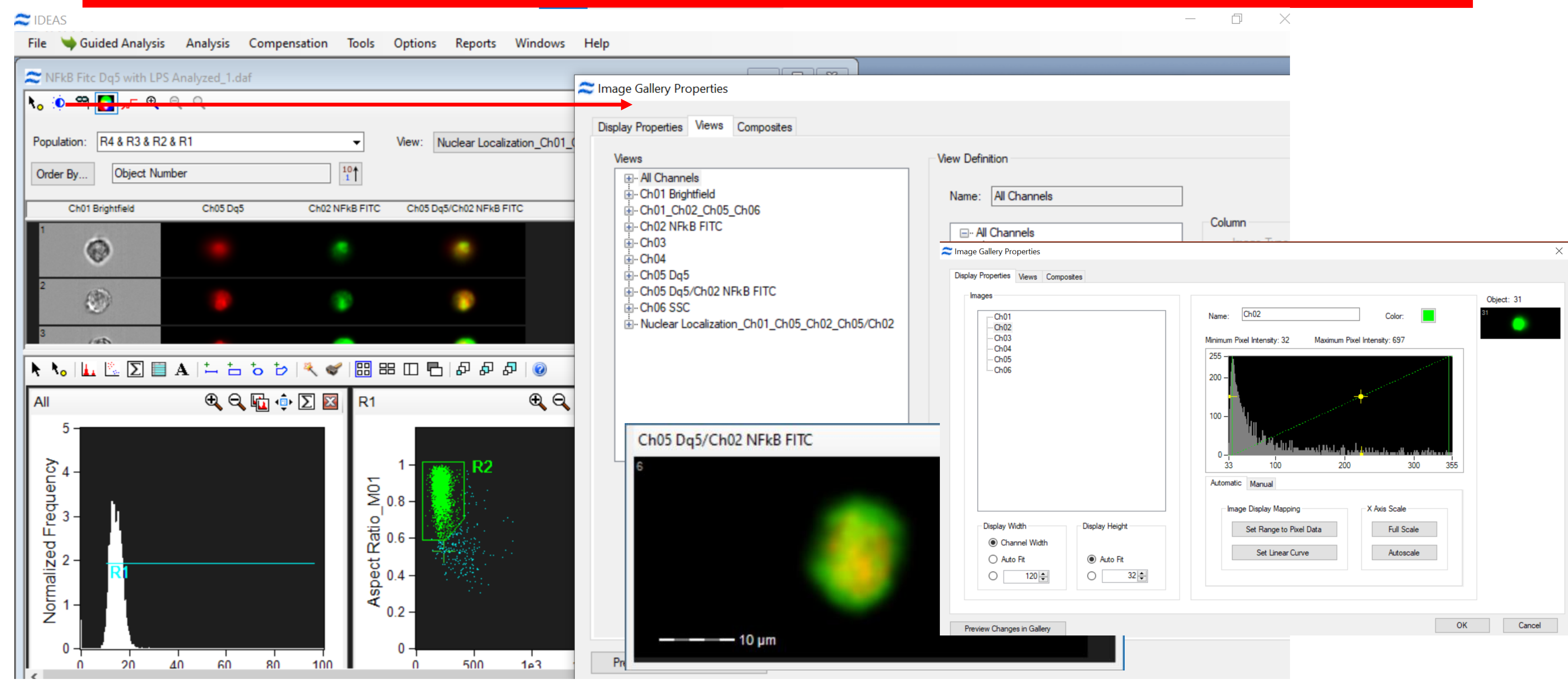
Gate target popualtions



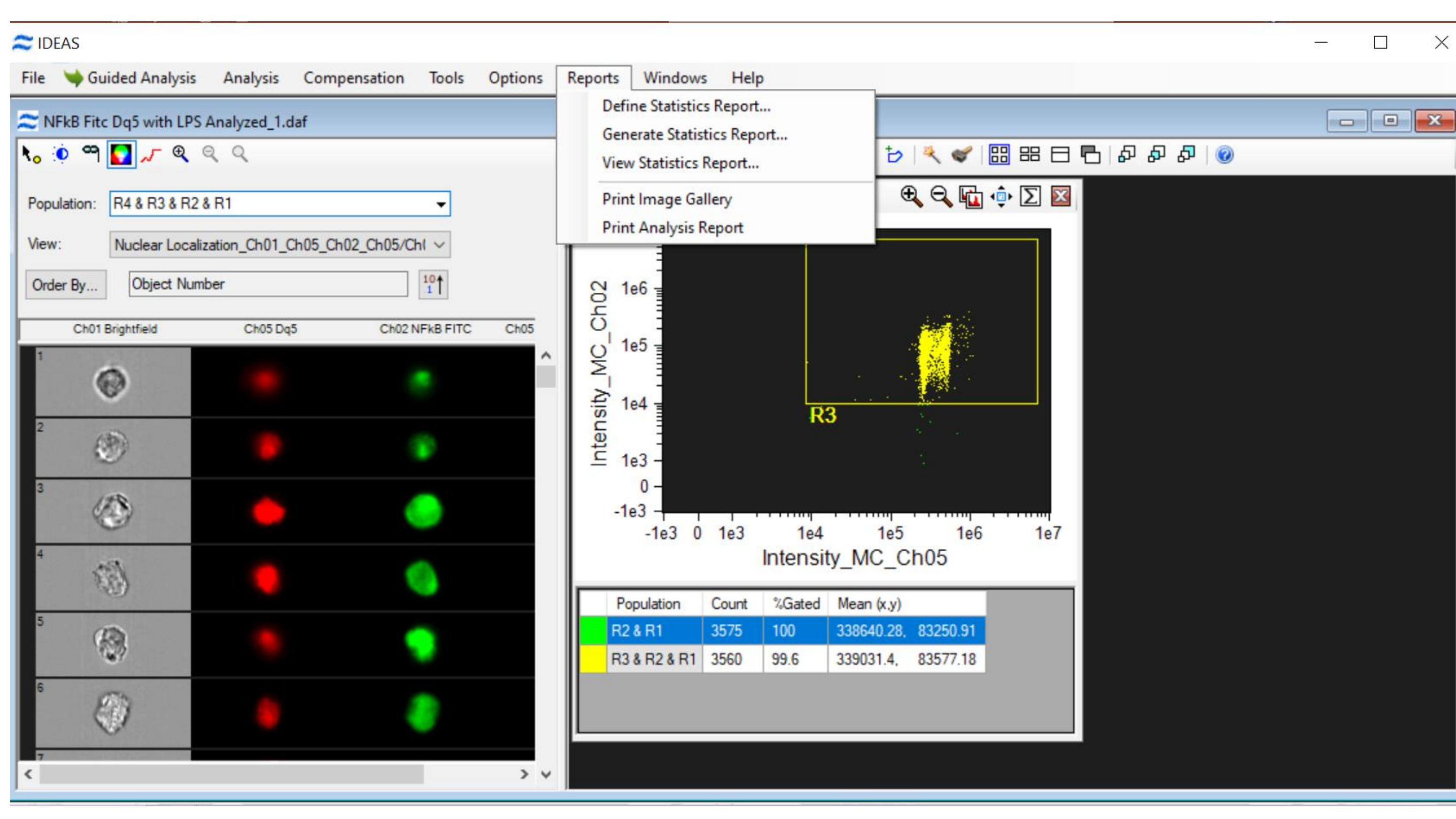
Set image display properties

- Choose population to display.
- Choose channels to display.
- Create composite images.
- Adjust image order.
- Match plot dots and images.
- Save single cell image.

Set image display properties



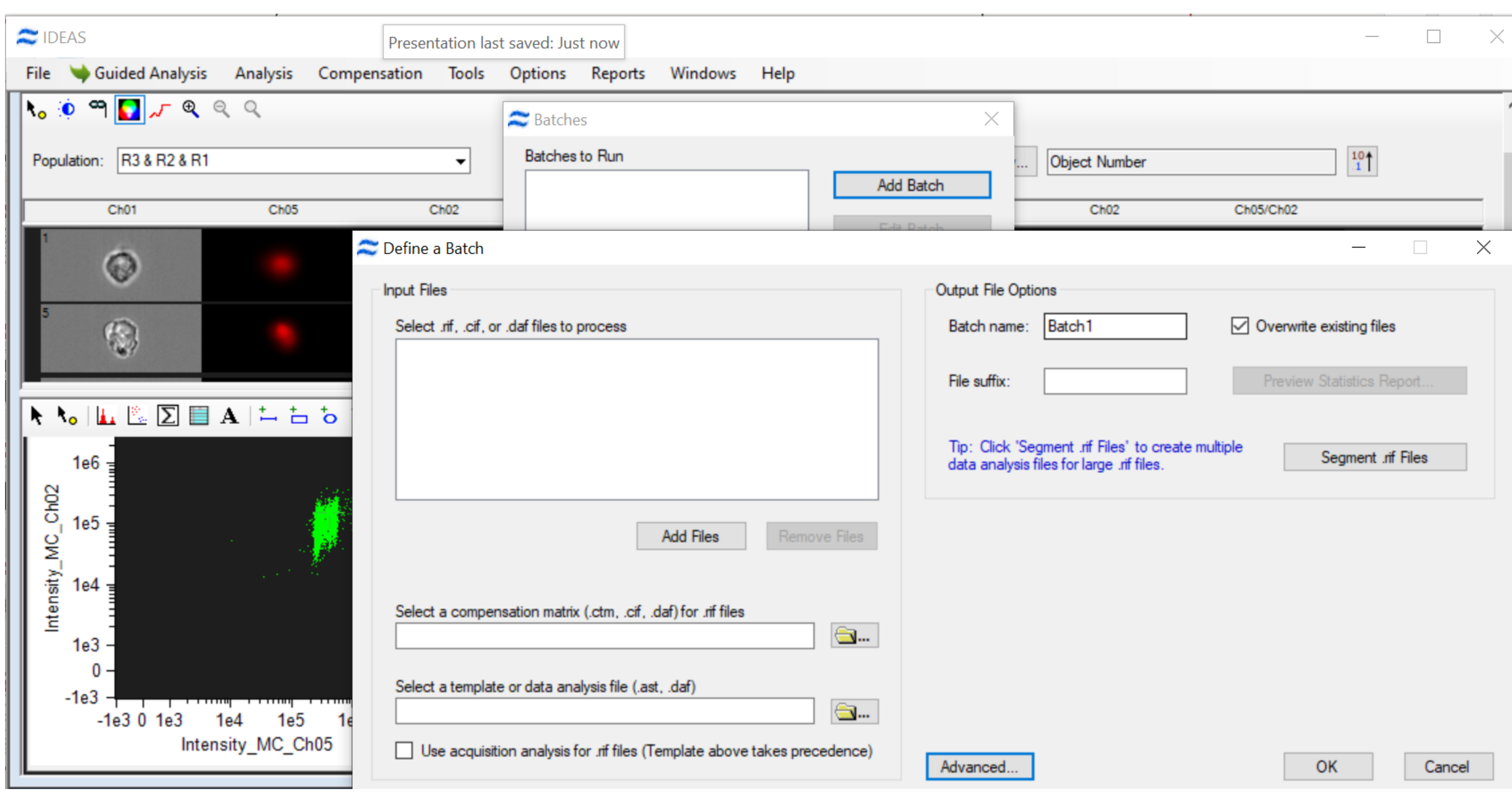
Statistics and report



Template and batch processing analysis

- **The software provides batch processing function that will save us a lot of time and energy.**
- **To perform batch process, We need to finish the first analysis and save it as a template.**
- **The batch process function use the same compensation matrix and template to process all the files in the experiments.**
- **The result is an easy to open .daf and a tabular statistics report.**

Template and batch processing analysis

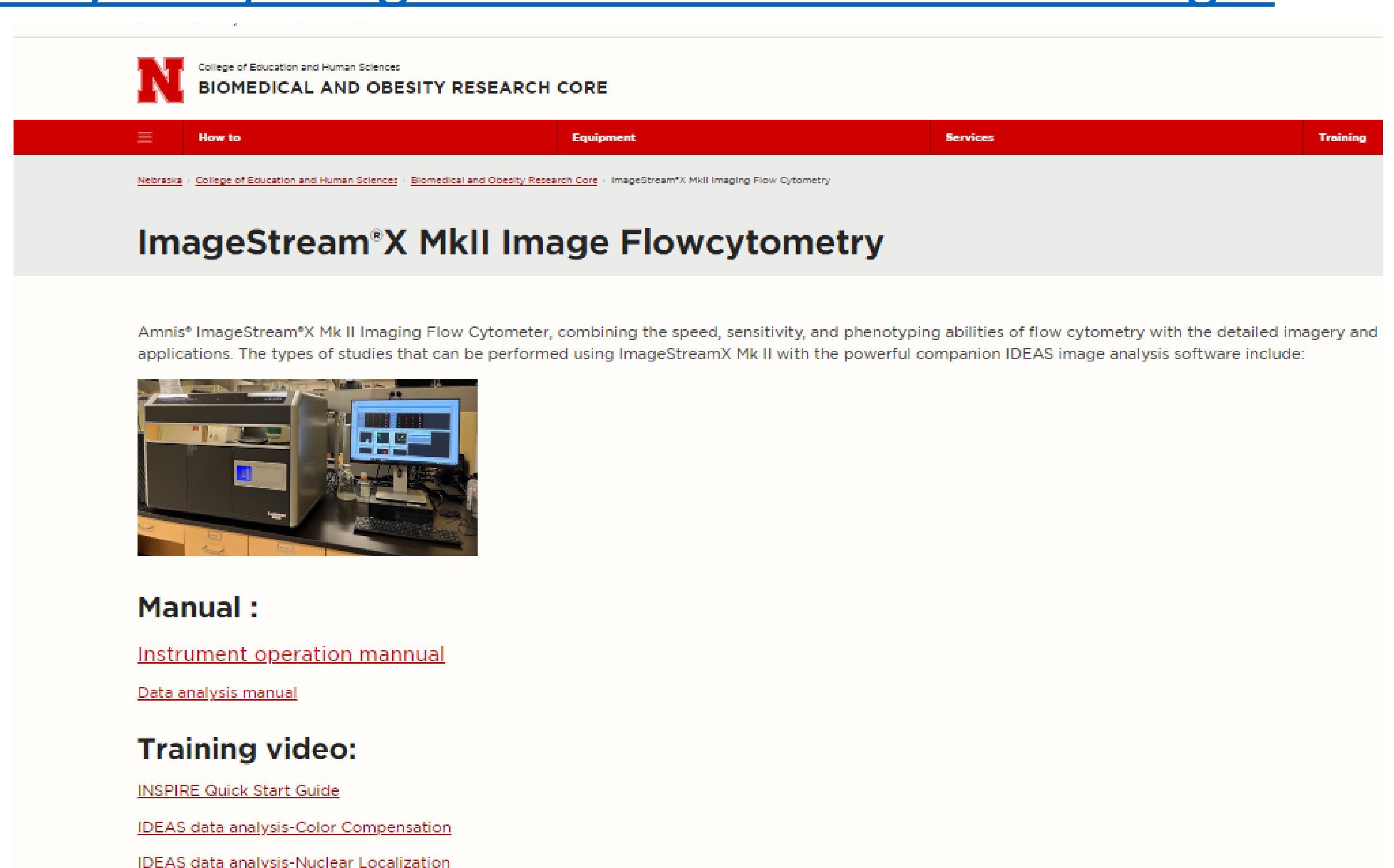


Masks and Features

- Masks are modules defined by the software to determine the region of interest. There 22 functional masks available to help users to quantify the region of interest. User also can create combined masks with multiple masks.
- Features are parameters predefined in IDEAS or defined by users to characterize cells. The quantification of features is based on masks.
- The software provides 22 masks and 86 features that can be used in the analysis to characterize cells. You may also define your own features.
- The profiling of features provides comprehensive information of cellular and subcellular location, morphology, signal intensity, etc.

Training documents and videos

https://cehs.unl.edu/borc/imagestream%C2%AEx-mkii-image-flowcytometry/



Feedback

- Feed back is welcome anytime.
- https://cehs.unl.edu/feedback-instrument/

Masks help to identify a region of interest

- The **Object** and the **Morphology** masks provide a tighter **boundary** around the image and are useful for a more accurate shape or size feature. Masks can also be eroded to reduce highlighted pixels at the periphery of the mask or dilated to expand the edge of the mask.
- The **Threshold** mask is useful for **highlighting a certain percentage of bright pixels** in the image (e.g., 30, 50, 70, etc.) and can be used for **apoptosis**, capping and other applications with condensed staining. For example, a Threshold of 75% highlights only the brightest 75% of pixels in the image.
- The **LevelSet** mask is useful for **highlight different pixel intensities** within an image (e.g., the brightest, dimmest and medium intensity pixels in this red blood cell can be highlighted separately).
- There are masks to help **identify backbones** in imagery (**Skeleton**) or **the point of contact** between two cells (**Valley and Interface**). **Holes** in other masks can also be filled in using the **Fill** mask.
- If an image contains multiple components that need to be analyzed individually, the **Component** mask can highlight each piece of the image separately.
- Masks can also be combined using Boolean logic. For example, the M1 mask has been shrunk by 3 pixels using the **Erode** mask and then two have been combined to create a **cytoplasm** mask. (M1 and not EM1_3)

Features help to characterize cells

- **Size features are in microns** (Area; Diameter; Width; etc.)
- **Shape features define the mask shape, units vary with the feature** (Aspect Ratio; Circularity; Symmetry; etc.)
- **Location features are in X,Y pixel coordinates** (Angle; Centroid X, Y; Spot Distance Min; etc.)
- **Texture features determine local intensity variations in images (** Contrast; Gradient Max; Gradient RMS etc. **)**
- **Signal Strength features are measured in counts (**Intensity; Raw Intensity; Raw Max Pixel; etc.**):**
- **Comparison features quantify intensity differences between masks or pixels (**Intensity Concentration Ratio; Internalization; etc. **):**
- **System features do not require a mask** (Camera Timer; Flow Speed; Object Number; etc.):
- **Combined features are created by using Boolean Logic**

Instrument settings- Lasers

Laser line	Common fluorophores used with laser	Application
Blue(488 nm)	FITC (491), iFluor® 488, Alpha Fluor™ 488 (516), PE, PE tandems (574), PerCP (675)	protein/antibody labeling dye
Blue(488 nm)	Nuclear Green™ LCS1 (526), PI (617), 7-AAD (647)	DNA stain
Blue(488 nm)	Calcein UltraGreen™ (514)	cell viability indicator
Blue(488 nm)	CytoTell™ Green (525), CytoTell™ UltraGreen (514)	cell proliferation indicator
Blue(488 nm)	Annexin V-iFluor® 488	early-stage apoptosis indicator
Red(642 nm)	iFluor® 633, iFluor® 647, AF647, APC (660), APC tandems, APC-iFluor 750 (791), APC-Cy7 (780)	protein/antibody labeling dye
Red(642 nm)	Nuclear Red™ LCS1 (645)	DNA stain
Red(642 nm)	Calcein Deep Red™ (515)	cell viability indicator
Red(642 nm)	CytoTell™ Red 650 (650)	cell proliferation indicator
Red(642 nm)	Annexin V-iFluor® 633, Annexin V-iFluor® 647	early-stage apoptosis indicator

- Be sure to collect compensation controls with the appropriate lasers on.