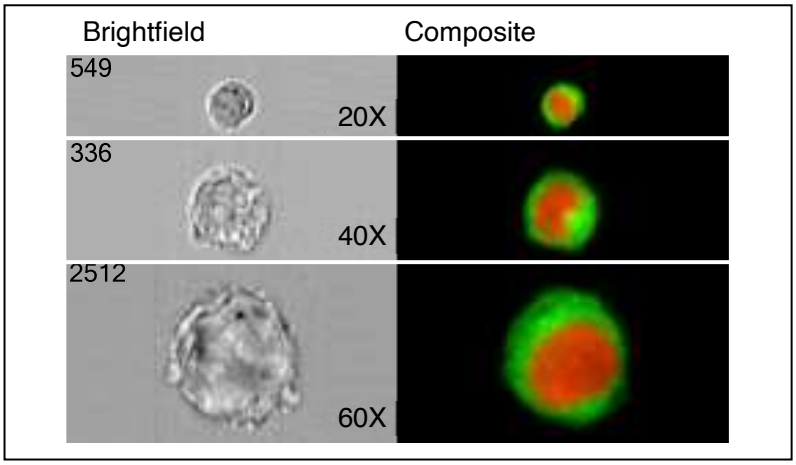
# Sample Preparation Guide

**Experimental Design**: The ImageStream system quantifies the intensity, location, and distribution of fluorescent signals within tens of thousands of cells per sample. It can perform most flow cytometric assays, but the best applications take advantage of the technology's imaging capabilities to discriminate subtle morphologic or signal distribution changes within individual cells and cell populations.

1. **Choice of Cell Type:** The particle size should be less than 120um using 20x magnification, 60um using 40x, and 40um using 60x. Images below are THP1 cells (~15um diameter) labeled with FITC NFkB and Draq5 displayed at each magnification.




2. **Final Sample Concentration and Volume:** At least 1 million cells in 50 uL ($2x10^7$ cells/ml) in PBS/2%FBS in a 1.5mL siliconized microcentrifuge tube. Will run ~400 cells per second on low speed.

3. **Protocols:** In general, any established labeling protocol used for flow cytometry will work with the ImageStream (see *Current Protocols in Cytometry* for general labeling techniques). Staining on ice in the presence of 0.1% Na-azide when possible will reduce non-specific capping of antibody. Use siliconized polypropylene tubes when possible.

4. **Choice of Fluorochromes:** Choose fluorochromes that are excited by the lasers in your ImageStream (405,488,642nm are most common). Use the chart on p.3 or look online for a spectra viewer that will help you plan which dyes will work the best.

5. **Compensation:** Have a sample of cells each labeled with a single-color for each fluorochrome used (i.e. FITC only cells, PE only cells, etc.).

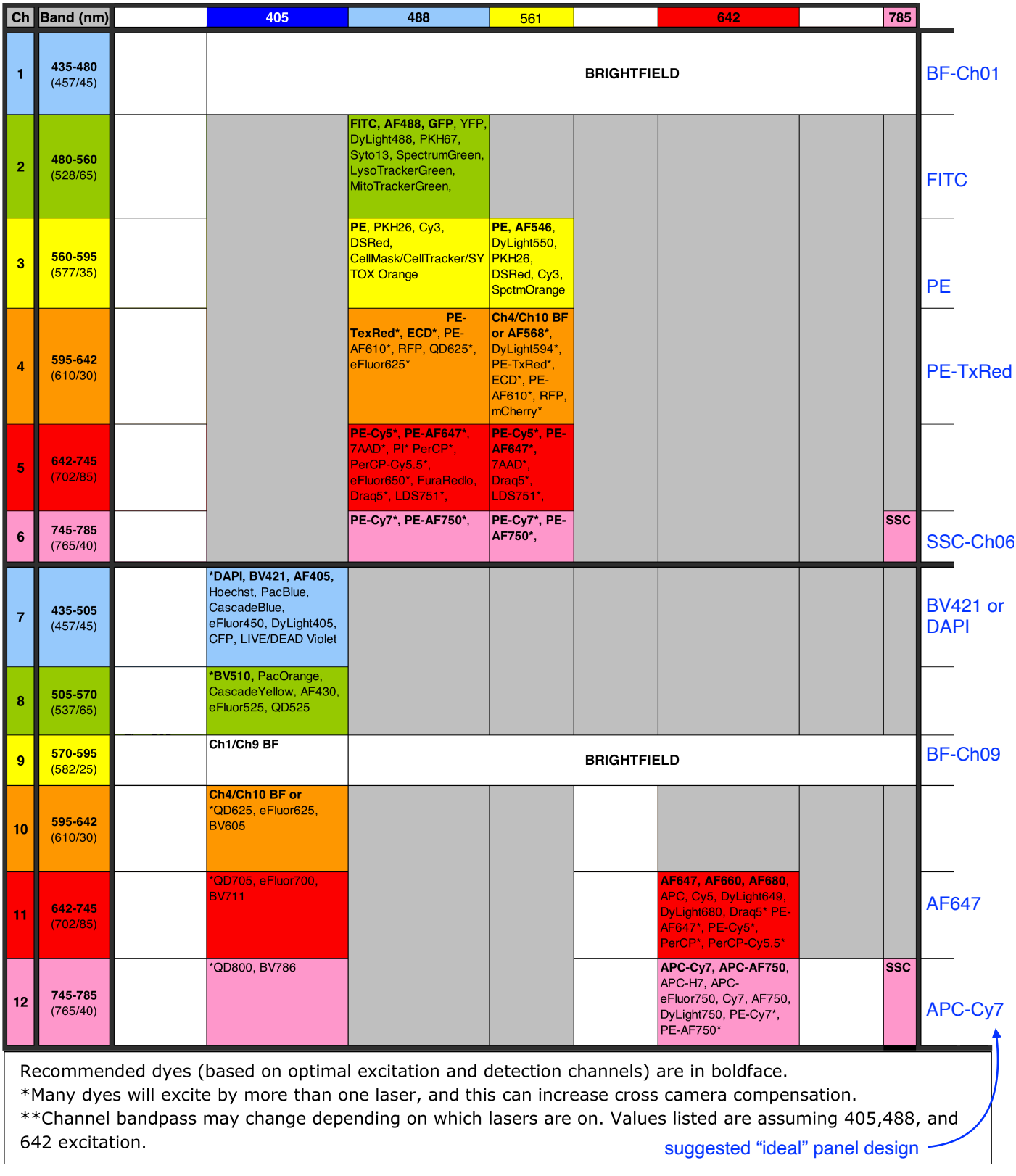
6. **Cell Aggregation:** Minimize aggregation problems by straining the sample through a 70um nylon mesh strainer, or by using an anti-clumping buffer such as EDTA or Accumax prior to fixation.

7. **Fixation:** If fixation is desired, fix cells with 1% PFA on ice for 20 min.

8. **Number of samples:** No more than 30 total for feasibility experiments. Please limit the samples to the following; Positive and Negative ***biologic controls***, ***compensation controls***, and ***experiment samples***.

# Sample Preparation Guide

9. **Brightness of Stain and Stain Balancing:** Quantifying the location and distribution of signals in an image is a demanding task that requires optimized labeling. Below are a few suggestions to help design the experiment:
    - Try to achieve at least a full log shift in fluorescence, as measured by FACS.
    - Use the brightest dye for the antigen with the smallest copy number.
    - The brightness of probes can be independently controlled by changing the laser power. However, data quality is enhanced when the brightness levels of all probes excited off a single laser are balanced to within a log of each other. Probe balancing avoids the saturation of bright stains when they are combined with dim stains in the same sample.



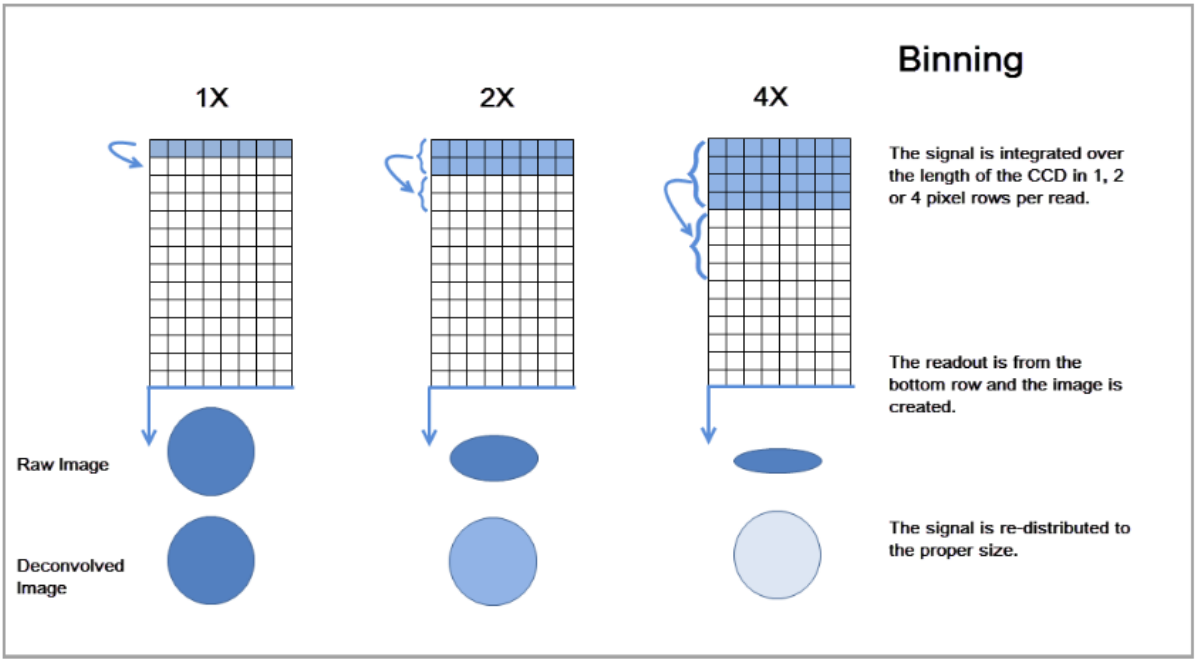

Recommended dyes (based on optimal excitation and detection channels) are in boldface.
*Many dyes will excite by more than one laser, and this can increase cross camera compensation.
**Channel bandpass may change depending on which lasers are on. Values listed are assuming 405,488, and 642 excitation.

suggested "ideal" panel design

| **20x** / 0.5 NA | **40x** / 0.75 NA | **60x** / 0.9 NA |
|---|---|---|
| 128 µm FOV (width of field) | 64 µm FOV (width of field) | 40 µm FOV (width of field) |
| 1 µm-square pixel area | 0.25 µm-square pixel area | 0.1 µm-square pixel area |
| 8 µm depth of field | 4 µm depth of field | 2.5 µm depth of field |

| Objective | Speed | Bin | Pixel Resolution | Sensitivity | Core Diameter | Velocity mm/sec | Sample obj/ml | Cells/Sec | Time to 10,000 |
|---|---|---|---|---|---|---|---|---|---|
| 20x | Low | 1x | 1 x 1 $um^2$ | High | 10um | 55 | $1x10^{7th}$ | 200 | 54 sec |
| | Med | 1x | 1 x1 $um^2$ | Med | 10um | 110 | $1x10^{7th}$ | 360 | 28 sec |
| | High | 2x | 1 x 2 $um^2$ | Low | 10um | 220 | $1x10^{7th}$ | 720 | 14 sec |
| | | | | | | | | | |
| 40x | Low | 1x | .5 x .5 $um^2$ | High | 10um | 55 | $1x10^{7th}$ | 200 | 50 sec |
| | Med | 2x | .5 x 1 $um^2$ | Med | 10um | 110 | $1x10^{7th}$ | 360 | 29 sec |
| | High | 4x | .5 x 2 $um^2$ | Low | 10um | 220 | $1x10^{7th}$ | 720 | 14 sec |
| | | | | | | | | | |
| 60x | Low | 1x | .3 x .3 $um^2$ | High | 7um | 40 | $1x10^{7th}$ | 60 | 160 sec |
| | Med | 2x | .3 x .6 $um^2$ | Med | 7um | 55 | $1x10^{7th}$ | 75 | 135 sec |
| | High | 4x | .3x1.2 $um^2$ | Low | 7um | 110 | $1x10^{7th}$ | 150 | 68 sec |

optimal concentration: **1 million cells per 50 µl**
minimum volume: **20 µl**
400 cells/sec = 24,000 cells/min
200 cells/sec = 12,000 cells/min
100 cells/sec = 6,000 cells/min
50 cells/sec = 3,000 cells/min



# INSPIRE® Quick Start Guide

## Amnis® ImageStream®X MK II Operation

***Operation of the ImageStream®X Mk II with software map on the reverse side:***

1. Power up the system (1 instrument, 2 large computer, 3 small computer)
2. Launch the **ISX** application.
3. Check to be sure the **buffer containers** are full and the waste tank is empty.
4. Select **Startup** and the instrument will load sheath in ~14min. “Calibrate with Assist” Checked.
5. Or under the Instrument/ Calibrate view, press **Start all calibrations and tests**.
6. When all tests pass, ASSIST light is green and the system is ready to run. **Close view**.
7. Select file and load **default template** or an experiment template from the File menu.
8. Press **Load** and, load an aliquot of a sample with each fluorochrome present.
9. Under Illumination, turn on the appropriate **lasers** for each fluorochrome.
10. Adjust the laser power to maximize brightness and **prevent saturation**.
11. Create dot plots and **regions** to identify the cells to collect, or collect all events. Typically Ch1_Area vs. Ch1_Aspect Ratio is used to eliminate SpeedBeads and debris, and collect cells.
12. Set the **acquisition parameters**: file name, destination, number of events and region to collect.
13. If needed save .fcs files as well as the image based .rif file.
14. **Compensate** data if needed. Data is normally compensated during **analysis**, however you can select the compensation drop down and load matrix, or create a new one.
    a. In the wizard’s directory, select the compensation wizard.
    b. Load the compensation control sample, press next, and verify the channel is correct, next.
    c. Verify the system finds the correct channel, press next.
    d. Set the acquisition file name, destination/ population to save, press acquire.
    e. Press next, and the wizard will cycle back to step B.
    f. Repeat steps B through E for each compensation control sample. Press exit to save matrix.
15. To manually collect **compensation controls**, turn off Brightfield and SSC, verify all channels are on, and collect 1000 All events (or suitable region) for each compensation control sample.
16. Continue collecting all **experiment files**. *(In general brightfield will be in Ch1 and 9, SSC ~5 mW in Ch6, and the cells to collect R1 using brightfield area vs. aspect ratio to identify single cells).*
17. Save an experiment **template** by selecting Save Template from the File menu.
18. Shut the system off by pressing the **Shutdown** button. The system will sterilize itself in ~40 min.

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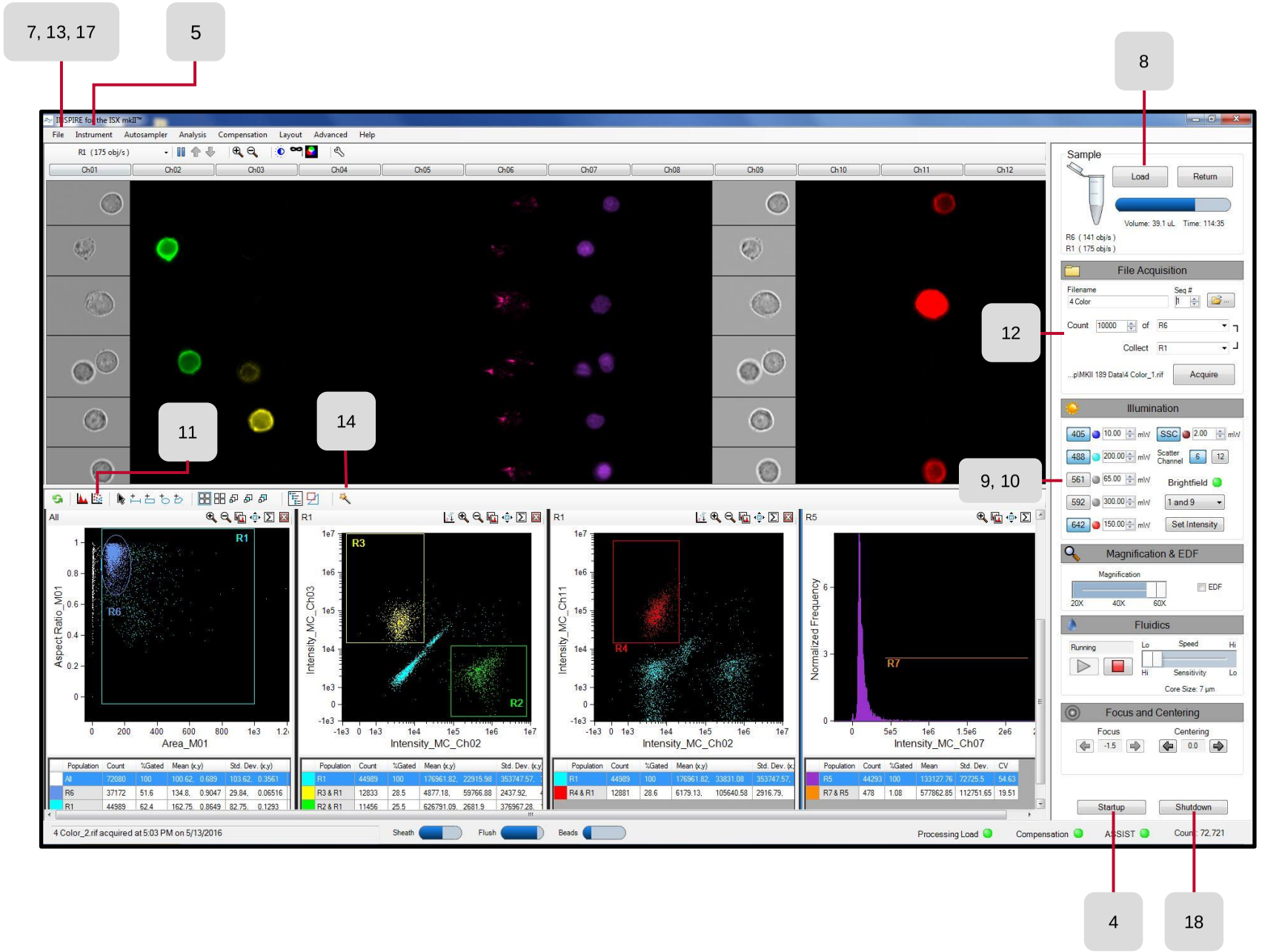




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# IDEAS® Quick Start Guide

## Analysis and Data Management

***Opening Data Files:*** There are three types of image file: Raw Image File (.rif), Compensated Image File (.cif), and Data Analysis File (.daf). FCS files can be analyzed in IDEAS or third party analysis software and will contain the feature data without the images.

***Opening files using the Start Analysis***


1. Launch **IDEAS®.**
2. Click **Start Analysis,** and browse for the appropriate **data file**. Or use "File" "Open" and choose a .rif, .cif, (skip to step 4) or .daf (skip to step 6). File extensions need to be visible in windows to know what the file type is.
3. Browse for an existing **compensation** matrix (.ctm file) or create a new one by clicking **New Matrix** and follow the steps outlined in the compensation QS guide.
4. Select an analysis **template** (.ast file) or a .daf with the analysis you wish to apply. If no template is chosen the default template will be used.
5. Select the active channels used in the experiment, and the **Image Gallery Display** properties will be applied to the images. They can be manually adjusted following the reporting QS guide.
6. Select the appropriate **wizard** for your application and follow the steps to completion. Basic Immunophenotyping can be done using the "**Begin Analysis**" wizard. Use the "**feature finder**" wizard if your application is not in the default list.
7. To add additional analysis use the **histogram** and **dot plot** icons in the tool bar, or select **Guided analysis** and choose a building block.
8. Add additional **Masks** as needed. (Analysis / Masks / New / Function).
9. Add additional **Features** as needed. (Analysis / Features / New).
10. When the analysis is complete **save** the data analysis file (.daf) and analysis temple (.ast) using the options in the file drop down list. This analysis can be applied to any data file following the steps above, or choosing "file" then "open" and applying the .ast and .ctm files.
11. When the wizard is complete, validate the regions by opening the control files using the saved analysis template and compensation matrix. Also verify the data in the statistics report under "reports" and "**define statistics report**".
12. To **batch process** additional files, select tools/ batch data files and refer to the Batching Multiple Files QS guide 3.
13. To **report** the data, right click plots or images, and chose copy to clipboard. You can then paste the data into any third party reporting software.


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***To save an analysis template file:***

1. From the File drop down option, Select **Save As Template File (.ast)**.
2. Enter the **name** of the analysis template to save.
3. Click **Save**. Any file can now be opened and analyzed using this template.

***To batch process data files:***

1. From the **Tools** menu, select **Batch data files**.
2. Click **Add Batch**.
3. Click **Add Files,** and ctrl select all the data files in the experiment.
4. Select the **compensation** and **analysis** template to use.
5. Click OK, and when all critical batches have been added, select **Submit Batch**, to start the batch.

***To merge Raw Image Files:***

1. From the **Tools** menu, select **Merge .rif Files**. The Merge .rif Files screen appears.
2. Click **Add Files**, and Ctrl-select the .rif files to merge.
3. Click **OK** and name the file with a unique file name.
4. Click **Save**.

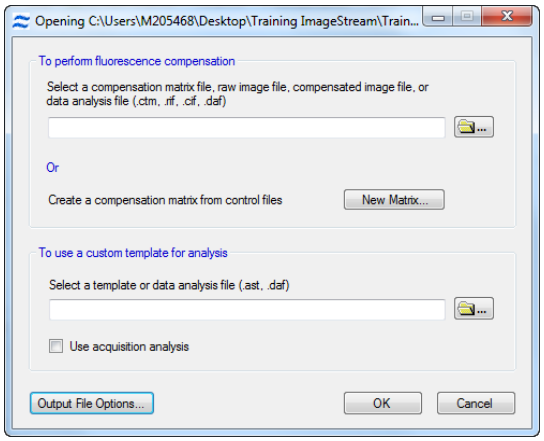
***To create a new data file from populations:***

1. From the **Tools** menu, select **Create Data File from Population**.
2. Check the **Raw Image File (.rif)** box to create a .rif file and click the browse button to select a name. Check the **Compensated Image File (.cif)** box to create a .cif.
3. Check the desired populations in the **Select population** list, and hit OK.

***To Export Image and Feature Data for multiple files:***

1. Under the **Tools** menu, select the type and format of the data to export.
2. To export **Feature data**, select export features, for images select export .tif Images.
3. Select the population and features to export.
4. Select **Add files**, and Ctrl-select the data files to export.
5. Chose the format and population of data to export (i.e. **FCS**), and press OK.

**File Structure Overview (.rif, .cif, .daf)**

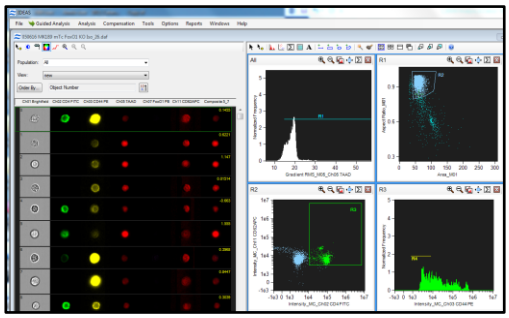



2.) Open a raw data file .rif

3.) Navigate to a compensation matrix.

4.) Navigate to an analysis template.

12.) Once analysis is validated **Batch** process experiment, and review statistics report.

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# IDEAS® Quick Start Guide

## Compensation

IDEAS® uses a wizard to automatically calculate a compensation matrix for your experiment. During data acquisition a file of between 500 and 1000 positively-stained events should be collected from a single color control sample for each fluorochrome in the experiment. Compensation control files should be collected with **all channels active and brightfield and SSC turned off**. Each compensation file is then loaded into IDEAS and used to measure the spectral crosstalk of each fluorochrome into each channel, and is applied to the experiment data when the file is opened or the experiment is batch processed.

***To create a compensation matrix:***

1. Select **New Matrix** when opening a .rif, or from the IDEAS toolbar select **Compensation** then **Create New Matrix**.
2. Click **Add Files** and control-select the appropriate –noBF control files. One for each fluorochrome in the experiment. Click **Next** to load the files.
3. Verify the active channels used in the experiment and click **next**.
4. IDEAS will automatically identify the **single color positive cells**, create adjacent channel intensity dot plots, and auto-generate a compensation matrix. Matrix values with greater than 1% error are flagged red. If all values are black click Finish.

***Validate the compensation matrix:***

5. If some values are flagged red, optimize the matrix. To do this:
   a. Double-click the red value in the matrix. The **Matrix Coefficient Intensity Plot** is displayed.
   b. If the green line does not fit the data points on the plot, click 

   c. Use a region tool  to select a new **positive population** that excludes any outliers.
6. Assign the **new population** to its channel using the appropriate drop down list.
7. A new matrix is calculated automatically and can be re-evaluated by double-clicking each cell in the matrix. Continue this process to obtain the lowest coefficient error value possible.
8. To view images with the applied matrix, select **Preview Images.** Double-click an image and IDEAS will display the compensated version of the image in the gallery window.
   a. If crosstalk images have black areas, the fluorochrome is **overcompensated,** and the corresponding matrix values should be manually reduced.
   b. If crosstalk images have bright areas, the matrix is **undercompensated,** and the corresponding matrix values should be manually increased. Repeat until crosstalk imagery is as close to background as possible and close the image gallery.
9. Click **Finish,** and save the compensation matrix


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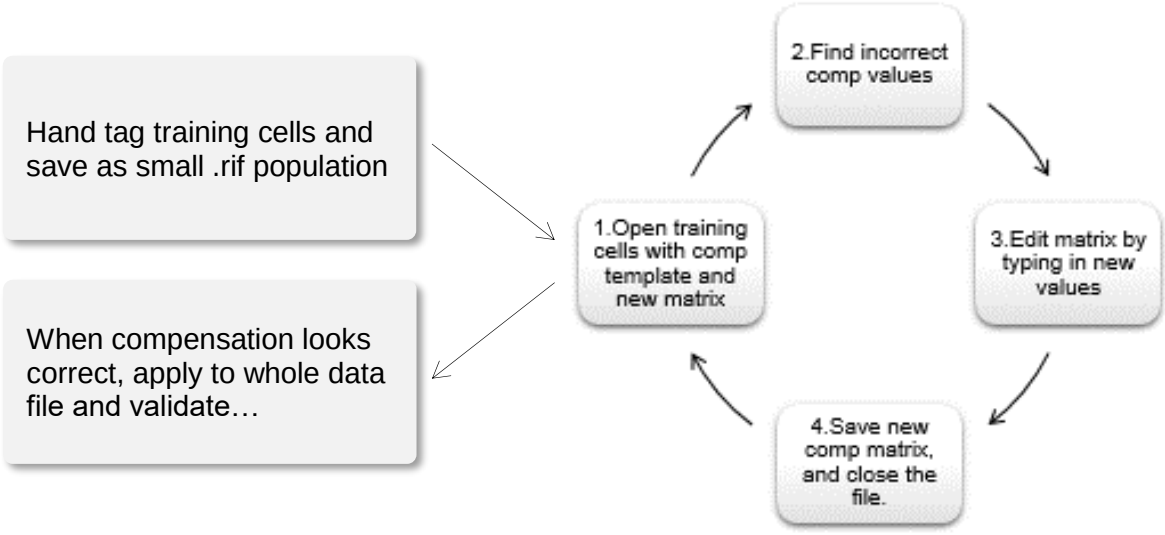
# Troubleshooting Compensation

Sometimes an applied matrix produces poorly compensated data. This can happen for a number of reasons including saturation, autofluorescence, or using the wrong population. Ideas lets you manually adjust the matrix using this method.

***To troubleshoot and repair a compensation matrix:***

1. Open the poorly compensated (comp) data and use the tagging tool to **create a training set** of poorly compensated events. Include blanks and cover a range of intensities.
2. Save the training data by selecting tools, then **create data file from population**.
3. Create and save a **Comp Template** or use the template in 9 color comp training data folder;
   a. Set the image gallery display properties for each channel from 0 to 150.
   b. Create adjacent channel Raw Max Pixel dot plots and gate out saturated events.
   c. Create adjacent channel Intensity dot plots using the non-saturated events.
   d. Save this template as an .ast file and use for future compensation troubleshooting.
4. **Open** the "training data set" with the "compensation" template and apply the current incorrect matrix.
5. Identify the matrix values that need adjusting using the **dot plots** and **images**:
   a. **Under-compensation** (crosstalk coefficient is too low):
      i. **Plots**: Single color positive population curves into the crosstalk channel.
      ii. **Images**: the crosstalk channel contains an apparent fluorescent mirror-image.
   b. **Over-compensation** (crosstalk coefficient is too high):
      i. **Plots**: Single color positive population curves off the plot.
      ii. **Images**: the crosstalk channel contains dark spots matching the bright spots in peak channel.
6. Go to the **Compensation** dropdown, select **View Edit Matrix**.
7. **Manually** change the incorrect matrix values from step5. Start with changes of ~.1 or ~.05.
8. **Save** the new matrix.
9. **Open** the "training data set" (step 1) using the "compensation" template (step 3), and apply the new compensation matrix (step 8).
10. **Repeat** this process using smaller and smaller increments as you refine the matrix. Continue until the data is properly compensated.
11. Save the new matrix by selecting **OK**, apply a unique name and then click **Save**.
12. Once the matrix is corrected and saved it can be used for any applicable data file.





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# IDEAS® Quick Start Guide

## Feature Finder Wizard

The Feature Finder wizard is used to analyze data outside of the core wizard set, to test novel masks/features, and assess existing analysis. It's applicable to any data set and works by guiding the user through the process of hand tagging two morphologies, identifying critical feature categories, then using the ratio of discrimination (**Rd**) to asses which of the tested features best separates the two cell types. In general **Rd** values greater than 2 have excellent separation power, between 1.5 and 2 is reasonable and below 1.2 is little separation.

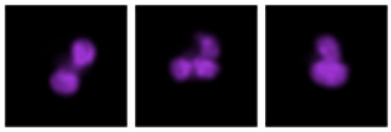
***Identify Lobed vs Round nuclei:***

Use a data file with both truth populations (control files may need to be merged before starting the wizard). This wizard only works for 2 populations, if more populations are needed multiple rounds of the wizard should be used. In the example below, we will find a feature to distinguish lobed vs round nuclei in human PBMCs.

1. Follow the wizard and identify the key **parent population** 1. Focused cells 2. Single events 3. Intensity plots (if needed). Manually create new masks and features as needed.

2. **Assign truth** populations by manually tagging 10-20 similar cells for each morphology. Focus on critical morphology (e.g. a range of lobed nuclei for one set, and round for the other).

Population 1: Lobed

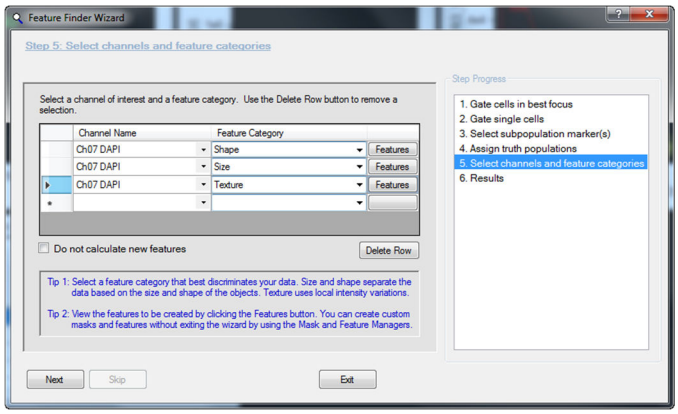


Population 2: Round

3. Select **channels and features**. To reduce errors use only critical channels, and features. (Ex. Use DAPI shape, size and texture to focus on nuclear shape, and omit surface markers and intensity so surface phenotype and DNA content don't obscure the shape analysis). *This can be done for all features, or to test two or three features or masks select only those few features.*

4. IDEAS® calculates new masks and features when processing the data. See p.2 for complete list.

5. **Review results**. Refine truth populations and features as needed. Verify the feature identified makes sense for your application by reading the feature definition in the IDEAS manual (F1). Refine and repeat the process if necessary.




Rd>2=excellent separation

RD Population Statistics — Use Short Names

| Features | RD Mean (Lobe,Round) |
|---|---|
| Circularity_Threshold(M07, Ch07, 50) | 2.18 |
| Shape Ratio_Threshold(M07, Ch07, 50) | 1.97 |
| Bright Detail Intensity R3_Morphology(M07, Ch07)_Ch07 | 1.92 |
| Thickness Min_Threshold(M07, Ch07, 50) | 1.66 |
| Compactness_Morphology(M07, Ch07)_Ch07 | 1.48 |
| Major Axis Intensity_M07_Ch07 | 1.40 |
| Major Axis_Morphology(M07, Ch07) | 1.37 |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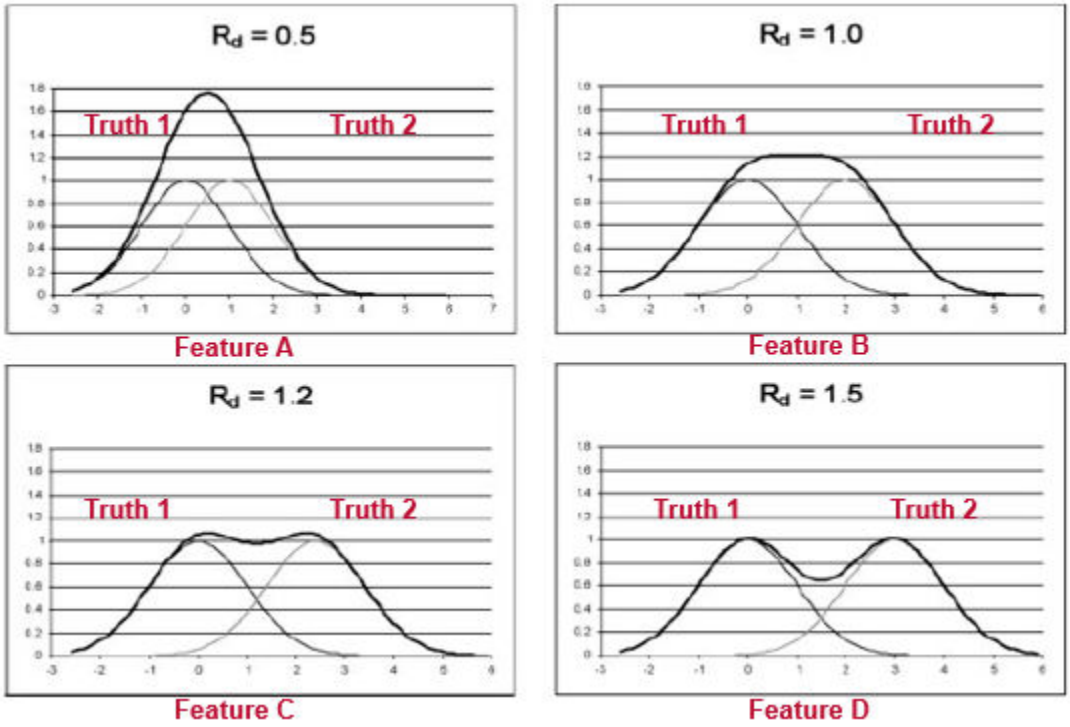
6. Features automatically calculated by IDEAS in addition to the 86 base features.

| Feature Category | Feature name | Mask used |
|---|---|---|
| **Shape** | **Shape Features** | |
| | Aspect Ratio | Object |
| | Circularity | Object |
| | Compactness | |
| | Elongatedness | Object |
| | Lobe Count | |
| | Shape Ratio | Object |
| | Symmetry 2, 3, 4 | |
| **Size** | **Size-based Features** | |
| | Area | Object, Threshold 30,50,70 % |
| | Height | Object |
| | Length | Object |
| | Major Axis and Minor Axis | Object |
| | Perimeter | Object, Threshold 30,50,70 % |
| | Thickness Max | Object |
| | Thickness Min | Object |
| | Width | Object |

| Feature Category | Feature name | Mask used |
|---|---|---|
| **Location** | **Location Features** | |
| | Delta Centroid XY | channel mask |
| **Texture** | **Texture features** | |
| | Contrast | Morphology, Object |
| | H Texture Features | Channel mask |
| | Modulation | Morphology, Object |
| | Spot Count | Channel mask |
| | Std Dev | Morphology, Object |
| **Signal Strength** | **Signal Strength Features** | |
| | Intensity | Morphology, Object, Threshold 30,50,70 % |

7. **Rd** is calculated using Fisher's Discriminant Ratio, for each feature in IDEAS where features with the highest Rd values have the best discrimination power. This method automatically compares up to 2000 features at once.

Rd = Signal / Noise
Rd = (Mean1 – Mean2) / (SD1 + SD2)





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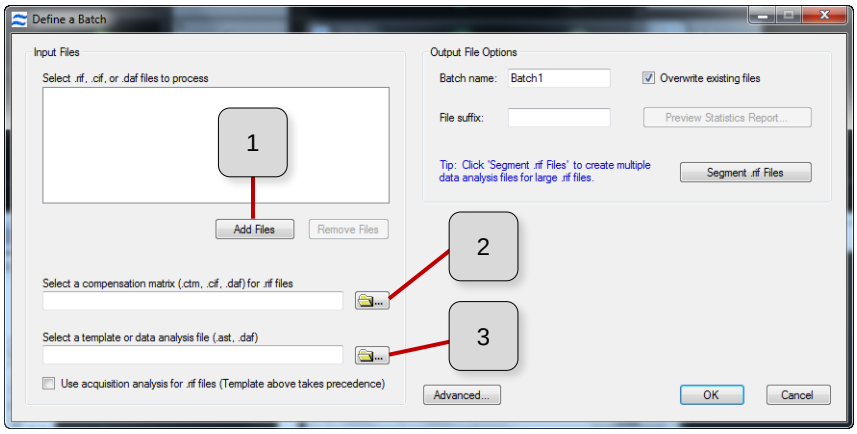
# IDEAS® Quick Start Guide

## Batch Processing Analysis

Batch processing allows you to automatically compensate and analyze a group of files with one analysis template and compensation matrix. IDEAS® will output .cifs and .dafs as well as a statistics table of relevant data defined in the statistics template.

### *To perform batch processing:*

1. Once the analysis is complete and verified, close all open data files and launch IDEAS.
2. Under the **Tools** menu select **batch data files**.
3. Select **Add Batch**.
4. Select **Add Files** and control select files to be batch processed. You can batch .rifs, .cifs or .dafs.
5. If data is not already compensated, browse for and select the **compensation** matrix to be applied.
6. Browse for and select the **template** to apply to the batched data.
7. For multiple batched data sets, you can apply a file suffix to prevent overwriting previous batches, however to simplify the directory it's better to **overwrite existing files**.
8. Select **OK**.
9. To begin a single batch, chose submit batches, or to add additional experiments select **Add Batch**.
10. When all the batches are queued up select **Submit Batches** and IDEAS will begin processing the data.

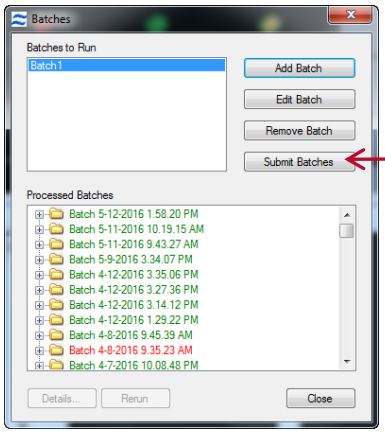



1.) Select **Add Files** and control-select the data files to batch.

2.) Select a compensation matrix (if needed).

3.) Select a template.

4.) If needed, Que up multiple experiments to batch.




5.) Once all experiments are defined, select **Submit Batches**.

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# Statistics Batching

Statistics batching is used to generate a report for analyzed data sets without the need to actively open every data file. First a template is created containing the statistics that will show up in the report. Next the template is applied to multiple data files, and a text file with the pertinent statistics is created. This text file can then be opened in excel and tables and charts can be generated.

***To generate a statistic report template:***

1. From the reports menu select **Define Statistics Report**.
2. Select **Add Column**.
3. Ctrl-select the **statistics** you want to use in the report.
4. Select the **population** and for some statistics, the **feature** used for that statistic.
5. Select **Add Statistics**.
6. Select **Close** when finished adding statistics.
7. To edit the **column name**, select the column and type a new name.
8. To edit the report title, enter a new name.
9. Select **OK** and save the analysis template using file / save as.
10. To generate the statistics report, follow the steps below or batch process the data.

***To generate a statistics report:***

1. Select **Generate Statistics Report** from the Report drop down menu.
2. Enter a report title and navigate to a **.daf** file containing the statistic report template.
3. Select **add files**, and Ctrl-Select the saved .dafs you wish to include in the stats report.
4. To order how stats appear in the report Ctrl-click in the order you wish, then right click and select “move here”.
5. Click **OK**, and agree to save and close the open data file if need be.
6. IDEAS will quickly go into each data file and extract the relevant statistics, and generate a **statistics table** as a text file.
7. To view the data, **right click** the .txt file.
8. Choose **open with**, and select Microsoft Excel.
    a. Alternatively, open Microsoft Excel and navigate to the .txt file, or drag it into a blank notebook. Excel will automatically delimit the data
9. The new statistic table will open in Excel and allow you to create new tables and graphs.


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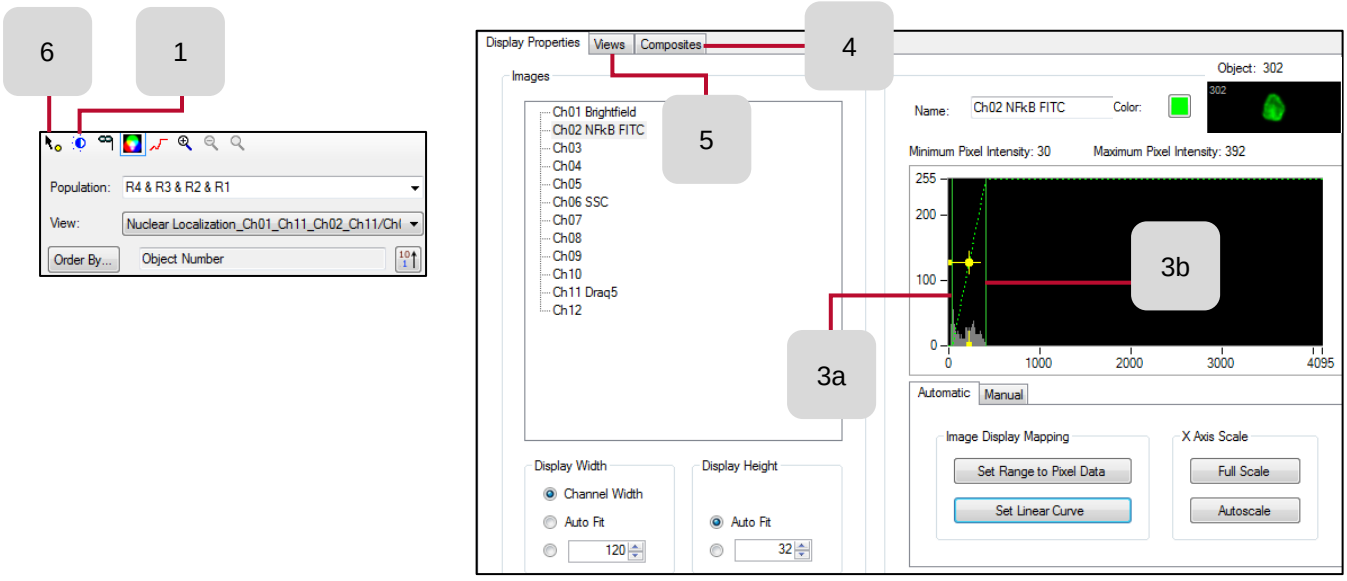
# IDEAS® Quick Start Guide

## Reporting

Reporting with IDEAS® is centered on Right-Click, copy to the clipboard and paste into reporting software like Word. This works for image galleries, single images, plots, and statistics. Using the print screen option can facilitate rapid reporting of single data files. FCS files from IDEAS can be analyzed in any third party software (like FlowJo) to generate plots. FCSexpress w/ imaging allows both images and plots to be processed for multiple data files.

***Reporting images and setting the Image Gallery Display Settings:***

1. Select **the gallery display** icon.
2. Select the **channel** to adjust. Edit the name and color as needed.
3. Optimize the **image contrast** for each channel: *The grey histogram displays the frequency of pixels at a given value between 0 and 4095 (12bit) in the raw image. IDEAS will display and print images on a scale between 0 and 255 (8bit). The green lines show how the software will convert raw 12 bit data to display at 8bits.*
   a.) Click on the **left vertical green line** and set to the left edge of the grey histogram. This sets the background limit, and should be at the dimmest pixel value in the image or slightly inside for black backgrounds.
   b.) Click on the **right vertical green line** and set to the right edge of the grey histogram. This sets the display saturation limit and should be at the brightest pixel in the image.
   c.) The **dashed green line** shows the look up table used to display the raw pixel data within the new boundaries. It can be adjusted, but should be set as a linear curve.
4. Create **composite overlays**. Select the composite tab and working left to right, select new, add image /image to overlay /add image again /add additional images.
5. Create **a gallery view**: Select the view tab, and working left to right, select add column, chose channel or composite images, masks, and ***feature values*** to display in the new view.
6. Use the hand tag tool to **select images** for your figure, save them as a population and display in the image gallery. *Select images that display well together or adjust contrast settings for each cell.*
7. Right-click and select **copy/save gallery**. Edit display to include measurement tool, feature values, object number, channel names and paste into reporting software.



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# Reporting

***Reporting plots:***

1. Right click on **plot** and select **Graph Properties**. Edit parent population, features, scaling, or density plot as needed. Intensity plots scale between -1000 and 10^7.
2. Select the **Statistics** icon, and add remove statistics.
3. Adjust **regions** as needed.
4. Right click on plot and select **Copy/Save Graph…** edit size, fonts, options, ***white / black background*** and include or remove statistics.
5. Save to clip board and paste into reporting software.

***Reporting statistics:***

1. **Individual plot stats**: Right click on plot and select export stats to clipboard.
2. **Sample stats**: In the workspace toolbar select stats, right click to edit and copy.
3. **Feature values**: In the workspace toolbar select table, right click to edit and copy.
4. **Experiment stats**: Select the Reports dropdown / generate statistics report.
   a. Add each .daf data file to include.
   b. Select OK and IDEAS will compile a .txt file with all the stats in the template in the experiment directory.
   c. Open the new .txt file in third party statistics software like excel.

***Create FCS files and export feature values:***

1. Select Tools / **Export Feature Values.**
2. Select **Add Files** and ctrl-select each data file to export.
3. Choose export to clipboard, .txt, or **.fcs format**.
4. Select the **features** to export and press OK.

***Export tif. images:***

1. Select Tools / **Export .tif Images.**
2. Select the **population** to export. For individual cells, use the hand tag tool to create a population of a single cell.
3. Select the **channels** to export
4. Choose the .tif **format**.
5. Press OK and IDEAS will export .tif files for each channel and image in the selected population.

***Histogram overlays in IDEAS:***

1. Select **Tools / merge .cif.**
2. Ctrl-select the data **files to overlay**. IDEAS generates one large combined .cif.
3. Open the merged .cif in the experiment **template**.
4. Create the populations to overlay for each sample in the merged data file.
5. Create a **histogram**, and in the histogram properties, Ctrl-select each population to overlay.

***Histogram overlays in Word:***

1. **Copy Paste** each histogram into Word, plot properties must be identical.
2. In Word, select the top plot, got to the “Picture” tab, select “color”, “**set transparent color**” and use the eye dropper to click on the plot background.
3. Repeat for each histogram making each plot below it **visible**.
4. **Stack the histograms** by Ctrl-selecting each plot, and in the “Picture” tab, align center, align middle, then group to create a single overlaid histogram


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