Technology that counts

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Quick Guide

FlexiCyte™

NucleoCounter®
NC-3000™

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Setting up the FlexiCyte Protocol

1. Open the ‘Select Protocol’ window opened with the  (Fig. 1A)
2. Select Organism: Mammalian, and Analysis: FlexiCyte, and Media Type: NC-Slide A2.
3. Select the FlexiCyte protocol for setting up a new FlexiCyte protocol (Fig. 1B), or select the protocol that should be modified.

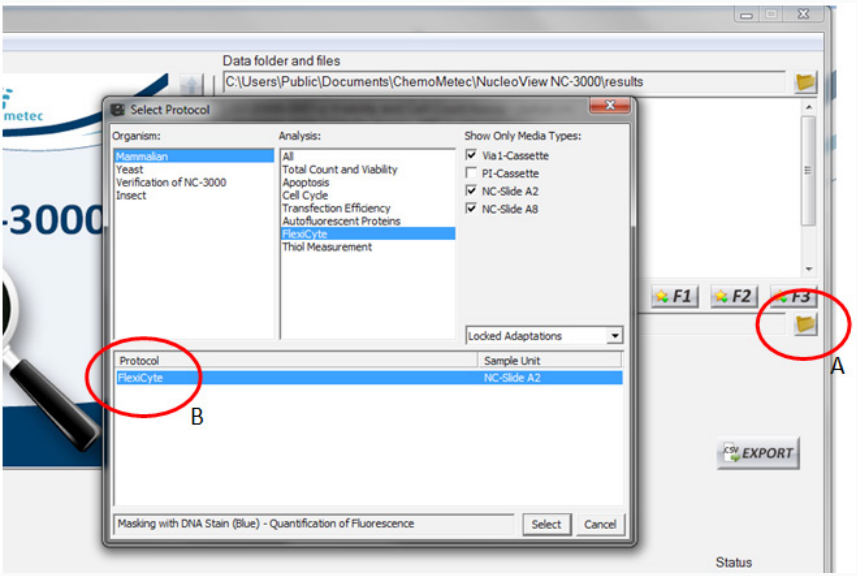


Figure 1. *Open ‘Select Protocol window (A), and select the FlexiCyte protocol (B).*

Editing Image Capture and Analysis Parameters

1. Open ‘Protocol Adaption Wizard’ (Fig. 2) located under ‘Tools’ on the main menu bar.
2. Follow the on-screen help to select:
    - The number of analytical channels (see fig. 3 for the available channels)
    - Masking method
    - Light sources (LEDs)
    - Exposure times
    - Emission filters
    - Minimum number of cells to analyze
    - Whether to include or exclude aggregated cells
3. A new title must be given to any adapted protocols.

Optional: If a sample has already been run, the wizard can be opened by right clicking the image file name, and generic data analysis can be set up (See Template Setup).

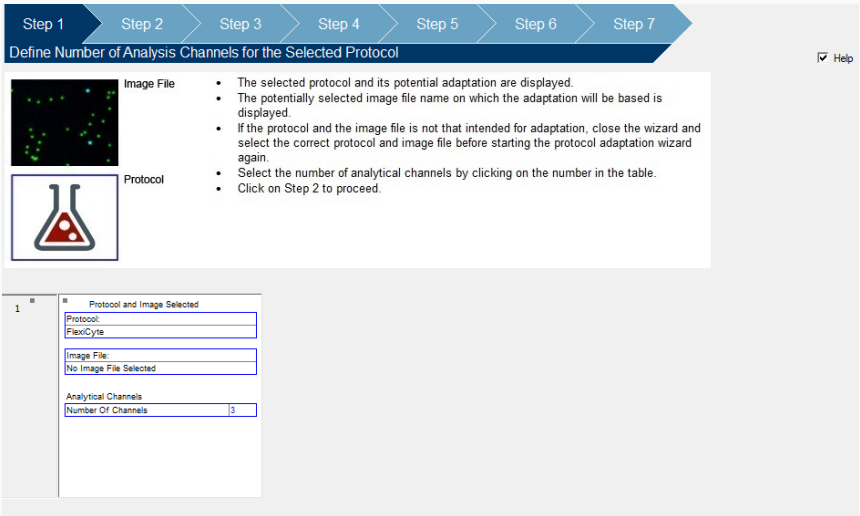


Figure 2.
Protocol Adaption Wizard allows the user to define protocols by adjusting the FlexiCyte™ parameters as described in the help section.

Figure 3. *A full list of possible LED/Emission filter combinations. The denotation of emission filter as e.g. Em530/15 means a bandpass filter that allows light of wavelength 530nm ± 15nm (515nm - 545nm) to pass.*

Optimising Exposure Time

Optimizing signal acquisition can easily be performed on the NucleoCounter® NC-3000™ by adjusting the exposure time in a manner similar to that used in photography. If the image is under-exposed, it will be darker and much of the finer detail may not be seen. Similarly, if it is over-exposed the pixels will become saturated and information will also be lost.

Optimizing the exposure time in the NC-3000™ needs to be determined empirically for the initial experiment but, once determined, the settings can be applied to all the following samples. That said, the default setting (200 milliseconds for LED(365) and LED(405), and 1000 milliseconds for LED(475), LED(530) and LED(630)) will fit most applications and optimization of exposure time may not be required.

As an example GFP, coupled to a highly expressed protein, has a significant chance of being over-exposed with the default of 1000 milliseconds. However most fluorochrome coupled antibodies bound to an expressed protein will be appropriately exposed with 1000 milliseconds.

1. Insert a sample stained with a single fluorophore of interest and masking stain (DAPI or Hoechst 33342) if flourescent masking is chosen into the NC-3000.
2. Set the exposure time to be evaluated in 'Protocol Adaption Wizard' (See section: editing Image Capture and Analysis Parameters).
3. Run the sample using the protocol with the adjusted exposure time.
4. The data will automatically open in 'Plot Manager'. Add a histogram to data by clicking on the histogram icon on the left-hand side of the data row.
5. Double-click on the small histogram to open the large histogram in editing mode. Change the x-axis to the appropriate channel and the parameter to 'Max Intensity'. This will display the signal intensity for the most intense pixel/cell rather than average intensity for the area defined as a cell when the scale is set to 'Intensity'.

6. To evaluate if the exposure time for the channel is appropriate for a particular channel, examine the distribution of the signal in the histogram. The NC-3000™ is based on 16-bit imaging that allows acquisition of signals from approx. 0 - 65,500. If the image is under-exposed 'normal distribution' curve may rest against the y-axis and lower intensity events will not be acquired (Fig 4A). If the image is over-exposed, the maximum intensity values will be close to 65,500 and a shoulder may be seen on the 'normal distribution' curve (Fig 4B).
7. If required, adjust the exposure time for your sample as appropriate and repeat step 2 - 6.
8. Step 2 - 7 should then be repeated for all channels used in the final assay.
9. Once the optimal exposure time has been determined for the individual channels, a final protocol with optimized exposure times for all the channels is saved in the 'Protocol Adaption Wizard'.

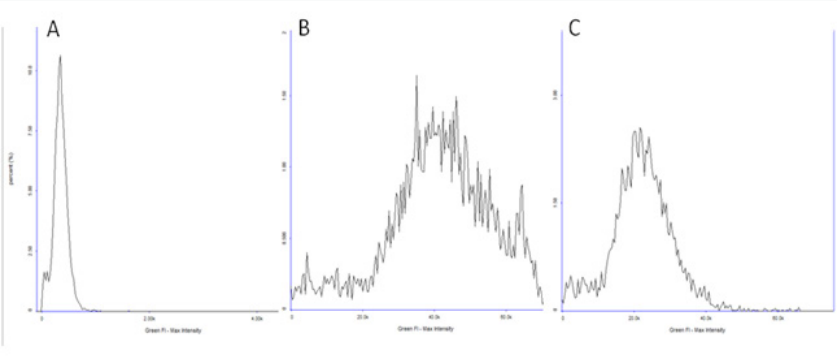


Figure 4. *Max intensity histograms for A) under-exposed image, B) over-exposed image with a shoulder indicating saturated pixels and C) an image with correct exposure. Note the smaller scale on the under-exposed image is different so that the peak can be more readily visualized.*

Compensation for Spectral Overlap

Even though fluorophores are designated a particular color, they emit light over a range of wavelengths. The color associated with a particular fluorophore refers to the wavelength where it has maximum fluorescence. However, the wavelengths where it has weaker emission may spill over into a neighboring channel giving a false increase in signal in this channel. For example, green fluorescent protein (GFP) mainly emits fluorescence in the green spectrum that is usually detected in a green channel. However, GFP also emits a small amount of fluorescence in the orange spectrum that can be weakly detected in an orange channel. To compensate for this fluorescent spillover into neighboring channels, a compensation factor can be set for each fluorophore and channel. Guidelines for approximate compensation factors to be applied for commonly used fluorophores is listed in the 'User Adaptable Protocols' application note, but the exact value should be determined as described below.

1. Load a sample labeled only with a single fluorophore of interest and masking stain (DAPI or Hoechst 33342) if flourescent masking is chosen.

2. Run the sample using the desired FlexiCyte™ protocol.

3. In Plot Manager, open a new scatter plot by clicking on the scatter plot icon on the left-hand side of the data row.

4. Double-click the small scatter plot to open the large scatter plot in editing mode. Change the x-axis to the colour of the fluorophore and the y-axis to the colour of the neighbouring channel. The axis settings for both axis should be 'Linear' and 'Intensity'. Click 'OK'. Tip: change the y-axis range to cover a larger, lower range so that the cell population is centred on the y-axis.

5. If there is spillover of fluorescence between the channels, the cell population will be placed diagonally across the graph area (Fig 5). If there is no spillover, the cell population will be placed horizontally across the graph area (Fig 6A).

6. Open the compensation matrix (Fig 6B) in Plot Manager by clicking on the icon on the left-hand side of the data row.

7. Enter an estimated compensation factor into the matrix box associated with the two channels of interest and click ‘Preview’ or use the scale bar for live updated adjustments. Inspect the cell population to ascertain that cell population is spread horizontally across the graph area. If the compensation factor is incorrect, click ‘Roll back’, enter a new value and inspect the cell population again. Repeat this procedure until the correct compensation factor is ascertained and click ‘Apply’.

8. Repeat step 1-7 for each individual fluorophore.

9. If multiple samples have been analyzed, enter the row number of the sample with the corrected compensation factor into the ‘Master Row’ box situated in the top left-hand corner of Plot Manager and click ‘Apply’. This will replicate the compensation in all other samples selected in Plot Manager.

Tip: after the final compensation has been set, a row with analysis including all compensation can be used as master row in the setup of the protocol (see Template Setup).

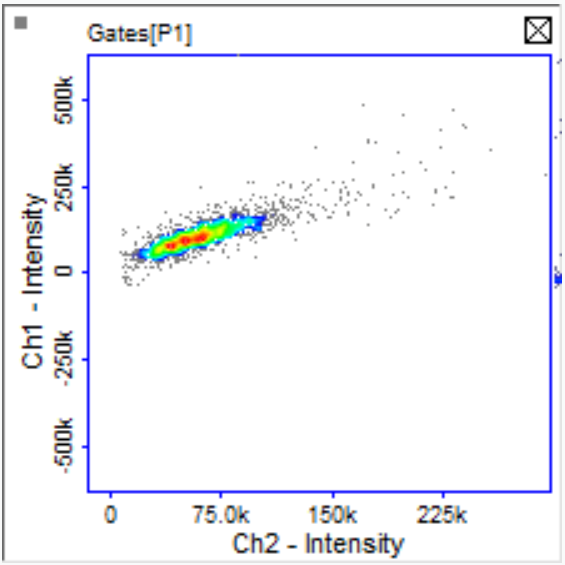


Figure 5. *Scatter plot of cells expressing GFP showing spillover of green fluorescence into the orange channel. An increase in orange fluorescence is seen as green fluorescence increases.*

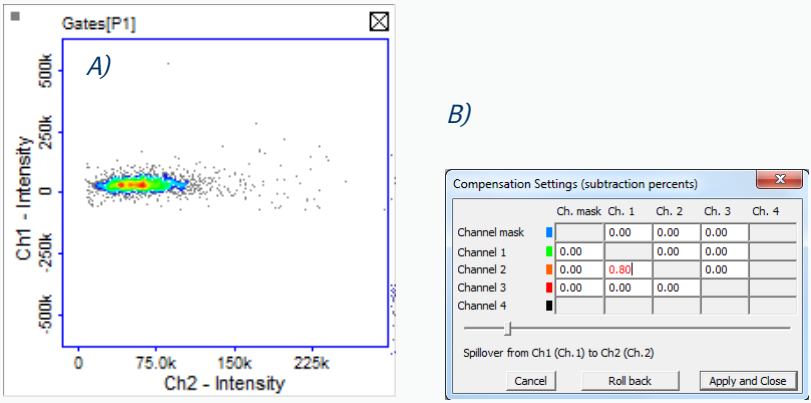


Figure 6. *A) Scatter plot showing GFP fluorescence in the green and orange channel after compensation. Note, no increase in orange fluorescence is seen as green fluorescence increases. B) Compensation matrix adjusted for 0.8% signal spillover from the GFP into the orange channel.*

Creating and Modifying Graphs

Default graphs representing each of the channels measured in a particular analysis or graphs defined by the master in the protocol are automatically opened in Plot Manager. However, these graphs may be modified for better viewing by changing the axis scale or parameter. Adding new scatter plots may be required for data analysis.

1. Double-click on the appropriate icon on the left-hand side of the data row representing either a scatter plot or a histogram .
2. A small plot with default settings will have been added to the corresponding data row in Plot Manager. Double-click on the small plot to open it in editing mode.
3. Axis scale and parameters can be changed and only those parameters available for that particular analysis will be available from the drop down menu. Axis scale can be returned to default setting by leaving fields blank or entering the number in the gray fields immediately below.
4. Click 'OK' to save changes and close the large graph.

Gating and Data Analysis

Data analysis will often require that debris or sub-populations of cells are identified and removed and statistics for the different populations gathered. Sub-populations of cells can be marked by either quadrants or polygons in scatter plots and markers in histograms.

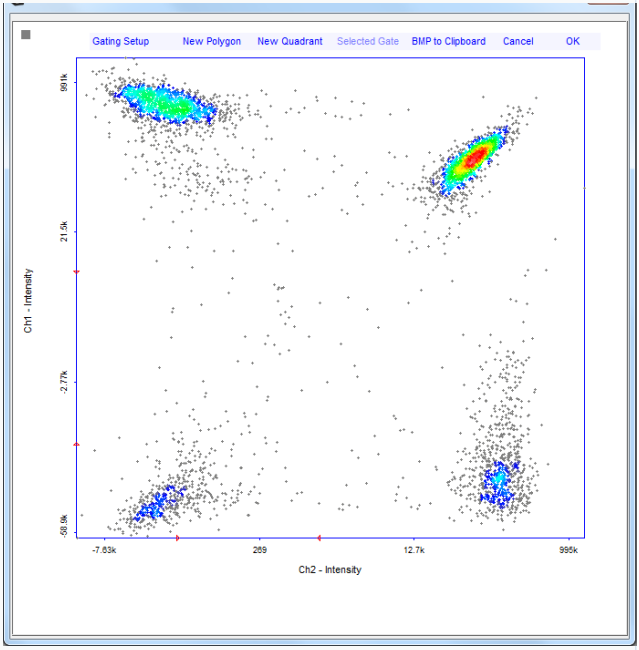
- *Polygons*

1. Open a large scatter plot in editing mode by double-clicking on the small graph.
2. Select 'New Polygon' (Fig. 7).
3. Place points encircling the population of interest by clicking in the graph area. Clicking in the grey area outside the scatter plot will remove the last point added. Click on the first point created to close polygon.
4. Polygons can also be copied between rows by right-clicking on a selected red polygon, or use the 'Selected Gate' menu point and copy to clipboard. The plot to which the polygon is to be added should then be opened by double-clicking followed by right-clicking and selecting 'Paste Gate'.

- *Quadrants*

1. Open a large scatter plot in editing mode by double-clicking on the small graph.
2. Select 'New Quadrant' (Fig. 7).
3. Click at the position in the scatter plot where the centre of the quadrant should be placed.
4. While the quadrants are highlighted in red, click the quadrant again. Red boxes will appear indicating that the quadrant is in editing mode. In this mode, the centre of the quadrant can be re-positioned and the angle of the quadrant boundaries can be adjusted.
5. Quadrants can also be copied between rows by right-clicking on a selected red Quadrant, or use the 'Selected Gate' menu point and copy to clipboard. The plot to which the quadrant is to be added should then be opened by double-clicking followed by right-clicking and selecting 'Paste Gate'.

Figure 7. *Large scatter plot of a FlexiCyte assay to measure GFP and RFP transfection.*



- ***Markers***

1. Markers can be inserted on histograms by clicking on the small histogram to open the large histogram in editing mode.
2. Select 'Create Marker' and the cursor will change from an arrow to a cross.
3. Create a marker by clicking on the positions in the histogram where the markers are to start and finish.
4. Marker position and length can be adjusted by dragging in the marker line or in the endpoints of the selected red marker.
5. Repeat step 2-4 to insert multiple markers into a single histogram.
6. Click 'OK' to save markers.
7. Markers can also be copied between rows by right-clicking on a selected red marker and selecting 'Copy Marker'. The histogram to which the marker is to be added should then be opened by double-clicking followed by right-clicking and selecting 'Paste Marker'.

Creating an Overlay of Histograms

Histograms from multiple analyses can be displayed in a single graph to allow better visual comparison of the results.

1. Right-click on the small histogram plot to be copied and select 'Copy Histogram'.
2. Right-click on the small histogram plot to where the histogram is to be pasted and select 'Paste Histogram'.
3. Repeat step 1-2 to insert overlay multiple histograms.
4. Double-click on the small histogram plot containing histogram overlay to open it in editing mode.
5. A new text input line will now be present and individual histograms can be colored and named by clicking this line (Fig. 8).
6. A 'red cross' button will also be present in the large histogram plot (Fig. 8). By clicking on the 'red cross' button, the associated histogram will be removed from the overlay.

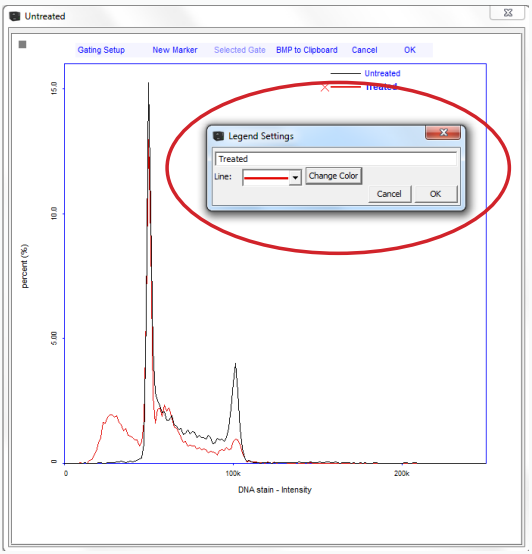
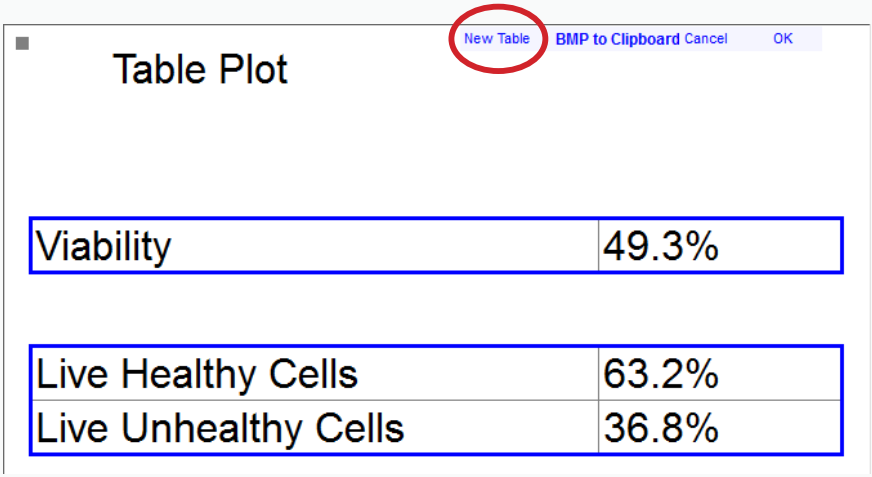


Figure 8. *Large histogram plot displaying an overlay of a standard cell cycle profile (red line) on a histogram of a cell cycle showing DNA fragmentation (black solid line). Buttons for removing histograms and a pop-up window for modifying line color and form along with text input fields encircled in red.*

- *Table plots*

1. Add and place a new table in your table plot. (Fig. 9)

Figure 9.



2. To fill in text, simply click in the table and write your text
3. To fill in a formula, right-click a table and fill in your calculation

    a. To select a value from a Quadrant, Polygon or Marker, use the

    Insert value ... button

4. To format cells with text colors and other settings, right click and select "Format Cell ..."

Identifying the Sub-Population in the Image Window

One of the advantages of image cytometry is that cells defined in a particular sub-population can be identified in the image acquired for analysis. This is done when the image file for the analysis is loaded in the main window and sub-populations have been defined by polygons, quadrants or markers.

1. Open the desired graph containing the polygon, quadrant or marker identifying the sub-population.
2. Select the sub-population by clicking on the gating lines so that it appears red.
3. Right-click and choose the appropriate option, for example 'Add cells inside/outside gate to image overlay'.
4. The events in a scatter plot will be highlighted and the corresponding cells in the image will be marked by colored boxes.
5. To de-select the cells in the image, right-click in the plot area and select 'Delete Image Overlay' or right-click on the image overlay button in the image window and select 'Delete Image Overlay'.

Gating Cell Populations for Inclusion/ Exclusion in Analysis

Particular sub-populations of observed events can be either included or excluded in the data analysis using gating options. The sub-populations are those defined by the polygons, quadrants and markers.

1. Double-click on the small graph to be analyzed to open the corresponding larger graph in editing mode.
2. Click the ‘Gating Setup’ in the upper left corner to open a list of gating options available for that particular analysis. Gate options are denoted as ‘P’ for polygon, ‘Q’ for quadrant and ‘M’ for markers followed by a number indicating the order of creation. The most commonly-used functions listed are to ‘Include’ or ‘Exclude’ sub-populations but more advanced settings may also be listed.
3. Check the box associated with the sub-population of interest and the desired action such as ‘Include’ or ‘Exclude’.
4. Click ‘Apply’ to show the effect of the gating chosen and ‘OK’ to save. If gating is not required, check the ‘Disable’ box followed by ‘Okay’.

Note: if multiple gates have been selected for inclusion or exclusion, they can be combined by either Union or Intersection of the populations.

- **Union:** this option is available when two gates have been set to either Include or Exclude.
  Selecting this option results in display of the sum of all events displayed for the two gates (included or excluded).
- **Intersection:** This option is available when two gates have been set to either Include or Exclude.
  Selecting this option results in display of the events displayed for both of the two gates (included or excluded).

Generic Gate

Once compensation factors, quadrants, polygons, markers and gating options have been defined for a sample, these settings can easily be applied to all future analyses acquired with a specific protocol.

1. Save the row with all adjusted compensations, markers, polygons, quadrants and gatings by clicking on the  button in the row dialog.
2. Right-click the file in the file list that represents the data just saved and select ‘Start Protocol Adaptation Wizard’.
3. In the wizard, skip directly to the step ‘Select and setup a master file’, the second last step.
4. Verify that all compensations, markers, polygons, quadrants and gatings are as desired.
5. Click on the row dialog to select the file as a master file.
6. Choose ‘Save’ to overwrite/update the existing protocol, or ‘Save as’ to create a new protocol.

Optional: Protocols can be locked to hinder future changes to the protocol (locked protocols must be un-locked to be changed).

Exporting Data to Spreadsheets

1. Data from post-processing can be exported in *.csv format (compatible with software such as Excel) by clicking on the  button at the top of Plot Manager.
2. All the data from each analysis and for each marker, polygon and quadrant will appear in a new pop-up window.
3. The data to be exported can be user-defined by right-clicking at the table heading and selecting the parameters of interest for each gate.
4. Select the table and right-click on it to copy it over to third-party spread sheet software for further analysis, if desired.

Exporting Data to Other Software

Data can be exported in '*.acs' or '*.fcs' formats which are compatible with most third-party cytometry analysis packages.

1. Select the file containing the data to be exported.
2. In the 'File' menu select 'Export Data'.
3. A new window will open allowing you to choose the format to be exported, which parameters are to be exported and the location where the file is to be saved.
4. Select the appropriate parameters and click on 'Export' to save the files to the desired location.

Tip: multiple files can be selected in the file browser and can be batch exported by right-clicking. Note that only files acquired with the same protocol can be batch exported together.

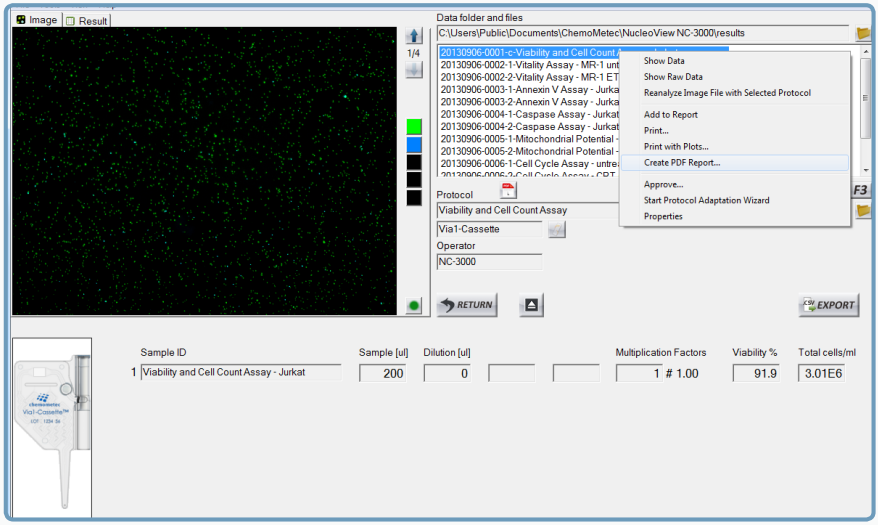
Exporting Graphics

All graphs along with cell images can be copied by either right-clicking on the cell image and selecting 'Copy BMP to clipboard' or for histograms and scatter plots, by clicking 'BMP to Clipboard'. The image can then be pasted into another document or presentation.

PDF reports

1. Right-click on file to create a PDF report

Figure 10

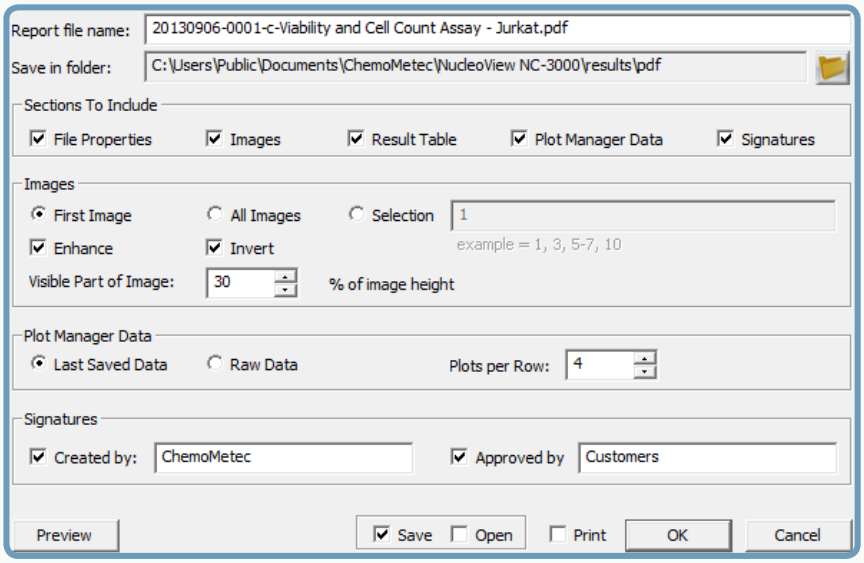


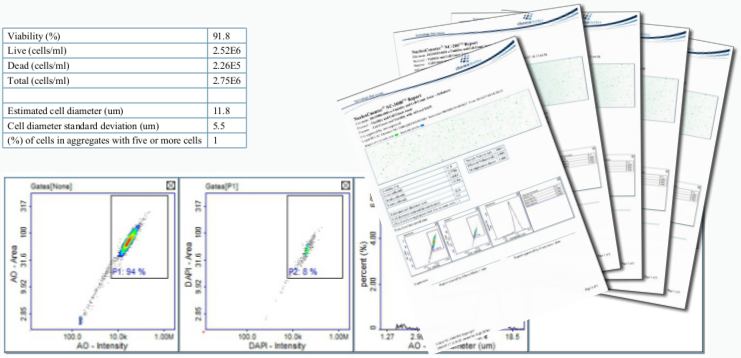
2. Select the parameters to be visible on the report and the properties of the parameters

3. Optional: preview your result

4. Save and/or print your report to the default printer

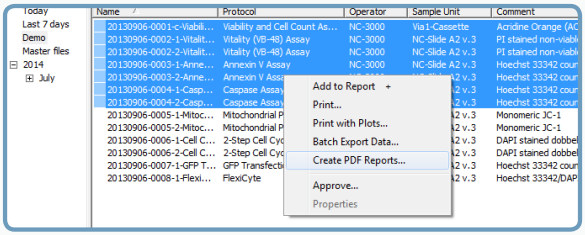
Figure 11





Tip: Batch exports can be done from the NucleoView™ File Browser.

Figure 12



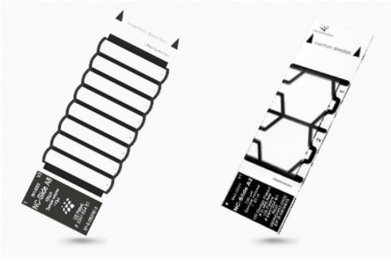
Additional Resources

Go to www.chemometec.com to find:

- Documentation
- SDS
- Certificates of analysis
- Application notes
- Articles
- Videos etc.

Consumables:

Item no.	Description
942-0003	NC-Slides A8™
942-0001	NC-Slides A2™
910-3012	Solution 12
910-3015	Solution 15



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