

Application note No. 3024. Rev. 1.3

## NucleoCounter<sup>®</sup> NC-3000<sup>™</sup>

### FlexiCyt<sup>™</sup> Protocol for the NucleoCounter<sup>®</sup> NC-3000<sup>™</sup> system

#### Product description

The NucleoCounter<sup>®</sup> NC-3000<sup>™</sup> system enables the user to perform advanced cell analyses of a broad range of mammalian cells.

#### Application

This application note for the NucleoCounter<sup>®</sup> NC-3000<sup>™</sup> system describes the usage of the FlexiCyt<sup>™</sup> protocol that can be modified by the user.

#### Staining of cells

Samples are stained with fluorophore(s) specified by the user. Note that the default FlexiCyt<sup>™</sup> protocol requires counterstaining of the sample with either DAPI or Hoechst-33342 in addition to the user specified staining. DAPI and Hoechst-33342 stain the nucleus and is used for detection (masking) of the total cell population. Hoechst-33342 stains both living and dead cells and is used for labeling samples with non-fixed cells. DAPI exclusively stains dead cells and is used for labeling cells fixed with either alcohols or aldehydes. For DAPI and Hoechst-33342 staining please refer to 'Procedure' section below. Counterstaining can be omitted if the dark field light source is selected as masking channel via the 'Protocol Adaptation Wizard'. Note that cell identification is less precise with the dark field masking method compared to using DAPI or Hoechst-33342 in the UV (LED365) channel. Furthermore, only single cells can be analyzed when using dark field masking.

For staining of cellular markers we recommend using Alexa488, Alexa568 and Alexa647. These dyes are bright and photostable and have minimal spectral overlap (see Table 3). Avoid using dyes that are prone to photobleaching, such as FITC, PE, APC and PerCP.

#### Selection of Protocol

In NucleoView NC-3000<sup>™</sup> open 'Select Protocol' window. Select Mammalian Organism and in 'Analysis' choose FlexiCyt<sup>™</sup>. Select the FlexiCyt<sup>™</sup> protocol.

#### Adaptation of the FlexiCyt<sup>™</sup> Protocol

In order to adjust the FlexiCyt<sup>™</sup> protocol, select the FlexiCyt<sup>™</sup> protocol and open the 'Protocol Adaptation Wizard' in the 'Tools' menu. The wizard is a guide for

the user to change masking method, the number of analytical channels, light sources, emission filters, channel names and exposure times for the fluorescence channels. The default method for masking is DAPI or Hoechst-33342 staining detected in the UV (LED365) channel. The default Channel 1 consists of the Blue (LED475) light source and the Em560/35 emission filter that collects light with a center wavelength of 560 nm and has cut-on at 525nm and cut-off at 595 nm. The default Channel 2 consists of the Green (LED530) light source and the Em675/75 emission filter. The default Channel 3 consists of the Red (LED630) light source and the Em740/60 emission filter. The light sources and emission filters can be changed by the user. However, it should be noted that there are differences in which filters are available for specific light sources. If the UV (LED365) or Violet (LED405) light source is selected the user should take care that there is no conflict if the default counterstaining with DAPI or Hoechst-33342 is used. This means that fluorophores used with these light sources should have a large stoke shift emitting light in the high nm range, in order to avoid spectral overlap. If a fluorophore only can be detected in the UV (LED365) Em479/55 or violet (LED405) Em475/15 channels, it is strongly recommended to use the dark field as the masking channel.

In addition to the selection of light sources and emission filters, the user can choose whether cell aggregates should be included or excluded in the analysis, if the UV (LED365) is selected for the masking channel. In the default setting cell aggregates will be excluded. In addition, the user can specify the minimal number of cells to be analyzed (please refer to 'Count statistics' section below). Finally, a master file can be set up that will be a template for how the data will be presented after each run in the Plot Manager. This requires an image file acquired with the FlexiCyt<sup>™</sup> Protocol.

In the final step of the Protocol Adaptation Wizard the adapted protocol can be saved with a new name.

#### Image acquisition

Default exposure time is 1000 msec. for the Blue (LED475), Green (LED530) and Red (LED630), whereas the default exposure time is 200 msec. for the UV

(LED365) and Violet (LED405). For many applications the default exposure times will be adequate due to the high dynamic range imaging technique used in the NC-3000™ system. If images are overexposed a warning box will appear and a histogram is displayed showing 'Max Intensity' of the pixels. The main population should be well below 65.500 (upper limit of the image range). Note: in case of overexposed images fluorescence signals are incorrectly quantified and compensation for spectral overlap is compromised.

### Image processing and analysis

After acquisition, images are automatically segmented and cell data are presented in the Plot Manager as scatter plots, histograms and table plots. In the plot manager data can be further processed and analyzed,

and facilitates e.g. counting, gating and spectral compensation. For more information about the Plot Manager refer to NucleoView NC-3000™ Software Users Guide.

### Count statistics

For reliable counting and quantification of fluorescence it is important that the samples loaded into NC-slides have a cell concentration within the range of  $5 \times 10^4$  to  $5 \times 10^6$  cells/ml. With the FlexiCyte™ protocol it possible to specify the number of cells to be analyzed. However, the actual cell concentration of the sample may have a limit on the number of cells analyzed (see Table 1). For example, if 10.000 counts are required the cell concentration should exceed  $\sim 7 \times 10^5$  cells/ml.

**Table 1: upper and lower limits of number of analyzed cells**

NC-slide	No. of views (individual images)	$5 \times 10^4$ cells/ml	$1 \times 10^6$ cells/ml	$5 \times 10^6$ cells/ml
A2	20	~ 800 counts	~ 16.000 counts	~ 80.000 counts

## Procedures

### Staining of non-fixed samples

#### Materials

- Cells to be stained\*
- Fluorophore(s) of choice and staining procedure\*
- Phosphate buffered saline (PBS) \*
- **Solution 15** (500 µg/mL Hoechst-33342)
- **NC-Slide A2™**

\* provided by the user

Important notes:

Counterstaining with Hoechst-33342 is mandatory when using the UV (LED365) as masking channel. Otherwise the assay will fail.

1. Stain cells with fluorophore(s) as specified by your protocol.
2. After staining harvest cells and resuspend pellet in PBS supplemented with 10 µg/ml Hoechst-33342 at a cell density of  $5 \times 10^4$  -  $5 \times 10^6$  cells/ml.
  - a. Note if  $>10.000$  counts are required keep the cell concentration in the range of  $2 \times 10^6$  -  $5 \times 10^6$  cells/ml.
3. Incubate cells at 37 °C for 15 minutes using a heating block. This step is crucial, thus comply with the specified incubation time and temperature!
4. Engage NucleoCounter® NC-3000™ by starting accompanying software.
5. Load  $\sim 30$  µl of each of the cell suspensions into the chambers of the **NC-Slide A2™**. Place the loaded slide on the tray of the NucleoCounter® NC-3000™ and select the FlexiCyte™ protocol and sample unit. Press RUN.
6. Cells are automatically imaged and processed. Fluorescence intensity is displayed as scatter plots or histograms.

## Staining of fixed and permeabilized samples

### Materials

- Cells to be stained\*
- Fluorophore(s) of choice and staining procedure\*
- Phosphate buffered saline (PBS) \*
- **Solution 12** (500 µg/ml DAPI)
- **NC-Slide A2™**

\* provided by the user

### Important notes:

Counterstaining with DAPI is mandatory when using the UV (LED365) as masking channel. Otherwise the assay will fail.

1. Stain cells with fluorophore(s) as specified by your protocol.
2. After staining harvest cells and resuspend pellet in PBS supplemented with 2 µg/ml DAPI at a cell density of  $5 \times 10^4$  -  $5 \times 10^6$  cells/ml.
  - a. Note if >10.000 counts are required keep the cell concentration in the range of  $2 \times 10^6$  -  $5 \times 10^6$  cells/ml.
3. Incubate cells at 37 °C for 5 minutes using a heating block. This step is crucial, thus comply with the specified incubation time and temperature!
4. Engage NucleoCounter® NC-3000™ by starting accompanying software.
5. Load ~30 µl of each of the cell suspensions into the chambers of the **NC-Slide A2™**. Place the loaded slide on the tray of the NucleoCounter® NC-3000™ and select the FlexiCyte™ protocol and sample unit. Press RUN.
6. Cells are automatically imaged and processed. Fluorescence intensity is displayed as scatter plots or histograms.

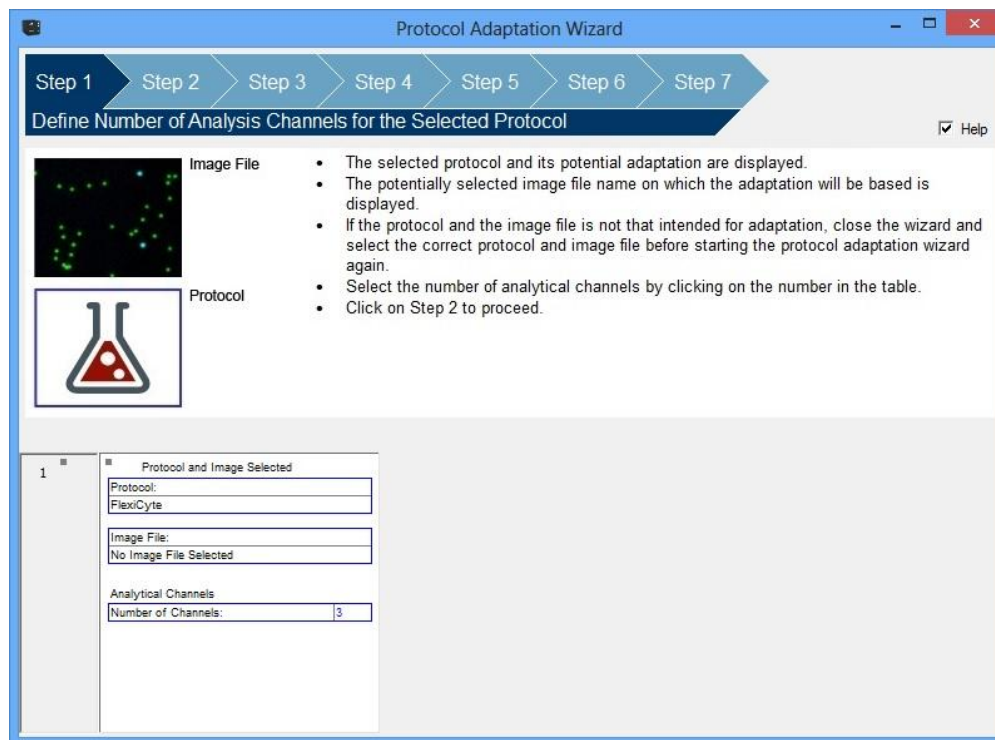
## Preparation of samples for analysis using dark field masking

### Materials

- Cells to be stained\*
- Fluorophore(s) of choice and staining procedure\*
- Phosphate buffered saline (PBS) \*
- **NC-Slide A2™**

\* provided by the user

1. Stain cells with fluorophore(s) as specified by your protocol.
2. After staining harvest cells and resuspend pellet in PBS at a cell density of  $5 \times 10^4$  -  $5 \times 10^6$  cells/ml.
  - a. Note if >10.000 counts are required keep the cell concentration in the range of  $2 \times 10^6$  -  $5 \times 10^6$  cells/ml.
3. Engage NucleoCounter® NC-3000™ by starting accompanying software.
4. Load ~30 µl of each of the cell suspensions into the chambers of the **NC-Slide A2™**. Place the loaded slide on the tray of the NucleoCounter® NC-3000™ and select the FlexiCyte™ protocol and sample unit. Press RUN.
5. Cells are automatically imaged and processed. Fluorescence intensity is displayed as scatter plots or histograms.



**Figure 1.** After selection of the FlexiCyte™ protocol open ‘Protocol Adaption Wizard’ (in the ‘Tools’ menu) and follow the instructions in the wizard.

**Table 2: Guidelines for selection of fluorophores for default selected light sources and emission filters.**

Default light source	Mask Channel <sup>1</sup> UV (LED365)	Channel 1 Blue (LED475)	Channel 2 Green (LED530)	Channel 3 Red (LED630)
Default emission filter <sup>2</sup>	Em470/55	Em560/35	Em675/75	Em740/60
<b>Non-fixed cells</b>	Hoechst-33342	Biomarker 1	Biomarker 2	Biomarker 3
<b>Fixed cells</b>	DAPI	Biomarker 1	Biomarker 2	Biomarker 3
<b>Recommended fluorophores<sup>3</sup></b>	NA	e.g. CF488A <sup>6</sup> , AlexaFluor488 <sup>6</sup> , Atto 488, FluoProbes488	e.g. CF568 <sup>6</sup> , AlexaFluor568 <sup>6</sup> , Cy3, atto565, FluoProbes547H	e.g. ,CF647 <sup>6</sup> , AlexaFluor647 <sup>6</sup> , Cy5, atto647N, FluoProbes647H
<b>Classical fluorophores<sup>4</sup></b>	NA	e.g. FITC, PE	e.g. PE, TRITC	e.g. APC
<b>Nucleic acid stains<sup>5</sup></b>	NA	e.g. AO <sup>6</sup>	e.g. PI <sup>6</sup> , 7-AAD <sup>6</sup> , EtBr	e.g. Draq5 <sup>6</sup> , Draq7, Nuclear-ID Red
<b>Fluorescent proteins</b>	NA	GFP <sup>6</sup> and green and yellow variants	DsRed, RFP <sup>6</sup> and orange/red variants	

<sup>1</sup> Masking channel. Used for identification and counting of cells. Counterstaining with Hoechst-33342 (non-fixed cells) or DAPI (fixed cells) is mandatory. Note that counterstaining can be omitted if dark field channel is used for masking.

<sup>2</sup> Bandpass of default selected emission filter: center wavelength/bandwidth. For example: Em560/35 has cut-on at 525nm and cut-off at 595 nm.

<sup>3</sup> Dyes optimized for image cytometry with increased brightness and photostability.

<sup>4</sup> Avoid using these dyes for analysis of low-abundance markers.

<sup>5</sup> High concentrations of these dyes may compete with Hoechst-33342/DAPI for DNA binding compromising the counterstaining.

<sup>6</sup> Information about spectral spill over is available for these dyes (see Table 3).

**Table 3: Spectral spill over percentages<sup>1</sup> (compensation settings) for selected fluorophores.**

	Mask Channel	Channel 1	Channel 2	Channel 3
Default light source	UV (LED365)	Blue (LED475)	Green (LED530)	Red (LED630)
Default emission filter	Em470/55	Em560/35	Em675/75	Em740/60
Fluorophore				
DAPI	NA	0%	0%	0%
Hoechst-33342	NA	0%	0%	0%
Alexa488	0%	NA	~1%	0%
CF488A	0%	NA	~0.5%	0%
Alexa546	0%	~60%	NA	0%
Alexa555	0%	~80%	NA	0%
Alexa568	0%	~7%	NA	0%
CF568	0%	~34%	NA	0%
Alexa633	0%	0%	~4.5%	NA
CF633	0%	0%	~4.5%	NA
Alexa647	0%	0%	~2%	NA
CF647	0%	0%	~2%	NA
GFP	0.75%	NA	0.4%	0%
YFP	0%	NA	5.5%	0%
RFP	0%	5%	NA	5.5%
AO	0%	NA	~3.5%	0%
PI	0%	~16%	NA	~0.25%
7-AAD	0%	~9%	NA	~5%
Draq5	0%	0%	~4%	NA

<sup>1</sup> Compensation settings were determined by exposing single-labeled samples for 1000 msec. in the default channel 1, channel 2 and channel 3 with their default filters selected. The given compensation settings are only rough guidelines and may need to be re-adjusted for each multi-color experiment. Furthermore, if a new filter is selected for a channel determination for compensation is needed.

## Troubleshooting

### Inappropriate loading of the NC-Slides:

Due to variations in chamber volumes the exact amount needed to fill the chamber may vary. Make sure that the chamber is completely filled and that no excess liquid spreads into other chambers or onto the top of the cover slip. Furthermore, avoid introduction of air bubbles into the chambers. Insufficient filling and air bubbles may cause cell movement compromising the quality of the image analysis.

### Inappropriate DNA-staining of cells:

The FlexiCyte™ protocol depends on successful DNA staining. In order to get adequate Hoechst staining of non-fixed cells it is crucial to follow the protocol carefully. Thus, in step 3 it is mandatory to incubate at 37 °C for 15 minutes using a heating block. Moreover, it is important to use Hoechst-33342 and not other variants such as Hoechst-33258 and Hoechst 34580. For fixed cells 5 minutes incubation with DAPI at 37 °C is sufficient for homogenous DNA staining.

**Handling and storage**

For handling and storage of ChemoMetec instruments and reagents and NC-Slides refer to the corresponding product documentation. For other reagents refer to the material data sheet from the manufacturer of the reagents and chemicals.

**Warnings and precautions**

For safe handling and disposal of the ChemoMetec reagents and NC-Slides refer to the corresponding product documentation and the NucleoCounter® NC-3000™ user's guide. For other reagents refer to the safety data sheet from the manufacturer of the reagents and chemicals required for this protocol. Wear suitable eye protection and protective clothes and gloves when handling biologically active materials.

**Limitations**

The NucleoCounter® NC-3000™ system is FOR RESEARCH USE ONLY. NOT FOR DIAGNOSTIC OR THERAPEUTIC USE. The results presented by the NucleoCounter® NC-3000™ system depend on correct use of the reagents, NC-Slide and the NucleoCounter® NC-3000™ instrument and might depend on the type of cells being analyzed. Refer to the NucleoCounter® NC-3000™ user's guide for instructions and limitations.

**Liability disclaimer**

This application note is for RESEARCH PURPOSES ONLY. It is not intended for food, drug, household, or cosmetic use. Its use must be supervised by a technically qualified individual experienced in handling potentially hazardous chemicals. The above information is correct to the best of our knowledge. Users should make independent decisions regarding completeness of the information based on all sources available. ChemoMetec A/S shall not be held liable for any damage resulting from handling or contact with the above product.

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