Cell fluorescence assays on the Agilent 2100 bioanalyzer – general use

Application

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Abstract

This Application Note describes the general use of cell fluorescence assays on the Agilent 2100 bioanalyzer. The cell assay extension together with the cell fluorescence LabChip® kit allows both the analysis of pre-stained cells and the analysis of cells by on-chip staining. Cell assays on the 2100 bioanalyzer are based on simple flow cytometric analysis. The technical principles, as well as the typical workflow in sample and chip preparation are described here. A typical dye optimization experiment was performed and the appropriate dye concentration for a specific cell line determined. The cell fluorescence software is easy-to-use and allows different forms of data evaluation and result presentation. Using an example, data evaluation is explained in detail. Specific advantages of cell fluorescence analysis with the Agilent 2100 bioanalyzer are low cell and reagent consumption, ease-of-use, automated data acquisition and evaluation of up to six samples.

Agilent Technologies

Introduction

The 2100 bioanalyzer was introduced by Agilent Technologies as the first commercially available microfluidic analysis system for the electrophoretic separation and analysis of DNA, RNA, and proteins. With the introduction of the cell assay extension, the instrument is now capable of measuring fluorescent labeled cells based on simple flow cytometric analysis [1]. Typical cell applications for this instrument are apoptosis detection, determination of transfection efficiency as well as monitoring protein expression by antibody staining.

Prerequisites

Dye selection for cell staining

A broad variety of fluorescent dyes are available for the staining of cells. Dyes can be directly or indirectly linked to antibodies or proteins (e.g. Cy5-labeled streptavidin). Functional dyes show a significant fluorescence when being metabolized within living cells and can therefore be used as live/dead cell stains. An example is the non-fluorescent, lipophilic molecule calcein-diacetate. As electrically neutral, or near neutral molecule, the calcein acetyloxymethyl (AM) ester freely diffuses into most cells. Inside living cells it is metabolized by unspecific esterases. Once the esters are cleaved, it becomes a fluorescent molecule. Due to the polarity and charge of the molecule, it is trapped within the cytoplasm after hydrolysis [2].

Some proteins have an intrinsic fluorescent property (e.g. GFP). This property can be utilized to monitor protein expression of cells. One example is to monitor transfection efficiency by means of a GFP fusion protein or by co-transfection of a GFP expression plasmid and a gene of interest. The Agilent 2100 bioanalyzer uses a two-color detection system. In contrast to flow cytometers, the hardware is not able to detect any scatter parameters. Therefore, one channel is used for a reference staining to select a specific cell population (e.g. all live cells). The other channel is used to assay a second specific cell property in question.

The type of dyes used for the cell fluorescence measurements, depend not only on the biology of the experiment but also on the technical requirements of the system. The Agilent 2100 bioanalyzer is capable of two-color fluorescence detection. The red laser diode has a maximum emission at 635 nm. The emission of the blue LED is at 470 nm. The detection windows are at 674 – 696 nm for the red and 510 – 540 nm for the blue channel. To use a specific dye or label with the 2100 bioanalyzer, the excitation as well as the emission spectra of the dye must coincide with these values. In addition, the fluorescence yield of the dye used must be intense enough. A commonly used blue dye is fluorescein isothiocyanate (FITC). FITC is compatible with the 2100 bioanalyzer optics but the intensity output for many applications is too low to be detected. The reason is the very low quantum yield of the FITC-molecule.

Table 1 lists Agilent 2100 bioanalyzer compatible dyes, which so far have been used successfully. It should be emphasized that staining conditions (e.g. concentration, time, and temperature) need optimization for each dye and cell line. An example for a typical dye optimization experiment is shown on page 7: “Dye optimization”.

Cell lines

A broad variety of eukaryotic cell lines were tested with the Agilent 2100 bioanalyzer (table 2). Suspension as well as adherent cells lines ran successfully. If cell lines are used that are known to form cell clumps and aggregates, the usage of sieves, e.g. cell strainers can help to remove the clumps. After clearing the clumps, the samples can be resuspended in cell buffer and run on the

Blue channel	Red channel
GFP/EGFP (Green Fluorescent Protein)	CBNF (live cell dye, Molecular Probes)
SYTO®16 (DNA dye, Molecular Probes)	SYTO®62 (DNA dye, Molecular Probes)
Calcein (live cell dye, Molecular Probes)	Cy5, PE-Cy5, Cy5.5 (antibody label, Amersham)
SYTOX® green (DNA dye, Molecular probes)	CyChrome (antibody label, BD)
CellTracker™ Green (live cell dye, Molecular Probes)	APC (antibody label, BD)

Table 1
Dyes used successfully with the Agilent 2100 bioanalyzer cell fluorescence assays.

2100 bioanalyzer. Once cells are transferred in cell buffer, they can no longer be pelleted by centrifugation . Therefore, the cell buffer, which is included in the cell fluorescence kit, cannot be used to wash the cells. The cell fluorescence kit has been optimized for eukaryotic cells. Analysis of prokaryotes and simple eukaryotes (e.g. yeast cells) with the 2100 bioanalyzer is currently under investigation. The specific advantage of low cell consumption makes the 2100 bioanalyzer the ideal tool to investigate primary cells [3].

Name	Type	Morphology	Source
CCRF-CEM	T-cell (CD4+)	Suspension & adherent	Human leukemia
CHO-K1	Epithelial	Adherent	Ovary, chinese hamster
M1WT3	Epithelial	Adherent	Ovary, chinese hamster
Jurkat	T-cell (CD4+)	Suspension	Human leukemia
H4-II-E-C3	Hepatocytic	Adherent	Rat
THP-1	Monocytic	Suspension	Human leukemia
HEK293	Epithelial	Adherent	Human kidney
U937	Monycyte	Suspension	Human lymphoma
HL-60	Myeloblast	Suspension	Human leukemia
A10	Myoblast	Adherent	Rat aorta smooth muscle
ZY(LCL)	Lymphoma	Suspension	Rhesus monkey lymphoma
K562	Myoblast	Suspension	Human leukemia
JS(LCL)	Lymphoma	Suspension	Rhesus monkey lymphoma
SMMC7731	Hepatoma	Adherent	Human hepatoma chinese cell line
H22	Hepatoma	Suspension	Mouse hepatoma cells
HUVEC	Primary Endothelial	Adherent	Human umbilical vein
PBMC	Lymphocytes	Suspension	Human blood

Table 2
Sampling of cell lines analyzed with the 2100 bioanalyzer cell fluorescence assays.

Sample and chip preparation

Instrument setup

Cell assays on the Agilent 2100 bioanalyzer are based on simple flow cytometric analysis. The cell fluorescence LabChip® kit together with the cell assay extension and the 2100 bioanalyzer enables the analysis of pre-stained cells and the analysis of cells by on-chip staining. Part of the cell assay extension is the pressure cartridge and the cell fluorescence software. To switch the instrument to the cell application, the electrode cartridge needs to be replaced by a pressure cartridge and the assay specific software needs to be loaded. Once this is done, the instrument firmware will automatically be updated. This process only takes a few seconds.

The cell fluorescence LabChip® kit includes 25 chips and all necessary reagents to run 150 cell samples, six samples per chip. Because it is a generic kit that can be used for different types of cell applications, it contains no application specific dyes or antibodies. Ordering information for reagents are given in the appropriate Application Notes which can be downloaded from the Agilent website (www.agilent.com/labonachip).

The system is designed that no cross talk between samples can occur and instrument maintainence is minimized (no wash steps), therefore chips are for on-time use only and have to be disposed after the run .

Sample preparation

After growing and harvesting the cells, they are stained with fluorescent dyes according to biologically relevant parameters. Several Application Notes are available that provide detailed protocols [4,5,6]. Compared to conventional cell fluorescence analysis methods, such as flow cytometry or fluorescence microscopy, there is no difference in cell preparation. After a washing step, stained cells are resuspended in an isobuoyant cell buffer. Cells should always be handled with care. Some cell lines are especially susceptible to vortexing or pipetting. To ensure good quality results, cells should be always resuspended carefully.

To achieve a statistically relevant number of events, a cell concentration of 2 million/ml is recommended. Higher cell concentrations may lead to the detection of doublets, i.e. more than one cell being in the detection window at a given time, and thus could affect data quality. Using a cell concentration of 2 million/ml will result in typically 500 to 1000 measured events during the default sample run time (4 minutes).
If higher event numbers for cell samples with a low number of positive cells needs to be measured, the data acquisition time can be extended up to a factor of 6. In those cases the number of samples on the chip needs to be reduced (see section “Data acquisition settings” on page 4).

Chip preparation and hydrodynamic focusing

The micro-fluidic channels of the cell chip are initially filled with a low viscosity priming solution. Capillary forces fill all of the channels within a few seconds. No external pressure is needed. With the help of a focusing dye solution, the Agilent 2100 bioanalyzer optics align with a dedicated dye channel. Afterwards, the instrument optics will move an incremental distance to the sample channels.

Once the chip run is started, a constant level of vacuum is generated by a pump inside the instrument and applied on the chip via the pressure cartridge. Cells flow inside the network of micro-fluidic channels in the chip. Individual channel systems for each of the six samples ensure that there is no cross-contamination among the individual samples. Each of the two cell buffer wells are connected to three sample channels. The sample channels are twice as long as the cell buffer channels. Since a constant level of vacuum is applied to the chip, the fluids in the cell buffer channels will move twice as fast as in the sample channels. The force of the cell buffer is used to hydrodynamically focus the cell samples. After the intersection of the cell buffer with the sample channel, both moving fluids do not mix immediately. Cells are focused to one portion of the channel. Here they pass the detector in single file. Figure 1 illustrates the hydrodynamic focussing process.

Data acquisition settings

The optics of the 2100 bioanalyzer scans each of the six sample channels consecutively. When the data acquisition of a sample is finished, the optics is adjusted horizontally and vertically to the following sample channel. To run a diluted sample or a sample where more than 1000 measured events are needed, the number of samples per chip needs to be lowered. The cell fluorescence software provides the flexibility to extend the run time of the specified samples (figure 2).

By reducing the number of cell samples to one, the run time can be extended up to 24 minutes. Alternatively, a fixed number of events can be specified. Particles, which can be present in the sample, may cause unspecific background signals. This can lead to differences between collected numbers of events and cells. However, the overall chip run time is fixed and limited to the amount of liquid that passes though the micro-fluidic channels. During the run, liquid is constantly moving through all of the channels and collected in one dedicated well.

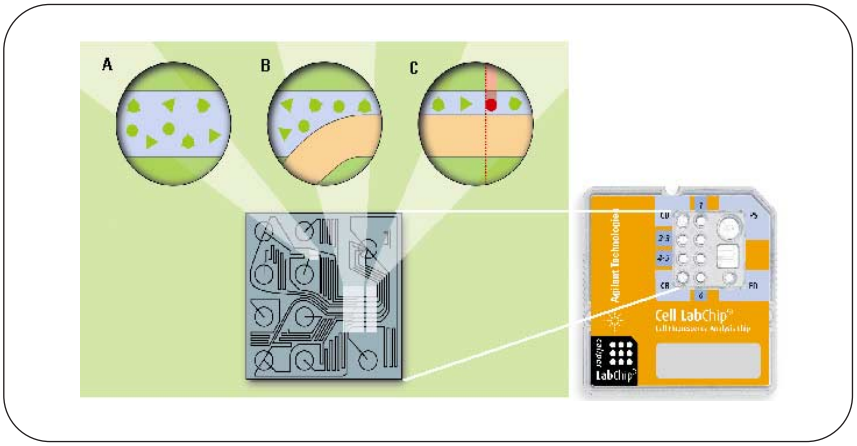

Figure 1
Hydrodynamic focusing on a cell chip. A: stained cells (symbolized as triangles and circles) are moving through the micro-fluidic channel. B: a flow of cell buffer forces the cells to one side of the channel. C: fluorescence is detected by the bioanalyzer optics as the cells move past in single file.

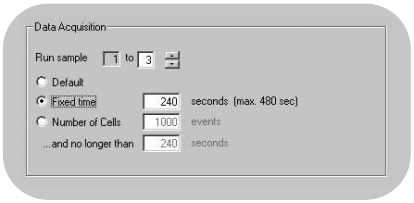

Figure 2
Data acquisition settings of the cell fluorescence software. In the default setting, 6 samples are run for 240 seconds each. In this example the number of samples analyzed on the chip was reduced to 3. Data acquisition time for these samples can be extended to a maximum of 480 seconds per sample.

On-chip staining

As an alternative to standard cell staining protocols, Agilent has developed on-chip staining procedures. The dye solution and/or dye-labeled antibody are directly added to the cell suspension in the sample wells of the chip. The chip is incubated at room temperature, to allow some time for the staining reaction. To reduce excessive evaporation during the staining, it is recommended to incubate the chip in a humified chamber.

Alternatively, a used chip can be stacked on top of the prepared chip which also reduces evaporation. After the cell samples are resuspended by vortexing, the chip can be directly inserted into the Agilent 2100 bioanalyzer and samples are analyzed with specific on-chip staining assays. Several Application Notes are available describing the on-chip staining for different applications [7,8]. The on-chip staining greatly streamlines the workflow in cell preparation. Compared to conventional staining methods, a significantly lower number of cells and amount of reagents (e.g. antibodies) are required.

Cell fluorescence checkout kit

To become familiar with the chip preparation and data evaluation of the cell fluorescence assays, a checkout kit is part of the cell assay extension. Included in the kit are synthetic beads, which are labeled with fluorescent dyes. A ready-to-use bead solution is easily prepared by diluting concentrated beads into cell buffer. Figure 3 shows a typical result of a bead run in the dot plot presentation. Red and blue beads were loaded in alternating order. The blue fluorescence is plotted on the x- and the red fluorescence on the y-axis. The fluorescence scale for both is over six decades. Bead samples can be added as an internal control on one chip together with cell samples. Especially if new staining methods are evaluated, bead samples can be added as positive controls to check chip and instrument performance.

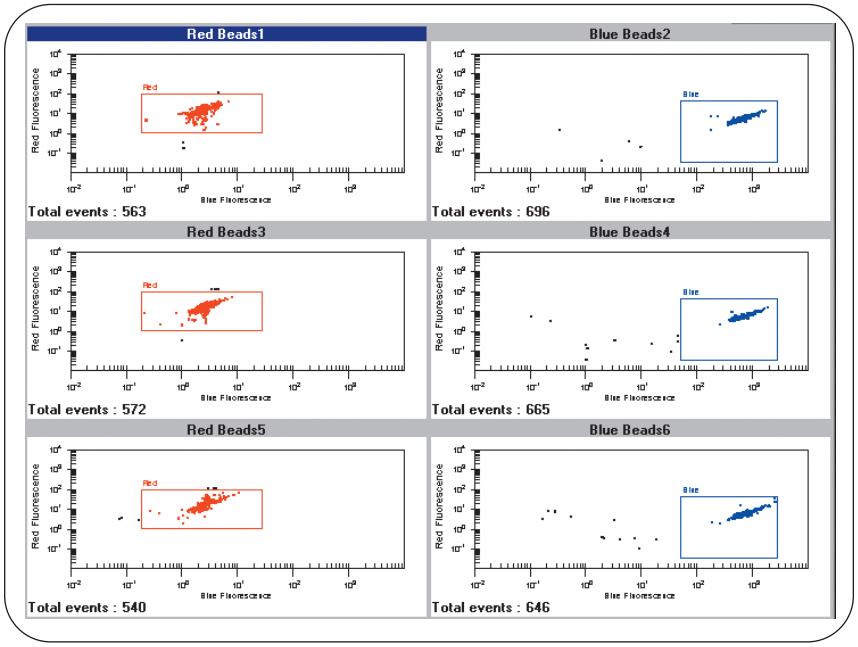

Figure 3
Typical data of a checkout bead run in dot plot view.

Data evaluation and result presentation

Cells pass the detector window on the chip in single file (figure 1). They are analyzed individually for their red and blue fluorescence properties. For each event detected, a fluorescence value in the red and blue channel is assigned and used for data evaluation. The detection of two fluorescence parameters allows the analysis of three types of cell events – cells that show only a red or blue fluorescence and cells that show fluorescence in both colors. In contrast to a flow cytometer, the 2100 bioanalyzer does not acquire scatter parameters and therefore non-fluorescent cells or particles are not detected. In the status bar at the lower part of the software screen, online information on the number of events detected per second is given. The progress of data acquisition can be followed in the dot plot view. The cell fluorescence software offers two different data representations: measured fluorescence values can be displayed in the histogram or dot plot view. After data acquisition is finished, the evaluation can be done using either data representation. The histogram presentation plots the number of events against the logarithmic scale of fluorescence. Since two fluorescence channels are measured, two histograms are displayed per sample (figure 4). To define dedicated sub-populations of cells within a measured cell sample, markers can be inserted and edited in the histogram. They are represented as dotted vertical lines. A marker sets a lower and upper fluorescence intensity value. The position of the marker can be adjusted individually for each histogram. In the pre-defined cell fluorescence assays, markers of the same channel are referenced to each other – if one marker is moved horizontally in one sample,

markers in the other samples will also be adjusted accordingly. Statistical data (e.g. number of events, mean fluorescence value, standard deviation, % CV) is shown in the data table below the histogram. The data table can be configured to show user defined headings. To decide if a stained cell sample shows a significant level of fluorescence, it is recommended to include a negative control on the same chip. The overlay function makes it easy to compare data from different sample wells (figure 4).

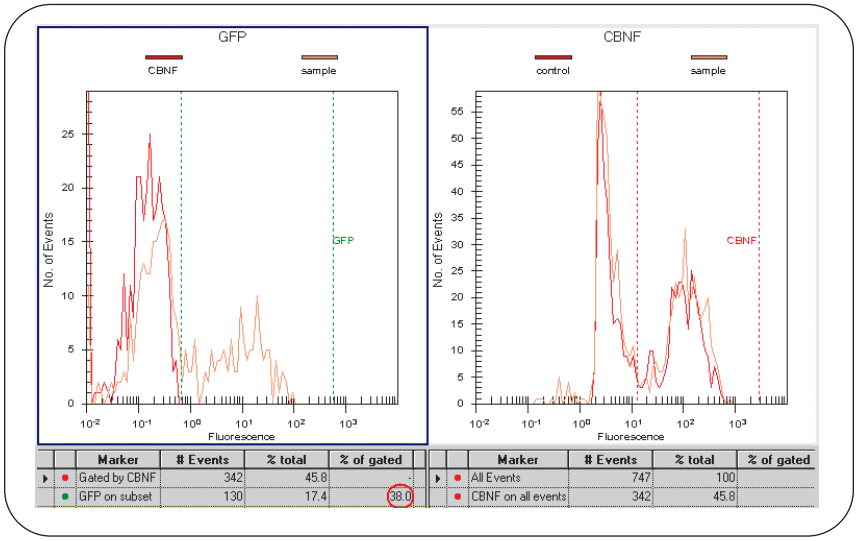

Figure 4
Histogram overlay of 2 CHO-K1 cell samples. CHO-K1 cells were mock transfected ('control') or transfected with an EGFP-containing plasmid ('sample'). Both cell populations were stained with the live cell dye CBNF. In 'sample', 38.0% of the CBNF positive cell population also show significant blue GFP-fluorescence. Experimental details are given in the GFP application note [6].

Cell fluorescence assays

The way data is analyzed depends on the biological background of the staining experiment. To facilitate data evaluation, a pre-defined set of assays, e.g. antibody staining, GFP and apoptosis are available within the cell fluorescence software. Markers and regions of pre-defined assays are set at positions where events are expected and gating direction is pre-determined (table 3). User-defined assays can be saved where individual sample information, data acquisition settings, positions and configurations of markers and regions are stored. The two assays *Red_to_Blue* and *Blue_to_Red* both have a pre-set gating direction and contain no other sample or staining specific information.

From data to result

To determine the percentage of GFP-expressing cells among the living cell population, the data evaluation follows a three-step process:

Step 1 – define reference sub-population

With the help of the mock-transfected cell sample ('contol'), the marker in the reference channel is adjusted. The decision which channel (or which color) is the reference channel depends on the application. The cell sub-population that exhibits a low red CBNF-fluorescence in Sample (figure 4, right histogram), results from unspecific staining or noise and can be excluded by adjusting the marker. Only events lying between the marker, CBNF-positive live cells, are considered for the data evaluation.

Step 2 – gating for second color

To determine the percentage of GFP-expressing cells, within the selected CBNF-stained sub-population, CBNF-positive cells are gated. In the default setting, only signals of the selected sub-population in the red are displayed in the other color (blue). In the example, the red CBNF-staining is the reference staining. The gating direction is from red to blue. If the generic assay has been selected, the gating direction must be set. In the pre-defined assays, the gating direction is fixed (table 3) and can only be changed by importing regions and markers from other assays. By defining the gating direction, events that are not included in the reference marker boundaries are excluded from the second channel.

Assay name	Reference channel	Gating direction
Antibody staining	Blue	Blue to Red
On-chip antibody staining	Blue	Blue to Red
Apoptosis	Blue	Blue to Red
Apoptosis-fast protocol	Blue	Blue to Red
GFP	Red	Red to Blue
On-chip GFP	Red	Red to Blue
Generic	User defined	User defined
Red_to_Blue	Red	Red to Blue
Blue_to_Red	Blue	Blue to Red

Table 3
Overview on existing cell fluorescence assays.

Step 3 – define the specific sub-population in second color: To obtain the final result, the marker in the second channel needs to be adjusted. With the help of the overlay function, the marker position can be adjusted appropriately. The overlay of the blue channel histograms (figure 4, left histogram) shows that lower intensity events ($<10^0$) are not only present in the sample but also in the control. Only the sample shows higher blue ($>10^0$) fluorescence. In the selected example 38.0 % of the CBNF-positive cell population also shows blue fluorescence resulting from the expression of the GFP-protein.

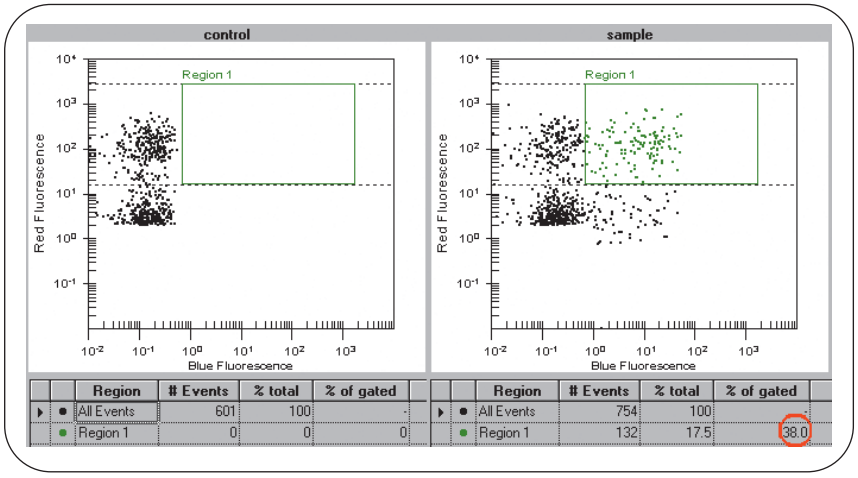

Figure 5
Dot plot of 2 CHO-K1 cell samples. CHO-K1 cells were mock transfected ('control') or transfected with an EGFP-containing plasmid ('sample'). Both cell populations were stained with the live cell dye CBNF. In the sample, 38.0% of the CBNF positive cell population also shows significant blue GFP-fluorescence. Experimental details are given in the GFP application note [6].

Alternatively, the data evaluation can be done in the dot plot view (figure 5). In the dot plot view each dot represents one detected event. Fluorescence values of the events are displayed in logarithmic scale in both colors. The size of the region can be manually adjusted. Gating directions are displayed as vertical (blue to red) or horizontal (red to blue) dotted lines. In pre-defined assays, regions are already configured and referenced among each other. In addition, the regions are linked to the markers that are displayed in the histogram view. Markers in the histogram will be adjusted, if the regions in the dot plot are changed and vice versa. This will lead to the same results, no matter in which representation (histogram or dot plot view) the data evaluation is done. In the generic assay the gating direction and number of regions can be defined. This is the only assay, where markers and regions are not referenced to each other. After data analysis is finished, results are automatically stored. Data can be re-analyzed at any later point of time. Histograms, dot plots and corresponding statistical data of all or selected samples can easily be printed. In addition, the cell fluorescence software offers the flexibility to export data in flow cytometry standard (FCS) format.

Dye optimization

All dyes and antibodies need to be optimized for specific experimental conditions. Figure 6 shows results of a typical optimization experiment. A CCRF-CEM (human leukemia) cell sample was harvested by trypsin treatment. Residual trypsin was removed by washing the cells with PBS-buffer. While the concentration of carboxynaphtofluorescein (CBNF) was held constantly at 1 µM, the concentration of CellTracker™ Green CMFDA (5-chloromethylfluorescein diacetate, Molecular Probes), a blue live dye, was varied over 4 orders of magnitude. All samples were incubated at 37 °C for 30 minutes. To remove unbound dye, the samples were washed with PBS after incubation. The cell density was adjusted to 2 million cells/ml by resuspending in the appropriate volume of cell buffer. The lipophilic CMFDA passes through the cell membrane and once inside the cell it is metabolized to a impermeable cell fluorescing product. CMFDA is especially useful for long-term tracing of living cells. To avoid a possible negative influence on the cell physiology, the concentration of the dye should be kept as low as possible. Therefore a concentration of 10 µM CMFDA was considered as the optimum concentration for CCRF-CEM cells.

Conclusion

The Agilent 2100 bioanalyzer offers a unique platform to analyze not only biomolecules such as DNA, RNA and proteins but also fluorescence parameters of cells. Core assays for the cell fluorescence applications are apoptosis detection, GFP transfection efficiency and antibody staining. A wide range

of different dyes and cell lines can be used with the system. The cell fluorescence software makes the data evaluation easy and straightforward. A typical dye optimization experiment was performed. By running just one chip, the optimal dye concentration for a selected dye and cell culture was found. The Agilent 2100 bioanalyzer hardware platform can be switched easily and quickly between the molecular and cell assays and thus represents a highly flexible tool in modern life science research. Other key advantages of the system are ease-of use, low cell and reagent consumption and automated analysis of up to six cell samples in less than 30 minutes.

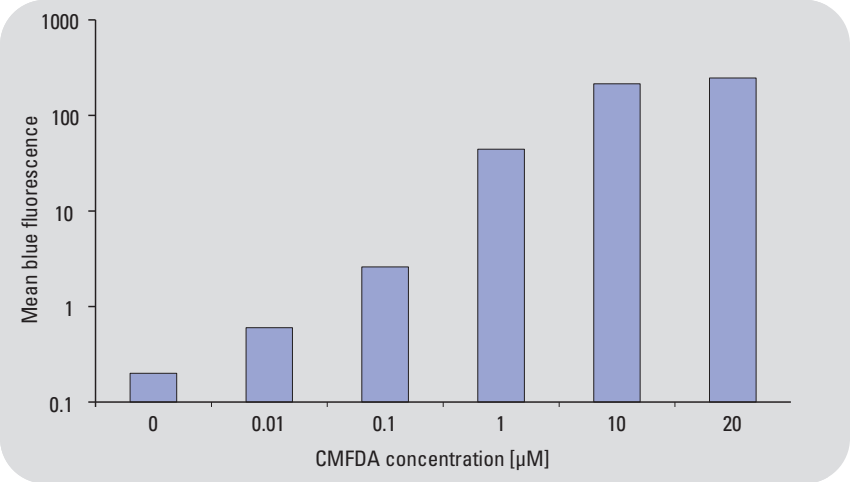

Figure 6
CCRF-CEM (human leukemia) cells were incubated with 1 µM carboxynaphtofluorescein (CBNF) and several concentrations of 5-chloromethylfluorescein diacetate (CMFDA) at 37 °C for 30 min in PBS-buffer. At the y-axis, the mean blue fluorescence of all live cell events is represented in log-scale.

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