Miltenyi Biotec

MACSQuant® Flow Cytometers

FRET Express Mode

Background

Fluorescence resonance energy transfer (FRET) is a mechanism describing an energy transfer between two fluorescent molecules. The efficiency of this energy transfer is inversely proportional to the distance between donor and acceptor, making FRET extremely sensitive to small changes in distance. Measurements of FRET efficiency can be used to determine if two fluorochromes are within a certain distance of each other. Therefore FRET can be used to detect molecular interactions in a number of systems and has hence often been applied in various assays in biology and chemistry.

FRET can be used to measure distances between two domains of a single protein and therefore to provide information about protein conformation. FRET can also detect two different interactions between proteins.

The FRET Express Mode of the MACSQuant® Flow Cytometers can be used to measure protein-protein interactions and conformational changes via FRET automatically. The proteins have to be marked with fluorescent proteins or dyes. During cell measurement, the transferred energy from the donor to the acceptor molecule is calculated according to Nagy *et al.* (1998)[1] and the FRET efficiency is returned by the program.

FRET calculation

Briefly, to calculate the FRET efficiency, the following calculations are carried out by the program:

Background subtraction

The background intensities are subtracted from each signal in donor, acceptor, and FRET channel:
→ this results in F_D, F_A, and F_F

Spillover factors

The crosstalk between the donor, acceptor, and FRET channel is calculated here:

$$S1 = \frac{F_F}{F_D} \qquad S3 = \frac{F_A}{F_D}$$

$$S2 = \frac{F_F}{F_A} \qquad S4 = \frac{F_D}{F_A}$$

Alpha factor

The rate of relative detection sensitivity of the excited acceptor compared to the excited donor is described by the alpha factor (α). The alpha factor has to be calculated manually (refer to section Material and methods).

FRET efficiency calculation

This calculation is run by the FRET Express Mode automatically. Here, the FRET efficiency (E) is calculated relative to the sensitivity of donor and acceptor dyes (α). In A, the FRET signal F_F is subtracted by spillover-corrected (S) donor and acceptor signal and divided by the intensity of the donor signal. As a result, the energy transfer per donor excitation is calculated here.

$$E = \frac{A}{1+A}$$

If S3 and S4 are approximately 0, which is usually the case in biological systems, then:

$$A = \frac{1}{\alpha} \times \frac{F_F - F_D \times S1 - F_A \times S2}{F_D}$$

Material and methods

For the FRET measurement, at least four samples are required: an unstained blank, an acceptor only and a donor only sample, and at least one FRET sample.

For the FRET pair, a blue donor (V1 channel) and a green acceptor (B1 channel) has to be chosen. The FRET transfer channel (donor excitation wavelength and acceptor emission wavelength) will then be the VioGreen™ (V2) channel.

Please note that the FRET Express Mode is only functional on the MACSQuant Analyzer 10 and the MACSQuant VYB.

Prepare cells according to standard protocol for flow cytometry measurements.

The samples are measured without compensation. Therefore, please avoid the staining of further markers or propidium iodide (PI), as the fluorescent signals might interfere with the FRET signals.

Before data acquisition, please calculate your alpha factor: The alpha factor gives you information about the rate of relative detection sensitivity of the excited acceptor compared to the excited donor. Therefore the number of photons absorbed by the donor and acceptor only sample are determined.

$$\alpha = \frac{I_{FinA}}{I_{DinD}} \times \frac{L_D}{L_A} \times \frac{B_D}{B_A} \times \frac{\varepsilon_D}{\varepsilon_A}$$

I = fluorescence intensity, L = labeling ratio, B = antigen ratio, ε = extinction coefficient at max. donor excitation

Please use antibodies directed against your protein of interest to determine the alpha factor. The labeling ratio should optimally be 1 for antibody conjugates, that means that the alpha factor is calculated by using the same antibody clone coupled to the donor and acceptor FRET fluorochromes.

In case you use fluorescent proteins and the expression levels vary between cells, it is most easy to use an antibody against a protein with uniform expression, which is similar to the examined proteins, and conjugated with the FRET dyes. Alternatively, α can be determined by using a donor-acceptor fusion protein, so an equal level of expression is ensured for both proteins and the B_D/B_A ratio would be 1.

As an example, the alpha factor for the FRET pair VioBlue® donor and FITC acceptor is calculated here.

- Look up both the extinction coefficient (ExC) at maximum and the quantum yield (QY) at donor excitation in the literature. Those values are multiplied to determine the ExC at donor excitation:
  efr = ExC at max × QY at donor excitation.

	ExC at max	QY at donor excitation	ExC at donor excitation
Donor	42860	0.97	D = 41574.2
Acceptor	79000	0.054	A = 4266
		efr =	= D/A = 9.75

- Determine the background (BG) corrected FRET intensity in the acceptor only labeled sample and the BG corrected intensity in the donor only sample.
- Look up the labeling ratios (F/P) for your donor and acceptor antibody conjugates.
- Determine the alpha factor according to formula above.

BG corrected FRET intensity in acceptor only sample	1
BG corrected donor intensity in donor only sample	5
Labeling ratio of donor (F/P ratio)	3.2
Labeling ratio of acceptor (F/P ratio)	5.87
Antigen ratio D/A	1
efr	9.75
Alpha =	**0.7351**

Data acquisition and analysis using the MACSQuant Analyzer 10 or MACSQuant VYB

1. Activate the parameter **height** (Channels → Advanced → Height).
   This parameter is required to enable the singlet gating in the Express Mode.
2. Set the forward scatter (FSC) trigger prior to measurement very close to your target population in order to eliminate debris.
3. Prepare four samples (blank, donor only, acceptor only, FRET sample) and put them into a Chill 5 Rack into the positions A1 to D1.
4. Select a **Chill 5 rack** from the drop-down menu.
5. **Group** the samples before the FRET Express Mode can be selected.
6. Select the FRET Express Mode from Settings → Express → Analysis → FRET.
7. Write your alpha factor in the description in the form of "alpha = *0.xxxx*". You may also add further descriptions for your samples here.
8. Then select the sample IDs for your samples in the drop-down menu.

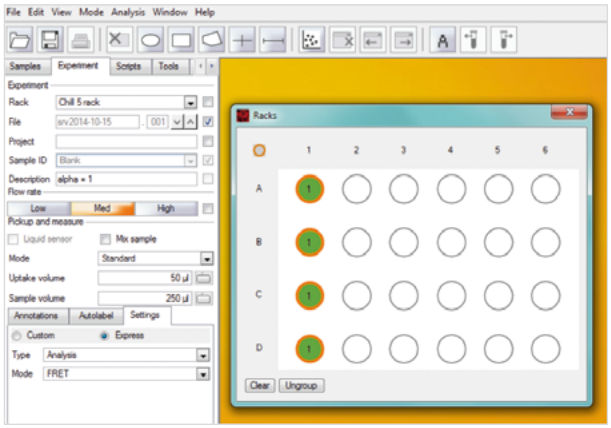

Figure 1: Settings for the FRET Express Mode.

9. Press **Start**.
   Compensations are deleted by the FRET Express Mode during the measurement and the samples are measured in log5 scale by default. The samples are now measured automatically and the FRET calculation is done by the Express Mode during the analysis.
   Please note that the log5 scale of the channels and the compensation are not reset to the previous settings after the Express Mode analysis has stopped.

Analysis

The FRET analysis is done completely automatically by the FRET Express Mode.

Analysis pages 1–4

For each of your samples, the Express Mode gives out an analysis page showing:

- The automatic gate on the target population in the SSC-FSC plot, which is calculated based on the donor and acceptor dye double positive cells.
- The gate on the singlet cells in the FSC-A versus FSC-H plot.
- Dot plots of the donor (V1) and acceptor (B1) channels versus the FRET (V2) channel: These are the spillover (S) factor plots. Automatic gates are applied on the positive populations.
- Intensity histograms for donor, acceptor, and FRET.
- Median intensities in donor, acceptor, and FRET channels.

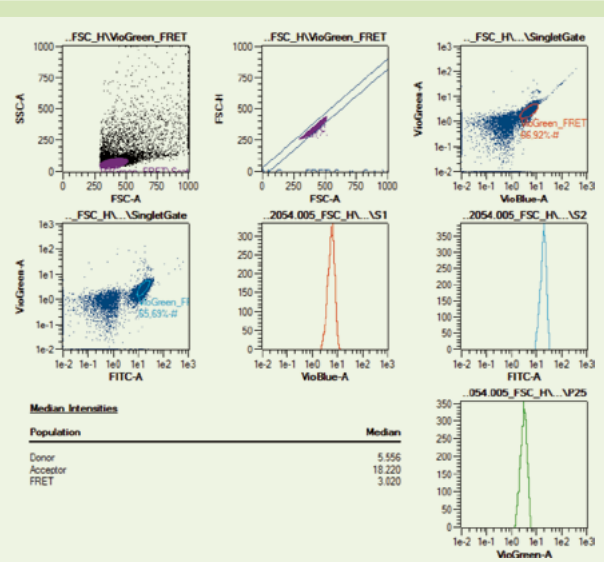

Figure 2: FRET Analysis Pages 1–4 for blank, donor only, acceptor only, and the FRET sample.

Analysis page 5

Page 5 shows an overview over all the calculations: the background subtraction, the spillover (S factors), and the FRET calculation.

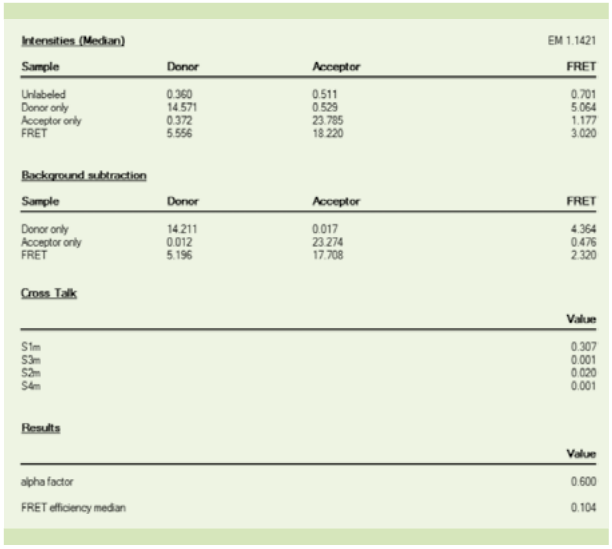

Figure 3: Analysis page 5: Overview on the FRET calculation.

Analysis page 6

The sixth page shows the most important results:

- The target population gating in the FSC-SSC and singlet gating in the FSC-A versus FSC-H plot.
- The S factor gatings.
- An overview over the intensities of donor, acceptor, and FRET in all four samples.
- As results, the alpha factor and the FRET efficiency median are displayed.

A manual change in the gates will result in the change of the median intensities and thus also in a change in the median FRET efficiency.

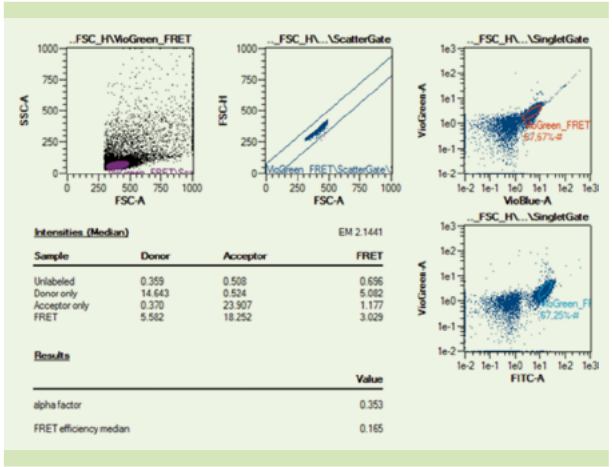

Figure 4: Analysis page 6: Result overview.

Analysis page 7

On the seventh page you can see the FRET efficiency histogram for FRET sample. The analysis page also includes the previous gating on the target population and on singlet cells.

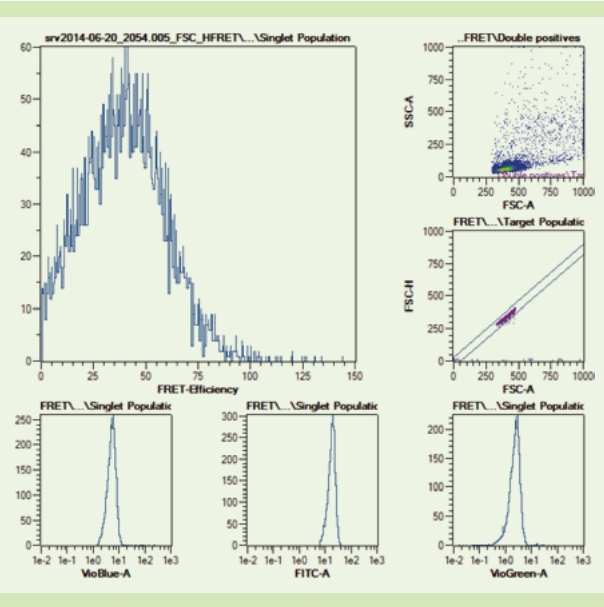

Figure 5: Analysis page 7: FRET Efficiency histogram on cell-by-cell basis.

Please note that the single-cell FRET histogram cannot be update after changing the gates, because the file, which is required for generating the FRET efficiency channel, has already been generated during the FRET EM analysis and thus the FRET calculation is completed.

Conclusion

The FRET Express Mode automatically measures FRET in living cells on the MACSQuant Flow Cytometers. This allows the identification of protein-protein interactions on large cell numbers in a minimum of time, in high throughput screenings, and is easy to use.

References

1. Nagy, P. *et al.* (1998) Intensity-based energy transfer measurements in digital imaging microscopy. Eur. Biophys. J 27: 377–389.

▶ **miltenyibiotec.com/macsquant**

Miltenyi Biotec

Miltenyi Biotec GmbH | Friedrich-Ebert-Straße 68 | 51429 Bergisch Gladbach | Germany | Phone +49 2204 8306-0 | Fax +49 2204 85197
macs@miltenyibiotec.de | **www.miltenyibiotec.com**

Miltenyi Biotec provides products and services worldwide. Visit **www.miltenyibiotec.com/local** to find your nearest Miltenyi Biotec contact.

