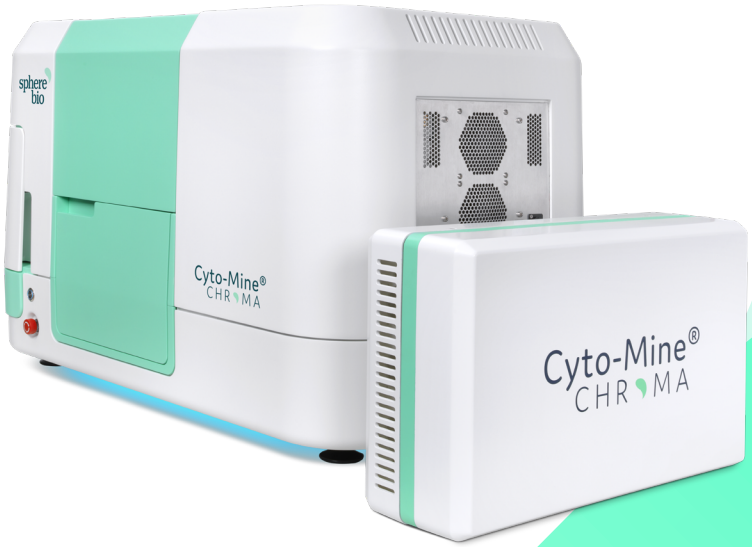
Customer Protocol

Antigen-Specific FRET Assay For Use With Cyto-Mine® Platforms





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1. Introduction

This protocol describes how to perform an antigen–specific Förster Resonance Energy Transfer (FRET) assay for use on the Cyto-Mine® single-cell analysis and screening platform to support antibody discovery (AbD) workflows. The assay enables rapid detection of secreted antibody binding to a target antigen at the single-cell level, by exploiting FRET between fluorescently labelled antigen and fluorescently labelled detection antibody, both specific to the secreted antibody.

Antigen-specific assays such as this are critical in AbD because they directly link antigen binding function to individual antibody-secreting cells. This specificity minimizes false positives from non-specific secretion and ensures that only relevant binders are selected for downstream development. By combining antigen-specific FRET detection with Cyto-Mine's high-throughput, automated single-cell screening, this approach provides a powerful and efficient platform for isolating rare, high-quality antigen- specific antibodies early in discovery campaigns.

In this protocol, TNF-α serves as a model antigen. Hybridoma cells secreting anti-TNF-α antibodies are encapsulated into picodroplets containing fluorescently labelled TNF-α (donor probe) along with fluorescently labelled anti-mouse IgG antibody (acceptor probe). When a secreted antibody binds to TNF-α, the close proximity of the donor-acceptor fluorophores induces FRET, generating a measurable emission signal detected by the Cyto-Mine's integrated optics. Positive droplets are automatically sorted for subsequent recovery and characterization (**Figure 1**).

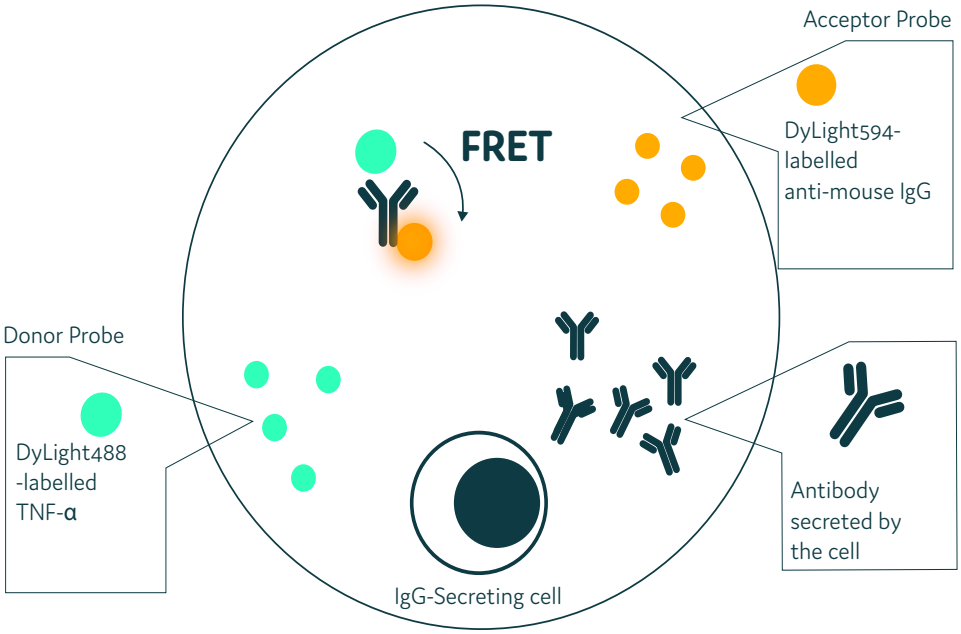


Figure 1. Schematic diagram for antigen-specific Förster Resonance Energy Transfer (FRET) assay. This method directly evaluates antigen-specificity of secreted antibody by using FRET, whereby the FRET donor (DyLight488 labelled antigen) is paired with a FRET acceptor (DyLight594 labelled anti-mouse IgG). A FRET response is only detectable when the two FRET probes bind antigen-specific secreted antibody and form a three-molecule complex.

Prior to single-cell screening, a plate reader-based assay is performed to optimize probe concentrations and ratios. This preliminary step is a fundamental part of the protocol to verify the signal specificity, establish assay sensitivity, and optimize labelling ratios prior to single-cell screening.

If other antigens are to be screened, the materials will need to be adapted for each target antigen, and this protocol should be used as a guide for the experimental work.

2. Equipment and Materials

Equipment

Item	Supplier	Catalogue Number
Plate Reader	BMG Labtech CLARIOstar®plus or equivalent	N/A Note: The plate reader must support excitation at 488 nm and emission detection at 520 nm and 620 nm.
Class II Microbiology Safety Cabinet	Scanlaf Mars 1200 or equivalent	N/A
NanoDrop™ 2000/2000c Spectrophotometers	Thermo Fisher Scientific	ND-2000
NucleoCounter NC-3000	ChemoMetec	970-300
Centrifuge with microtiter plate rotor- 5804R	Eppendorf or equivalent	022623508
Multipette M4	Eppendorf or equivalent	4982000012
Pipettes (10 µL , 20 µL, 200 µL, 1,000 µL)	Various suppliers	N/A
Cyto-Mine® or Cyto-Mine® Chroma	Fluidic Sciences	S003 or S008

Software

Item	Supplier	Catalogue Number
Cyto-Mine® Studio Suite – Instrument Control	Fluidic Sciences	N/A
Cyto-Mine® Data Analysis Software (v2.6.20)	Fluidic Sciences	N/A
FlowJo™ (v10.10)	Becton, Dickinson and Company	N/A

Consumables

Product Name	Supplier	Catalogue Number
Corning® 384 Well Plates, Black (Low volume)	Corning	4511
96 Well flat-bottom microtiter plate	Corning	3595
Micro centrifuge tubes 1.5 mL or 2 mL	Eppendorf or equivalent	N/A
Pipette tips (10 µL , 20 µL, 200 µL, 1,000 µL)	Various suppliers	N/A
10 mL Stripettes	Costar or equivalent	N/A
NC Slide A8	ChemoMetec	942-0003
Pierce™ Dye Removal Columns	Thermo Fisher Scientific	22858
Cell strainer (30 - 70 µm mesh)	Sysmex	04-004-2326
25 mL reservoir	Integra	4382
50 mL tubes	SLS	352070
Cyto-Surf® A	Fluidic Sciences	C303
Cyto-Surf® B	Fluidic Sciences	C304

Reagents

Product Name	Supplier	Catalogue Number
DyLight® 488 NHS Ester	Thermo Fisher Scientific	46402
PBS, pH 7.4, sterile filtered	Thermo Fisher Scientific	10010023
Recombinant Human TNF-α Protein	Bio-Techne Ltd	NBP2-35076-1mg
Goat Anti-Mouse IgG Fc (DyLight® 594) pre-absorbed	Abcam	Ab97017
Anti-TNF-α antibody [52B83]	Abcam	Ab1793
Mouse IgG Isotype Control	Invitrogen	31903
BupH™ Borate Buffer Packs	Thermo Fisher Scientific	28384

DMSO	Merck	67-68-5
RPMI-1640	ATCC	30-2001
Hybridoma 357-101-4	ECCAC	92030603
Glutamine	Merck	G7513-100ML
Solution 13 (AO-DAPI)	Chemometec	910-3013
Foetal Bovine Serum (FBS)	Gibco	A5670701
OptiPrep™ Density Gradient agent	Sigma-Aldrich	D1556
Poloxamer 188 Non-ionic Surfactant	Thermo Fisher Scientific	24040032

3. Method

3.1 Important notes before the start of the experimental work

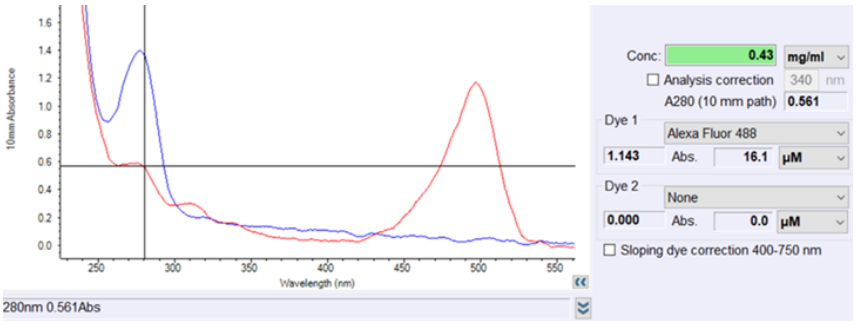
- Make sure the pipettes used have a valid calibration.
- Use best practice when pipetting, to ensure precision and accuracy.
- All steps involving handling any stock solution should be carried out in a Class II Microbiology Safety Cabinet (MBSC), except centrifugation and shaking operations (if relevant). During centrifugation and shaking all material must be contained within sealed containers to maintain sterility.
- For antigen labelling follow the supplier's manual (Themo Fisher Scientific, DyLight Amine-Reactive Dyes Instruction Manual). Antigen can be labelled using other optimized methodology and dyes with similar spectra.

3.2 Antigen labelling with DyLight® 488 (DL488)

Experimental Procedure

Step	Operation	Details
1	Prepare 1 mg/mL **antigen stock**	• Dissolve the TNF-α protein in 0.05 M sodium borate buffer (pH 8.5) to a final concentration of 1.1 - 2 mg/mL. ***Note:*** *Primary amines (e.g., ammonium ions, Tris, glycine, ethanolamine, triethylamine, glutathione), or imidazole will significantly inhibit protein labelling. Therefore, desalting or dialysis may be required prior to antigen labelling if the product contains these amines. Note that the presence of contaminant or additives (such as BSA) will also result in inefficient labelling due to competition with target protein.* *When calculating the volume of buffer to add, account for the subsequent dilution caused by adding the* ***DL488 dye stock (step 4)***
2	Prepare 10 mg/mL **DL488 dye stock**	• Take a vial of DyLight® 488 NHS Ester (DL488; 1 mg) from -20 °C, let it equilibrate to room temperature before opening to prevent moisture condensation. Spin briefly. • Open the vial, add in 100 µL of anhydrous dimethylformamide (DMF) or dimethyl sulfoxide (DMSO) • Mix well. • Spin briefly.
3	Calculate amount of dye needed for **Labelling Reaction**	• Use the following equation to calculate the appropriate volume of reactive dye to use. $\frac{mg\ \ protein}{MW\ \ of\ \ protein} \times\ 15 \times MW\ \ of\ \ fluor\ \ =\ \ mg\ \ of\ \ fluor$ MW: Molecular Weight; 15:Molar-fold excess of the NHS-ester dye to protein (calculated based on DyLight® Amine-Reactive Dyes Instructions, Thermo Fisher Scientific). Different antigen might require a different Molar excess. • Information required: - Weight of protein (TNF-α): 1 mg - MW of protein (TNF-α): 17,000 Dalton - MW of fluor: 1,011 Dalton • According to this equation, 1 mg of TNF-α requires addition of 0.892 mg of DL488 (89.2 µL of **DL488 dye stock from Step 2**). $\frac{1\ \ mg\ \ of\ \ \ TNF-\propto}{17{,}000\ \ \ MW} \times\ 15 \times 1{,}011\ \ =\ \ 0.892\ \ mg\ \ of\ \ DL488$

4	Make **Labelling Reaction**	• In a 1.5 mL Eppendorf tube, add TNF-α **antigen stock (Step 1)** and 89.2 µL of **DL488 dye stock (Step 2),** to make a final TNF-α concentration of 1 mg/mL. • Mix well and spin briefly. • Keep the tube in dark, at room temperature for **2 hours**. ***Note:*** *After the labelling reaction, it is crucial to purify the labelled protein to remove unreacted NHS ester, hydrolysed NHS ester products and other impurities.*
5	Purify **DL488-labelled antigen** from impurities and free dye	• Calculate the number of spin columns required for the purification as follows: $$No.\ columns = \frac{Volume\ of\ labelling\ reaction\ (\mu L)}{250\ \mu L}$$ • For the Labelling Reaction made in Step 4, the number of spin columns required is 5. • Insert the calculated number of spin columns into 2 mL Eppendorf tubes. • Mix the resin suspension well. • Pipette 400 µL of resin into the columns. • Spin the columns at 1,000×g for 30 seconds to remove the storage solution, discard the flow-through, replace with new 2 mL Eppendorf tubes. • Mark side of the spin columns where resin is slanted upward. For subsequent centrifugation steps, make sure that side is facing away from the rotor centre. • Add 400 µL PBS pH7.2 to each spin column, centrifuge for 2 minutes at 1,000xg, discard flow-through. • Immediately before protein purification, spin the columns again at 1,000×g for 30 seconds, discard the flow-through, replace with new 2 mL Eppendorf tubes. • Gently pipette 250 µL Labelling Reaction mix into the columns. Do not disrupt the resin bed. • Spin the columns again at 1,000×g for 30 seconds, to collect the purified labelled antigen. • Pool the eluents, transfer each sample to a fresh low-protein binding 1.5 mL Eppendorf tube. • Store at 4°C.

		Note: *For long term storage divide into single-use aliquots, freeze at -20 °C. For better sample preservation consider adding glycerol (final 50%). (Read Thermo Fisher Fluorescent Dye Removal Columns Instruction Manual)*
6	Determine **Degree of Labelling (DOL)**	· Load 2 µL of the **Purified DL488- labelled antigen (Step 5)** in the NanoDrop to measure its concentration by absorbance at 280 nm (A280) and at 493 nm (A493): - Follow instructions provided by instrument supplier - Blank: sample buffer - Molar Extinction coefficient for TNF-α: 21555 or 21430 ***Note:*** *Molar Extinction Coefficient can be calculated using the ProtParam tool on the Expasy Website using protein sequence or UniProt ID: https://web.expasy.org/protparam/. Two molar extinction coefficients are listed (according to the oxidation state of the Cys residues, which only differ marginally; use the average if in doubt.* · Data from a typical NanoDrop measurement is shown below. ***Note:*** *The recorded spectrum should show a peak at 280 nm for the protein (**see Figure 2**), otherwise the measured protein concentration is unreliable.*  **Figure 2. Typical quantification of protein concentration and dye concentration using NanoDrop.** Red Line represents absorption of protein at 280nm, Blue Line represents measurement of DL 488. · Using the data extracted from NanoDrop, follow instructions provided by instrument supplier of the labelling product (Thermo Fisher Scientific, Cat# 46402) to calculate protein concentration in µM using the following equation: $$[protein,\ \mu M] = \frac{[protein(\frac{mg}{mL})]}{MW(protein,\ Da)} \times 10^6$$ · Calculate the degree of labelling (DOL): $$DOL = [dye,\ \mu M] \div [protein,\mu M]$$ ***Note:*** *Ideal DOL (indicating the molar ratio of dye to protein) can fall within 0.5-1 (low DOL), or higher range (2-10), however the optimal DOL is highly dependent on nature of the target protein and the application of interest. Care should be taken especially with FRET assay, as over-labelling might result in fluorescent dye molecules quenching, or negatively affect the protein's biological activity or solubility.*

3.3 FRET Probe Optimization Using Plate Reader

The following section describes the procedure for evaluating donor-to-acceptor ratios using a plate reader. This is a critical step in the protocol to ensure good assay performance when running Cyto-Mine® and maximize value from the Cyto-Mine® experiment.

In our experience, a donor-to-acceptor FRET probe ratio of 1 µg : 20 µg (donor: TNF-α antigen; acceptor: anti-mouse IgG Ab) provides a strong FRET signal in the presence of secreted anti-TNF-α antibodies and enables successful isolation of antigen-specific Antibody Secreting Cells (ASCs). However, as DOL of the antigen may vary between users, we recommend testing a range of donor-to-acceptor ratios to optimize assay sensitivity before running samples on the Cyto-Mine®.

Experimental Procedure

<table>
<tr><th>Step</th><th>Operation</th><th>Details</th></tr>
<tr><td>1</td><td>Prepare Master Mix</td><td>
• Allow probes to equilibrate to room temperature:
- Donor: DL488- labelled TNF-α antigen from section 3.2 (Stock: 1 mg/mL).
- Acceptor: DL594- Goat anti-mouse IgG Fc (Stock: 0.5 mg/mL)
- Target anti-TNF-α IgG stock 0.1 mg/mL

• Recommended Donor-to-Acceptor mass ratio to be tested
- Ratio 1 – Donor-to-Acceptor ratio 1 : 20
- Ratio 2 – Donor-to-Acceptor ratio 2 : 20
- Ratio 3 – Donor-to-Acceptor ratio 4 : 20

• Using a 1.5 mL Eppendorf tube prepare the following Master Mix (final reaction volume 500 µL):
<table>
<tr><th rowspan="2">Component</th><th colspan="3">Volume (µL)</th></tr>
<tr><th>Ratio1</th><th>Ratio2</th><th>Ratio3</th></tr>
<tr><td>Culture medium</td><td>379.5</td><td>379</td><td>378</td></tr>
<tr><td>Goat Anti-Mouse IgG Fc (DL594)</td><td>20</td><td>20</td><td>20</td></tr>
<tr><td>Antigen (DL488)</td><td>0.5</td><td>1</td><td>2</td></tr>
<tr><td>Total</td><td>400</td><td>400</td><td>400</td></tr>
</table>
• Note: An isotype mouse IgG control antibody with no specificity to TNF-α may be used as negative control.
</td></tr>
<tr><td>2</td><td>Label dilution plate</td><td>
• Label 1x 96 well microtiter plate for the dilutions as shown to the right :
<table>
<tr><th></th><th>1</th><th>2</th><th>3</th><th>4</th><th>5</th><th>6</th><th>7</th><th>8</th><th>9</th><th>10</th><th>11</th><th>12</th></tr>
<tr><td>A</td><td colspan="8">Ratio 1</td><td></td><td></td><td></td><td></td></tr>
<tr><td>B</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>C</td><td colspan="8">Ratio 2</td><td></td><td></td><td></td><td></td></tr>
<tr><td>D</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>E</td><td colspan="8">Ratio 3</td><td></td><td></td><td></td><td></td></tr>
<tr><td>F</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>G</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>H</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
</table>
</td></tr>
</table>

<table>
<tr>
<td>3</td>
<td>Prepare the first dilution</td>
<td>

Make up the first dilution for each donor-to-acceptor ratio using the relevant Master Mix (Step 1) and purified mouse anti-TNF-α antibody (Abcam, Ab1793)
- Ratio 1: well A1
- Ratio 2: well C1
- Ratio 3: well E1

<table>
<tr><th>Component</th><th>Volume (µL)</th></tr>
<tr><td>Master Mix</td><td>80</td></tr>
<tr><td>Mouse anti-TNF-α antibody</td><td>20</td></tr>
<tr><td>Total</td><td>100</td></tr>
</table>
Note: The concentration of the mouse anti-TNF-α antibody for the first dilution is 20 µg/mL (133 nM).
</td>
</tr>
<tr>
<td>4</td>
<td>Adjust concentration of Master mix</td>
<td>

Add 80 µL fresh buffer or culture medium used to prepare the Mouse anti-TNF-α to the remaining Master Mix.

Note: This step serves to adjust the remaining Master Mix to the same concentration as in Well A1. The dilution factor (20/100=0.2) is applied to the Master Mix (500 µL) without the volume of the added target antibody (20 µL) (500*0.2-20=80 µL) giving a volume of 80 µL to be added.
</td>
</tr>
<tr>
<td>5</td>
<td>Serial Dilution</td>
<td>

Add 50 µL of the Master Mix from Step 4 into the following wells:
- Ratio 1: well A2 – A8
- Ratio 2: well C2 – C8
- Ratio 3: well E2 – E8
To make a ½ serial dilution of the target IgG, transfer 50 µL:
- Ratio 1: from well A1 to A2
- Ratio 2: from well C1 to C2
- Ratio 3: from well E1 to E2
Mix contents by pipetting up and down before transferring 50 µL:
- Ratio 1: from well A2 to A3
- Ratio 2: from well C2 to C3
- Ratio 3: from well E2 to E3
Continue serially diluting the anti TNF-α IgG to prepare 0.31 µg/mL (Well A7/C7/E7).
Keep wells A8/C8/E8 as Blank (no IgG).
This will give the following concentration of mouse anti-TNF-α antibody in the wells.

<table>
<tr><th></th><th>1</th><th>2</th><th>3</th><th>4</th><th>5</th><th>6</th><th>7</th><th>8</th></tr>
<tr><td>A/C/E</td><td>20 µg/mL, 133.3 nM</td><td>10 µg/mL 66.7 nM</td><td>5 µg/mL, 33.3 nM</td><td>2.5 µg/mL 16.7 nM</td><td>1.25 µg/mL 8.3 nM</td><td>0.63 µg/mL 4.2 nM</td><td>0.31 µg/mL 2.1 nM</td><td>0 µg/mL, 0 nM</td></tr>
</table>
</td>
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</table>

<table>
<tr>
<td>6</td>
<td>Label assay plate</td>
<td>

Label 1x black bottom 384 well microtiter assay plate, as shown below:

<table>
<tr><th></th><th>1</th><th>2</th><th>3</th><th>4</th><th>5</th><th>6</th><th>7</th><th>8</th><th>9</th><th>10</th><th>11</th><th>12</th><th>13</th><th>14</th><th>15</th><th>16</th><th>17</th><th>18</th><th>19</th><th>20</th><th>21</th><th>22</th><th>23</th><th>24</th></tr>
<tr><td>A</td><td colspan="8" rowspan="3">Ratio 1</td><td colspan="16"></td></tr>
<tr><td>B</td><td colspan="16"></td></tr>
<tr><td>C</td><td colspan="16"></td></tr>
<tr><td>D</td><td colspan="8"></td><td colspan="16"></td></tr>
<tr><td>E</td><td colspan="8" rowspan="3">Ratio 2</td><td colspan="16"></td></tr>
<tr><td>F</td><td colspan="16"></td></tr>
<tr><td>G</td><td colspan="16"></td></tr>
<tr><td>H</td><td colspan="8"></td><td colspan="16"></td></tr>
<tr><td>I</td><td colspan="8" rowspan="3">Ratio 3</td><td colspan="16"></td></tr>
<tr><td>J</td><td colspan="16"></td></tr>
<tr><td>K</td><td colspan="16"></td></tr>
<tr><td>L</td><td colspan="8"></td><td colspan="16"></td></tr>
<tr><td>M</td><td colspan="8"></td><td colspan="16"></td></tr>
<tr><td>N</td><td colspan="8"></td><td colspan="16"></td></tr>
<tr><td>O</td><td colspan="8"></td><td colspan="16"></td></tr>
<tr><td>P</td><td colspan="8"></td><td colspan="16"></td></tr>
</table>
</td>
</tr>
<tr>
<td>7</td>
<td>Transfer the samples in triplicate into assay plate</td>
<td>

Transfer 3×15 µL of each sample from dilution plates from Step 5, into the assay plate to set up triplicate wells for each condition.
The layout of samples in the 384 well assay plate is shown below:

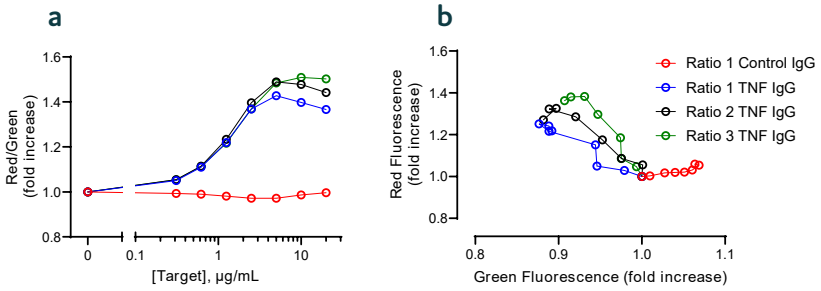
<table>
<tr><th></th><th>1</th><th>2</th><th>3</th><th>4</th><th>5</th><th>6</th><th>7</th><th>8</th></tr>
<tr><td>A</td><td rowspan="3">20 µg/mL, 133.3 nM</td><td rowspan="3">10 µg/mL 66.7 nM</td><td rowspan="3">5 µg/mL, 33.3 nM</td><td rowspan="3">2.5 µg/mL 16.7 nM</td><td rowspan="3">1.25 µg/mL 8.3 nM</td><td rowspan="3">0.63 µg/mL 4.2 nM</td><td rowspan="3">0.31 µg/mL 2.1 nM</td><td rowspan="3">0 µg/mL, 0 nM</td></tr>
<tr><td>B</td></tr>
<tr><td>C</td></tr>
<tr><td>D</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>E</td><td rowspan="3">20 µg/mL, 133.3 nM</td><td rowspan="3">10 µg/mL 66.7 nM</td><td rowspan="3">5 µg/mL, 33.3 nM</td><td rowspan="3">2.5 µg/mL 16.7 nM</td><td rowspan="3">1.25 µg/mL 8.3 nM</td><td rowspan="3">0.63 µg/mL 4.2 nM</td><td rowspan="3">0.31 µg/mL 2.1 nM</td><td rowspan="3">0 µg/mL, 0 nM</td></tr>
<tr><td>F</td></tr>
<tr><td>G</td></tr>
<tr><td>H</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>I</td><td rowspan="3">20 µg/mL, 133.3 nM</td><td rowspan="3">10 µg/mL 66.7 nM</td><td rowspan="3">5 µg/mL, 33.3 nM</td><td rowspan="3">2.5 µg/mL 16.7 nM</td><td rowspan="3">1.25 µg/mL 8.3 nM</td><td rowspan="3">0.63 µg/mL 4.2 nM</td><td rowspan="3">0.31 µg/mL 2.1 nM</td><td rowspan="3">0 µg/mL, 0 nM</td></tr>
<tr><td>J</td></tr>
<tr><td>K</td></tr>
</table>

Briefly, spin the assay plate, without a lid, in a centrifuge to remove any bubbles in the samples.

</td>
</tr>
<tr>
<td>8</td>
<td>Measure FRET response in the Plate Reader</td>
<td>

Load the assay plate into the plate reader and measure the fluorescence in both green and red channel, using the following recommended settings:
Excitation: 488 nm
Emission: λmax= 520 nm (Green Channel), and λmax= 620 nm (Red Channel)

</td>
</tr>
</table>

		Note: *Do not excite with 594 nm laser, as this laser would directly excite the acceptor probe. No further incubation is necessary, as the binding equilibrium is quickly reached. However, if an incubation step is desired, it should be performed at room temperature in the dark.*
9	Generate **Standard Curve**	<u>Average fluorescent intensity:</u> · Calculate the average fluorescence intensity for both Green & Red Channels using the triplicate measurements for each condition. <u>Red/Green ratio:</u> · Divide **Average Red fluorescence intensity by Average Green fluorescence intensity** for each mouse anti-TNF-α antibody concentration. <u>Normalize the Red/Green ratio:</u> · Divide the **Red/Green ratio** for each mouse anti-TNF-α antibody concentration with the ratio obtained for 0 nM (Blank). <u>Standard curve:</u> · Plot the **Normalized Red/Green ratio** against mouse anti-TNF-α antibody concentrations (**Figure 3a**). <u>Fluorescence v Green Fluorescence:</u> · Plot **Average Red fluorescence intensity** versus **Average Green fluorescence** intensity to estimate what the FRET response signal will look like on the Cyto-Mine® scatter plots (**Figure 3b**).  **Figure 3. Analysis of FRET assay in plate reader.** (a) Ratio between Red/Green fluorescence whereby fixed amount of FRET probes were tested with increased concentrations of recombinant target antibody. Notably, reaction of same FRET probes with unrelated mouse antibody (Ratio 1 control IgG) did not result in FRET response. (b) Alternative plot, shown as XY plot of Red vs Green fluorescence, respectively, for better visualization of predicted Cyto-Mine® detection.
10	Choose the optimal **Donor:Acceptor FRET probe**	<u>Signal trend:</u> · The Red/Green (R/G) ratio should rise with increasing IgG concentration on the standard curve. <u>Detection threshold:</u> · To achieve robust detection performance in Cyto-Mine® the minimum recommended increase in normalized R/G ratio is 1.1 across the 0 – 20 µg/ml IgG range.

		Assay performance expectations: • Well-performing donor-to-acceptor ratio pairs typically produce a **1.2–5-fold increase** in R/G ratio within the **0–20 µg/mL** IgG range. • Example: TNF-α assay shows ~1.5-fold increase in this range. Hook effect: • At very high IgG concentrations, the R/G ratio may **drop due to probe saturation by the analyte** ("hook effect"). • Commonly seen around **5 µg/mL** in the TNF-α assay. Data interpretation: • Plotting **red vs. green fluorescence intensities** helps visualize FRET behaviour. • **Low IgG**: high green, low red (bottom right of plot). • **Increasing IgG**: signal shifts toward **higher red and lower green** (top left). General considerations: • Minor fluctuations can occur at low target levels. • Hook effect may reappear at very high concentrations. Optimal Donor:Acceptor FRET probes: • The assay performance was evaluated based on the following acceptance criteria: - The FRET Red/Green signal must be at least 1.1-fold greater than the background. - No saturation of the FRET signal should be observed at antigen-specific antibody concentrations below 5 µg/mL. • All tested ratios satisfied these criteria. Ratio 1 (1:20), which utilized the lowest donor concentration, was therefore selected for subsequent assay on the Cyto-Mine® platform.

3.4 Assay performance on Cyto-Mine®

This section describes the procedure for performing a Cyto-Mine® experiment based on the assay performance demonstrated using the plate reader.

This protocol provides summary information regarding experimental conditions for sample preparation and operating Cyto-Mine® and is intended for users that are already familiar with operating the Cyto-Mine®.

For more information of how to operate the Cyto-Mine® please refer to the Cyto-Mine® User Manual.

<table>
<tr><th>Step</th><th>Operation</th><th>Details</th></tr>
<tr><td>1</td><td>Cyto-Mine® Set Up
System Warm Up</td><td>• Start Cyto-Mine® GUI software and start System Warm Up Step (Hands Off Step).

Note: To save time, run the System Warm Up before preparing cells</td></tr>
<tr><td>2</td><td>Prepare
Encapsulation Medium</td><td>• Prepare the Encapsulation Medium as follows (1.5 mL total):

<table>
<tr><th>Component</th><th>Made in Section</th><th>Stock Conc.</th><th>Final Conc.</th><th>Volume (µL)</th></tr>
<tr><td>Culture medium</td><td></td><td>N/A</td><td>74% v/v</td><td>1050</td></tr>
<tr><td>Opti-Prep</td><td>N/A</td><td>60% w/v</td><td>16% v/v</td><td>240</td></tr>
<tr><td>Pluronic™ F-68</td><td>N/A</td><td>10%</td><td>10% v/v</td><td>150</td></tr>
<tr><td>DL488- labelled TNF-α antigen</td><td>3.2</td><td>1 mg/mL</td><td>1 µg/mL</td><td>1.5</td></tr>
<tr><td>DL594- Goat anti-mouse IgG Fc</td><td>N/A</td><td>0.5 mg/mL</td><td>20 µg/mL</td><td>60</td></tr>
</table>

Note: Protocol is shown for 1.5 mL of sample mix to accommodate for sample loss during the preparation. Only 1 mL of sample mix is required for the Cyto-Mine® experiment.

Do not use any media that is expected to contain antibodies (e.g. conditioned media) as it will affect the FRET assay.

Culture media containing supplements required for cell growth may be used to make up the Encapsulation Medium, however, please do not use more than 10% foetal bovine serum (FBS) and any detergents as they may affect droplet integrity.</td></tr>
<tr><td>3</td><td>Prepare Antibody Secreting Cells (ASC)</td><td>• Resuspend Antibody Secreting Cells (ASC; 357-101-4) in tissue culture flask

• To count cells using NucleoCounter® NC300:
- Take out 19 µL of cell culture
- Add 1 µL Solution 13 (AO-DAPI)
- Add 10 µL of this mix into a glass slide
- Run the measurement to obtain cell density and viability

• Calculate the volume of cells corresponding to 4.5 x10^5 cells (for final cells concentration of 3 x10^5 cells in 1.5 mL of sample

• Transfer the calculated volume into clean 1.5 mL Eppendorf tube

• Spin cells down at 300xg for 5 minutes at room temperature

• Gently remove medium from the tube without disturbing the pellet

• Gently resuspend the pellet in 1 mL of fresh culture medium

• Spin cells down at 300xg for 5 minutes at room temperature</td></tr>
</table>

		• Gently remove medium from the tube without disturbing the pellet • Gently resuspend the pellet in 1.5 mL of Encapsulation Medium (from **Step 2**) • Filter the mixture through a 30 µm mesh filter to eliminate any cell clumps or aggregates and collect into a sterile 1.5 mL Eppendorf tube. • Count and record cell number in the final sample using the NucleoCounter® NC300 ***Note:*** *Cells are kept at room temperature during the washes to avoid 'cold capture'.* *Cell encapsulation in droplets is a stochastic process that follows a Poisson distribution of randomly dispersed entities. This relationship can be utilized to predict droplet occupancy by single cells at a specified cell concentration. The Poisson model can be applied to determine the optimal experimental parameters—namely, the cell concentration required to achieve a desired proportion of droplets containing single cells. Please contact Technical Support for more details.*
4	**Load** cells into Cyto-Cartridge®	• Load 1 mL of prepared sample into the Cyto-Cartridge, then place the Cyto-Cartridge® into the Cyto-Mine®. • Follow the instructions shown on screen. ***Note:*** *Load the Cyto-Cartridge® aseptically in a Biosafety Cabinet.* ***Cyto-Cartridge® is single-use*** *and disposable to prevent any cross-contamination.*
5	Cyto-Mine® Set Up & Run	<u>Experimental Parameters</u> • Number of picodroplets to generate: 1.5 x 10^6 • Time and temperature for incubation: 120 minutes at 37°C. • Type of microtiter plates (MTPs) for dispensing: 96-Well Plate. • Input final cell density loaded based on the information obtained from **Step 3**. • Set up cameras focus and other parameters (please refer to Cyto-Mine® User Manual for further information) <u>Cyto-Cartridge® priming</u> • Hands-off step. • Time: 30 minutes.

Picodroplet generation/cell encapsulation

- Hands-off step.
- Picodroplet size: 450 pL.
- Time: 36 minutes.

Picodroplet incubation/antibody secretion

- Hands-off step.
- Incubation temperature: 37 °C.
- Incubation time: 2 hours

Note: *according to predicted cell secretion rate, a shorter or longer incubation can be applied*

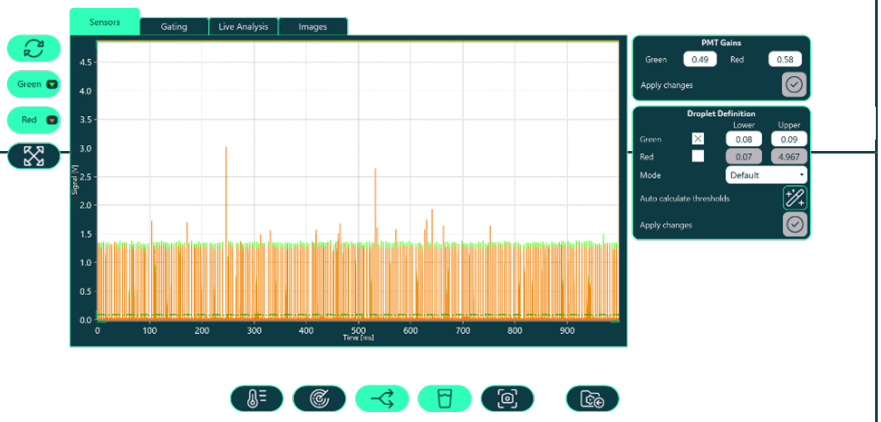
Flow Stabilization before Sorting

- Wait until this step is automatically finished

Note: *Flow of picodroplets stabilizes following 3-4 minutes of run.*

Sorting

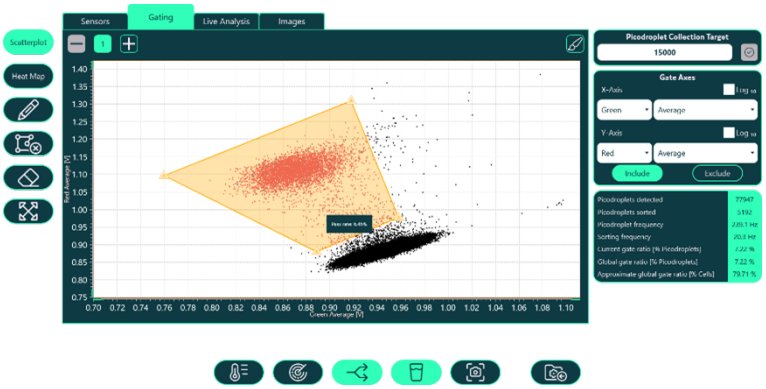
- Set up detection:
  - Set up PMT Gains for Green & Red to achieve the readout peak (y axis=Signal) at the height of 1-1.5 voltage (for representative example see below)
  - Detection trigger: Green above Threshold.



- Auto-Thresholds for detection of fluorescence

Note: *If using Cyto-Mine® Chroma, turn ON the Blue Laser (488 nm) for excitation of FRET donor and use Green &* **Orange** *PMTs.*

Make sure that if Cyto-Mine® Chroma is used, the Orange laser is switched off, as this laser will directly excite the acceptor probe.

		Gating · Max. Picodroplets to Collect: 15,000 · Y-Axis: Red Average · X-Axis: Green Average · Draw a polygon on the scatter plot to gate the FRET positive events  Acquire date · Sort 15,000 positive droplets if the aim is to dispense (**see Step 6**). To analyse antibody secretion within the cell population without dispensing, acquire 5 x10^5 total droplets. · The data acquired from this step will be used for further analysis (**see Data Analysis**)
6	End of experiment or Dispensing	· Dispensing of droplets is not required if the aim is solely to evaluate cell secretion. · If dispensing antigen-specific ASC is required, please follow the Cyto-Mine® user manual. · If cells are required to be dispensed for downstream PCR analysis, please contact the Fluidic Sciences support team (support@fluidic.com). ***Note:*** *Keep the Orange laser switched off during dispensing on the Cyto-Mine® Chroma to avoid direct excitation of the acceptor fluorescence.*

4. Data Analysis

This section describes how to use the FlowJo™ software to analyze data generated in Cyto-Mine®.

To quantify the FRET-positive population using FlowJo™:

- Open the experiment file using Cyto-Mine® Data Analysis software.
- Export .csv format files of the sorting and/or dispensing data.
- Open the .csv files in FlowJo™ software.
- Plot the sorting graph using Green Average Fluorescence and Red Average Fluorescence as X and Y axes, respectively.
- Identify the negative population of picodroplets (the main population found in the lowest right corner of the scatterplot, see **Figure 4**).
- Identify the FRET positive population (population of picodroplets that extend towards top left corner – reduced red signal and increased green signal).
- Draw a polygon around FRET positive population to quantify the percentage of FRET positive picodroplets (an example is shown in **Figure 4**).
- This information can be used to determine the percentage of cells that show antigen-specific antibody secretion. An example is shown below where 0.1 x10^6 cells were encapsulated in Cyto-Mine®:
    - Estimated proportion of ASC in total cell population (Poisson distribution):
        - 95.6% of empty droplets
        - 4.3% of droplets containing single cells
        - 0.1% of droplets containing ≥ 2 cells
    - % FRET positive droplets: 0.8% gated FRET positive using FlowJo™ data analysis

$$\%ASC = \frac{0.8}{4.4} \times 100 = 18.18\%$$

Figure 4 shows example Cyto-Mine® data acquired for the hybridoma cell line 357-101-4 using the TNF-α antigen-specific FRET assay described in this protocol. A detectable FRET response (**Figure 4** gated population) was observed after only 15 minutes' incubation of encapsulated cells with FRET probes in Cyto-Mine®. To assess whether extending the antibody secretion time increased signal intensity, droplets were incubated for a further 45–165 minutes. An additional 45 minutes (60 minutes total) resulted in increased FRET signal and a higher percentage of droplets showing a positive response. Extending incubation to 120 and 180 minutes did not produce any further increase, indicating that 60 minutes is sufficient.

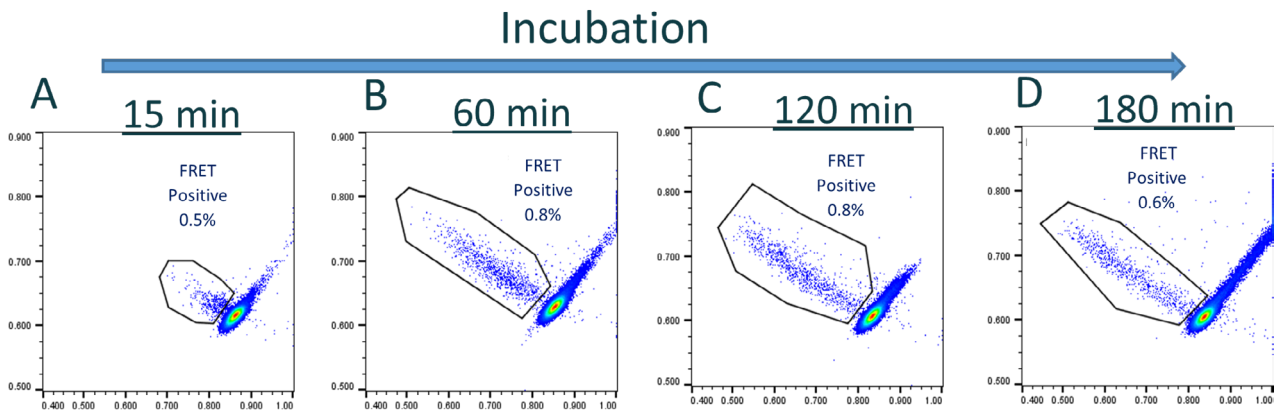


Figure 4. Time course analysis of ASC FRET response measured on Cyto-Mine® over 15 - 180 minutes of incubation. Hybridoma cell line 357-101-4 was incubated for different lengths of time and scatter plots of picodroplets were analysed using FlowJo™ software. FRET positive population (gated by a polygon) was detected using TNF-α specific FRET assay and represents picodroplets containing cells secreting anti-TNF-α mouse IgG.

5. Experiment Optimization and Troubleshooting

Step	Operation	Details
Antigen labelling	Poor labelling efficiency/low DOL	· Make sure that the antigen buffer does not contain primary amines or additional proteins. Proceed with desalting or purification if required.
Antigen labelling	High DOL	· Make sure that most of unbound dye was removed. Proceed with another round of purification using spin columns or gel filtration
Antigen labelling (DOL calculation)	No apparent peak during Nanodrop measurement	· Check if the buffer used as blank matches the buffer in which the protein is dissolved. · Check for presence of leftover reagents from the labelling procedure as it may interfere with the absorption measurement at 280 nm. Further purification of the conjugate, e.g. by extensive dialysis may help in removing interfering substances.
FRET assay in plate reader	Suboptimal performance/no FRET response	· Make sure that antigen was not over labelled, resulting in loss of function or fluor quenching. · Use different analytical methods to evaluate protein integrity and function such as protein gel, or ELISA · Determine FRET response using wider range of Donor : Acceptor ratio. The concentrations/ratios suggested in this protocol tend to work well, but lower or higher concentrations of Donor (or Acceptor) may result in better sensitivity or assay range. Lower donor concentrations tend to decrease the lower limit of detection (LLOD) at the expense of assay range. Higher donor concentrations increase the LLOD but may increase assay range.

		• Consider the use of an anti-Fab antibody as the acceptor to ensure the distance between donor and acceptor is not greater than the optimal Förster radius for FRET • Unbound dye was not completely removed, causing high green background. Proceed with additional purification using spin columns or gel filtration methods
FRET assay in plate reader or in Cyto-Mine®	Suboptimal performance	• Confirm assay compatibility with experimental conditions by testing it in the presence of all the additives required for cell culturing (FBS, Glutamine, etc).
FRET assay in Cyto-Mine®	Low proportion of positive picodroplets / no positive population	• Test cell productivity in Cyto-Mine® by using general IgG FRET assay (use Cyto-Cellect Mouse®, Fluidic Sciences, C312). • If possible, test compatibility of the antigen-specific FRET assay by testing the cell culture supernatant containing secreted antibody. • Make sure to avoid any Ab contamination in the encapsulation media (i.e. conditioned media) and to wash the secreting cells during sample preparation.
FRET assay in Cyto-Mine®	Low proportion of positive picodroplets	• Assure no cell loss in the final Cyto-Mine® sample. • Ensure all required additives (Opti-Prep, PF-68 and FRET probes) have been added to Encapsulation medium.
FRET assay in Cyto-Mine	High level of fused / destabilized droplets	• Make sure your Encapsulation Media does not contain any detergents or more than 10% FBS.

Notes

Notes

sphere bio

work small, think big

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