Analysis of individual mitochondria via fluorescent immunolabeling with Anti-TOM22 antibodies

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A. IgG1 isotype control images

Fig. S1. Control ruling out major colocalization of Atto-488 IgG1 isotype antibody with mito-DsRed2. (i) Atto-488 IgG1 isotype control, (ii) DsRed2, and (iii) overlay of (i) and (ii). Note that DsRed2 localization is not equally distributed in all mitochondria. Scale bar: 100 µM. This figure is relevant to subsection “LIF Analysis of Individual Immunolabeled Mitochondria” of this manuscript
B. Crosstalk Corrections

Flowgrams of mitochondria labeled with only one fluorophore, DsRed2 or Atto-488, were obtained to determine a correction factor for cross-talk between PMTs used to detect green and red events, respectively. (See subsection “Data Analysis” in the manuscript and Figure S2 below). This factor was determined empirically by fitting a third order polynomial to peak intensities. Mitochondrial samples had 50 µg/mL in protein contents and were either isolated from: (i) DsRed2 143B osteosarcoma cells or (ii) 143B osteosarcoma cells and then immunolabeled with 32 µg/mL (2.1×10^{-7} M) Atto-488 Anti-TOM22 Antibodies. Due to possible variations in crosstalk due to native fluorescence, these single-fluorescent controls were taken daily and were used to normalize fluorescence of data collected on the same day.

As an example, the polynomial curve correction for red-to-green cross-talk described in the upper graph of Figure S2 is:

\[ \text{DsRedFit}(x) = p_1 x^2 + p_2 x + p_3 \]  

(Equation S1)

Where DsRedFit(x) corresponds to the \( C_{(Rm)} \) in Equation 1 of the manuscript, \( x \) represent red intensities of coincident events used in the fitting, and each coefficient, including its 95% confidence bounds in parenthesis, is

\[ p_1 = 0.0097 \ (0.0083, \ 0.0110) \]
\[ p_2 = 0.0015 \ (-0.0105, \ 0.0136) \]
\[ p_3 = 0.049 \ (0.026, \ 0.0714) \]

Similarly, an example of the polynomial curve correction for green-to-red cross-talk described in the lower graph of Figure S2 is:

\[ \text{Atto488Fit}(x) = p_1 x^2 + p_2 x + p_3 \]  

(Equation S-2)

Where Atto488Fit(x) corresponds to the \( C_{(Gm)} \) in Equation 2 of the manuscript, \( x \) represents the green intensities of coincident events used in the fitting, and each coefficient, including its 95% confidence bounds in parenthesis, is

\[ p_1 = 0.16 \ (0.08, \ 0.25) \]
\[ p_2 = -0.056 \ (-0.170, \ 0.058) \]
\[ p_3 = 0.078 \ (0.045, \ 0.111) \]
**Fig. S2.** Determination of cross-talking. The green and red intensities of coincident peaks of red only and green only controls are included in these plots. Red lines represent polynomial fits used to for crosstalk correction. For DsRed2 corrections (upper graph) to the green channel, red fluorescent peak intensities of coincident events were plotted on the x-axis while green fluorescent peak intensities of coincident events were plotted on the y-axis. Likewise, for Atto-488 corrections (lower graph) to the red channel, green fluorescent peak intensities of coincident events were plotted on the x-axis while red fluorescent peak intensities of coincident events were plotted on the y-axis.

**C. Calculation of the Number Atto-488 Anti-TOM22 Antibodies per Mitochondrion**

As mentioned in the manuscript, to estimate the number of Atto-488 Anti-TOM22 antibodies per mitochondrion the following steps were taken: (i) Assigning molecular equivalent of soluble fluorophore (MESF) values to a fluorescein microbead standard, (ii) determining the relative fluorescence response between fluorescein and Atto-488, (iii) comparing the fluorescent response of fluorescein microbeads and Alignflow 488 flow cytometry alignment beads, and (iv) calculating the number of Atto-488 Anti-TOM22 antibodies labeling each mitochondrion.

(i) Assigning MESF values to a fluorescein microbead standard. The total number of 0.22-µm diameter fluorescein microspheres in suspension was determined as:

\[
\frac{\text{Microspheres}}{\text{mL}} = \frac{C \times 10^{12}}{\rho \left(\frac{4}{3}\pi r^3\right)}
\]

(Equation S3)
Where C is the concentration of microspheres in grams per milliliter, \( \rho \) is the density of polystyrene (1.05 g/mL) and \( r \) is the radius of the microspheres in micrometers. \( C \) was determined by drying 100 µL aliquots of microspheres overnight at 60°C and measuring the mass of each aliquot. The concentration was \( 3.3 \times 10^8 \) microspheres/mL.

The fluorescence of a fluorescein microbead suspension in MIB (diluted 10,000 \( \times \) from the initial concentration) was measured in a spectrofluorometer set for excitation at 488-nm and emission at 517-553 nm. These excitation/emission conditions are equivalent to those used in the home-built capillary cytometry-LIF setup. The fluorescence readings covering the entire emission range were integrated and used to estimate MESF by comparison with calibration curves of fluorescein and Atto-488 ranging in concentrations from \( 6 \times 10^{-9} \) to \( 5 \times 10^{-8} \) M. These curves were prepared using GraphPad Prism 5 software. The equivalent fluorescein concentration of the microbead suspension was 21 nM. Therefore, the MESF was calculated as

\[
MESF_{\text{fluorescein}} = \frac{2.1 \times 10^{-15} \text{mol}}{3.3 \times 10^8 \text{microspheres/mL}} = 6.4 \times 10^{-20} \text{mol/microsphere} = 3.8 \times 10^4 \text{molecules/microsphere}
\]

(Equation S4)

(ii) Determining the relative fluorescence response between fluorescein and Atto-488. The integration of the fluorescence response of equal concentrations of fluorescein and Atto-488 measured over 517-553 nm was used to estimate relative fluorescence response under the excitation/emission conditions used in the home-built capillary cytometry-LIF setup. The relative fluorescence response and the relationship between MESF\textsubscript{fluorescein} and the MESF for Atto-488 (MESF\textsubscript{488}) was

\[
\frac{MESF_{\text{Atto-488}}}{MESF_{\text{fluorescein}}} = \frac{\text{Atto-488 Emission}}{\text{Fluorescein Emission}} = 1.5 \pm 0.1
\]

(Equation S5)

Thus, this relationship makes possible to convert MESF of fluorescein to MESF of Atto-488.

(iii) Comparing the fluorescent response of fluorescein microbeads and Alignflow 488 flow cytometry alignment beads. The latter were used to calibrate the instrument on different days. In order to use only the fluorescence response of Alignflow 488 beads in the calculations that follow, the median fluorescent peak intensities of these beads and fluorescein microspheres were compared. The medians were 1.1 and 3.0 Relative fluorescent units (RFU), respectively. On subsequent days, the median of the fluorescence of the Alignflow 488 beds (\( M_{\text{Align}} \)), was used to calculate a projected fluorescence response for fluorescein microspheres (\( P_{\text{fluorescein}} \)) using the equation

\[
P_{\text{fluorescein}} = \frac{3.0}{1.1} M_{\text{Align}}
\]

(Equation S6)
(iv) Calculating the number of Atto-488 Anti-TOM22 antibodies labeling each mitochondrion. When the fluorescence intensity of a single mitochondrion labeled with Atto-488 Anti-TOM22 is $F_{\text{Atto-488}}$, the projected number of equivalent fluorescent molecules ($P_{\text{Atto-488-mol}}$) in this mitochondrion can be calculated as

$$P_{\text{Atto-488-mol}} = \frac{\text{MESF}_{\text{fluorescein}}}{1.5 \times P_{\text{fluorescein}}} F_{\text{Atto-488}}$$  \hspace{1cm} (Equation S7)

where MESF_{fluorescein} and P_{fluorescein} have been defined by Equations S4 and S6, respectively and the factor 1.5 accounts for the relationship described by Equation S5.

Because the Atto-488:antibody molar ratio is 3:1 (provided by the vendor) the total number of Anti-TOM22 antibodies bound to each mitochondrion ($N_{\text{Anti-TOM22}}$) is

$$N_{\text{Anti-TOM22}} = \frac{P_{\text{Atto-488-mol}}}{3}$$  \hspace{1cm} (Equation S8)

For instance, when $M_{\text{Align}}$ is 3.8 RFU and the median of $F_{\text{Atto-488}}$ is 2.7 RFU, $P_{\text{fluorescein}}$ is 10.4 RFU (Equation S6), the median $P_{\text{Atto-488-mol}}$ is $6.6 \times 10^4$ Atto-488 molecules (Equation S7), and the median $N_{\text{Anti-TOM22}}$ is $2.2 \times 10^3$ Anti-TOM22 antibodies.

To use Equation S7 to estimate $P_{\text{Atto-488-mol}}$ at the lowest $F_{\text{Atto-488}}$ value observed (60 and $6.7 \times 10^2$ respectively), the ratio of MESF_{fluorescein} to $P_{\text{fluorescein}}$ must be constant when corresponding $P_{\text{fluorescein}}$ are much lower than median values used in Equation 6. We examined this requirement by comparing the median fluorescence intensities (capillary cytometry-LIF) and the MESF of microspheres with different fluorescein contents (Figure S3). Using Equation S5, for the lowest observed $F_{\text{Atto-488}}$ values, the corresponding fluorescein intensity is $(4.4 \times 10^2)$ falls within the linear range defined by the standards used in Figure S3. (Median capillary cytometry-LIF intensities are $3.6 \times 10^1 \pm 5.3 \times 10^1$, $1.1 \pm 1.4$, and $4.7 \pm 4.6$ RFU for beads with corresponding MESF values of $22 \pm 7$, $4.2 \times 10^3 \pm 2.2 \times 10^3$, and $2.5 \times 10^4 \pm 7.6 \times 10^3$, respectively).
Calibration Curve Demonstrating Linearity of Bead Response via Capillary Cytometry

**Fig. S3.** Relationship between fluorescence intensities of individual microspheres determined by capillary cytometry-LIF and and their respective MESF. Fluorescein conjugated bead standards were 0.2, 0.1, and 0.02 micron in diameter. The trend line is \( y = (1.8 \pm 0.01) \times 10^{-4} \pm 1 \times 10^{-6} x + 0.37 \pm 0.02 \) \( (r^2 = 0.999) \). X error bars represent the variation (~30-50%) determined by propagation of error. Y error bars represent variation of relative fluorescent intensity of beads obtained via capillary cytometry (RSD ~100%)

**D. Isotype Controls**

Mitochondria isolated form 143B cells were incubated with various concentrations of Atto-488 IgG1 mouse isotype antibodies. The samples were then analyzed by capillary cytometry using the conditions described under “Capillary Cytometry” subsection in the manuscript. The level of non-specific binding of Atto-488 IgG1 isotype antibodies to DsRed labeled mitochondria was calculated as either (i) the ratio of coincident events detected when DsRed2 mitochondria were labeled with Atto-488 IgG1 isotype antibodies relative to the total of number of DsRed2 labeled mitochondria (Table 1) or (ii) the comparison of the Atto-488 fluorescence intensities of individual coincident events when using the Atto-488 IgG1 isotype or the Atto-488 Anti-TOM22 Antibodies. Based on the ratio of coincident events and the median Atto-488 fluorescence intensities of individual events, non-specific binding to mitochondria is 10±6% (N=5 concentrations) and ~4% (N =5 concentrations), respectively.
### Table S1. Non-specific binding of isotype IgG to mitochondria

<table>
<thead>
<tr>
<th>Non-Specific Binding%</th>
<th>[Isotype IgG] (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>2.7×10⁻⁸</td>
</tr>
<tr>
<td>13</td>
<td>5.4×10⁻⁸</td>
</tr>
<tr>
<td>3</td>
<td>1.1×10⁻⁷</td>
</tr>
<tr>
<td>9</td>
<td>1.6×10⁻⁷</td>
</tr>
<tr>
<td>7</td>
<td>2.1×10⁻⁷</td>
</tr>
</tbody>
</table>

The isotype antibody concentrations tested were identical to those used for Atto-488 Anti-TOM22 Antibody incubations.

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### Fig. S4. Atto-488 fluorescence intensities of coincident events detected when mitochondria were labeled with either isotype or anti-TOM22 antibodies. Medians are indicated as a line in the middle of a bar with lower and upper limits of 25 and 75 percentiles, respectively. Whiskers represent lower and upper limits of individual fluorescence intensities. The median green fluorescence of mitochondria labeled with the Atto-488 IgG1 isotype antibodies is 0.1 ± 0.4 (N=58), while the median green fluorescence of mitochondria labeled with Atto-488 anti-TOM22 antibodies 2.7 ± 2.7 (N=512)
E. Volumetric Flow Rate

To determine volumetric flow rates, 1 nM fluorescein solution in MIB was delivered through a 50-cm long, 30-µM I.D. capillary at various pressures (9.5-11 kPa). The measurements were done with a commercially available Beckman Coulter P/ACE MDQ instrument. The time taken to reach the detector was determined by noticing an increase in fluorescence intensity using excitation from an argon-ion laser at 488 nm and collecting fluorescence with a green bandpass filter (535DF35); see Figure S3. The correspondence between volumetric flow rate and applied pressure (Table S2) was used to determine the volumetric flow rates observed in the custom-built capillary cytometry-LIF setup.

**Fig. S5.** Pressure driven delivery of 1 nM fluorescein. The increase in fluorescence intensity indicates was used to determine the travel time at each pressure used (indicated on the right) and to calculate the volumetric flow rate.
### Table S2. Relationship between pressure and travel time through a 30-µm capillary

<table>
<thead>
<tr>
<th>Pressure (psi)</th>
<th>Detection time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.4</td>
<td>18.3±0.5</td>
</tr>
<tr>
<td>1.5</td>
<td>17.3±0.4</td>
</tr>
<tr>
<td>1.45*</td>
<td>17.8*</td>
</tr>
</tbody>
</table>

Two different pressures (1.4 and 1.5 psi) spanning the pressure used in the custom-built instrument (1.45 psi) were used to predict the travel time in this instrument (17.8 min). A star indicates data corresponding to the custom built instrument. Because the capillary volume is 350 nL, the volumetric flow rate is ~ 20 nL/min.