**SUPPLEMENTAL INFORMATION FOR:**

Linear Behavior of the Phase Lifetime in Frequency-Domain Fluorescence Lifetime Imaging of FRET Constructs

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**Supplementary Table 1:**

Table T1: Modulation frequencies used in the study

|  |  |
| --- | --- |
| **f (MHz)** | **w (rad/s)** |
| 14.0625 | 88,357,293.4 |
| 21.0938 | 132,535,940 |
| 23.4375 | 147,262,156 |
| 28.1250 | 176,714,587 |
| 32.8125 | 206,167,018 |
| 35.1563 | 220,893,233 |
| 42.1875 | 265,071,880 |
| 49.2188 | 309,250,527 |
| 51.5625 | 323,976,742 |
| 56.2500 | 353,429,174 |
| 60.9375 | 382,881,605 |
| 63.2813 | 397,607,820 |
| 65.6250 | 412,334,036 |
| 67.9688 | 427,060,251 |
| 70.3125 | 441,786,467 |

**Supplementary Note 2: Methods and data pertaining to EFRETtrue values**

The data in Supplementary **Table T2** were collected with the microscope and filters described in Menaesse et al [1]. The microscope was equipped with a mercury arc source and excitation and emission filter wheels (Ludl) mounted with the appropriate filters for the mTFP1/mVenus FRET pair: mTFP1 excitation filter (Chroma, ET450/30x), Venus excitation filter (Chroma, ET514/10x), mTFP1 emission filter (Chroma, ET485/25m), Venus emission filter (Semrock, FF01-571/72). The excitation and emission filters were used with a single two-band dichroic mirror (Chroma, T450/514rpc) mounted in the filter reflector turret of the microscope. We used two neutral density filters OD=1.0 and OD=0.5 to limit lamp intensity below 7.5 mW·mm-2 at the sample and avoid photobleaching. Images in the donor, acceptor and FRET channels were collected with the same exposure time on either one of two cameras, a Roper Scientific Cascade 512B CCD and a sCMOS (PCO Edge 4.2 bi).

In every case, we calibrated the instrument using the Fc/IDD slope (ratio of corrected FRET signal to quenched donor signal) and the published [2] FRET efficiency of TSMod (ETSMod= 0.286) using the method described in Menaesse et al. For each sample, FRET efficiency, E, was obtained as follows:

with Equ. E1

For each image, was obtained by fitting a line to a pixel-by-pixel 2D histogram of Fc vs IDD as described in [1]. Each value in Table T2 corresponds to one imaged field-of-view in the data set.

Representative images of the constructs are shown in **Fig. S1**.

**Fig. S1**: Representative FRET efficiency images of the 4 FRET constructs overlaid on the acceptor channel signal (normalized intensity in arbitrary units (a.u.)). The field of view is 204mm x 204mm.



We list in **Table T2** the G factor and the FRET efficiency data used to generate the values in Supplementary Table T3. To obtain the mean values of in **Table T3**, we averaged the EFRET means of the 3 experimental repeats corresponding to each construct. For each construct, the standard error was calculated as the standard deviation of these 3 means.

Table T2: E FRET data used to obtain the average EFRET true values in Table T3

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| 1 repeat (Roper Cascade 512B) | | |  | 2 repeats (PCO Edge 4.2 bi) | | |  |  |  |  | 3 repeats (PCO Edge 4,2bi) | | |
| E FRET (G= 2.08) | | |  | E FRET (G= 2.6814) | | | | | |  | E FRET (G= 2.6814) | | |
| **TSMod** | **GGS2** | **TRAF** |  | **TSMod** | **GGS2** | **TRAF** | **TSMod** | **GGS2** | **TRAF** |  | **GGS1** | **GGS1** | **GGS1** |
| 0.298 | 0.474 | 0.029 |  | 0.271 | 0.481 | 0.085 | 0.274 | 0.449 | 0.027 |  | 0.516 | 0.601 | 0.613 |
| 0.295 | 0.521 | 0.103 |  | 0.325 | 0.540 | 0.041 | 0.284 | 0.526 | 0.046 |  | 0.539 | 0.601 | 0.538 |
| 0.276 | 0.560 | 0.041 |  | 0.264 | 0.502 | 0.039 | 0.274 | 0.549 | 0.023 |  | 0.537 | 0.575 | 0.528 |
| 0.264 | 0.548 | 0.038 |  | 0.234 | 0.514 | 0.107 | 0.290 | 0.551 | 0.040 |  | 0.565 | 0.581 | 0.564 |
| 0.283 | 0.542 | 0.054 |  | 0.223 | 0.519 | 0.036 | 0.300 | 0.524 | 0.028 |  | 0.612 | 0.561 | 0.540 |
| 0.305 | 0.511 | 0.041 |  | 0.229 | 0.548 | 0.019 | 0.325 | 0.545 | 0.022 |  | 0.587 | 0.593 | 0.563 |
| 0.325 | 0.488 | 0.056 |  | 0.362 | 0.515 | 0.027 | 0.293 | 0.549 | 0.017 |  | 0.586 | 0.596 | 0.550 |
| 0.288 | 0.536 | 0.040 |  | 0.288 | 0.446 | 0.057 | 0.313 | 0.538 | 0.030 |  | 0.591 | 0.621 | 0.569 |
| 0.256 | 0.550 | 0.037 |  | 0.313 | 0.473 | 0.048 | 0.298 | 0.525 | 0.055 |  | 0.609 | 0.644 | 0.492 |
| 0.307 | 0.529 | 0.046 |  | 0.287 | 0.502 | 0.017 | 0.290 | 0.500 | 0.015 |  | 0.561 | 0.650 | 0.486 |
| 0.285 | 0.513 | 0.027 |  | 0.302 | 0.529 | 0.040 | 0.288 | 0.557 | 0.040 |  | 0.538 | 0.604 | 0.549 |
| 0.229 | 0.536 | 0.030 |  | 0.294 | 0.476 | 0.079 | 0.315 | 0.429 | 0.026 |  | 0.566 | 0.594 | 0.558 |
| 0.291 | 0.561 | 0.021 |  | 0.231 | 0.503 | 0.046 | 0.334 | 0.525 | 0.022 |  | 0.607 | 0.585 | 0.558 |
| 0.255 | 0.551 | 0.023 |  | 0.278 | 0.503 | 0.030 | 0.277 | 0.511 | 0.043 |  | 0.584 | 0.530 |  |
| 0.295 | 0.530 | 0.056 |  | 0.288 | 0.567 | 0.026 | 0.281 | 0.530 | 0.032 |  | 0.624 | 0.592 |  |
| 0.291 | 0.552 | 0.124 |  | 0.236 | 0.498 | 0.028 | 0.306 | 0.514 | 0.033 |  | 0.562 | 0.549 |  |
| 0.287 | 0.527 | 0.027 |  | 0.253 | 0.510 | 0.105 | 0.273 | 0.522 | 0.034 |  | 0.616 | 0.618 |  |
| 0.320 | 0.551 | 0.039 |  | 0.268 | 0.480 | 0.087 | 0.286 | 0.478 | 0.020 |  | 0.500 | 0.561 |  |
| 0.275 | 0.548 | 0.041 |  | 0.285 | 0.492 | 0.046 | 0.300 | 0.519 | 0.015 |  | 0.551 | 0.561 |  |
| 0.294 | 0.561 | 0.029 |  | 0.273 | 0.513 | 0.063 | 0.283 | 0.505 | 0.055 |  | 0.553 | 0.569 |  |
| 0.265 | 0.522 | 0.024 |  | 0.311 | 0.522 | 0.099 | 0.277 | 0.574 | 0.030 |  | 0.594 | 0.584 |  |
| 0.295 | 0.538 | 0.034 |  | 0.351 | 0.499 | 0.026 | 0.277 | 0.550 | 0.035 |  | 0.546 | 0.604 |  |
| 0.292 | 0.577 | 0.128 |  | 0.305 | 0.536 | 0.049 | 0.251 | 0.540 | 0.052 |  | 0.517 | 0.591 |  |
| 0.262 | 0.462 | 0.051 |  | 0.281 | 0.543 | 0.042 | 0.282 | 0.538 | 0.025 |  | 0.581 | 0.662 |  |
| 0.322 | 0.539 | 0.041 |  | 0.309 | 0.531 | 0.028 | 0.289 | 0.550 | 0.037 |  | 0.522 | 0.550 |  |
| 0.286 | 0.558 | 0.037 |  | 0.245 | 0.534 | 0.032 | 0.314 | 0.535 | 0.047 |  |  | 0.507 |  |
| 0.277 | 0.496 | 0.095 |  | 0.315 | 0.485 | 0.034 | 0.351 | 0.564 | 0.039 |  |  |  |  |
| 0.308 | 0.523 | 0.071 |  | 0.239 | 0.506 | 0.042 | 0.245 | 0.545 | 0.015 |  |  |  |  |
| 0.276 | 0.557 | 0.024 |  | 0.247 | 0.558 | 0.087 | 0.268 | 0.528 | 0.036 |  |  |  |  |
| 0.283 | 0.528 | 0.031 |  | 0.270 | 0.558 | 0.041 | 0.270 | 0.531 | 0.036 |  |  |  |  |
| **Mean:** |  |  |  | **Mean** |  |  |  |  |  |  | **Mean** |  |  |
| **0.286** | **0.533** | **0.048** |  | **0.279** | **0.513** | **0.050** | **0.290** | **0.527** | **0.033** |  | **0.567** | **0.588** | **0.547** |

**Supplementary Table T3:**

Table T3: FRET efficiency, EFRET true, and expected amplitude-weighted average lifetime of the constructs (See Methods Section). EFRET true is obtained with the intensity-based sensitized emission method. is calculated from Equ. 2 using EFRET true and tD= 3.1143 ns.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | **TFP** | **TRAF** | **TSMod** | **GGS2** | **GGS1** |
| EFRETtrue | 0 | 0.044 | 0.285 | 0.524 | 0.567 |
| EFRETtrue standard error |  | 0.01 | 0.006 | 0.01 | 0.021 |
|  |  | 2.9773 | 2.2267 | 1.4824 | 1.3485 |
|  |  | 0.0311 | 0.0187 | 0.0311 | 0.0654 |

**Supplementary Note 3: FLIM data**

In Supplementary **Table T4**, 626 images were collected in separate experimental repeats as follows:

* mTFP1: 7 experimental repeats
* TRAF: 4 experimental repeats
* TSMod: 4 experimental repeats
* GGS2: 5 experimental repeats
* GGS1: 3 experimental repeats

Several frequencies were tested in each experiment. However, not all frequencies were tested each time. As a result of this, the number of experimental repeats for each frequency considered individually is not the same.

Fig. S2 shows representative distributions of fluorescence lifetimes for coumarin (a) and mTFP1 and our FRET constructs (b). We observed that our histograms were mono-modal and usually skewed to the right with what appeared to be outliers with very long lifetimes. Gates et al [2] have reported that skewed distributions in FRET data can originate from experimental errors and found that the mode (i.e. the most frequent value of a distribution) resulted in a more robust measurement than the mean or median values under these circumstances. Thus, for each imaged field-of-view, we estimated the mode of the distribution of fluorescence lifetime pixel values by plotting a histogram of the data with bins of 0.01ns width and taking the center of the bin with the maximum number of pixels as the mode of the distribution. A typical histogram included more than 12,000 pixel values and we report the mode without bootstrapping.

While the estimation of the mode is prone to noise in the histogram, our estimated mode values were closer to the expected lifetime value (which is known for Coumarin 6) than the mean. In contrast with the mode, the mean of the histogram varied depending on the extent of the outliers for a given sample and was systematically higher than the expected value at higher frequencies. The use of the mode (most frequent value) to estimate the expected lifetime value of our constructs was justified in our case since our image pixel distributions were monomodal. It is however important to point that a histogram with a more complex shape would require a more detailed analysis of the image pixel data to quantify the shape of the histogram or the specific spatial distribution of the pixels.

**Fig. S2**: (a) Distribution of pixel values in one image of Coumarin 6 in ethanol acquired at modulation frequency 42MHz. (b) Distribution of pixel values corresponding to the lifetime images of mTFP1 and the FRET constructs depicted in Fig. 1 of the manuscript.

Once the mode value is obtained for each image, we list in **Table T4** the mean value of the images obtained in each independent experiment performed at a given modulation frequency and for a given construct. *Finally, we report in the figures of the manuscript the average lifetime and standard deviation over the ‘N’ experimental repeats (values labeled in red in Table T4)*.

Table T4: Measured phase lifetimes(ns). N is the number of independent experiments performed at each frequency.

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**Supplementary Figure S3: Comparison of Coumarin lifetimes**

**Fig. S2**: Box and whisker plots of lifetimes of Coumarin 6 (Sigma Aldrich 546283) in ethanol (10 images), Coumarin 6 in methanol (22 images), and Coumarin 153 (Sigma Aldrich 546186) in methanol (18 images).

**Supplementary Figure S4: Double exponential fit to mTFP1 phase data**

The following Equation was fit to the mTFP1 phase data using Equ. 5.38 in [3] with the constraint (a1+a2)=1. The fit is shown in **Fig. S4**.

**Fig. S4**: Double exponential decay model fit to mTFP1 phase data. The fit results in two indistinguishable lifetimes (t1D=t2D= 3.088e-9 ns, a1: 0.5, c2: 0.0683.)

Equ. E2

**References:**

1. Menaesse, A., et al., *Simplified Instrument Calibration forWide-FieldFluorescence Resonance Energy Transfer (FRET) Measured by the Sensitized Emission Method.* Cytometry Part A, Early Access Aug. 2020 DOI: 10.1002/cyto.a.24194: p. .

2. Gates, E.M., et al., *Improving Quality, Reproducibility, and Usability of FRET-Based Tension Sensors.* Cytometry Part A, 2019. **95A**(2): p. 201-213.

3. Lakowicz, J.R., *Principles of Fluorescence Spectroscopy, Third edition*. 2006, New York, NY: Springer.