Supporting Information

Fluorescence Quenching of Xanthene Dyes during Amide Bond Formation using DMTMM

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Materials
Chemicals (unless otherwise stated) including fluorescent dyes, solvents, and coupling agents were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA) and used without further purification. Chemicals include Eosin Y, Cy3 Azide, Cy5 Azide, Pyronin Y, 5-DTAF, Sulforhodamine 101 acid chloride (Texas Red), Rhodamine 110 chloride, Rhodamine B, Dimethyl sulfoxide-d6, Deuterium oxide (Water-d2), Fluoresceinamine isomer I (FAM), 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM), 4-Morpholinepropanesulfonic acid (MOPS), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), N-hydroxysuccinimide (NHS) and phosphate buffered saline, pH 7.4. AFDye 647 NHS Ester (Equivalent to Alexa Fluor® 647) was obtained from Click Chemistry Tools (Scottsdale, AZ, USA) and used without further purification. 5-OG488 acid (equivalent to Oregon Green® 488 carboxylic acid, 5-isomer) was obtained from AAT BioQuest Inc. (Sunnyvale, CA, USA) and used without further purification. DNA sequences (Tables S1) were synthesized from the University of Utah DNA/Peptide Synthesis Core (Salt Lake City, UT, USA) and from Integrated DNA Technologies (Coralville, IA, USA). We would like to note that the use of NHS esters for some dyes was not to harness their reactivity, but rather because these were the most easily available and cost-effective versions of these small-molecule fluorophores.

Methods
Fluorescence quenching experiments.
In performing bioconjugation reactions where in FAM labeled aptamers were coupled to solid support surfaces, we identified that fluorescence of FAM, regardless of attachment to aptamer, was quenched in the presence of DMTMM. After method development with FAM, the general protocol for all plate-based dye experiments is as follows;

1 μM dye in 1% DMSO was exposed to DMTMM concentrations of 1 M, 500 mM, 250 mM, 100 mM, 10 mM, 1 mM, 100 μM, 10 μM and 0 μM in a 96-well plate. Solutions ranging between 0-2.0 μM dye were made from each dye solution in triplicate to generate a calibration curve on the same plate as the reaction mixtures. Raw fluorescence values were compared to the calibration curve and normalized to fluorescence intensity at t=0 h, and formulas for calibration curves as well as concentrations were quantitatively determined using Excel. All fluorescence experiments were performed at N=6 and error bars represent standard deviation. All fluorescence measurements were taken using a Biotek Cytation 5 imaging reader. In between measurements, the 96-well plate was covered with an opaque lid and placed under a box to minimize photobleaching. Each well in the perimeter of the 96-plate was filled with 200 μL water to minimize evaporation over the 24-hour observation period.

Scheme S1. General Mechanism of Reactivity for DMTMM
Figure S1. Experimental layout of 96-well plate (a) at t=0, (b) at t=24, and (c) with concentrations of dye and DMTMM listed.
Figure S2. Fluorescence of a) 5-FAM, b) Rhodamine 110, c) Rhodamine B, d) 5-OG 488, e) Texas Red, f) FITC, g) 5-DTAf, h) Eosin Y, i) Pyronin Y j) Cy5 Azide, k) Cy3 Azide, l) Af 647 NHS Ester when treated with varying concentrations of DMTMM and monitored for 24 h.

Spectral Scan
1 μM dye in 1% DMSO was exposed to DMTMM concentrations of 1 M, 500 mM, 250 mM, 100 mM, 10 mM, 1 mM, 100 μM, 10 μM and 0 μM in a 96-well plate. Solutions ranging between 0-2.0 μM dye were made from each dye solution in triplicate to generate a calibration curve on the same plate as the reaction mixtures. Raw fluorescence values were compared to the calibration curve and normalized to fluorescence intensity at t=0 h, and formulas for calibration curves as well as concentrations were quantitatively determined using Excel. All fluorescence experiments were performed at N=6 and error bars represent standard deviation. All fluorescence measurements were taken using a Biotek Cytation 5 imaging reader with the following parameters;

5-FAM
- Fluorescence Spectra
- Scale to peak: Scaled to three wells containing 2 μM dye in calibration curve, 80,000 RFU, expected peak: 518 nm
- Excitation: 462 nm
- Emission Start: 490 nm, Emission Stop: 700 nm
- Steps: 5
- Read Height: 7.00 mm
Cy3 Azide
- Fluorescence Spectra
- Scale to peak: Scaled to three wells containing 2 μM dye in calibration curve, 80,000 RFU, expected peak: 590 nm
- Excitation: 500 nm
- Emission Start: 540, Emission Stop 700
- Steps: 5
- Read Height: 7.00 mm

In between measurements, the 96-well plate was covered with an opaque lid and placed under a box to minimize photobleaching. Each well in the perimeter of the 96-plate was filled with 200 μL water to minimize evaporation over the 24-hour observation period.

Characterization of reactivity.
50 μL of FAM stock was added to 50 μL of DMTMM such that the reaction between DMTMM and FAM in 10:20 mM, 10:10 mM, 1:2 μM and 1:1 μM ratios were made in triplicate in a 96 well plate. Standards of 10 mM and 1 μM DMTMM as well as 10 mM, 20 mM, 1 μM and 2 μM FAM were added to well plates in triplicate, and fluorescence was observed over the course of 24 hours. As two well plates were run per dye, in total N=6. With values normalized to fluorescence at t=0, fluorescence intensity tends decrease with higher concentrations of both FAM and DMTMM which supports concentration being a more significant factor than molar ratio in determining depletion. Following this, reactivity was explored in MOPS buffer at pH 8.0, phosphate buffer at pH 7.4, and MilliQ water at pH 6.9. The first two of these conditions were chosen, as they represent typical buffers used for bioconjugation reactions with DMTMM.

![Graphs showing fluorescence of FAM after exposure to various concentrations of DMTMM in different buffers.](image)

**Figure S3.** Fluorescence of FAM after exposure to various concentrations of DMTMM in a) MilliQ Water, pH=6.9 b) phosphate buffer, pH=7.4, and c) MOPS buffer, pH=8.0. All experiments were performed at N=3. Points below zero removed from plot.

Fluorescence quenching experiments in biomolecules
Kanamycin A binding aptamer (Ky2) modified with a 5’ FAM (Table S1) and monitored fluorescence quenching in MOPS buffer at pH 8 in the presence of 30 μL of 300 mM DMTMM for 24 hours. DNA concentration (via absorbance at 260 nm) and fluorescence intensity at Ex/Em=498/518 nm were quantified using a Biotek Cytation 5 imaging reader prior to and 24 hours following DMTMM exposure. Similarly, Goat Anti-Dog IgA antibody labeled with FITC was incubated in PBS buffer at pH=7.4 with 30 μL of 300 mM DMTMM for 24 hours. DNA concentration (via absorbance at 280 nm) and fluorescence intensity at Ex/Em=491/516 nm were quantified using a Biotek Cytation 5 imaging reader prior to and 24 hours following DMTMM exposure. Experiments were performed at N=2 and error bars represent standard deviation.

Buffer Recipes are as follows;
10X PBS Buffer:
- 137 mM NaCl
- 2.7 mM KCl
- 8 mM Na₂HPO₄
- 2 mM KH₂PO₄
  Adjusted to pH = 7.0

0.3 L 10X MOPS Buffer:
- 0.2 M MOPS free acid
- 0.05 M Sodium Acetate
- 0.01 M Disodium EDTA
  Adjusted to pH = 8.0

Table S1. DNA Sequences Used in this Study

| CODE   | Sequence (5'→3')                                      |
|--------|-------------------------------------------------------|
| FAM Ky2 | 5'-/FAM/-TGGGGGTGAGCTAAGCGCA/C6NH2/-3'               |
| Cy3 Ky2 | 5'-/CY3/-TGGGGGTGAGCTAAGCGCA/C6NH2/-3'              |
| FAM BPA | 5'-/FAM/-GGATAGCGGGTTCC/C6NH2/-3'                   |
| Cy3 BPA | 5'-/Cy3/-GGATAGCGGGTTCC/C6NH2/-3'                   |

Surveying reversibility.
Surveying wash surveyed using UV-VIs and NMR. Regarding UV-Vis, samples were prepared at a concentration of 50 mM FAM and 50 mM DMTMM in DMSO at 25°C in 8-well cuvettes and run on a Shimadzu UV-1800 spectrophotometer to monitor absorbance. At t=0, 0.5, 1, 2, 4, and 24 hours.

For NMR analysis, a solution was prepared with 100 mM FAM and 100 mM DMTMM in deuterated DMSO and ¹H NMR measurements were taken at t=0, 0.5, 1, 2, 4, and 24 hours on a Varian 600 MHz Varian INOVA 600 MHz NMR. 300 µL solutions of 100 mM FAM and 100 mM DMTMM in deuterated DMSO were measured at these time points as controls, to monitor degradation over the course of 24 hours.

Figure S4. Absorbance spectra of independent solutions of 50 mM FAM and 50 mM DMTMM after 24 hours. FAM was solubilized in a 1% DMSO solution.
Figure S5. $^1$H NMR Spectrum of FAM at $t=0$.

Figure S6. $^1$H NMR Spectrum of DMTMM at $t=0$.

Figure S7. $^1$H NMR Spectrum of FAM and DMTMM at $t=0$. 

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Figure S8. $^1$H NMR Spectrum of FAM and DMTMM at $t=24$. 
Figure S9. \(^1\)H NMR comparison of FAM and DMTMM at t=0 (green) and t=24 (purple), 2.0-4.5 ppm.

Figure S10. \(^1\)H NMR comparison of FAM and DMTMM at t=0 (green) and t=24 (purple), 5.5-7.5 ppm.

Figure S11. \(^1\)H NMR comparison of FAM and DMTMM at t=0 (green) and t=24 (purple), 9.5-10.5 ppm.
Figure S12. $^1$H NMR Spectrum of FAM and DMTMM at $t=1$h.

Figure S13. $^1$H NMR Spectrum of FAM and DMTMM at $t=2$h.
Figure S14. \(^1\)H NMR Spectrum of FAM and DMTMM at t=4h.
Figure S15. $^1$H NMR comparison of Pyronin Y and DMTMM at $t=0$ (green) and $t=24$ (purple).

Figure S16. $^1$H NMR comparison of Pyronin Y and DMTMM at $t=0$ (green) and $t=24$ (purple), 2.8-4.0 ppm.
Figure S17. $^1$H NMR comparison of Pyronin Y and DMTMM at t=0 (green) and t=24 (purple), 6.7-8.9 ppm.

Figure S18. Time Lapse $^1$H NMR Spectra of FAM and DMTMM over 24 hours, -2.0-14 ppm.

Figure S19. Time Lapse $^1$H NMR Spectra of FAM and DMTMM over 24 hours, 2.3-4.5 ppm.

Figure S20. Time Lapse $^1$H NMR Spectra of FAM and DMTMM over 24 hours, 5.7-8.0 ppm.
Figure S21. Time Lapse $^1$H NMR Spectra of FAM and DMTMM over 24 hours, 5.7-8.0 ppm.

Mass Spectrometry

Positive and negative ion mass spectrometry were detected on FAM, DMTMM, and in 50:50 MeOH:EtOAc. Major peaks observed in spectra include the following:

DMTMM $[M+H]^+$
242 [M+], 227 [M+ -15(CH$_3$)], 197 [M+ -45], 158 [M+ -82], 130 [M+ -112],

DMTMM $[M-H]^-$
274 [M-] 142[M- -132], 216[M- - 58], 249[M- -25], 352 [M- +78], 433 [M- +159] 479[M- +205]

FAM $[M+H]^+$
348, [M+], C$_{20}$H$_{14}$NO$_5$+, 242 [M+ -106], 227 [M+ -121], 214 [M+ -133], 197 [M+ -151], 184[M+ -164, (C$_6$COC$_6$)], 172[M+ -176] (C$_6$OC$_6$), 102 [M+ -246]

FAM $[M-H]^-$
346[M-], 306[M- -40], 290[M- -56], 112[M- - 78], 248[M- -98], 216[M- -130], 174[M- -172], 142 [M- -204], 112[M- -234]

FAM and DMTMM
FAM-DMT adduct: 487, [M+H]+, C$_{25}$H$_{19}$N$_4$O$_7$+.

Impurity
[M+ - 40], 308.982.
Figure S22. Positive Ion Mass Spectrometry Data of FAM and DMTMM in 50:50 Methanol: Ethyl Acetate at t=0 hours
**Figure S23.** Positive Ion Mass Spectrometry Data of FAM and DMTMM in 50:50 Methanol: Ethyl Acetate at t=1 hours

| Sample Name | fem-dronm | Position | P-E3 | Instrument Name | Instrument 1 |
|-------------|-----------|----------|------|----------------|--------------|
| User Name   |           |          |      |                |              |
| Sample Type | Sample    | Inj Vol | 5    | fem-dronm-pas-mesh-ethyl_ac | fem-dronm-pas-mesh-ethyl_ac |
| ACQ Method  | Positive 100-700 176 |    | | 2,292,428, 1,233,586 | 1,233,586, 2,292,428 |
| Comment     |           | 5       | 5    | 0.012000, 0.013036 | 0.013036, 0.012000 |

**Figure S24.** Positive Ion Mass Spectrometry Data of FAM and DMTMM in 50:50 Methanol: Ethyl Acetate at t=2 hours

| Sample Name | fem-dronm | Position | P-E3 | Instrument Name | Instrument 1 |
|-------------|-----------|----------|------|----------------|--------------|
| User Name   |           |          |      |                |              |
| Sample Type | Sample    | Inj Vol | 5    | fem-dronm-pas-mesh-ethyl_ac | fem-dronm-pas-mesh-ethyl_ac |
| ACQ Method  | Positive 100-700 176 |    | | 2,292,428, 1,233,586 | 1,233,586, 2,292,428 |
| Comment     |           | 5       | 5    | 0.012000, 0.013036 | 0.013036, 0.012000 |
Figure S25. Positive Ion Mass Spectrometry Data of FAM and DMTMM in 50:50 Methanol: Ethyl Acetate at t=4 hours

Figure S26. Positive Ion Mass Spectrometry Data of FAM and DMTMM in 50:50 Methanol: Ethyl Acetate at t=24 hours
Figure S27. Negative Ion Mass Spectrometry Data of FAM and DMTMM in 50:50 Methanol: Ethyl Acetate at t=0 hours

Figure S28. Negative Ion Mass Spectrometry Data of FAM and DMTMM in 50:50 Methanol: Ethyl Acetate at t=1 hours
Figure S29. Negative Ion Mass Spectrometry Data of FAM and DMTMM in 50:50 Methanol: Ethyl Acetate at t=2 hours

Figure S30. Negative Ion Mass Spectrometry Data of FAM and DMTMM in 50:50 Methanol: Ethyl Acetate at t=4 hours
Figure S31. Negative Ion Mass Spectrometry Data of FAM and DMTMM in 50:50 Methanol: Ethyl Acetate at t=24 hours

Figure S32. Positive Ion Mass Spectrometry Data of DMTMM in 50:50 Methanol: Ethyl Acetate at t=0 hours
Figure S33. Positive Ion Mass Spectrometry Data of DMTMM in 50:50 Methanol: Ethyl Acetate at t=1 hours

Figure S34. Positive Ion Mass Spectrometry Data of DMTMM in 50:50 Methanol: Ethyl Acetate at t=2 hours
Figure S35. Positive Ion Mass Spectrometry Data of DMTMM in 50:50 Methanol: Ethyl Acetate at $t=4$ hours

Figure S36. Positive Ion Mass Spectrometry Data of DMTMM in 50:50 Methanol: Ethyl Acetate at $t=24$ hours
**Figure S37.** Negative Ion Mass Spectrometry Data of DMTMM in 50:50 Methanol: Ethyl Acetate at t=0 hours

**Figure S38.** Negative Ion Mass Spectrometry Data of DMTMM in 50:50 Methanol: Ethyl Acetate at t=1 hour

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Figure S39. Negative Ion Mass Spectrometry Data of DMTMM in 50:50 Methanol: Ethyl Acetate at t=2 hours

Figure S40. Negative Ion Mass Spectrometry Data of DMTMM in 50:50 Methanol: Ethyl Acetate at t=4 hours
Figure S41. Negative Ion Mass Spectrometry Data of DMTMM in 50:50 Methanol: Ethyl Acetate at t=24 hours

Figure S42. Positive Ion Mass Spectrometry Data of FAM in 50:50 Methanol: Ethyl Acetate at t=0 hours
Figure S43. Positive Ion Mass Spectrometry Data of FAM in 50:50 Methanol: Ethyl Acetate at t=1 hours

Figure S44. Positive Ion Mass Spectrometry Data of FAM in 50:50 Methanol: Ethyl Acetate at t=2 hours
Figure S45. Positive Ion Mass Spectrometry Data of FAM in 50:50 Methanol: Ethyl Acetate at t=4 hours

Figure S46. Positive Ion Mass Spectrometry Data of FAM in 50:50 Methanol: Ethyl Acetate at t=24 hours
Figure S47. Negative Ion Mass Spectrometry Data of FAM in 50:50 Methanol: Ethyl Acetate at t=0 hours

Figure S48. Negative Ion Mass Spectrometry Data of FAM in 50:50 Methanol: Ethyl Acetate at t=1 hours
Figure S49. Negative Ion Mass Spectrometry Data of FAM in 50:50 Methanol: Ethyl Acetate at t=2 hours

Figure S50. Negative Ion Mass Spectrometry Data of FAM in 50:50 Methanol: Ethyl Acetate at t=4 hours
Figure S51. Negative Ion Mass Spectrometry Data of FAM in 50:50 Methanol: Ethyl Acetate at t=24 hours