Fluorescence Quenching Immunoassay Performed in an Ionic Liquid

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Synthesis of [bmim][BF_4]. All reactions were conducted under inert conditions using standard Schlenk line techniques. 1-Methylimidazole (Sigma-Aldrich, ≥99%, purified by redistillation) was re-distilled from KOH and 1-bromobutane (Sigma-Aldrich, *ReagentPlus*™, ≥99%) was washed with concentrated H_2SO_4 and distilled from P_2O_5 before use. The preparation of 1-butyl-3-methylimidazolium bromide, [bmim]Br, followed methods similar to those reported earlier by Gardinier et al.[S1] Briefly, using an addition funnel, 1.25 mol of 1-bromobutane was slowly added to 1.00 mol of vigorously stirred 1-methylimidazole at 0 °C over 30 min. The reaction flask was immediately covered with aluminum foil. After 4 h, the ice bath was allowed to melt and warm to room temperature and was then heated to 40 °C for 3 days. The semi-solid formed was washed several times with cold ethyl acetate and then re-crystallized from acetonitrile or ethyl acetate to yield a white crystalline solid which was dried under vacuum to yield [bmim]Br in roughly 90% yield. ^{1}H-NMR (400 MHz, Acetone-d_6) δ: 10.19 (1H, s, NC$\underline{H}$N), 8.03 (1H, m, CH_3NCHC$\underline{H}$N), 7.95 (1H, m, CH_3NC$\underline{H}$CHN), 4.47 (2H, t, J=7.2 Hz, NC$\underline{H}_2$(CH_2)$_2$$CH_3$), 4.12 (3H, s, NC$\underline{H}_3$), 1.93 (2H, m, NC$H_2C\underline{H}_2$$CH_2$$CH_3$), 1.36 (2H, m, N($CH_2$)$_2C\underline{H}_2$$CH_3$), 0.93 (3H, t, J=7.2 Hz, N(CH_2)$_3$C$\underline{H}_3$).

The as-prepared and dried [bmim]Br was dissolved at ~0.75 g mL^{-1} in doubly distilled, deionized water (18.2 MΩ cm resistivity, Millipore) and refluxed for 4 h with decolorizing carbon. The charcoal was subsequently removed by centrifugation at 10,000*g* for 15 min followed by decantation into a fresh centrifuge tube. This was repeated twice followed by filtration through a 0.45 μm regenerated cellulose syringe

filter. The clear solution was frozen in liquid nitrogen and lyophilized to near-dryness over 2 days.

Metathesis was carried out by combining 50 g [bmim]Br (0.228 mol) in 100 mL Millipore-purified water with 25 g $NaBF_4$ (0.228 mol, ≥98%, Fluka) in 100 mL water and stirring at room temperature overnight. [bmim][BF_4] was isolated in excellent yield, typically better than 85%, using DCM as the extraction solvent in a Gregar-type liquid–liquid continuous extractor for 12 h. This extraction step was repeated twice in order to ensure complete removal of the bromide salt. This was confirmed using silver nitrate titration. The methylene chloride was then removed on a rotary evaporator and the residual [bmim][BF_4] passed once through a DryDisk™ filtration membrane (Horizon Technology, Inc.) and then through a short plug of silica to give [bmim][BF_4] as a colorless fluid. ^{1}H-NMR (400 MHz, Acetone-d_6) δ: 8.98 (1H, s, NC<u>H</u>N), 7.75 (1H, m, CH_3NCHC<u>H</u>N), 7.69 (1H, m, CH_3NC<u>H</u>CHN), 4.34 (2H, t, J=7.2 Hz, NC<u>H</u>$_2$(CH_2)$_2$$CH_3$), 4.03 (3H, s, NC<u>H</u>$_3$), 1.91 (2H, m, NCH_2C<u>H</u>$_2$C$H_2$$CH_3$), 1.38 (2H, m, N($CH_2$)$_2$C<u>H</u>$_2$$CH_3$), 0.97 (3H, t, J=7.2 Hz, N(CH_2)$_3$C<u>H</u>$_3$).

Prior to use in fluoroimmunoassay, [bmim][BF_4] was additionally dried on a vacuum line (~25 microns Hg) at 60 °C for 2 days and samples subsequently transferred to crimp-top bottles lined with PTFE/red rubber septa in a dry argon-filled inflatable glove bag (Cole-Parmer). The water content as determined by Karl-Fischer coulometric titration was <400 ppm.

Antibody Array Fabrication and Fluorescent Imaging. Glass microscope slides (75 × 25 × 1 mm, Fisher Scientific, Pittsburgh, PA) were cleaned by soaking in 1:1 (v/v) MeOH/HCl for 30 min followed by exhaustive rinsing with Millipore-purified water until no “Schlieren lines” were observed. Slides were dried under a stream of nitrogen and inserted into a fused silica holder that allowed for the surface to be exposed to various reaction media. At this point, a typical water contact angle of less than 4° was observed for 1.0 μL sessile drops. Slides that did not meet this criterion were re-cleaned following exposure to a plasma source (Harrick Plasma, Ithaca, NY). Fused silica was used to avoid the possible interference of metal ions. Clean, dried slides were incubated with 2.0 vol% (3-aminopropyl)diethoxymethylsilane (Gelest, Inc., Morrisville, PA) in anhydrous

toluene under a nitrogen atmosphere for 12 h at room temperature. Slides were always handled with clean forceps to avoid manual deposition of skin oils. After the silanization, slides were rinsed three times in toluene and baked for 30 min at 120 °C. Subsequently, they were sonicated in toluene, 1:1 (v/v) toluene/MeOH, and finally MeOH followed by drying under vacuum. Wettability studies showed advancing contact angles with water of 62 ± 2°, typical of an aminosilylated surface. Surface aminosilane uniformity was confirmed by imine formation with 4-nitrobenzaldehyde which gives a strong absorption band at about 285 nm.

In order to produce high-fidelity aminosilylated surface patterns, freshly prepared SAMs on glass slides were selectively exposed to deep-UV light from a medium-pressure Hg-discharge grid lamp (10–20 mW cm^{-2}; UVP, Inc., Upland, CA) using an in-house chromium-over-quartz lithography mask pressed against the slide. The assembly was tightly held together with the aid of two strong paper clamps and was then mounted in front of the beam outlet of the Hg lamp with the Cr mask facing the light source for a 30 min exposure in a closed chamber. Micropatterned substrates were rinsed thoroughly using several repetitive washes with absolute EtOH, Millipore water, and then phosphate-buffered saline (PBS, 50 mM, pH 7.40, 137 mM NaCl). Finally, SAM patterns were rinsed with PBS in an ultrasonic bath for 10 s. From this point onward, samples were protected from light during all preparation steps prior to imaging.

Anti-BODIPY FL antibody attachment was carried out by placing the amine patterned slides vertically in the racks of a screw-cap top polypropylene Coplin staining jar (Ted Pella, Inc., Redding, CA) and immersing the slides in PBS containing 2.50 vol% glutaraldehyde and 40 mM sodium cyanoborohydride ($NaCNBH_3$) for 2 h at room temperature followed by several extensive washes with Millipore water. The activated patterns were then soaked in a 0.15 mL of 200 μg mL^{-1} anti-BODIPY IgG (Invitrogen Corp., Carlsbad, CA) in PBS with 40 mM $NaCNBH_3$ for 16 h at 4 °C using 20-mm diameter press-to-seal™ silicone gaskets with 1-mm wells (Invitrogen Corp.) mounted on the same face of the slide that touched the photolithography mask earlier. To help reduce non-specific background staining, antibody solutions were briefly centrifuged at 5000*g* for 10 min and only the supernatant used for surface immobilization. The aim of this step is to eliminate protein aggregates that may have formed during storage. After incubation,

anti-BODIPY solutions were flushed from the patterning templates using ice cold PBS. To decrease nonspecific binding, residual amino groups were deactivated by adding blocking solution (1% bovine serum albumin (an "irrelevant" protein), 1% sucrose, 0.05% NaN_3, and 0.05% Tween-20 in PBS) and incubating overnight at 4 °C. Again, 40 mM $NaCNBH_3$ was used for reductive amination. Slides were then rinsed with Millipore water, washing solution (0.05% Tween-20 in water), and pure water again. Final immunopatterned surfaces were stored in PBS containing 0.05% NaN_3 at 4 °C and used within one week of preparation.

Before use, samples were rinsed several times with PBS then Millipore water and then dried under a stream of nitrogen. For ionic liquid (IL) studies, neat IL was first introduced followed by labeled IL containing 100 nM BODIPY® FL (4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-propionic acid; Invitrogen Corp.). After a 1.5 h incubation period, the surface was again rinsed with dry, BODIPY-free IL and the pattern imaged using a Zeiss Axiophot fluorescence microscope coupled to a cooled 12-bit CCD camera (CE200A; Photometrics, Tucson, AZ). This microscope used a 63× oil immersion Plan Neofluar objective (Carl Zeiss, Germany), a 200-W Hg/Xe short-arc lamp (OptiQuip, Highland Mills, NY) for excitation, and a FITC specific filter/dichroic set for spectral filtering. A commercial software package (ISee Imaging Systems, Raleigh, NC) was used for CCD image acquisition and to control the microscope's electronic systems.

Figs. S1–S6:

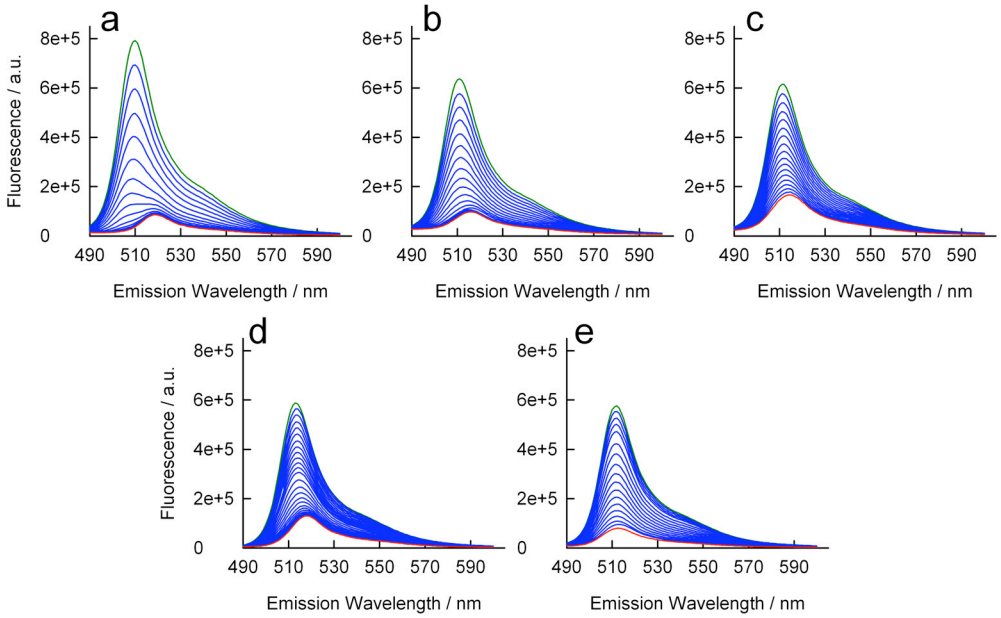

Fig. S1 FQIA titrations using 100 mM, pH 8.0 PB containing 0 (a), 10 (b), 25 (c), 50 (d), and 75 vol% [bmim][BF_4] (e). In any given panel, the green profile denotes the fluorescence spectrum of free BODIPY FL in PB and the red profile indicates the fully antibody-bound BODIPY.

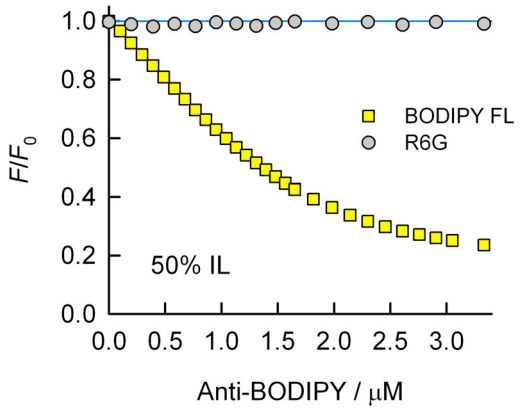

Fig. S2 Control experiment showing the specificity in anti-BODIPY IgG binding and subsequent quenching toward BODIPY FL in 50 vol% [bmim][BF_4]; other volume fractions of IL have similar results. We note that the data for BODIPY FL as haptenic target is the same as in Fig. 1b only recast with an ordinate axis of F/F_0 to show fractional intensity remaining instead of (F_0–F), the total magnitude of quenching. As is

evident, no quenching occurred for rhodamine 6G (R6G) across this [anti-BODIPY] range. Not shown are the Texas Red (TR) results which are, within error, the same as for R6G.

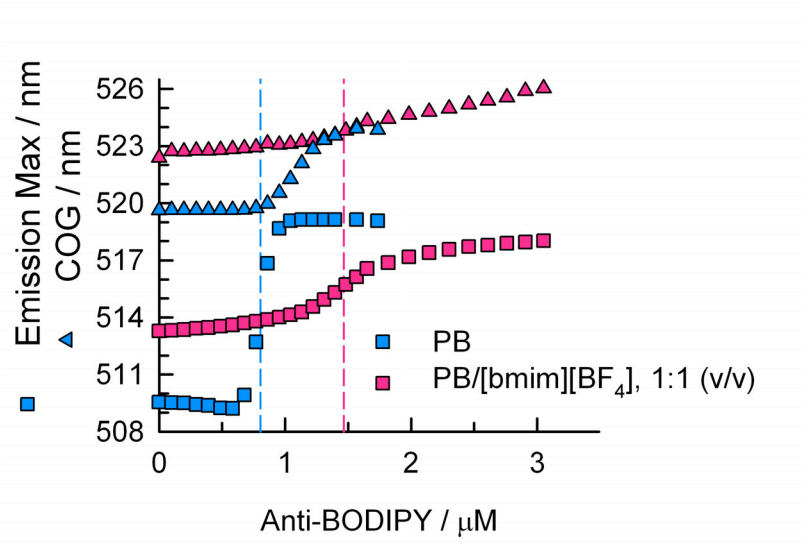

Fig. S3 BODIPY FL emission maxima and centers-of-gravity in PB vs. 50 vol% [bmim][BF_4] as a function of [anti-BODIPY] for A = 66 nM. The vertical dashed lines denote the inflection points determined for sigmoidal fits to the emission max data shown in squares.

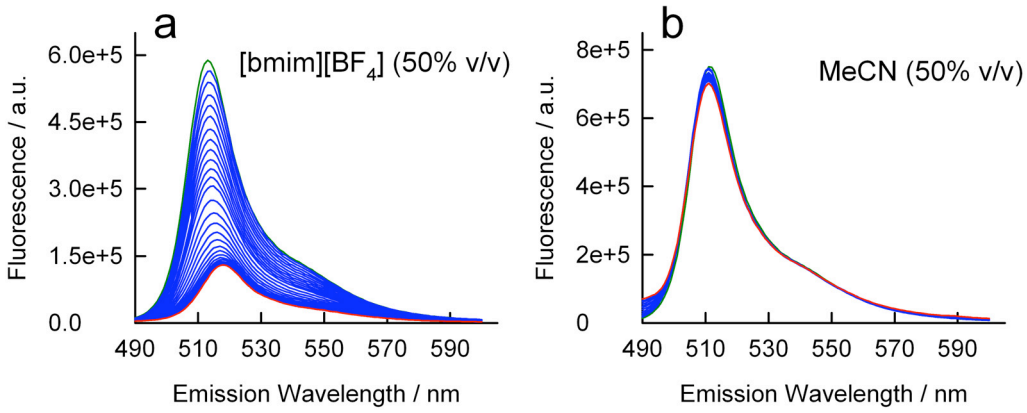

Fig. S4 Comparison between the steady-state emission profiles as anti-BODIPY FL is incrementally added to 66 nM BODIPY FL in 50 vol% [bmim][BF_4] versus 50 vol% MeCN, both in PB. λ_{ex} = 457 nm.

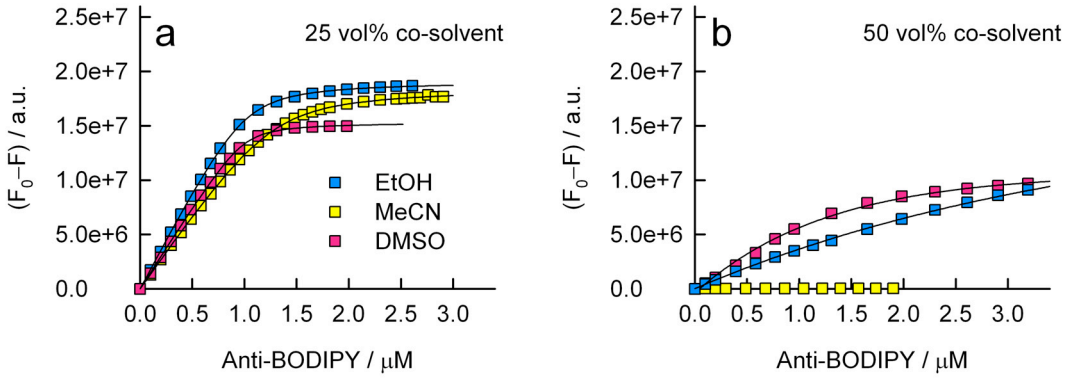

Fig. S5 FQIA binding isotherms for 25 vol% (a) and 50 vol% (b) of different organic solvents in PB.

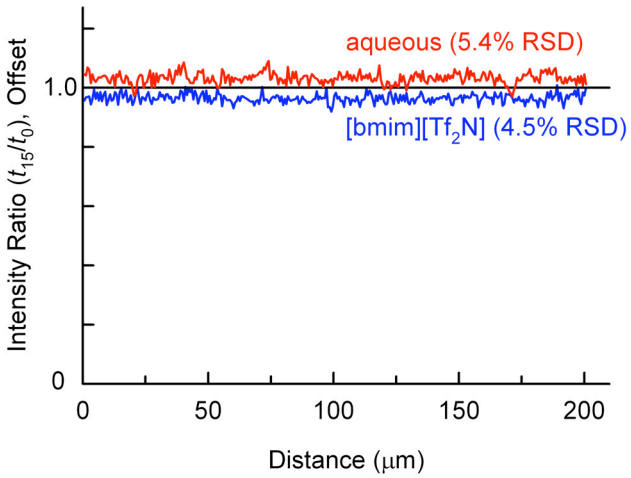

Fig. S6 Fluorescence intensity profiles taken at t = 15 min normalized to the level at t = 0, the time immediately after the stained immuno-labeled slides were rinsed with a flow of neat IL to remove excess and nonspecifically-bound BODIPY FL. Shown are composite line scans produced by "splicing" together eight ~25-μm segments taken from the vertical strokes of "L"s in micrographs similar to those shown in the inset of Fig. 3. These results are important as they not only reveal that the immobilized anti-BODIPY IgG maintains affinity for its hapten against a concentration gradient over time *in pure IL* but that, in addition, the signal stability is comparable to that in an aqueous environment.

References

S1. Gardinier, W. E.; Baker, G. A.; Baker, S. N.; Bright, F. V. *Macromolecules* **2005**, *38*, 8574.