Supporting Information

Detection of Protein-Protein Interactions by Proximity-Driven S_NAr Reactions of Lysine-Linked Fluorophores

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Experimental section

General. $^1$H (500 MHz) and $^{13}$C (125 MHz) NMR spectra were acquired on Bruker DRX-500 or Bruker Avance AV-III 500 instruments. Chemical shifts are reported in ppm and are referenced to CD$_3$CN (1.94 ppm for $^1$H and 118.3 ppm for $^{13}$C) or CD$_3$OD (3.31 ppm for $^1$H and 49.0 ppm for $^{13}$C). $^1$H and $^{13}$C NMR spectra of compounds 7-9 were acquired at 65 °C. Coupling constants $J_{HH}$ are in hertz and are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, dd = doublet of doublets), coupling constant, and integration. Melting points were acquired using a Thomas Hoover Capillary Melting Point Apparatus and are uncorrected. Infrared spectra (IR) were recorded with a Perkin-Elmer Spectrum 100 FT-IR spectrophotometer. UV/Vis measurements employed an Agilent 8452A diode array spectrophotometer (Figure S1). Fluorescence measurements used a Perkin-Elmer LS55 spectrometer (Figure S1). High-resolution mass spectra were obtained at the Mass Spectrometry Laboratory at the University of Kansas. Low-resolution mass spectra were acquired on a Waters Micromass ZQ instrument with electrospray ionization (ESI+). Thin layer chromatography (TLC) used EMD aluminum-backed silica plates (0.20 mm, 60 F-254), and flash chromatography used ICN silica gel (200-400 mesh). Plates were visualized by UV or staining with ceric sulfate/molybdic acid. Preparative HPLC was performed with an Agilent 1200 instrument equipped with a Hamilton PRP-1 reverse phase column (250 mm length, 21 mm ID, 15-50 µm particle size) with detection by absorbance at 215, 254, and 350 nm. All non-aqueous reactions were carried out using flame- or oven-dried glassware under an atmosphere of dry argon or nitrogen. Dichloromethane used as reaction solvent was purified via filtration through two columns of activated basic alumina under an atmosphere of Ar using a solvent purification system from Glass Contour. Other commercial reagents were used as received unless otherwise noted. Yields are reported based on isolated material.

Synthetic procedures and compound characterization data

General procedure A: Synthesis of bromopyrions (4-6) from xanthones (1-3). Xanthones, prepared as previously reported,$^1$ were added to round-bottom flasks containing dry CH$_2$Cl$_2$ (2-3 mL). Trifluoromethanesulfonic anhydride (1 M in CH$_2$Cl$_2$, 1.5 eq.) was added dropwise with stirring, and the reaction mixture transitioned from pale yellow to bright red. The reaction was stirred at room temperature (22 °C) for 5 min. After this period, tetrabutylammonium bromide (3 eq.) was added, and the reaction was stirred at room temperature for an additional 30 min. The reaction mixture was loaded onto silica gel and subjected to flash chromatography (eluent: CH$_2$Cl$_2$:CH$_3$CN, 20:1 to 3:1). Eluted fractions containing pure material were combined and concentrated by rotary evaporation. The resulting residue was dissolved in a minimal amount of CH$_2$Cl$_2$ and precipitated with excess diethyl ether. The resulting red solid was filtered, washed extensively with diethyl ether, and dried under high vacuum to yield pure products as trifluoromethanesulfonate salts.

General procedure B. Synthesis of ethylaminopyrions (7-9) from bromopyrions (4-6). The bromopyrin starting material was added to a round-bottom flask containing dry CH$_2$Cl$_2$ (1-2 mL). Gaseous ethylamine was bubbled through the solvent with stirring for 1 min or until the bright red color was eliminated and the solution became yellow. The solvent was removed under vacuum, the residue was dissolved in DMSO, and the product purified by preparative RP-HPLC (Gradient: H$_2$O:CH$_3$CN (9:1) to (0:100) with added TFA (0.1%) over 20 min; elution time = 11-13 min). Pure fractions were collected, combined, and dried under high vacuum.
N-(9-bromo-6-(ethylamino)-2,7-difluoro-3H-xanthen-3-ylidene)ethanaminium trifluoromethanesulfonate (4). Following general procedure A, 3,6-bis(ethylamino)-2,7-difluoro-9H-xanthen-9-one (1, 104 mg, 0.327 mmol) yielded compound 4 (128 mg, 0.241 mmol, 73%), a red solid, as the trifluoromethanesulfonate salt. mp 222-226 °C; \(^1\)H NMR (500 MHz, CD\(_3\)CN) \(\delta\) 7.81 (d, \(J = 11.7\) Hz, 2H), 7.15 (s, 2H), 6.87 (d, \(J = 6.8\) Hz, 2H), 3.51 (qd, \(J = 7.2, 5.7\) Hz, 4H), 1.36 (t, \(J = 7.2\) Hz, 3H); \(^{13}\)C NMR (126 MHz, CD\(_3\)CN) \(\delta\) 155.77, 151.81 (d, \(J = 251.6\) Hz), 149.37 (d, \(J = 15.6\) Hz), 148.10 (t, \(J = 5.6\) Hz), 122.05 (q, \(J = 320.8\) Hz), 115.24 (d, \(J = 9.5\) Hz), 113.73 (d, \(J = 23.4\) Hz), 96.33 (d, \(J = 4.2\) Hz), 39.39, 13.72; IR (film) \(\nu_{\max}\) 3272, 2978, 1621, 1582, 1524, 1480, 1321, 1253, 1139, 1032, 856 cm\(^{-1}\); HRMS (ESI) \(m/z\) 409.0714 (M\(^+\), C\(_{17}\)H\(_{16}\)BrF\(_2\)N\(_2\)O\(^+\) requires 409.0409).

N-(9-bromo-6-(isopropylamino)-2,7-difluoro-3H-xanthen-3-ylidene)propan-2-aminium trifluoromethanesulfonate (5). Following general procedure A, 2,7-difluoro-3,6-bis(isopropylamino)-9H-xanthen-9-one (2, 90 mg, 0.268 mmol) yielded compound 5 (95 mg, 0.170 mmol, 63%), a red solid, as the trifluoromethanesulfonate salt. mp 214-218 °C; \(^1\)H NMR (500 MHz, CD\(_3\)CN) \(\delta\) 7.78 (d, \(J = 11.7\) Hz, 2H), 6.93 (s, 2H), 6.89 (d, \(J = 6.9\) Hz, 2H), 4.01 (dq, \(J = 13.0, 6.5\) Hz, 2H), 1.36 (d, \(J = 6.4\) Hz, 12H); \(^{13}\)C NMR (126 MHz, CD\(_3\)CN) \(\delta\) 155.82, 151.78 (d, \(J = 251.8\) Hz), 148.53 (d, \(J = 15.6\) Hz), 147.91 (t, \(J = 5.6\) Hz), 122.05 (q, \(J = 320.9\) Hz), 115.18 (d, \(J = 9.5\) Hz), 113.83 (d, \(J = 23.6\) Hz), 96.58 (d, \(J = 4.1\) Hz), 46.92, 21.83; IR (film) \(\nu_{\max}\) 3255, 2980, 1655, 1624, 1580, 1537, 1523, 1487, 1426, 1360, 1324, 1271, 1203, 1151, 1034, 871 cm\(^{-1}\); HRMS (ESI) \(m/z\) 409.0714 (M\(^+\), C\(_{19}\)H\(_{20}\)BrF\(_2\)N\(_2\)O\(^+\) requires 409.0722).

N-(9-bromo-6-(ethylamino)-2,7-difluoro-3H-xanthen-3-ylidene)-2-methylpropan-2-aminium trifluoromethanesulfonate (6). Following general procedure A, 3,6-bis(tert-butylamino)-2,7-difluoro-9H-xanthen-9-one (3, 84 mg, 0.231 mmol) yielded compound 6 (82 mg, 0.139 mmol, 60%), a red solid, as the trifluoromethanesulfonate salt. mp 194-199 °C; \(^1\)H NMR (500 MHz, CD\(_3\)CN) \(\delta\) 7.84 (d, \(J = 12.0\) Hz, 2H), 7.12 (d, \(J = 6.9\) Hz, 2H), 6.58 (s, 2H), 1.56 (s, 18H); \(^{13}\)C NMR (126 MHz, CD\(_3\)CN) \(\delta\) 155.27, 152.33 (d, \(J = 251.9\) Hz), 148.70 (t, \(J = 5.6\) Hz), 147.94 (d, \(J = 14.2\) Hz), 122.07 (q, \(J = 320.9\) Hz), 115.40 (d, \(J = 9.8\) Hz), 113.46 (d, \(J = 24.6\) Hz), 98.16 (d, \(J = 3.3\) Hz), 54.92, 28.71; IR (film) \(\nu_{\max}\) 2964, 2877, 1620, 1520, 1471, 1344, 1260, 1147, 1030, 868 cm\(^{-1}\); HRMS (ESI) \(m/z\) 437.1019 (M\(^+\), C\(_{21}\)H\(_{24}\)BrF\(_2\)N\(_2\)O\(^+\) requires 437.1035).
**N-(6,9-bis(ethylamino)-2,7-difluoro-3H-xanthen-3-ylidene)ethanaminium 2,2,2-trifluoroacetate (7).** Following general procedure B, compound 4 (53 mg, 0.100 mmol) yielded compound 7 (40 mg, 0.087 mmol, 87%), a yellow solid, as the TFA salt. mp 136-140 °C; \(^1\)H NMR (500 MHz, CD\(_3\)OD) \(\delta\) 7.86 (d, \(J = 13.3\) Hz, 2H), 6.68 (d, \(J = 7.4\) Hz, 2H), 4.00 (q, \(J = 7.2\) Hz, 2H), 3.35 (q, \(J = 7.2\) Hz, 4H), 1.52 (t, \(J = 7.2\) Hz, 3H), 1.33 (t, \(J = 7.2\) Hz, 6H); \(^{13}\)C NMR (126 MHz, CD\(_3\)OD) \(\delta\) 162.93 (q, \(J = 293.8\) Hz), 110.19, 101.60, 97.64 (d, \(J = 4.5\) Hz), 44.17, 38.68, 14.83, 13.98; IR (film) \(\nu_{\text{max}}\) 3441, 3264, 2985, 1686, 1650, 1624, 1570, 1514, 1470, 1320, 1246, 1142, 797, 718 cm\(^{-1}\); HRMS (ESI) \(m/z\) 346.1698 (M\(^+\), C\(_{19}\)H\(_{22}\)F\(_2\)N\(_3\)O\(^+\) requires 346.1725).

**N-(9-(ethylamino)-2,7-difluoro-6-(isopropylamino)-3H-xanthen-3-ylidene)propan-2-aminium 2,2,2-trifluoroacetate (8).** Following general procedure B, compound 5 (34 mg, 0.061 mmol) yielded compound 8 (24 mg, 0.050 mmol, 82%), a yellow solid, as the TFA salt. mp 174-178 °C; \(^1\)H NMR (500 MHz, CD\(_3\)OD) \(\delta\) 7.90 (d, \(J = 13.2\) Hz, 2H), 6.77 (d, \(J = 7.4\) Hz, 2H), 4.03 (q, \(J = 7.2\) Hz, 2H), 3.85 (hept, \(J = 6.4\) Hz, 2H), 1.52 (t, \(J = 7.2\) Hz, 3H), 1.34 (d, \(J = 6.4\) Hz, 12H); \(^{13}\)C NMR (126 MHz, CD\(_3\)OD) \(\delta\) 162.94 (q, \(J = 293.8\) Hz), 156.11, 155.58, 150.02 (d, \(J = 243.1\) Hz), 145.62 (d, \(J = 14.6\) Hz), 118.46 (q, \(J = 306.2\) Hz), 110.37, 101.68, 98.15 (d, \(J = 4.3\) Hz), 45.80, 44.16, 22.14, 14.81; IR (film) \(\nu_{\text{max}}\) 3265, 2985, 1686, 1650, 1624, 1589, 1518, 1479, 1324, 1168, 1142, 875, 816, 760 cm\(^{-1}\); HRMS (ESI) \(m/z\) 374.2060 (M\(^+\), C\(_{21}\)H\(_{26}\)F\(_2\)N\(_3\)O\(^+\) requires 374.2038).

**N-(6-(tert-butylamino)-9-(ethylamino)-2,7-difluoro-3H-xanthen-3-ylidene)-2-methylpropan-2-aminium 2,2,2-trifluoroacetate (9).** Following general procedure B, compound 6 (46 mg, 0.078 mmol) yielded compound 9 (32 mg, 0.061 mmol, 79%), a yellow solid, as the TFA salt. mp 166-170 °C; \(^1\)H NMR (500 MHz, CD\(_3\)OD) \(\delta\) 7.95 (d, \(J = 13.5\) Hz, 2H), 7.04 (d, \(J = 7.6\) Hz, 2H), 4.05 (q, \(J = 7.2\) Hz, 2H), 1.54 – 1.50 (m, 21H); \(^{13}\)C NMR (126 MHz, CD\(_3\)OD) \(\delta\) 162.98 (q, \(J = 31.4\) Hz), 156.34, 154.89, 150.53 (d, \(J = 242.8\) Hz), 144.76 (d, \(J = 13.4\) Hz), 118.74 (d, \(J = 294.3\) Hz), 110.16, 101.88, 100.18 (d, \(J = 3.5\) Hz), 53.26, 44.16, 29.13, 14.75; IR (film) \(\nu_{\text{max}}\) 3435, 3260, 2922, 2851, 1677, 1648, 1622, 1514, 1470, 1320, 1199, 1170, 1132, 827, 798, 718 cm\(^{-1}\); HRMS (ESI) \(m/z\) 402.2336 (M\(^+\), C\(_{23}\)H\(_{30}\)F\(_2\)N\(_3\)O\(^+\) requires 402.2351).
Measurement of extinction coefficients ($\epsilon$). Beer’s Law plots of absorbance versus concentration were measured in aqueous PBS (pH 7.0) with increasing concentrations of sample (Figure S2). Values for absorbance $\lambda_{\text{max}}$ were determined for all samples. Linear least squares fitting of the data (including a zero intercept) was used to determine the slope, which corresponds to the extinction coefficient. Molar absorptivity ($\text{M}^{-1} \text{cm}^{-1}$) was calculated using the following equation ($b =$ path length $= 1$ cm): Absorbance = [$\epsilon$] [b] [concentration (M)]

Measurement of quantum yields ($\Phi$). Samples were excited at absorbance $\lambda_{\text{max}}$ and the integrated fluorescence emission was quantified (a 1 cm path length quartz cuvette was used). Fluorescein ($\Phi = 0.92$ in 0.1 M NaOH) and carboxyfluorescein ($\Phi = 0.925$ in 0.1 M NaOH) provided standards. The integrated fluorescence emission at a given concentration was plotted against the maximum absorbance of the sample at that concentration determined by extrapolation based on absorbance measurements at higher concentrations (Figure S3). Linear least squares fitting of the data (including a zero intercept) was used to calculate the slope, which is proportional to the quantum yield. Quantum yields were calculated with the following equation using the average of the values measured for fluorescein and carboxyfluorescein as standards: $\Phi_x = \Phi_{\text{st}}(\text{Grad}_x/\text{Grad}_{\text{st}})$, where $\Phi_{\text{st}}$ represents the quantum yield of the standard, $\Phi_x$ represents the quantum yield of the unknown, and Grad is the slope of the best linear fit.

Measurement of pKa values. Absorbance spectra for pyronins 7-9 (10 µM in PBS) were obtained between pH 4-12. Samples were analyzed immediately upon adjustment to final pH values with aqueous HCl or NaOH to avoid potential hydrolysis. The absence of hydrolysis under these conditions was confirmed by analysis of absorbance spectra upon reacidification of aliquots subjected to basic pH. The absorbance spectra of 7-9 did not change appreciably in the range of pH 4-9 (data not shown), but substantial changes in absorbance were observed at more basic pH values (Figure S4). Values for pKa were calculated using non-linear regression of pH-dependent changes in absorbance at 415 nm.

Analysis of the kinetics of reaction of pyronins 7-9 with methylamine. To ethylaminopyronins 7-9 (50 µM) at 37 °C in either MeOH/H$_2$O (9:1), or H$_2$O, was added aqueous methylamine (final [MeNH$_2$]=10 mM). The pH of the reaction in pure H$_2$O was adjusted to 7.4 by addition of conc. aq. HCl. Low-resolution mass spectra were collected from three independent reactions of each pyronin over time. The integrated peak intensities of the ethyl-substituted starting materials and methyl-substituted products were summed and normalized to 100% to quantify changes in starting material and product over time. The resulting curves, corresponding to pseudo first order kinetic profiles, were analyzed with an exponential one-phase association model (Prism 6 software) to calculate half-time values.
Figure S1. Absorbance and emission spectra in PBS (pH 7.0) and measured properties for pyronins 4-9.
Figure S2. Determination of extinction coefficients ($\varepsilon$) in PBS (pH 7.0).

Figure S3. Determination of quantum yields ($\Phi$). Fluorescein and 5-carboxyfluorescein in aqueous NaOH (0.1 M, $\Phi = 0.92$) provided standards. Values for 4-9 were obtained in PBS (pH 7.0).
Figure S4. Determination of pKa values of aminopyronins 7-9. The absorbance at 415 nm was plotted as a function of pH and analyzed by non-linear regression (Prism 6 software). Samples were analyzed immediately upon adjustment to final pH values with aqueous HCl or NaOH to avoid any potential hydrolysis.
Figure S5. Analysis of transfer of pyronin from full-length human IgG (A), the IgG Fc region (B), and the IgG Fab fragment (C) to SpA. The IgG-derived proteins were conjugated to pyronin 5, added at 5 µM to PBS (pH 7.4) containing unlabeled SpA (5 µM), allowed to react at 37 °C, and analyzed by SDS-PAGE with fluorescence detection. The bands shown are from a single gel where the proteins were analyzed in parallel. Quantification of background-subtracted total fluorescence of each SpA band is shown on the bottom.

Figure S6. Examination of the extent of transfer of other fluorophores from IgG to SpA. Panels A-C: Comparison of the structures of the fluorophores of IgG conjugates. Panel D: Transfer of fluorophores from full-length human IgG to SpA as analyzed by SDS-PAGE with fluorescence detection. Human IgG was conjugated to pyronin 5, 5-carboxyfluorescein, or FITC. These conjugates were purified, added to PBS at 5 µM (pH 7.4) containing unlabeled SpA (5 µM), and allowed to react at 37 °C for 16 h. The bands shown in Panel D are from a single gel where the proteins were analyzed in parallel. In contrast to the pyronin conjugate 14, no transfer of fluorophore was observed with the amide-linked or thiourea-linked conjugates 18 or 19.
Figure S7. $^1$H NMR (500 MHz) of 4 in CD$_3$CN.

Figure S8. $^{13}$C NMR (125 MHz) of 4 in CD$_3$CN.
Figure S9. $^1$H NMR (500 MHz) of 5 in CD$_3$CN.

Figure S10. $^{13}$C NMR (125 MHz) of 5 in CD$_3$CN.
**Figure S11.** $^1$H NMR (400 MHz) of 6 in CD$_3$CN.

**Figure S12.** $^{13}$C NMR (125 MHz) of 6 in CD$_3$CN.
Figure S13. $^1$H NMR (500 MHz) of 7 in CD$_3$OD at 65 °C.

Figure S14. $^{13}$C NMR (125 MHz) of 7 in CD$_3$OD at 65 °C.
Figure S15. $^1$H NMR (500 MHz) of 8 in CD$_3$OD at 65 °C.

Figure S16. $^{13}$C NMR (125 MHz) of 8 in CD$_3$OD at 65 °C.
Figure S17. $^1$H NMR (400 MHz) of 9 in CD$_3$OD at 65 °C.

Figure S18. $^{13}$C NMR (125 MHz) of 9 in CD$_3$OD at 65 °C.
Biological assays and protocols

General. Reagents were purchased from Sigma-Aldrich, Fisher Scientific, and Acros Organics. Cell culture reagents were from Sigma-Aldrich. Staphylococcal Protein A (SpA, recombinant) was obtained from GenScript. Human immunoglobulin G and bovine ribonuclease A (from bovine pancreas) were from Sigma-Aldrich. Human IgG Fc region and human IgG Fab fragment were purchased from Athens Research and Technology. Ribonuclease inhibitor (recombinant, human placental) was from New England BioLabs. Absorbance data for protein quantification were collected on a Thermo Scientific NanoDrop 1000 spectrophotometer. Analysis by SDS-PAGE employed an XCell II module with Bis-Tris 4-12% polyacrylamide gradient gels (1.5 mm, Life Technologies) and MOPS-SDS running buffer (Life Technologies). An Alpha Imager MultImage II Light Cabinet (DE-500) with UV excitation (365 nm) and a 560 ± 40 nm emission filter was used for fluorescence imaging of gels. Sequencing of tryptic peptides by mass spectrometry was performed by the KU Analytical Proteomics Laboratory (APL) with a Thermo Finnigan LTQ-FTICR hybrid mass spectrometer.

Cell Culture. HeLa cells (ATCC #CCL-2) were cultivated in DMEM containing fetal bovine serum (FBS, 10%), penicillin (100 units/mL), and streptomycin (100 µg/mL). Cells were grown in a humidified incubator (5% CO₂) at 37 °C.

Procedure for labeling of human IgG with pyronins. Lyophilized human IgG was reconstituted in sterile water, and the protein concentration was determined by absorbance at 280 nm (ε E1%= 13.7 L g⁻¹ cm⁻¹).² The corresponding bromopyronin (4-6) was dissolved in ethanol at 10 mM. Aqueous sodium bicarbonate (final concentration = 50 mM, pH 8) was added to the protein solution, followed by the bromopyronin (10 equiv.) in ethanol. This reaction was allowed to proceed at 37 °C for 20 min. To purify the conjugate, Sephadex G-25 resin (Superfine, Sigma) was suspended in PBS (pH 7.0). The resulting slurry (900 µL) was added to a mini-spin column (USA Scientific) and centrifuged at 16,000 x G to remove the buffer and pack the resin. After the 20 min conjugation reaction, the solution containing the bromopyronin and IgG was loaded onto the packed resin of the spin column (50-70 µL per column) and centrifuged at 16,000 x g for 1 min to separate the labeled protein from the small molecule retained by the resin. The absorbance at 280 nm and 425 nm of the eluent was determined to quantify the degree of protein labeling. This protocol typically afforded 3 to 4 molecules of pyronin per IgG molecule.

Procedure for labeling of human IgG with commercially-available amine-reactive fluorophores. Lyophilized human IgG was reconstituted in sterile water, and the protein concentration was determined by absorbance at 280 nm (ε E1%= 13.7 L g⁻¹ cm⁻¹).² Aqueous sodium bicarbonate (final concentration = 50 mM, pH 8) was added to the protein solution. The commercially available fluorophores 5-carboxyfluorescein NHS-ester (10 equiv. from a 10 mM DMSO stock solution) or fluorescein isothiocyanate isomer I (FITC, 10 equiv. from a 10 mM DMSO stock solution) were added and the reaction was incubated at 37 °C for 20 min. The conjugated IgG protein was separated from unreacted fluorophores using Sephadex G-25 loaded spin columns as described previously. Proteins were labeled with 5 molecules of 5-carboxyfluorescein per molecule of IgG and 4 molecules FITC per molecule of IgG.
Procedure for labeling of human Fc and Fab regions with pyronins. Human Fc and Fab regions were obtained as aqueous solutions ([Fc] = 1.5 mg/mL, [Fab] = 5 mg/mL). Aqueous sodium bicarbonate (final concentration = 50 mM, pH 8) was added to this protein solution, followed by bromopyronin 5 in ethanol (10 equiv., from a 10 mM stock solution). The reaction was allowed to proceed for 20 min at room temperature (21-23 °C), and the labeled protein was purified using Sephadex G-25 and the spin column protocol described for purification of full-length human IgG conjugates. The final protein concentration was determined by absorbance at 280 nm (ε E1% = 13.5 L g⁻¹ cm⁻¹ used for both proteins). Both the Fc and Fab regions were typically labeled with 1.5 to 2 molecules of pyronin per molecule of protein.

Procedure for labeling of ribonuclease A with pyronin. Bovine ribonuclease A was reconstituted in sterile water and the protein concentration determined from absorbance at 280 nm (ε = 8,640 M⁻¹ cm⁻¹, calculated with the peptide property calculator: http://www.basic.northwestern.edu/biotools/proteincalc.html based on the sequence for bovine ribonuclease A (UniProt: P61823). Aqueous sodium bicarbonate (final concentration = 50 mM, pH 8) was added to this protein solution, followed by bromopyronin 5 in ethanol (2 equiv., from a 10 mM stock solution). NOTE: Unlike the more soluble IgG, extensive labeling of ribonuclease A was found to cause this protein to precipitate from solution, greatly reducing the yield. The reaction was allowed to proceed for 20 min at room temperature (21-23 °C), and the labeled protein was subsequently separated from excess fluorophore using the spin column protocol described for purification of human IgG conjugates. Ribonuclease A was typically labeled with 0.3-0.6 molecules of pyronin per molecule of ribonuclease A.

Analysis of transfer of pyronin from human IgG to SpA. Human IgG was labeled with bromopyronins 4-6 and purified as previously described. These IgGs were combined with bovine serum albumin (BSA) and *Staphylococcus* protein A (at a final concentration of 5 µM for each protein) in phosphate-buffered saline (PBS) at pH 7.4. These protein mixtures were incubated at 37 °C for 0 to 16 h and analyzed by SDS-PAGE with fluorescence imaging. Quantitative analysis of the fluorescence of SpA was performed using Adobe Photoshop (CS6). The total fluorescence intensity was measured as the average pixel density of a fixed rectangular region of interest (ROI) encompassing each SpA band. Contributions from background fluorescence were removed by measuring the fluorescence intensity at t = 0 and subtracting this value from the fluorescence measured at subsequent time points.

Analysis of transfer of pyronin from human Fc and Fab regions to SpA. The full-length human IgG, Fc region, and Fab fragment were labeled with bromopyronin 5 and purified as previously described. These proteins were combined with *Staphylococcus* protein A (at a final concentration of 5 µM for each protein) in phosphate-buffered saline (PBS, pH 7.4). These protein mixtures were incubated at 37 °C for 0 to 16 h and analyzed by SDS-PAGE with fluorescence imaging. Quantitative analysis of SpA fluorescence was performed as previously described for transfer of pyronin from human IgG to SpA.

Analysis of lysine residues on SpA that accept pyronins from IgG. Human IgG was conjugated to bromopyronin 5 and purified as previously described. This labeled IgG (25 µM) was allowed to react with SpA (10 µM) in PBS (pH 7.4) at 37 °C for 18 h. Following this incubation period, excess Protein A agarose (Pierce Biotechnology) was added to immobilize the IgG. The resulting slurry was centrifuged at 2,000 rpm for 5 min, and the supernatant containing enriched SpA was removed for analysis by sequencing of tryptic peptides. Protein
samples were diluted with ammonium bicarbonate (0.2 M) and treated with DTT (10 mM) at 37 °C for 30 min. Iodoacetamide was added (final concentration = 20 mM) and samples were further incubated at room temperature for 30 min to alkylate cysteine residues. Following this incubation period, sequencing-grade Trypsin (0.5 µg, Promega) was added and the samples incubated at 37 °C overnight (16 h). Trifluoroacetic acid (0.1% final concentration) was added to neutralize the trypsin, and samples were stored at 4 °C prior to analysis by MS/MS sequencing. Tandem mass spectra were extracted using Xcalibur version 2.1. All MS/MS samples were analyzed using Mascot software (Matrix Science, London, UK; version 2.3.02). Mascot was used to search the uniprot_sprot database (538010 entries) assuming digestion by trypsin. Mascot was searched with a fragment ion mass tolerance of 0.50 Da and a parent ion tolerance of 20 PPM. Carbamidomethyl cysteine was specified in Mascot as a fixed modification. Modification of lysine by the pyronin was specified in Mascot as a variable modification. Scaffold (version 4.0.4, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identification. Peptide identities were accepted if they could be established at greater than 95.0% probability by the Peptide Prophet algorithm3,4 with Scaffold delta-mass correction. Protein identities were accepted if they could be established at greater than 99.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm.3,4 Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

Transfer of pyronin from ribonuclease A to endogenous ribonuclease inhibitor present in a lysate of HeLa cells. Ribonuclease A was labeled with bromopyronin 5 as previously described. HeLa cells (~10^7) in a T75 culture flask were trypsinized, centrifuged at 2,000 rpm for 2 min, and washed with PBS (pH 7.4). The cell pellet was re-suspended in PBS (200 µL) containing DTT (10 mM), EDTA (1 mM), and PMSF (1 mM). The cell suspension was cooled to 4 °C and homogenized by brief ultrasonication (one 3 sec. pulse). The resulting lysate was centrifuged (18,000 x g, 10 min, 4 °C). The supernatant containing the cytosolic fraction was removed and the total protein concentration was determined with a Pierce BCA5 Protein Assay Kit. An aliquot containing 50 µg of total protein was added to PBS (pH 7.4) containing pyronin-labeled ribonuclease A (20 µM) and incubated at 37 °C for 18 h. As a positive control, labeled ribonuclease A (20 µM) was co-incubated with purified human recombinant ribonuclease inhibitor (200 units) at 37 °C for 18 h. Following incubation, the protein mixtures were subjected to SDS-PAGE with fluorescence imaging. The gel was subsequently stained with Coomassie blue to reveal all proteins and allow examination of the specificity of fluorophore transfer.

References for the Supporting Information

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