Supplementary Information

Antibody-based multiplex analysis of structurally closely related chiral molecules

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1. Antibody Production

Antibodies were generated as previously described.[1-3] For the production of stereoselective anti-amino acid (anti-AA) antibodies, *p*-amino-D- and -L-phenylalanine, respectively, were coupled to the protein keyhole limpet hemocyanin (KLH) via the *p*-amino group by diazotization; the resulting conjugates, *p*-azo-D-phenylalanine-KLH and *p*-azo-L-phenylalanine-KLH (Figure SI 1), were first used to immunize rabbits to obtain polyclonal antibodies.[1]

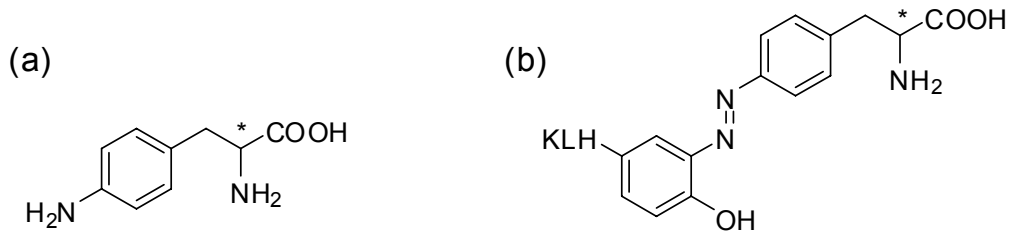

Figure SI1. Structure of (a) *p*-amino-phenylalanine and (b) the immunogen *p*-azo-phenylalanine-KLH.

In order to produce stereoselective anti-hydroxy acid (anti-AHA) antibodies, the same immunization strategy was adopted. *p*-Amino-D- and -L-phenyllactic acid, respectively, were synthesized from the corresponding phenyllactic acid enantiomers, and separately coupled via the *p*-amino group to KLH by diazotization. The resulting hapten-carrier conjugates were first used for the production of polyclonal antibodies in rabbits.[3]

Monoclonal anti-AA and anti-AHA antibodies were produced according to standard procedures.[2-4] In brief, eight week old BALB/c mice were immunized three times, at intervals of two weeks, with 50 μg of the immunogens. Final boosts were given intraperitoneally four and three days prior to fusion. The spleens of mice displaying strong immune responses were isolated, and splenocytes were fused with either NS0 or P3X63-AG8.653 myeloma cells using polyethylene glycol. Hybridomas were selected in hypoxanthine/aminopterin/thymidine medium. Supernatants were screened by enzyme-linked immunosorbent assay (ELISA), and positive hybridomas were cloned at least twice by limiting dilution. Large quantities of antibodies were produced by the preparation of ascites fluid. Monoclonal antibodies were purified on Protein G-agarose or on DEAE-Sephacel following ammonium sulfate precipitation.

2. Labeling of Antibodies

Four antibodies were selected for conjugation to four different fluorophores: the anti-D-AHA antibody secreted by the clone 8E10.9 (anti-D-AHA 8E10.9), anti-D-AA polyclonal antibody, the anti-L-AHA

polyclonal antibody from rabbit #65 (anti-L-AHA 65), and the anti-L-amino acid antibody secreted by the clone 18.3 (anti-L-AA 18.3).

The compounds used for labeling the aforementioned antibodies were DyLight™ 547 NHS ester, DyLight™ 647 NHS ester, DyLight™ 800 NHS ester, and DyLight™ 490 NHS ester, respectively (all from Pierce Biotechnology; Rockford, IL). Antibodies were first dialyzed against 50 mM sodium borate buffer, pH 8.5. Labeling was then carried out as follows: a 15-fold excess of DyLight™ 547 (24.3 μL of a 10 mg/mL solution in DMF) was added to 1.5 mL of anti-D-AHA 8E10.9 (at 2.3 mg/mL). A 7.5-fold excess of DyLight™ 647 (12.6 μL of a 10 mg/mL solution in DMF) was added to 1.5 mL of the anti-D-AA polyclonal antibody (at 2.3 mg/mL). A 6-fold excess of DyLight™ 800 (15 μL of 10 mg/mL solution in DMSO) was added to 1 mL anti-L-AHA 65 (at 1.4 mg/mL). A 5-fold excess of DyLight™ 490 (21 μL of a 10 mg/mL solution in DMSO) was added to 1.5 mL of anti-L-AA 18.3 (at 2.1 mg/mL). The solutions were briefly vortexed and the reactions were allowed to proceed at 4°C for 2 hours under continuous rotation. Reactions were carried out in the dark with all microcentrifuge tubes wrapped in aluminum foil to protect the fluorophores from light. After completion of the reactions, the solutions were dialyzed extensively against phosphate buffered saline (PBS), pH 7.4, in 2 L Erlenmeyer flasks wrapped in foil in order to remove unreacted dyes.

Antibody concentrations were determined spectrophotometrically by measuring the absorbance at 280 nm. The dye-to-protein ratio (Table SI1) was calculated based on the absorbance of the protein conjugates at the following wavelengths: 494 nm for the DyLight™ 490-labeled anti-L-AA 18.3, 547 nm for the DyLight™ 547-labeled anti-D-AHA 8E10.9, 647 nm for the DyLight™ 647-labeled anti-D-AA polyclonal antibody, and 774 nm for the DyLight™ 800-labeled anti-L-AHA 65. The extinction coefficients of the respective dyes were taken from the manufacturer's product information.

Table SI1. Fluorophore-to-antibody ratios of DyLight™ conjugated antibodies

Fluorophore-antibody conjugate	number of fluorophores per antibody
anti-D-AHA 8E10 DyLight™ 547 conjugated	6
anti-L-AHA 65 DyLight™ 800 conjugated	11
anti-D-AA polyclonal antibody DyLight™ 647 conjugated	4
anti-L-AA 18.3 DyLight™ 490 conjugated	8

3. Fluorescence Immunoassays

Reacti-bind™ white opaque 96-well plates (Pierce Biotechnology; Rockford, IL) were coated with the following antigens: *p*-azo-D-phenyllactic acid-BSA for DyLight™ 547-labeled anti-D-AHA 8E10.9, *p*-azo-D-phenylalanine-BSA for DyLight™ 647-labeled anti-D-AA polyclonal antibody, *p*-azo-L-phenyllactic acid-BSA for DyLight™ 800-labeled anti-L-AHA 65, and *p*-azo-L-phenylalanine-BSA for

DyLight™ 490-labeled anti-L-AA 18.3 (100 µL/well; 1 µg/mL in 50 mM carbonate buffer, pH 9.6; overnight at 4 °C). Following a washing step with phosphate buffered saline (PBS) containing 0.05% Tween 20 (PBS/0.05% Tween), unoccupied spaces on the plastic surface were blocked with a 1% gelatin solution in PBS/0.05% Tween 20 (250 µL/well) by incubation at 37°C for 2 hours.

Noncompetitive fluorescence immunoassays were first conducted to confirm that the antibodies still selectively bound to only the correct coating after conjugation to their respective fluorophores. Microtiter plates were divided into five sections; each section was coated with one of five antigens: *p*-azo-D-phenyllactic acid-BSA, *p*-azo-L-phenyllactic acid-BSA, *p*-azo-D-phenylalanine-BSA, *p*-azo-L-phenylalanine-BSA, or underivatized BSA. Serial dilutions of antibody in PBS were dispended onto the plate (100 µL/well) and incubated for 1 hour at room temperature. The plate was washed three times with PBS/Tween 20 and then rinsed with PBS. One hundred µL/well of PBS were applied to the plate prior to measuring fluorescence in a FLUOstar plate reader (BMG Labtechnologies, Offenburg, Germany). Excitation and emission wavelengths were as follows: DyLight™ 490: 491/518 nm, DyLight™ 547: 557/570 nm, DyLight™ 647: 652/673 nm, and DyLight™ 800: 770/794 nm.

All four antibodies were tested on all five coatings; results are presented in Figure SI2 and Figure SI3.

The DyLight™ 647-labeled anti-D-AA polyclonal antibody only bound to the *p*-azo-D-phenylalanine-BSA coating (Figure SI2a). The DyLight™ 490-labeled anti-L-AA 18.3 bound only to the *p*-azo-L-phenylalanine-BSA coating (Figure SI2b). The anti-AHA antibodies, likewise, bound only to the "correct" coatings. The DyLight™ 547-labeled anti-D-AHA 8E10 bound only to the *p*-azo-D-phenyllactic acid-BSA (Figure SI3a), and the DyLight™ 800-labeled anti-L-AHA 65 bound only to the *p*-azo-L-phenyllactic acid-BSA coating (Figure SI3b). None of the four antibodies showed any significant binding to the BSA coating.

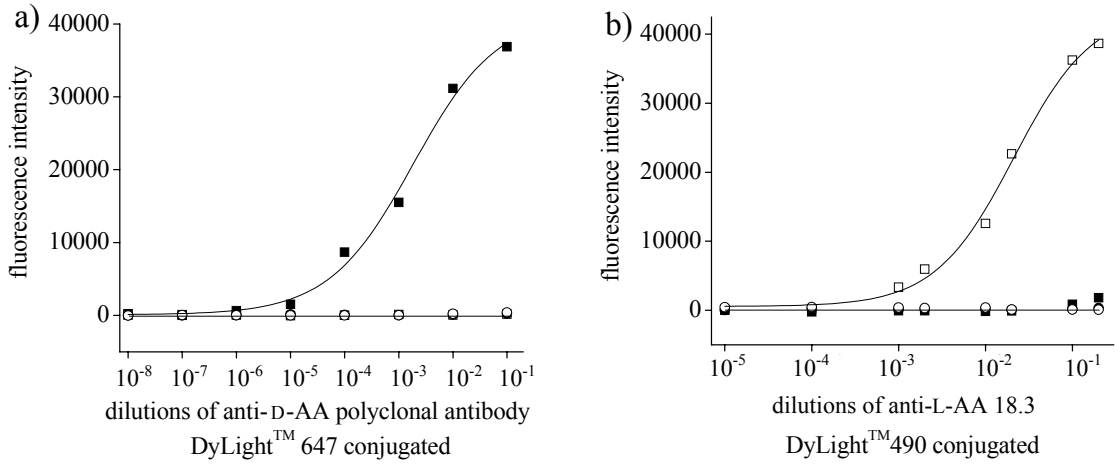

Figure SI2. Noncompetitive fluorescence immunoassay results for (a) anti-D-AA polyclonal antibody DyLight™ 647 conjugated (stock 0.5 mg/mL) and (b) anti-L-AA 18.3 DyLight™ 490 conjugated (stock 1 mg/mL). *p*-Azo-D-phenylalanine-BSA (■), *p*-azo-L-phenylalanine-BSA (□), *p*-azo-D-phenyllactic acid-BSA (●), *p*-azo-L-phenyllactic acid-BSA (○), and BSA were used as solid-phase coatings. At each dilution, the values obtained with the BSA coating were subtracted from those obtained on the other coatings.

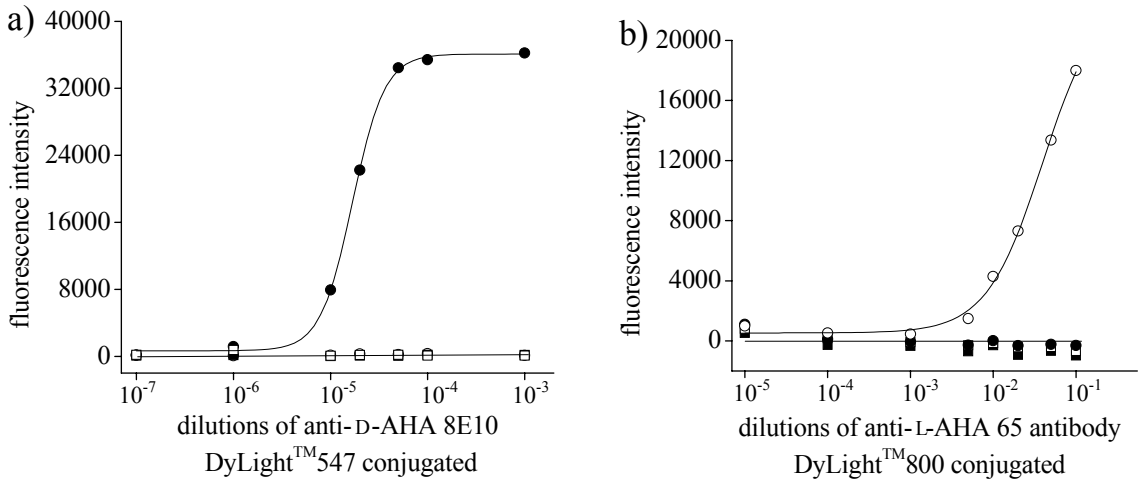

Figure SI3. Noncompetitive fluorescence immunoassay results for (a) anti-D-AHA 8E10 DyLight™ 547 conjugated (stock 0.5 mg/mL) and (b) anti-L-AHA 65 DyLight™ 800 conjugated (stock 1 mg/mL). *p*-Azo-D-phenyllactic acid-BSA (●), *p*-azo-L- phenyllactic acid-BSA (○) *p*-azo-D-phenylalanine-BSA (■), *p*-azo-L-phenylalanine-BSA (□), and BSA were used as solid-phase coatings. At each dilution, the values obtained with the BSA coating were subtracted from those obtained on the other coatings.

Next, competitive tests were performed with the individual analytes at varying concentrations in either the absence or presence of the other compounds and corresponding antibodies. It was verified that analyte detection was unaffected by the presence of any of the other compounds at concentrations up to 10 mM (not shown).

For the simultaneous detection of amino and hydroxy acid enantiomers in a competitive setup, a mixture of *p*-azo-D-phenyllactic acid-BSA, *p*-azo-D-phenylalanine-BSA, *p*-azo-L-phenyllactic acid-BSA, and *p*-azo-L-phenylalanine-BSA was used to coat microtiter plates (100 μL/well). All four of the antigens were in 50 mM carbonate buffer pH 9.6; each antigen was at a concentration of 1 μg/mL. The proteins were allowed to adsorb overnight at 4 °C, and plates were washed and blocked as described above. Mixtures of serial dilutions of D- and L-phenylalanine and D-and L-phenyllactic acid were applied in triplicate onto the plate (12.5 μL/well in PBS for each competitor) and antibody solution containing all four fluorophore-conjugated antibodies at a constant concentration in PBS was applied (50 μL/well). The plates were washed three times with PBS/Tween 20 and then rinsed with PBS. One hundred μL/well of PBS were applied to the plate prior to measuring as described above. It is noteworthy that in such competitive tests no direct qualitative or quantitative comparisons were made between antibodies labeled with different fluorophores; therefore, disproportionate labeling or different quantum yields do not have to be taken into consideration.

All tests were performed at least in triplicate and data were analyzed using the program Origin (OriginLab Corporation; Northampton, MA). Curves were fitted using the sigmoidal fit function. Intra- and interassay standard deviations were typically less than 5%.

4. Microarray Experiments

PATHplus protein microarray slides (Gentel Biosciences; Madison, WI) were printed using a Cartesian PixSys 4200 microarrayer (GenomicSolutions; Ann Arbor, MI) equipped with Telechem SMP15 pins (Sunnyvale, CA). One quadrant of each of the 14 subarrays was printed with 2 nL of one of the four BSA conjugates (i.e., *p*-azo-D-phenyllactic acid-BSA, *p*-azo-D-phenylalanine-BSA, *p*-azo-L-phenyllactic acid-BSA, and *p*-azo-L-phenylalanine-BSA) at a concentration of 50 μg/mL. Printed slides were stored at 4°C in a desiccator and were equilibrated to room temperature in the desiccator prior to blocking. To block unoccupied spaces, the printed slides were placed in a screw cap slide holder filled with a solution of 1% BSA and 5% sucrose in PBS for 15 min at room temperature. After centrifuging slides in a clean screw cap slide holder to remove excess solution, slides were placed in a frame and washed with PBS/0.05% Tween. A mixture of D- and L-phenylalanine and D- and L-phenyllactic acid at varying concentrations (25 μL in PBS) was applied to the slide, followed by a mixture of the four antibody-fluorophore conjugates at a fixed concentration (25 μL in PBS). Slides were incubated for 1 hour at room temperature and then washed three times with PBS/0.05% Tween. After removing slides from the frame, slides were rinsed by brief dipping in 0.25 x PBS. Slides were centrifuged in a clean screw cap slide holder until dry.

Visualization of the DyLight™ 490, DyLight™ 547, and DyLight™ 647 fluorophores was accomplished using a Typhoon 9410 Imager and ImageQuant software (both GE Healthcare Bio-Sciences Corp.; Piscataway, NJ), while for the DyLight™ 800 a Licor Odyssey system with included software (LI-COR, Lincoln, NE) was used since the Typhoon Imager was not equipped with the necessary 800 nm filter.

References

1 O. Hofstetter, H. Hofstetter, V. Schurig, M. Wilchek, B. S. Green, *J. Am. Chem. Soc.*, 1998, **120**, 3251-3252.
2 O. Hofstetter, H. Hofstetter, M. Wilchek, V. Schurig, B. S. Green, *Int. J. Biochromatogr.*, 2000, **5**, 165-174.
3 T. K. Zeleke, J. M. Zeleke, H. Hofstetter, O. Hofstetter, *J. Mol. Recognit.*, 2005, **18**, 334-340.
4 Eshhar Z., in Hybridoma Technology in the Biosciences and Medicine, ed. T. A. Springer, Plenum Press, New York, 1985, pp. 3-41.