Supporting Information

Imaging of protein crystals with two−photon microscopy†

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Supplementary Figures and Legends

Figure S1. Non−protein crystals can exhibit SHG. Bright field images **(1),** TPM images **(2)**, X−ray diffraction frames **(3)**, and two−photon−excited emission spectra **(4)** are shown. **(A)** GR125487 crystal grown from vapor−diffusion crystallization trials of 5−HT4R. **(B)** KH_2PO_4 salt crystals. Spectra in **(A4)** were recorded at three different excitation wavelengths: 810 nm (open circles), 850 nm (closed circles) and 910 nm (closed triangles). Laser for the spectrum in **(B4)** was tuned at 850 nm. Scale bars for **(A2)** and **(B2)** are 150 µm.

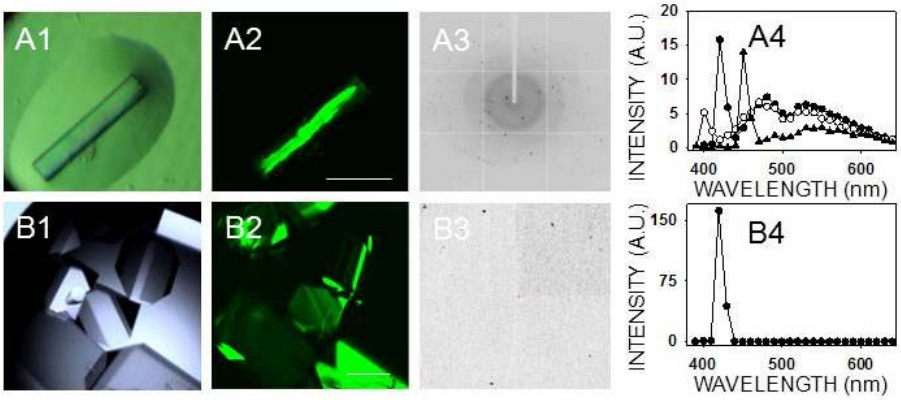

Figure S2. Comparison of TPM images of a tetragonal lysozyme crystal obtained in the epi and transmission configuration. (A) Bright field image, **(B)** epi TPM image, **(C)** transmission TPM image. Scale bar is 150 μm.

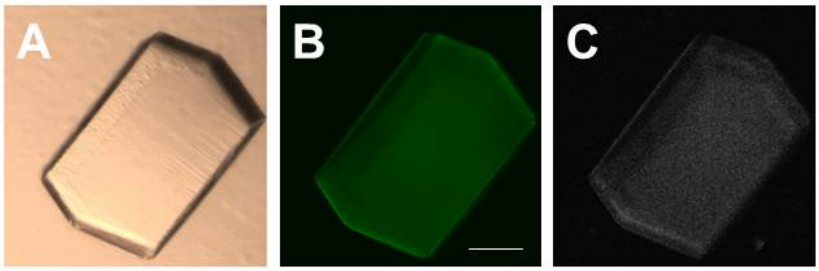

Figure S3. TPM imaging of aromatic amino acid crystals. Bright field images **(1)**, TPM images **(2)** and emission spectra **(3)** of aromatic amino acid crystals of tryptophan **(A)**, tyrosine (**B)**, and phenylalanine **(C)** are shown. Sharp peaks at 420−430 nm corresponded to SHG, whereas fluorescence was weak or absent. Scale bars are 150 µm.

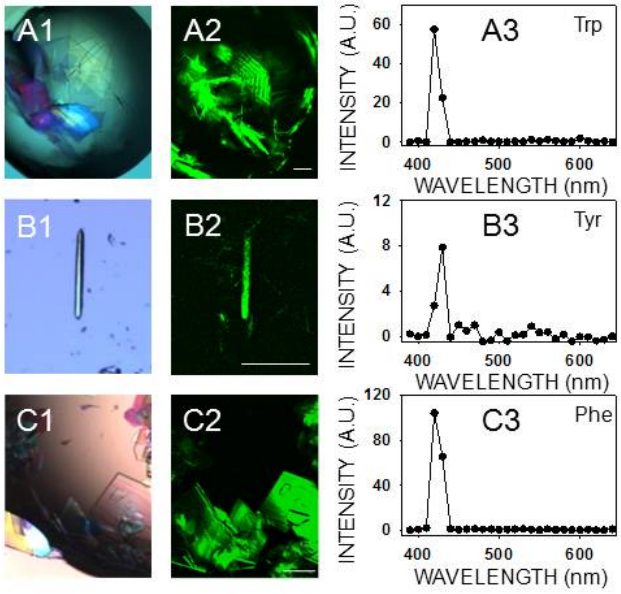

Figure S4. **Increase of visible TPEF in a fresh tetragonal lysozyme crystal upon treatment with APS. (A)** Bright field image (top) and TPM images, 1.5 min after adding 17 mM APS (middle) and after 20 min of incubation with 17 mM APS at room temperature (bottom). Scale bar represents 150 μm. The exact control image of the crystal without APS could not be obtained, because the crystal moved during APS addition and the image was refocused to a somewhat different focal plane. **(B)** Two-photon emission spectra corresponding to the crystal at the two time points of incubation with APS. Excitation wavelength was 730 nm.

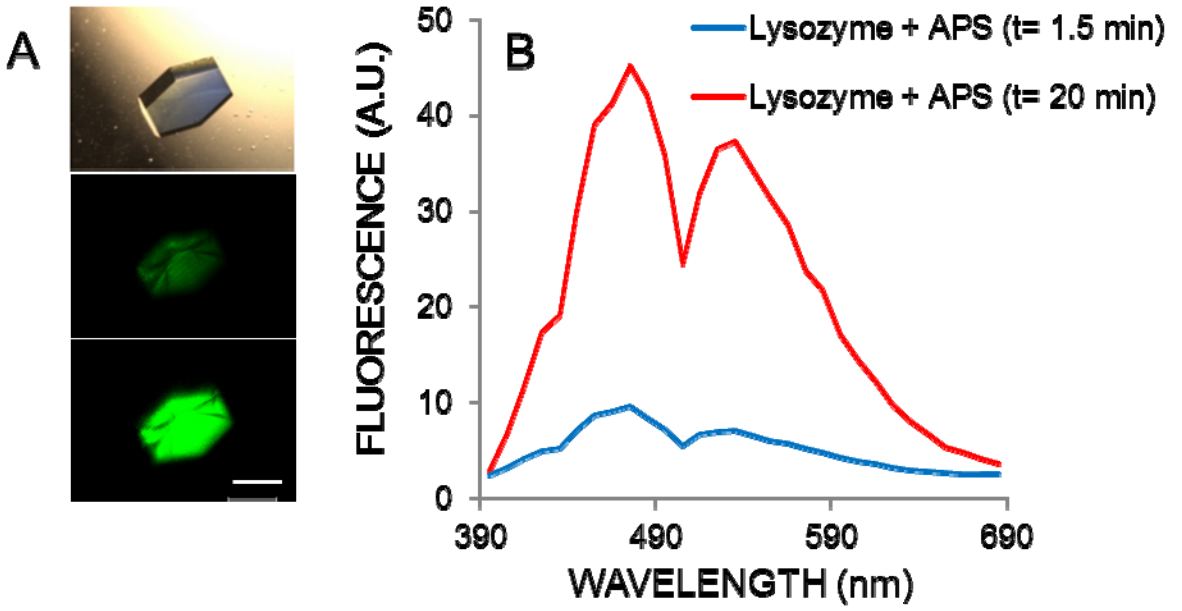

Figure S5. UV-A absorbance of lysozyme in solution, treated with APS. Lysozyme (1 mg/ml) in 0.1 M NaCl + 0.1 M buffer (sodium acetate, pH 4.5 or Tris, pH 7.4) was incubated with 1 mM or 2 mM APS at room temperature for 6 h. Spectra of 2 mM APS and of lysozyme without APS, are shown as controls. The top spectra were measured with a 1-mm path length, whereas the bottom spectra (focusing on the 300-500 nm range) were obtained with a 1-cm path length.

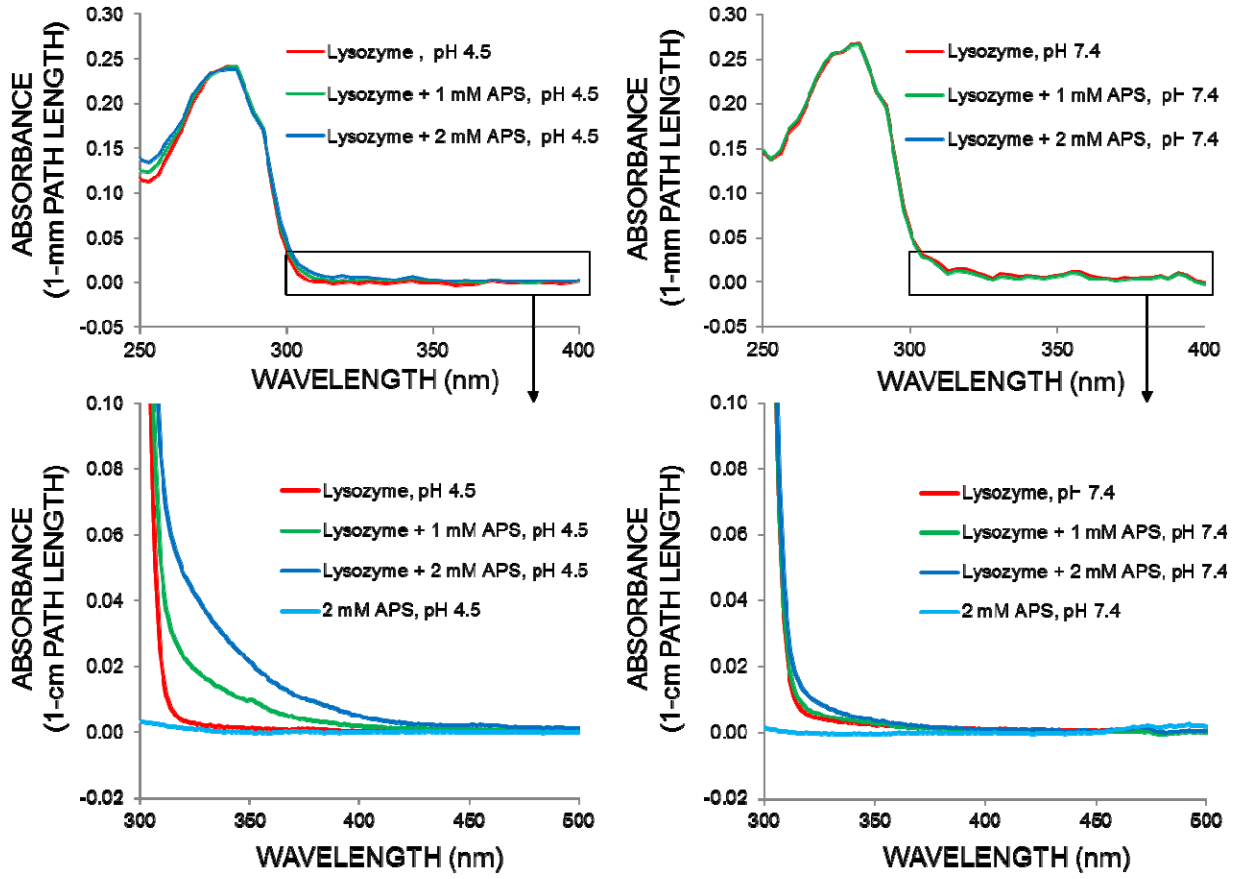

Figure S6. Visible one-photon fluorescence of lysozyme in solution treated with APS. Lysozyme (1 mg/mL) in 0.1 M sodium acetate, pH 4.5, and 0.1 M NaCl was incubated with 2 mM APS. Excitation **(A)** and emission **(B)** spectra were measured after 7 and 24 h of incubation at room temperature. Spectra of 2 mM APS and of lysozyme alone are shown as controls and the spectrum of buffer was substracted from all spectra shown. The emission maximum is ~450 nm for all the lysozyme samples, although the emission intensity of non-APS-treated lysozyme (inset) was ~55 times smaller than that of protein incubated with APS for 24 h.

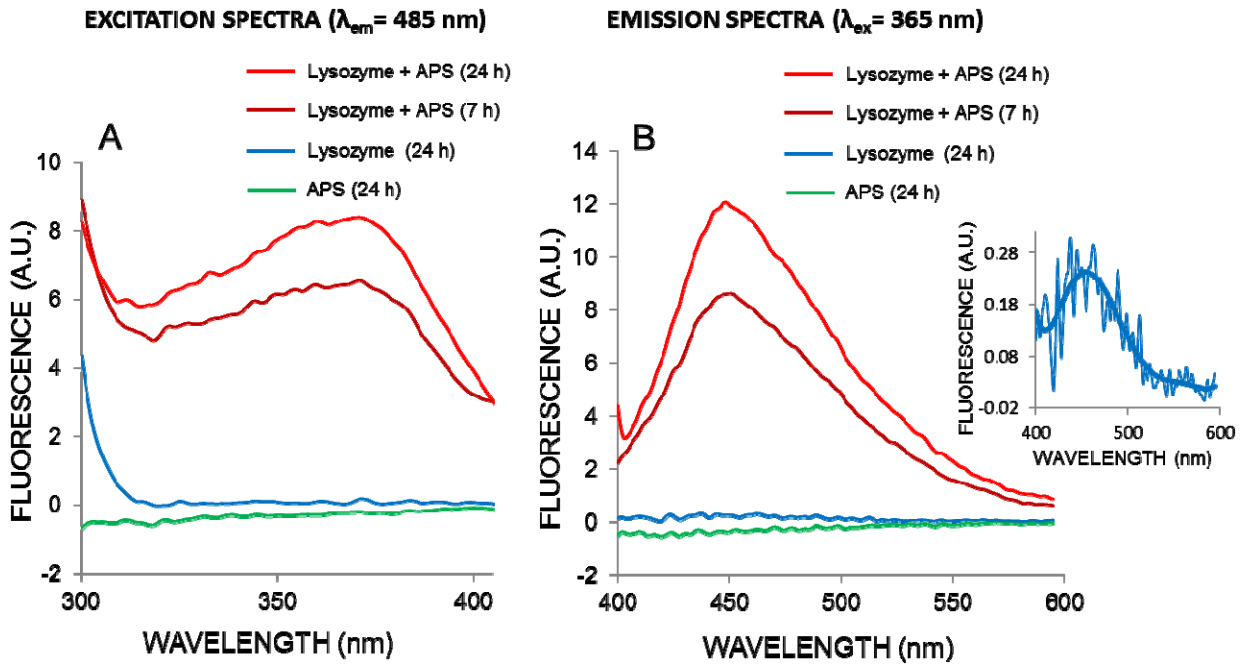

Figure S7. Visible two-photon fluorescence of aromatic amino acids in solution, treated with APS.

(A, B) Two-photon fluorescence image of a mixture of aromatic amino acids (Ar aas) (13.6 mM Trp, 16.8 mM Phe, 0.77 mM Tyr) upon incubation with 167 mM APS (bottom-right drop). TPM images were taken right after sample preparation (A) and after 15 min of incubation at room temperature (B). Amino acid mix (bottom-left drop) and 167 mM APS (top drop) are shown as negative controls. Weak, stable fluorescence emission was observed for the Ar aas drop, but no signal was observed for the drop containing APS solution. Scale bar represents 300 μm. **(C)** Two-photon emission spectra of the bottom drops in panel B (Ar aas with and without APS) after 20 minutes of incubation. The excitation wavelength was 730 nm.

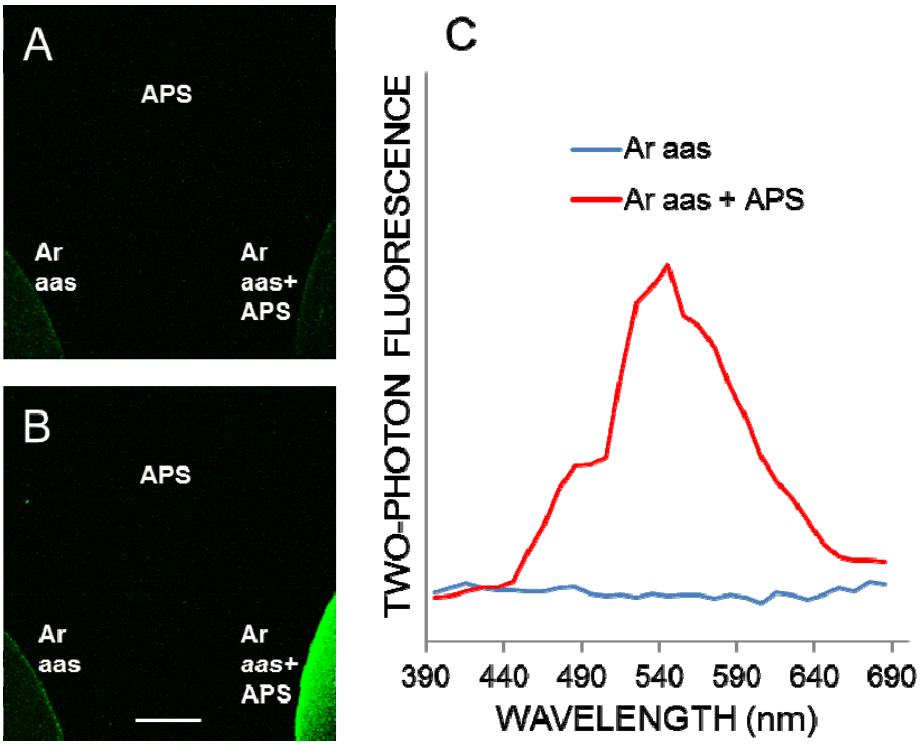

Figure S8. UV-A absorbance and visible one-photon fluorescence of aromatic amino acids in solution treated with APS. Solutions of individual amino acids (7.3 mM Trp, 9.1 mM Phe or 1.24 mM Tyr) and a mixture of the three aromatic amino acids (Ar aas, 7.3 mM Trp, 9.1 mM Phe, 0.41 mM Tyr) were prepared in 0.45 M sodium acetate, pH 4.5, with or without 50 mM APS. Samples were incubated at room temperature for 40 min before measuring their absorbance with a 1-mm path length **(A)**. Samples were then incubated overnight and diluted 100 times to measure their one-photon fluorescence emission with an excitation wavelength of 365 nm **(B)**. The inset shows the same spectra except those corresponding to the Trp and Ar aas mix treated with APS. A buffer blank was substracted from each spectra.