Appendix B

COMMON PROTOCOLS

B.1. Instrumentation

Polymerase Chain Reaction (PCR) was performed on a MJ Research PT150 Minicycler. UV-visible absorbance spectra were obtained on an Agilent 8453 diode array spectrophotometer. Mass spectrometry was performed using MALDI-TOF in the Mass Spectrometry Facility at Caltech, or LC-MS at the Caltech Protein/Peptide MicroAnalytical Laboratory (PPMAL). Fast protein liquid chromatography (FPLC) was performed on an AKTApurifier FPLC (GE Healthcare) using HiPrep desalting, HiPrep Q anion exchange, and Superdex 75 gel filtration columns. Steady-state fluorescence spectra were acquired on a Jobin Yvon Spec Fluorolog®-3-11 fluorometer.

B.2. Site-directed mutagenesis

Mutations to the P450 gene were made using Qiagen QuikChange site-directed mutagenesis kit. Samples were prepared on ice:

38.5 μL milliQ water
5 μL Buffer stock (10X)
2 μL parent plasmid,
1.25 μL of forward primer
1.25 μL of reverse primer
1 μL dNTPs.

1 μL of enzyme was added to this mixture as the final component.

The following PCR protocol was used:

1. 95 °C 1 min
2. 55°C 1 min
3. 68°C 8 min
4. Repeat steps 1-3, x 17
5. 4°C 4 hrs (until pickup)

Digestion of methylated DNA (parent plasmid) is achieved by addition of 1 μL dpn1 enzyme and incubation at 37 °C for one hour, followed by denaturation at 65 °C for 15 minutes. The PCR mixtures are stored at –20 °C until needed.

B.3. Transformation Protocol

Plasmid for the desired mutant (1-2 μL) is incubated on ice with (ultra)competent cells (30-50 μL) for 30 min, followed by 45 seconds of heat shock at 42 °C. Following an additional 2 minute incubation on ice, NZY$^+$ broth (250 μL) is added and the transformation mixture is incubated for 1 hour at 37 °C with gentle agitation. 5-150 μL of the transformation mixture are plated on LB/Agar culture plates containing 100 μg/mL ampicillin. These are incubated overnight at 37 °C; BL21-DE3 cells are incubated ~16 hours, XL-1 Blue/Nova Blue are incubated 24 hours.

B.4. Amplification and purification of plasmid DNA

To amplify plasmid DNA, such as for sequencing, the purified plasmid DNA or PCR mixture is transformed into XL-1 Blue or Nova Blue supercompetent cells (see Transformation Protocol).

Overnight cultures containing 5 mL Luria Bertani broth, 100 μL/mL ampicillin, and a single *E. coli* colony are grown for 16 hours at 37 °C. Cells are pelleted by centrifugation (10 min, 5000 rpm), and supernatant is discarded. Plasmid DNA is extracted using a Qiagen miniprep kit. To verify successful mutagenesis, 10 μL samples are submitted for sequencing (Laragen) along with sequencing primers. A combination of forward (ptac-ptac promoter: TTGACAATTAATCATCGGC, T_M=53.7 °C) and reverse (pCWrev: CTTTCGTCTTCAAGCAGATCTG, T_M=60.8 °C) primers are used to sequence the entire P450 gene.

B.5. P450 overexpression in *E. coli*, extraction and purification

All P450 mutants were expressed in *E. coli* BL21(DE3) cells, with minor differences for P450-BM3 and CYP119 variants.

Expression

Overnight cultures of Luria Bertani broth (25 mL) containing 100 μg/mL ampicillin and a single *E. coli* colony (transformed with the mutant plasmid of interest) are incubated at 37 °C overnight, shaking at 180-200 rpm. Induction cultures of TB (1x TB for P450 BM3, 2x TB for CYP119; 1L in 4L flask or 2 L in 6L flask) containing 200 μg/mL ampicillin, 1 μM thiamine, 0.4% glycerol, and 250 μL mineral supplements (100 mM $FeCl_3$, 10 mM $ZnCl_2$, 8.5 mM $CoCl_2$, 8.5 mM Na_2MoO_4, 7 mM $CaCl_2$, 7.5 mM $CuCl_2$, 8 mM H_3BO_3), are inoculated with the overnight culture and incubated at 37 °C until reaching an optical density of ~1 at 600 nm. Cultures are induced by addition of 1 mM IPTG, and 0.5 mM α-aminolevulenic acid, a heme precursor, is added. The temperature is lowered to 30 °C; the P450 BM3 expression period is 24 hours, CYP119 expression period is 40 hours. Following expression, cells are harvested by centrifugation (5000 rpm, 10 min), and cell pellets are stored at –80 °C until needed.

Extraction and purification

Cell pellets are resuspended in cold Wash Buffer (50 mM Tris pH 8, 300 mM sodium chloride, 20 mM imidazole). A small spatula tip each of two protease inhibitors (benzamidine hydrochloride and Pefabloc SC) are added, and cells are lysed by two to three cycles of probe-tip sonication (0.5 s on, 0.5 s off, for 5 minutes), cooled by an ice-water bath.

Centrifugation (15,000 rpm, 1 hr, 8 °C) pellets cellular debris, and the supernatant is loaded directly onto a Ni batch column. After thorough washing with Wash Buffer (1.5-2 L), protein is eluted (200 mM imidazole in Wash Buffer), and the colored (red/orange) fractions are collected and concentrated in 30 kDa centrifugal filters. Gel filtration chromatography is used to remove fragmented proteins, followed by buffer exchange into 20 mM Tris, pH 8 with 20 mM dithiothreitol (DTT) added to reduce intermolecular disulfide bonds. Purity is determined by UV-vis absorption (A_{420}/A_{280}), SDS-PAGE, and mass spectrometry. Protein not intended for immediate use is flash-frozen in liquid nitrogen (with 40% glycerol added to solution as cryoprotectant) and stored at -80°C.

B.6. Ru-P450 conjugation

The complex $[Ru(bpy)_2(IA\text{-}phen)]^{2+}$ (IA-phen = 5-iodoacetamido-1,10-phenanthroline) was covalently coupled to P450 mutants containing a single, surface exposed cysteine, to afford the respective Ru^{II}-Fe^{III}P450 conjugates.

Approximately three-fold excess of $[Ru(bpy)_2(IA\text{-}phen)]^{2+}$ is added to a 20 μM solution of P450 mutant in 20 mM Tris buffer (pH 8), and shaken in the dark at 4 °C. Labeling progress can be monitored by MALDI mass spectrometry; no further increase in the peak corresponding to the predicted mass of Ru^{II}-P450 is observed after two hours. Excess $[Ru(bpy)_2(IA\text{-}phen)]^{2+}$ is removed during concentration in 30 kDa filters, followed by desalting on an FPLC HiPrep column.

To separate photosensitizer-labeled and unlabeled enzymes, protein samples are loaded onto an anion exchange MonoQ or HiPrep Q column equilibrated with 20 mM Tris buffer, pH 8 (Q Wash Buffer). The column is washed with Q Wash Buffer until UV-visible absorbances return to baseline. The gradient is ramped

quickly to 59% Q Elution Buffer (Q Wash Buffer + 250 mM sodium chloride), followed by a slow gradient of 59-65% Q Elution Buffer over 60 minutes.

B.7. Preparation of laser samples

Laser samples are composed of approximately 10 μM Ru^{II}-Fe^{III}P450 or the model complex $[Ru(bpy)_2(IAphen)]^{2+}$, with and without oxidative quencher (17 mM $[Ru^{III}(NH_3)_6]^{3+}$) in buffered solution (pH 6: 20 mM sodium acetate; pH 7: 20 mM sodium acetate; pH 8: 50 mM sodium borate or 50 mM Tris). High ionic strength (100-200 mM sodium chloride) helps to prevent protein precipitation in the presence of quencher. Samples are placed in a specialized, high-vacuum, four-sided quartz cuvette (Starna Cells) with a high-vacuum Teflon valve (Kontes) and 14/20 adaptor (**Figure B.1**), equipped with a small stir bar (1 x 3 mm). Solutions are stirred constantly during deoxygenation and data collection. Deoxygenation is achieved via 3 x 10-15 gentle pump-backfill cycles with argon on a Schlenk line, with 15 minutes of equilibration between each set of cycles.

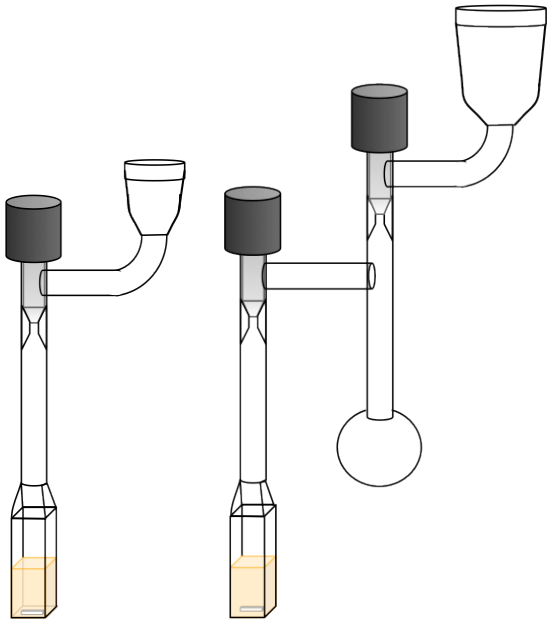

Figure B.1. Two high-vacuum cuvettes for laser studies. Left: typical cuvette for flash-quench. Right: cuvette for Fe^{II}-CO photolysis and rebinding.

B.8. Laser details

Nanosecond-to-second transient spectroscopies

Excitation for nanosecond-to-millisecond transient luminescence and absorption experiments was provided by visible light (e.g., 480 nm) pulses from a tunable optical parametric oscillator (Spectra Physics, Quanta-Ray MOPO-700) pumped by the third harmonic from a Spectra Physics Q-switched Nd:YAG laser (Spectra-Physics, Quanta-Ray PRO-Series, 8 ns pulse width) operated at 10 Hz.

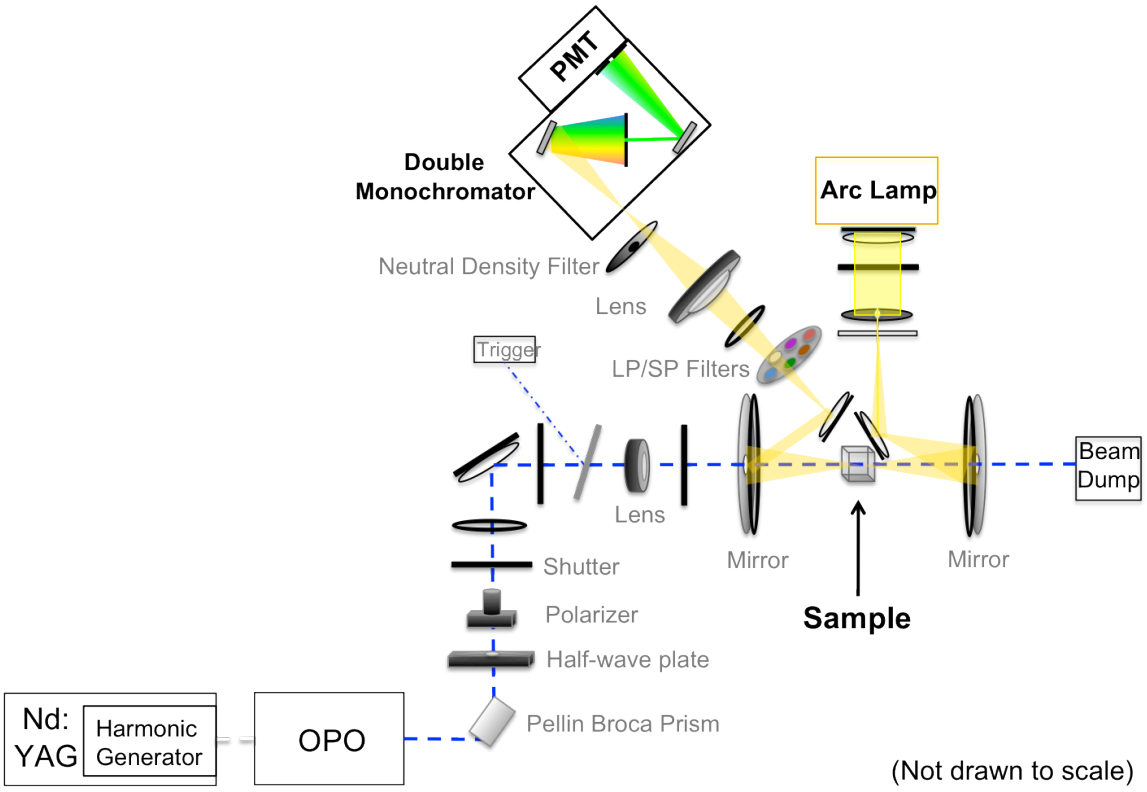

Figure B.2. Nanosecond-pulsed single-wavelength transient luminescence and absorption setup: NSI.

The setup for single-wavelength transient luminescence and absorption studies ("NSI") is shown in **Figure B.2**. Probe light was provided by a 75-W arc lamp (PTI Model A 1010) that could be operated in continuous or pulsed mode, and passed

through the sample collinearly with the excitation pulse. After rejection of scattered light by appropriate long- and short-pass filters, and intensity modulation by a neutral density filter, probe wavelengths were selected by a double monochromator (Instruments SA DH-10) with 1 mm slits. Transmitted light was detected by a photomultiplier tube (PMT, Hamamatsu R928), and amplified by a 200 MHz wideband voltage amplifier DHPVA-200 (FEMTO).

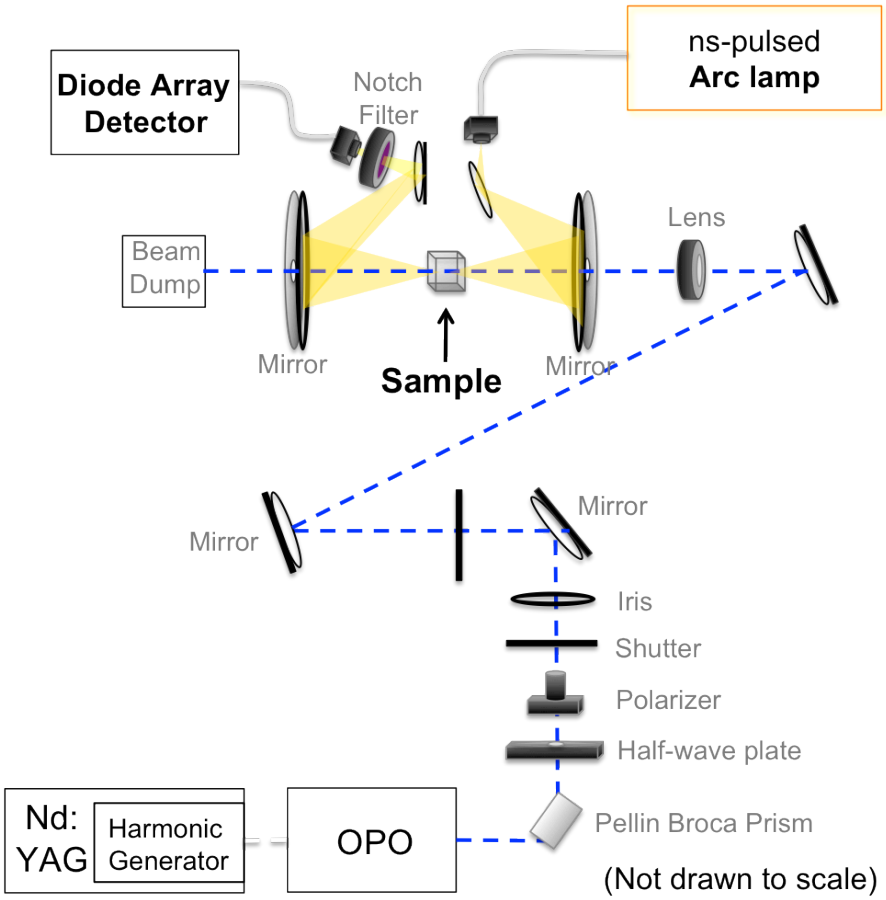

Figure B.3. Nanosecond-pulsed multi-wavelength transient absorption setup: NSII.

The setup for multi-wavelength transient absorption studies ("NSII") is shown in **Figure B.3.** Probe light was provided by white flashlamp, and transmitted to the sample by fiberoptic cables. A partial reflector directed approximately 30% of the probe light around the sample as a references beam, while the majority was aligned

through the sample, collinearly with the excitation beam. Scattered excitation light was rejected by a narrow notch filter (centered at 486 nm), and the transmitted probe light was detected into two photodiode arrays (Ocean Optics S1024DW Deep Well Spectrometer). Measurements were made with: neither probe light nor excitation light (dark correction), probe light only (background), excitation light only (fluorescence correction), and probe light and excitation light (TA). The timing of laser fire, flashlamp fire, and photodiode array readout were controlled by a series of timing circuits, and the readout was interfaced with a PC via a National Instruments multifunction input/output card. Difference spectra were averaged over approximately 150 shots.

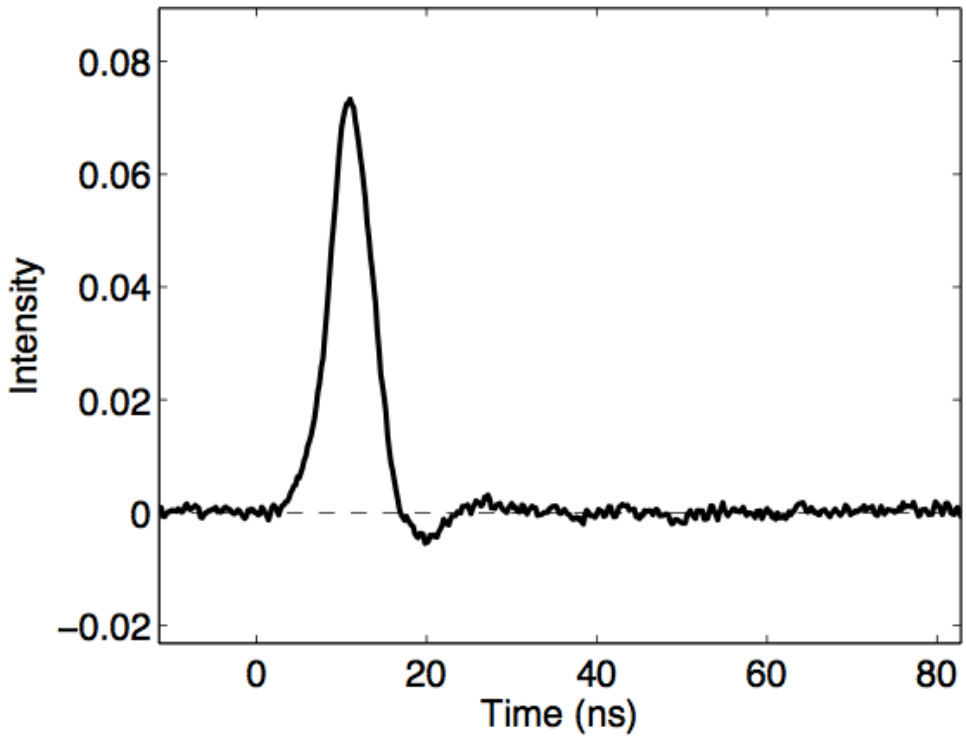

Figure B.4. NSI instrument response to scattered laser light, collected on a 2 μs window (pulse width: 8 ns).

Picosecond-to-nanosecond transient spectroscopy

The setup for picosecond-pulsed single wavelength transient absorption measurements is shown in **Figure B.5.**

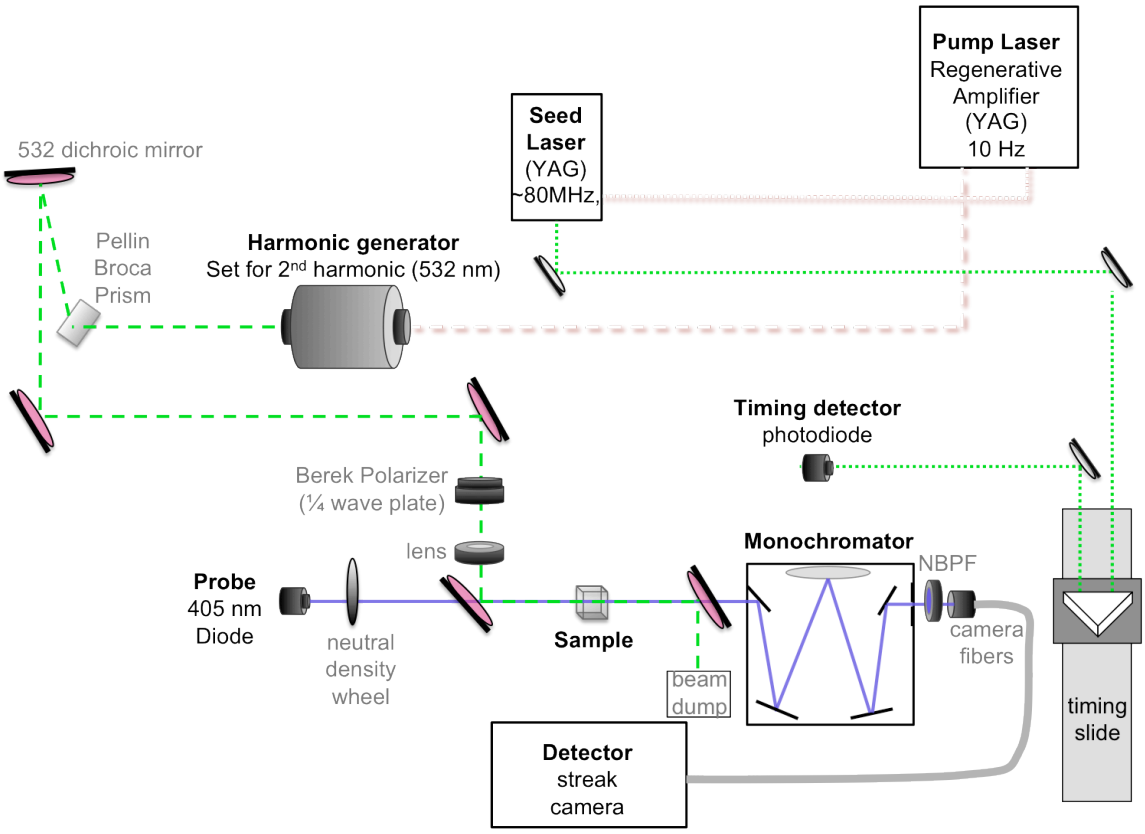

Figure B.5. Picosecond-pulsed single-wavelength transient absorption setup: PSI.

Excitation pulses for picosecond single-wavelength transient absorption measurements were generated by a Continuum Regenerative Amplifier (RGA 60 Series) operated at 10 Hz, seeded by a 70 MHz train provided by the first harmonic (1064 nm) of a mode-locked DPSS Nd:YAG (Spectra Physics Vanguard 2000 HM532). A small portion of the seed laser (2nd harmonic, 532 nm output) was used for timing. The excitation pulse (pulse width ~10 ps) was tuned to the second harmonic (532 nm) by a harmonic generator, and converted to circularly polarized

light by a Berek Retardation Polarizer Compensator (5540) operating in ¼ wave plate mode, before being directed through the sample. Continuous probe light was provided by a Sony 405 nm 100 mW focusable laser diode. After passing through the sample, excitation pulses were rejected using a 532 diochroic mirror, and probe light was tramsitted through a monochromator and into a picosecond streak camera (C5680 Hamamatsu Photonics) in photon counting mode, operated by a Hamamatsu controller. Samples were stirred continuously, with typical laser powers of 0.5-0.75 mJ/pulse. Kinetics traces were collected on 5 ns and 50 ns time windows, averaged over 10,000 exposures.

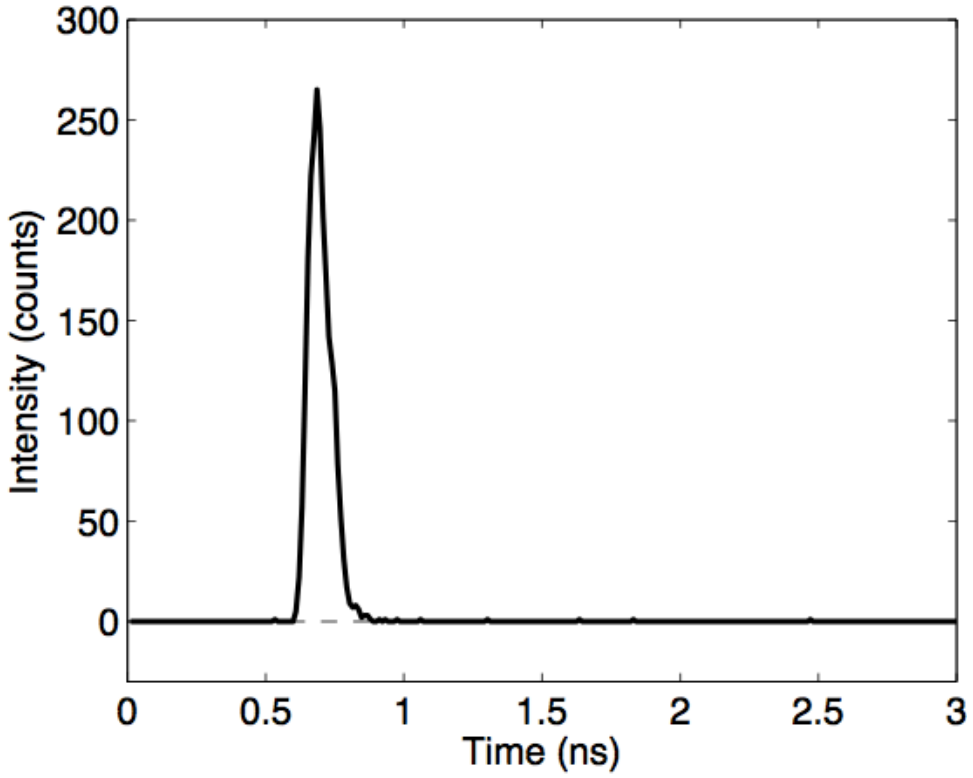

Figure B.6. PSI instrument response to scattered laser light, collected on a 5 ns window.

B.9. Data workup

To generate a complete kinetics picture of TA data on the picosecond-to-second timescale, individual kinetics traces were log-compressed, overlaid, and spliced together.

On the NSI system, timescales 400 μs and shorter were amplified and digitized by the fast amp/digitizer system, while timescales 10 ms and longer are amplified and digitized by the slow amp/digitizer. It was noted that transient absorption data using the slow amp had inconsistent magnitudes that could vary by a factor of 2-3 when compared between different time-windows. Fast-amp traces were more self-consistent. Thus, the magnitude of the fastest trace (2 μs window) was taken as the standard amplitude for each data set; TA data at other time-windows multiplicatively scaled to achieve overlap.

Data collected on the picosecond streak camera had significantly lower signal-to-noise ratios, and showed larger variations in intensity compared to ns1 data. When overlaying ps1 and ns1 data, the magnitude of the ns1 trace from the 2 μs window was taken as the standard amplitude for each data set, and other time-windows were multiplicatively scaled to achieve overlap.