Supplementary material

High-precision background correction and artifact suppression for ultrafast spectroscopy by quasi-simultaneous measurements in a split-sample cell

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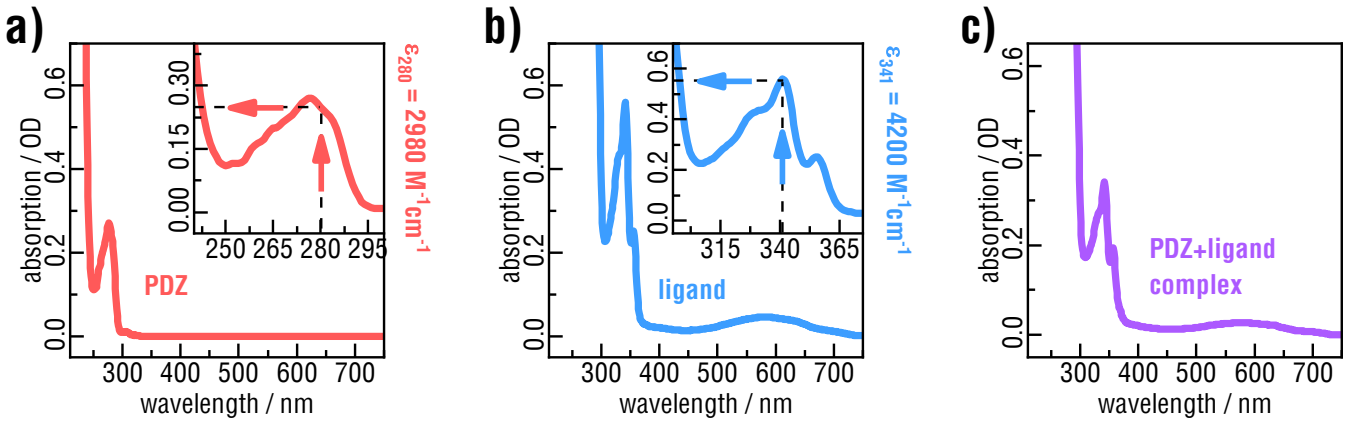
1. Sample preparation, concentration determination

PDZ3 wt and PDZ3 Aha mutant expression and purification were performed according to a protocol described elsewhere (manuscript submitted). The VET-donor AzAla was synthesized enzymatically.[1] The modified PDZ-ligand containing the VET-donor (AzAla-KQTSV) was synthesized by *sb-peptide* (Saint Egrève, France) via solid phase peptide synthesis to HPLC purity of > 95% and used without further purification.

For an accurate reproduction of background signals it is crucial to equalize as much parameters as possible for the sample and reference solution. Careful adjustment of concentrations thus played a major part in sample preparation. Protein concentrations were determined spectroscopically by measuring the absorption at 280 nm (see SFig. 1a) in a 60 µm CaF_2 groove cuvette and employing Lambert-Beer's law. The required extinction coefficient for PDZ3 was calculated with EXPASY to 2980 $M^{-1}cm^{-1}$. In our case, accurately determining the protein concentration was important to calculate the required amount of ligand to achieve sufficient protein-ligand binding.

The concentration of the ligand containing the VIS-absorber azulenylalanine (AzAla) was first determined before mixing with the PDZ3 solution. The ligand stock solution was diluted 600fold and measured in a quartz cuvette with 1 cm optical pathlength. The characteristic absorption feature at 341 nm (see SFig. 1b) was used to calculate the concentration with the corresponding extinction coefficient of 4200 $M^{-1}cm^{-1}$ known from literature.[2]

Since our background signal is directly linked to the amount of the AzAla in the final sample and background solutions, because it determines how much energy is deposited, their AzAla-concentrations were checked one more time via the 341 nm absorption (see SFig. 1c) after mixing the PDZ3 and ligand solutions.

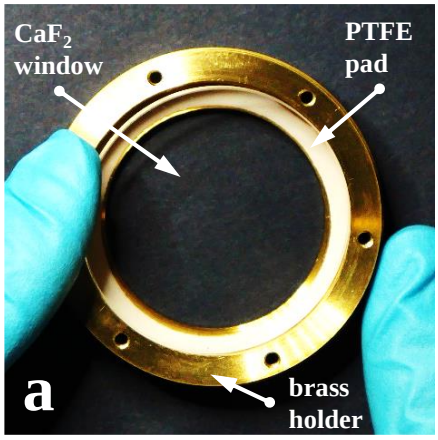


SFig 1 Exemplary UV/VIS spectra of PDZ3 and the ligand before and after mixing a) UV/VIS spectra of PDZ3 measured in a 60 µm cuvette. The absorption band around 280 nm is characteristic for proteins and routinely used for concentration determination. The inset displays a close-up view of the band. b) UV/VIS spectra of the 600fold diluted ligand stock solution measured in a 1 cm quartz cuvette. The absorption features around 340 nm and 600 nm come from the AzAla-residue of the modified ligand. The inset displays a close-up view of the sharp bands in the near-UV region. The peak at 341 nm is used for concentration determination. c) UV/VIS spectra of the PDZ3+ligand complex measured in a 60 µm cuvette. Since the 341 nm band does not interfere with the protein absorption at 280 nm it is used for AzAla-concentration determination of the final sample and background solutions.

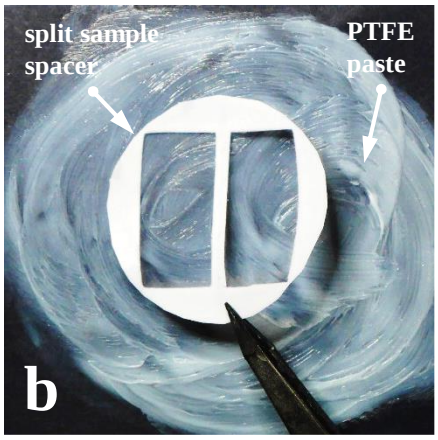
1. E. J. Watkins, P. J. Almhjell, and F. H. Arnold, Chembiochem : a European journal of chemical biology 21, 80 (2020).
2. M. Venanzi, A. Valeri, A. Palleschi, L. Stella, L. Moroder, F. Formaggio, C. Toniolo, and B. Pispisa, Biopolymers 75, 128 (2004).

The challenge when assembling and loading the cell is to reliably avoid mixing of the sample and background solutions. As the compartments are only separated by a thin bridge of PTFE spacer, the careful use of PTFE paste for sealing is important. The procedure is shown in the following.

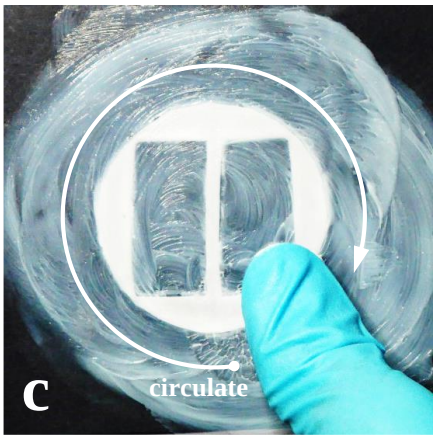
1. Cell assembly protocol – *cell preparation*



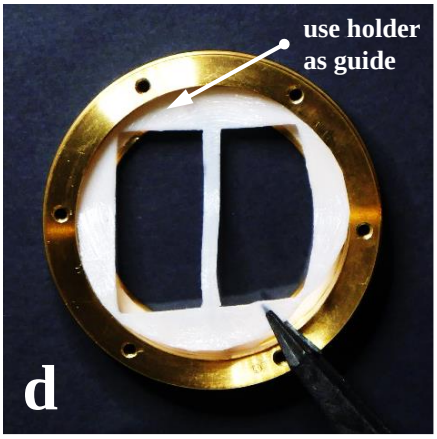
Place the PTFE pad into the brass holder, then let the CaF_2 window slide into it.



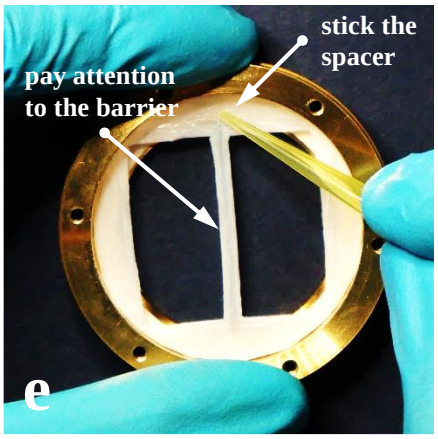
Cover a clean plastic sheet with PTFE paste and place the PTFE split sample spacer onto it.



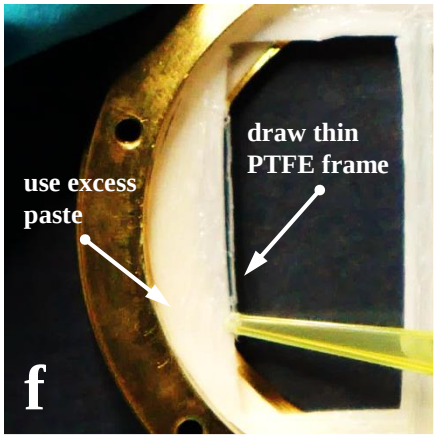
Apply some additional paste on top of the spacer and grease thoroughly using your finger tip.



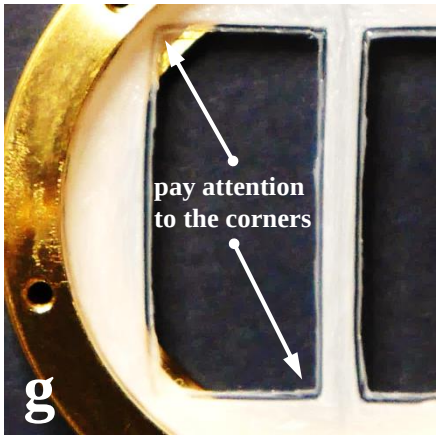
Put the greased spacer on top of the window using the holder as a guide for a centered placement.



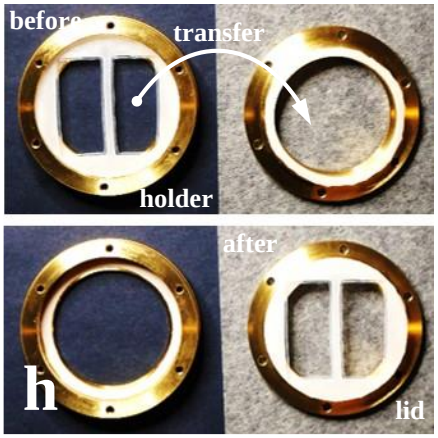
Pad the spacer down using a plastic pipette tip and make sure it sticks completely to the window by squeezing out trapped air.



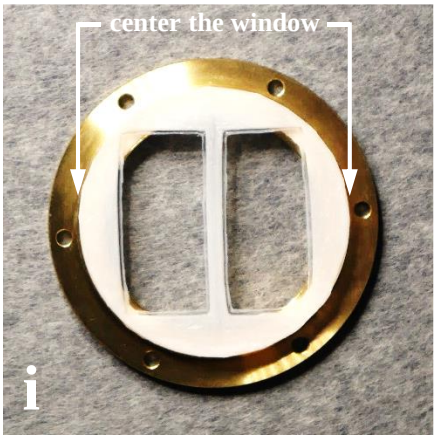
With the pipette tip, gently and carefully apply paste around the inner edges of the compartments.



Create a closed (!) frame out of paste for both compartments. Take your time.

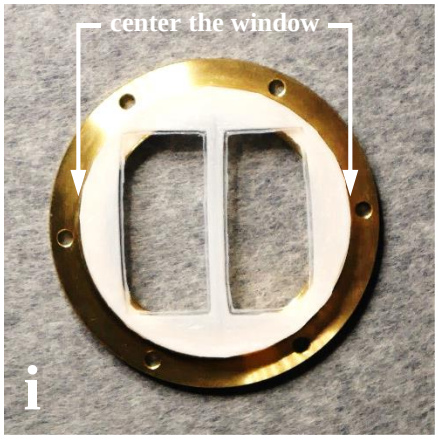


Transfer the window with the pasted spacer from the holder to the lid. Lift the window from below by pushing it with a lens tissue.



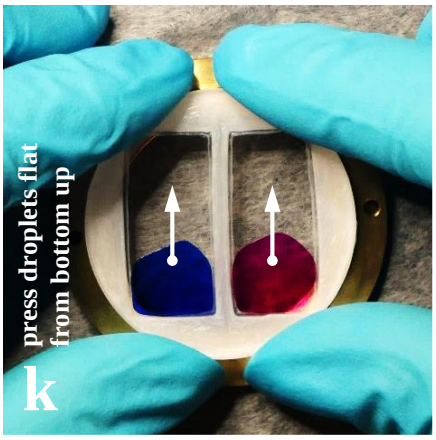
Check that window, spacer and lid are perfectly centered and the spacer is still sticking completely.

2. Cell assembly protocol – *sample loading*

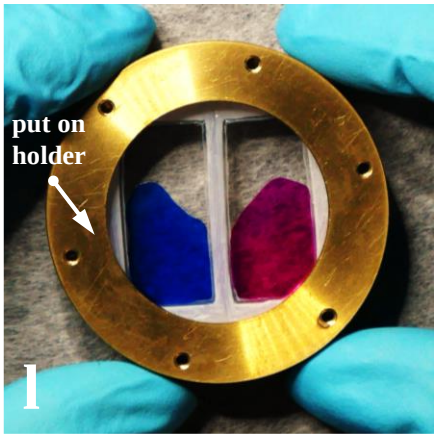


Before loading, check again that window, spacer and lid are centered and the spacer is still sticking.

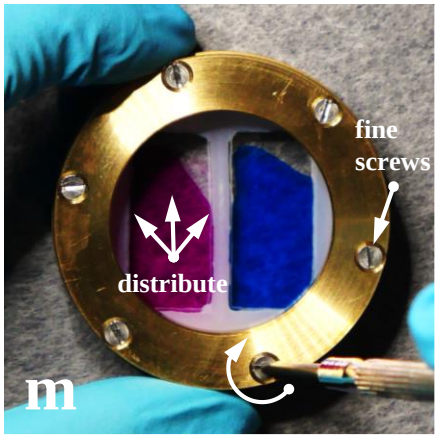
Pipette the sample and background solution onto the lower edges of the respective compartments. (The white spots in the droplets are reflections.)



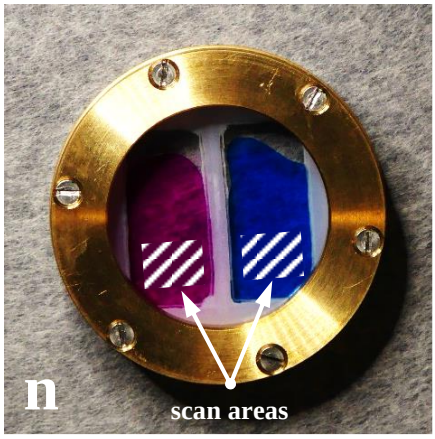
Carefully place the second window on top with a slight tilt so that both droplets touch the top window first, then press them flat.



Gently put on the holder and encase the sticking windows.



Turn the cell over and insert all fine screws. Tighten the screws, alternating between them to evenly distribute the solutions.



It is on purpose that the compartments are not fully loaded to avoid leakage. The split sample cell is ready to go.

2. Cell assembly protocol – *window cleaning*

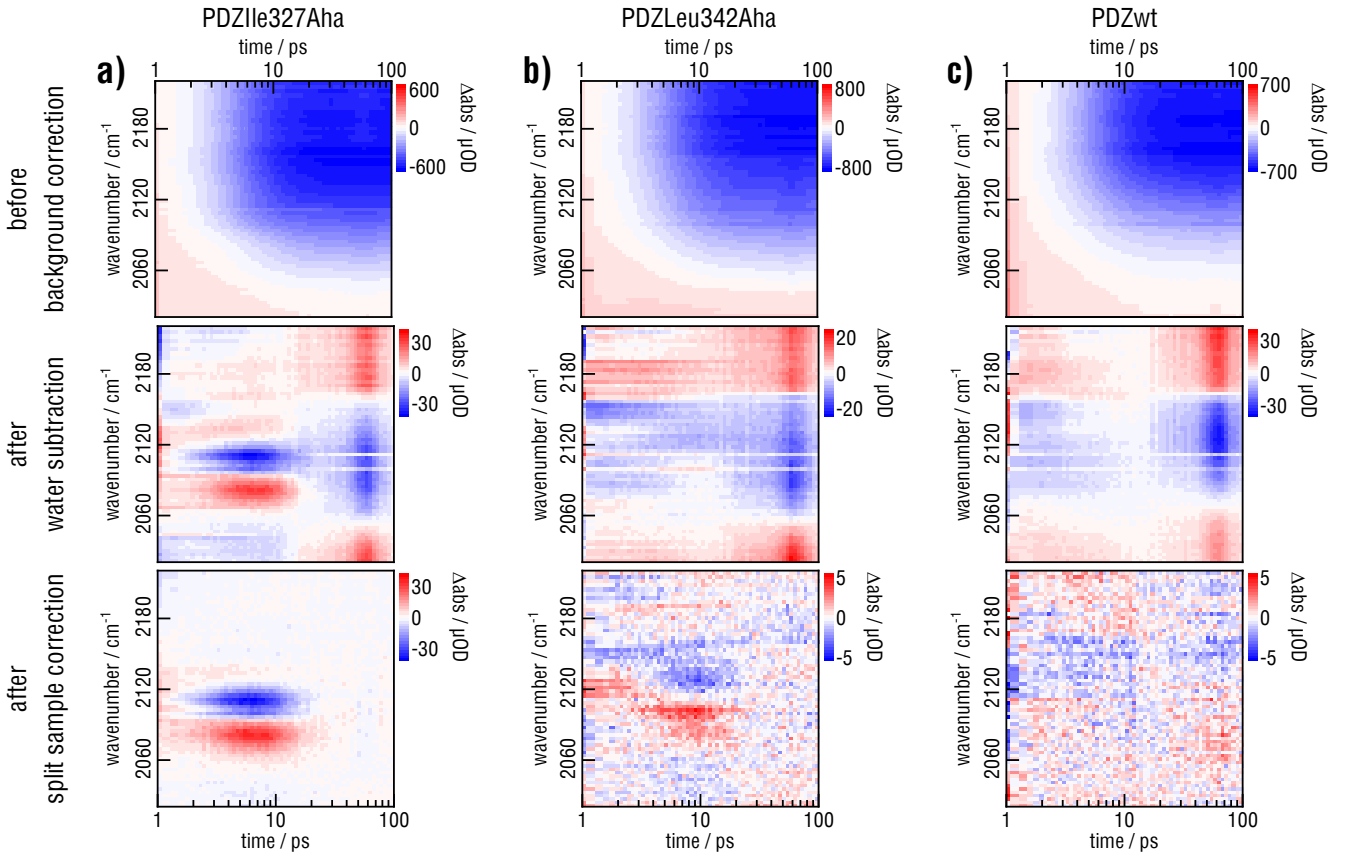
1.5” CaF_2 windows for the split sample cell are expensive. During cell assembly larger areas of the windows are smeared by the required generous amount of PTFE paste. Thus, a reliable cleaning procedure is required for reuse. With the protocol described below, the layer of PTFE paste can be removed without mechanical intervention.

- take measurement cell apart
- separate both windows by carefully sliding them against each other
- peel off the spacer and save for reuse. Rinse off sample solutions from the windows
- put both windows upright into a beaker with ~200 ml distilled water plus ~5% Hellmanex®
- let windows in the Hellmanex®-bath gently shake overnight at room temperature
- thoroughly rinse with distilled water and blow dry with compressed air
- check – and if necessary wipe off any traces of paste with methanol

3. Water background correction

In our vibrational energy transfer (VET) measurements, vibrational energy also dissipates into the solvent resulting in a time-dependent and long-lived heating signal. In the probed spectral region centered around the azide stretching frequency of 2110 cm^{-1}, the response from water heating is broad and 1–2 orders of magnitude stronger than the much narrower azide signal. As can be seen in SFig. 2 below, transient IR spectra of PDZ3 before background correction are completely dominated by the water response and no azide signal can be recognized whatsoever.

The water signal lives much longer than the azide signal. After 50 ps the azide signal has decayed to zero, while the heating signal still contributes to the transient spectrum. To get rid of the water background, we take the well-averaged spectral slice at the latest measured time point and subtract this representative transient water signal from every spectral slice in a scaled fashion together with a time-dependent offset. The two parameters are obtained by a best fit. This works reliably and reproducibly, because in the time range investigated the water heating signal changes only its intensity as a function of time, while its spectral shape remains unchanged. Although this procedure removes the major background contribution from water heating, an additional correction step is necessary for the finely structured and dynamically complex artifacts, which we attribute mainly to thermal lensing effects, as discussed in the main text.



SFig. 2: TRIR spectra of PDZ3 variants before and after background correction steps. Top row spectra are before background subtraction and, thus, completely dominated by a broad, intense and long-lived water heating signal. Middle row spectra are after subtracting the water contribution, revealing spectrally sharp and dynamically complex absorption changes. The bottom row spectra are corrected with the split-sample approach to remove the obscuring layer of any artifacts. Please note the different color scales of individual panels.