

Multi-scale photoacoustic tomography using reversibly switchable bacterial phytochrome as a near-infrared photochromic probe

Supplementary Materials

Supplementary Note 1. Sub-diffraction photoacoustic microscopy of reversibly switchable phytochrome BphP1 (RS-PAM)

In optical-resolution photoacoustic microscopy (OR-PAM), if the excitation laser pulse is in both thermal and stress confinement, the PA signal amplitude detected by the single-element focused ultrasonic transducer is given by

$$P \propto \Gamma \eta_{\text{th}} \mu_a F . \quad (\text{S1})$$

Here, Γ is the Grueneisen coefficient of the target, and η_{th} is the percentage of the absorbed photon energy that is converted into heat, μ_a is the optical absorption coefficient and F is the optical fluence. Since F is proportional to the excitation intensity I with a given pulsewidth, and μ_a is proportional to the number of molecules N with a given excitation spot size, Eq. S1 can be rewritten as

$$P \propto \Gamma \eta_{\text{th}} N \sigma_a I , \quad (\text{S2})$$

where σ_a is the absorption cross section of a single molecule.

Photoswitching is a stochastic process, where the number of ON-state molecules that are switched off by any excitation pulse is proportional to the total number of ON-state molecules present at the beginning of this excitation. This type of switching process can be modeled by an exponential decay function as

$$N_i = N_0 \exp(-\beta i) , \quad (\text{S3})$$

where N_i is the number of remaining ON-state molecules after the i th excitation pulse, N_0 is the initial number of ON-state molecules, and β denotes the switching-off rate.

The switching-off rate has a strong dependence on the excitation intensity. For most molecules, the switching-off rate has an excitation intensity power dependence of one for one-photon absorption, and at least two for two-photon absorption. Collectively, the switching-off rate can be expressed as

$$\beta = kI^b, \quad (\text{S4})$$

where k is a constant factor and b is the excitation intensity power dependence with $b > 0$.

Substituting Eqs. S3-S4 into Eq. S2, we get

$$P_i \propto \Gamma \eta_{\text{th}} N_0 \sigma_a I \exp(-kI^b i). \quad (\text{S5})$$

The Taylor expansion of Eq. S5 yields

$$P_i \propto \underbrace{\Gamma \eta_{\text{th}} N_0 \sigma_a I}_{\text{Conventional PAM}} - \underbrace{\Gamma \eta_{\text{th}} N_0 \sigma_a \sum_{n=1}^{\infty} \frac{(-1)^{n+1} k^n I^{nb+1} i^n}{n!}}_{\text{Switching-off}}. \quad (\text{S6})$$

Note that the first part in the right-hand side of Eq. S6 denotes the conventional PAM signal without photoswitching, while the second part denotes the PA signal decay induced by switching-off the ON-state molecules over consecutive laser pulses.

An m th polynomial function can be used to fit the measured PA signal decay as a function of time. For one-photon photoswitching of BphP1 (i.e., $b = 1$), the contrast of the RS-PAM comes from the fitted m th-order coefficient, expressed as

$$P_{\text{RS-PAM}} \propto k^m \Gamma \eta_{\text{th}} N_0 I^{m+1}. \quad (\text{S7})$$

Eq. S7 indicates that, on the one hand, the RS-PAM contrast is linear to the optical absorption, which maintains its linearity to the BphP1 molecule concentration. On the other hand, the RS-PAM contrast is non-linear to the excitation intensity, which enables sub-diffraction imaging. It is worth noting that higher order polynomial fitting can certainly be applied in Eq. S7, as long as

the signal-to-noise ratio permits. The resolution enhancement of RS-PAM is explained in the following notes.

Supplementary Note 2. Lateral resolution of RS-PAM

In OR-PAM, since the focused ultrasonic traducer has a focal spot size on the level of tens of micrometers, the lateral resolution is determined predominantly by the excitation point spread function (PSF). Because RS-PAM relies on non-linear optical measurement, where the signal is given by the m th-order coefficient in Eq. S7, the lateral PSF of RS-PAM can be expressed as

$$PSF_r^{RS-PAM} = PSF_r^{\text{excitation}} \times PSF_r^{\text{switching}}, \quad (\text{S8})$$

where $PSF_r^{\text{excitation}}$ is the excitation fluence distribution, and $PSF_r^{\text{switching}}$ is the photoswitching profile (switching-off profile).

If the excitation profile can be approximated by a Gaussian function, we obtain

$$PSF_r^{\text{excitation}}(r) = \exp\left(-\frac{2r^2}{w_e^2}\right) \quad (\text{S9})$$

$$PSF_r^{\text{switching}}(r) = \exp\left(-\frac{2mr^2}{w_e^2}\right), \quad (\text{S10})$$

where r is the radial distance from the center of the Airy disk, and w_e is the Gaussian width of the excitation beam where the beam intensity drops to $1/e^2$ of its center value.

The full-width-at-half-maximum (FWHM) of the excitation beam can be expressed as

$$FWHM_r^{\text{excitation}} = \sqrt{2 \ln 2} w_e \approx 0.51 \frac{\lambda_0}{NA}, \quad (\text{S11})$$

where λ_0 is the excitation wavelength and NA is the numerical aperture of the optical objective. In fact, Eq. S11 shows the diffraction-limited lateral resolution of conventional PAM, where the signal contributions of the molecules within the excitation spot are proportional to the local excitation fluence.

From Eqs. S8-11, we obtain the lateral resolution of RS-PAM as

$$FWHM_r^{RS-PAM} = \sqrt{\frac{2\ln 2}{1+m}} w_e \approx \frac{0.51}{\sqrt{1+m}} \frac{\lambda_0}{NA} = \frac{1}{\sqrt{1+m}} FWHM_r^{\text{excitation}}. \quad (\text{S12})$$

Eq. S12 indicates that, compared with conventional diffraction-limited PAM, the lateral resolution of RS-PAM is improved by a factor of $\sqrt{1+m}$. Therefore, with a third-order polynomial fitting (i.e., $m=3$), RS-PAM has a two-fold improvement on the lateral resolution.

Supplementary Note 3. Optical sectioning of RS-PAM

Similarly, if we approximate the excitation beam as a Gaussian beam, the axial PSF of RS-PAM for point targets can be expressed as

$$PSF_z^{\text{point}}(z) = \left[1 + \left(\frac{z}{Z_R}\right)^2\right]^{-(1+m)}, \quad (\text{S13})$$

where Z_R is the Rayleigh range of the Gaussian beam, which is given by

$$Z_R = \pi \frac{w_e^2}{\lambda_0} \approx 0.9 \frac{\lambda_0}{NA^2}. \quad (\text{S14})$$

The axial resolution of RS-PAM for point targets is then given by the FWHM of the axial PSF as

$$FWHM_z^{\text{RS-PAM, point}} = 2\sqrt{2^{1/(1+m)} - 1} Z_R = 1.8\sqrt{2^{1/(1+m)} - 1} \frac{\lambda_0}{NA^2}, \quad (\text{S15})$$

which indicates RS-PAM has an axial resolution improvement by a factor of $1/\sqrt{2^{1/(1+m)} - 1}$ for point targets.

For large (or planar) targets, conventional PAM lacks sectioning capability because its axial PSF is constant.

By contrast, for RS-PAM, the axial PSF for planar targets is given by

$$PSF_z^{\text{planar}}(z) = \left[1 + \left(\frac{z}{Z_R}\right)^2\right]^{-m}. \quad (\text{S16})$$

Therefore, the optical sectioning capability of RS-PAM can be expressed as the FWHM of the axial PSF:

$$FWHM_z^{\text{RS-PAM, planar}} = 2\sqrt{2^{1/m} - 1}z_R = 1.8\sqrt{2^{1/m} - 1}\frac{\lambda_0}{NA^2} . \quad (\text{S17})$$

Eq. S17 shows that, with a third-order polynomial fitting (i.e., $m = 3$), RS-PAM has an optical sectioning capability of approximately the Rayleigh range of the Gaussian beam.

Supplementary Video 1. Whole-body photoacoustic computed tomography of mouse internal organs *in vivo*. The light fluence on the animal skin was 8 mJ/cm^2 at 780 nm. Stepping along the animal trunk with a step size of 0.3 mm, a total of 250 cross-sectional images were acquired with a field of view of 2 cm by 3 cm.

Supplementary Video 2. Elevational-scanning whole-body photoacoustic computed tomography of a BphP1-expressing U87 tumor *in vivo* with a scanning step size of 1 mm. The differential PA signals from the tumor (shown in color) were superimposed on top of the OFF state PA signals from blood (shown in gray). A global threshold was applied to all the differential images with a threshold level at three times the noise level.

Supplementary Video 3. Depth-scanning photoacoustic microscopy (PAM) of multiple layers of BphP1-expressing U87 cells with a scanning step size of $0.25 \text{ }\mu\text{m}$. The reversibly switchable PAM (RS-PAM) with optical sectioning was able to resolve cells at different depths, while conventional PAM with acoustic sectioning could not.