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

\[ P \propto \Gamma \eta_{th} \mu_{a} F . \]  
(S1)

Here, \( \Gamma \) is the Grueneisen coefficient of the target, and \( \eta_{th} \) is the percentage of the absorbed photon energy that is converted into heat, \( \mu_{a} \) 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 \( \mu_{a} \) is proportional to the number of molecules \( N \) with a given excitation spot size, Eq. S1 can be rewritten as

\[ P \propto \Gamma \eta_{th} N \sigma_{a} I , \]  
(S2)

where \( \sigma_{a} \) 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

\[ N_i = N_0 \exp(-\beta i) , \]  
(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 $\beta$ 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

$$\beta = kI^b,$$  \hspace{1cm} (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

$$P_i \propto \Gamma \eta_{th} N_0 \sigma_a I \exp(-kI^b i).$$  \hspace{1cm} (S5)

The Taylor expansion of Eq. S5 yields

$$P_i \propto \Gamma \eta_{th} N_0 \sigma_a I - \Gamma \eta_{th} N_0 \sigma_a \sum_{n=1}^N \frac{(-1)^{n+1} k^n I^{nb+1} i^n}{n!}.$$  \hspace{1cm} (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

$$P_{RS-PAM} \propto k^m \eta_{th} N_0 I^{m+1}.$$  \hspace{1cm} (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

$$PSF_{r}^{RS-PAM} = PSF_{r}^{\text{excitation}} \times PSF_{r}^{\text{switching}},$$

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

$$PSF_{r}^{\text{excitation}}(r) = \exp(-\frac{2r^2}{w_e^2}),$$

$$PSF_{r}^{\text{switching}}(r) = \exp(-\frac{2mr^2}{w_e^2}),$$

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

$$FWHM_{r}^{\text{excitation}} = \sqrt{2 \ln 2}w_e \approx 0.51\frac{\lambda_0}{NA},$$

where $\lambda_0$ 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

\[ FWHM_{rs-pam}^r = \sqrt{\frac{2\ln 2}{1 + m}} w_e \approx \frac{0.51}{\sqrt{1 + m}} \frac{\lambda_0}{NA} = \frac{1}{\sqrt{1 + m}} FWHM_{excitation}^r. \]  \hspace{1cm} (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

\[ PSF_{z, point}^r(z) = \left[ 1 + \left( \frac{z}{Z_R} \right)^2 \right]^{-(1-m)}, \]  \hspace{1cm} (S13)

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

\[ Z_R = \frac{w_e^2}{\lambda_0} \approx 0.9 \frac{\lambda_0}{NA^2}. \]  \hspace{1cm} (S14)

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

\[ FWHM_{z, rs-pam, point} = 2\sqrt{2^{1/(1+m)} - 1}Z_R = 1.8\sqrt{2^{1/(1+m)} - 1} \frac{\lambda_0}{NA^2}, \]  \hspace{1cm} (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

\[ PSF_{z, planar}^r(z) = \left[ 1 + \left( \frac{z}{Z_R} \right)^2 \right]^{-m}. \]  \hspace{1cm} (S16)
Therefore, the optical sectioning capability of RS-PAM can be expressed as the FWHM of the axial PSF:

\[
FWMH_{z, \text{RS-PAM, planar}} = 2\sqrt{2^{1/m} - 1}z_R = 1.8\sqrt{2^{1/m} - 1} \frac{\lambda}{NA^2}.
\]  

(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 µm. 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.