Supplementary Information

Alginate beads as a highly versatile test-sample for optoacoustic imaging

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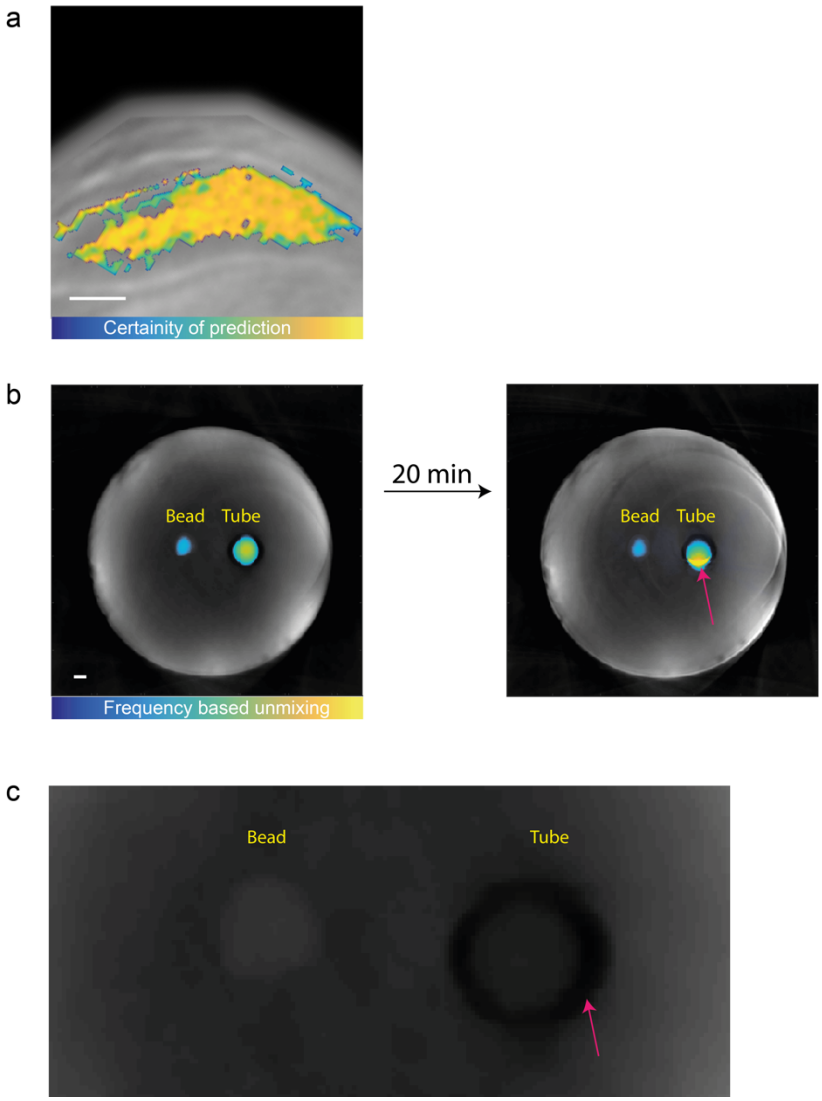
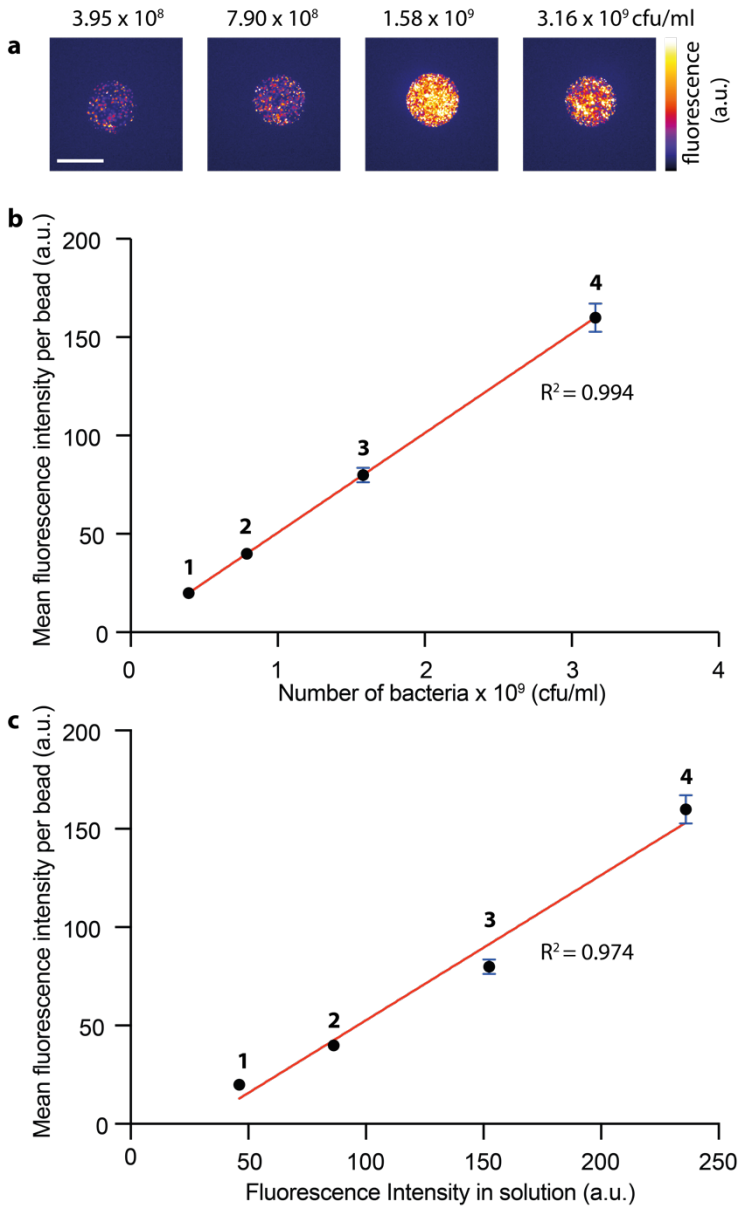


Supplementary Figure 1: **Challenges of alternative *in vivo* test-samples. (a)** Inhomogeneity and frayed edges of a Matrigel subcutaneous implant of 3,200 cells\µl Jurkat cells expressing *Re*BphP-PCM. Unmixing is done by a machine learning approach described in[15]. The colorbar shows the certainty of prediction, scalebar is 1 mm. Image and data are taken from Mishra et al., *Science Advances*, 2020[15] published under the CC BY-NC license. **(b)** Sedimentation of cells in tubing. Shown is a blood (2%) / intralipid (3%) phantom with two samples at the center: left a 1.6 mm diameter AlBe containing *E. coli* expressing 1x10^6 cells/µl *Re*BphP-PCM (conc), right a tube of 2 mm inner diameter filled with the same *E. coli* cells. The first and second dataset are recorded separated by 20 min of wait-time. It is apparent that the cells in the tube are sedimenting with time (pink arrow) while the bead stays homogenous. Data was recorded with MSOT and unmixed based on the frequency component of the photo-switching. The coloring shows the strength of the first harmonic of the Fourier transform of the time-series per pixel. Scalebar is 1 mm. **(c)** Outtake of b, left with only the raw image at 680 nm excitation shown. For the tube a transition zone between the agent signal and the phantom material is visible (pink arrow).

Supplementary Info 1: Alginate bead preparation step by step procedure is the following:

1) Pour 2 g of alginate powder on 100 ml of Mili Q water under stirring and keep the mix under constant stirring at least 24 h to ensure the polysaccharide is fully hydrated and dissolved. In parallel, prepare a solution of 0.2 M calcium chloride.
2) Produce a stock of cells (which can e.g. carry your label of interest).
3) Centrifuge a desired number of cells. Resolve the pellet in 1 ml of alginate solution (1) with gentle stirring to avoid bubbles formation.
4) Load the alginate solution into a 1 ml syringe. Insert the syringe in the 3D-printed falcon cap until the 0.6 ml mark is inside the cap (Figure 1b). Insert the needle with the desired size.
5) Add 5 ml of calcium 0.2M inside the falcon tube.
6) Place the tube with the cap in a falcon centrifuge and run at 300 rpm for at least 10 min.
7) Leave the beads to rest and sediment for 10 min, then remove the calcium solution and replace it with water. Perform this washing procedure at least three times to ensure the removal of calcium excess.



Supplementary Figure 2: Four (1-4) preparations of AlBes with different number of *E. coli* expressing fluorescent *Re*BphP-PCM. Shown are exemplary images of beads **(a)** and the correlation between the AlBes fluorescence and the number of bacteria **(b)** or the fluorescence signal of the bacterial solution before preparation of the beads **(c)**. To analyze the AlBes fluorescence N = 20 beads were analyzed under a fluorescence microscope. Shown are the mean value and standard deviation. AlBes have been prepared using a 25G needle. Scalebar is 500 µm.

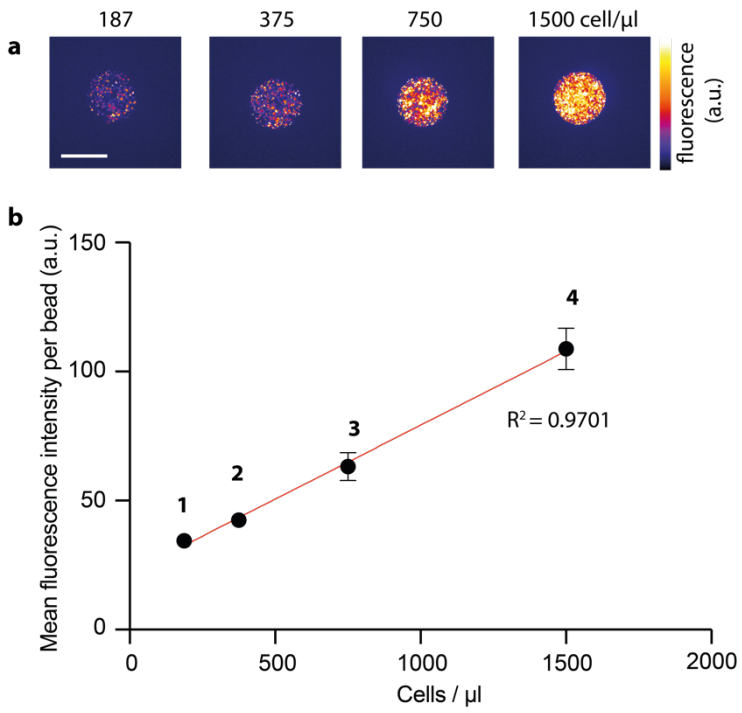
Supplementary Table 1: Bead stability under different conditions shown as diameter **(a)** and fluorescence **(b)**. All buffers 1x, HEPES is 5 mM HEPES and 100 mM NaCl. The values are reported as mean and standard deviation of N = 20 beads normalized to 0 h in water.

a

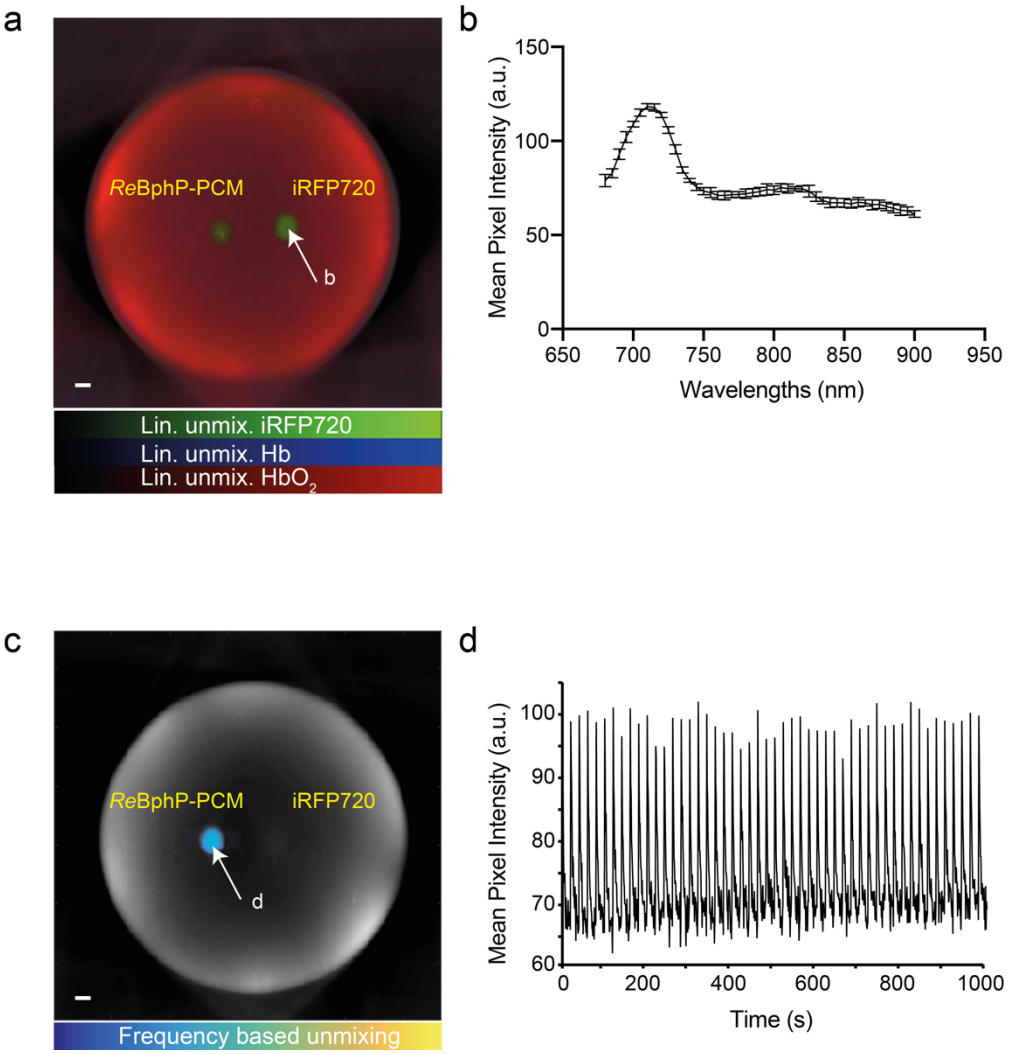
after x h in	water	PBS	MOPS	HEPES	HBSS
0	1.00 ± 0.01				
24	1.00 ± 0.01	1.06 ± 0.06	1.02 ± 0.04	1.13 ± 0.06	1.03 ± 0.04
48	1.00 ± 0.02	1.06 ± 0.05	1.02 ± 0.04	1.11 ± 0.06	1.03 ± 0.03
72	1.00 ± 0.02	1.06 ± 0.04	1.02 ± 0.04	1.11 ± 0.05	1.03 ± 0.04

b

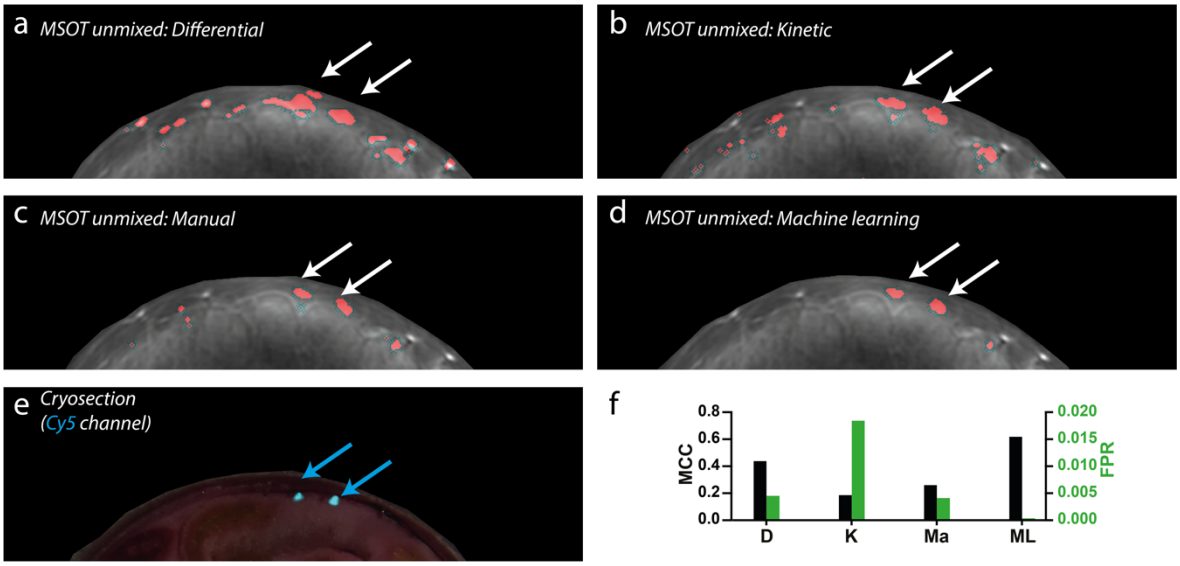
after x h in	water	PBS	MOPS	HEPES	HBSS
0	1.00 ± 0.04				
24	1.03 ± 0.11	0.55 ± 0.04	0.83 ± 0.07	0.57 ± 0.07	0.54 ± 0.04
48	1.01 ± 0.05	0.55 ± 0.04	0.85 ± 0.04	0.57 ± 0.05	0.53 ± 0.04
72	1.02 ± 0.04	0.54 ± 0.04	0.86 ± 0.05	0.57 ± 0.06	0.54 ± 0.06



Supplementary Figure 3: Four (1-4) preparations of AlBes with different number of HCT116 cells expressing fluorescent GFP. Shown are exemplary images of beads **(a)** and the correlation between the AlBes fluorescence and the number of cells **(b).** To analyze the AlBes fluorescence N = 20 beads were analyzed under a fluorescence microscope. Shown are the mean value and standard deviation. AlBes have been prepared using a 25G needle. The mammalian cells are no monoclonal preparation leading to the varying brightness of cells which are actually different as individual cells with ~20 µm. Scalebar is 500 µm.



Supplementary Figure 4: Blood (2%) / Intralipid (3%) phantom with two AlBes at the center. Left a bead filled with *E. coli* expressing photo-switching *Re*BphP-PCM, right a bead with *E. coli* expressing NIR-absorbing protein iRFP720. **(a)** Multispectral tomography data (680 nm – 900 nm) and linear unmixing of the data. Note that oxygenated and deoxygenated blood are equally picket up. Further note, that the *Re*BphP-PCM AlBe is also positively unmixed for iRFP720 since it shares spectral features in the 700 nm region. Scalebar 1 mm. **(b)** Mean pixel intensity spectra of a region of interest (ROI) in the center of the bead indicated with arrow and b in figure a. The slight offset of the iRFP720 spectra in intensity and wavelength stems from the spectral coloring and background intensity of the surrounding blood. **(c)** The same phantom recorded with alternating illumination of 680 nm and 770 nm to drive photo-switching and unmixed based on the frequency component of the photo-switching (intensity of the first harmonic of the Fourier transform of the time series per pixel). **(d)** Mean pixel intensity over time of ROI in the center of the bead indicated with arrow and d in figure c.



g

Measure	Differential	Kinetics (exponential decay fitting)	Manual (kinetics + rsqr)	ML
ACC	0.9953	0.9814	0.9951	0.9989
TPR	0.8824	0.6941	0.4824	0.5882
FPR	0.0046	0.0185	0.0042	4.5255 e-04
TNR	0.9954	0.9815	0.9958	0.9995
MCC	**0.4418**	**0.1894**	**0.2634**	**0.6216**
BA	0.9389	0.8378	0.7391	0.7939
F1	0.3555	0.0994	0.2240	0.6211

ACC = Accuracy
TPR = True Positive Rate
FPR = False Positive Rate
TNR = True Negative Rate
MCC = Matthews Correlation Coefficient
BA = Balanced Accuracy
F1 = F1-score

Supplementary Figure 5: Additional data showing the *in vivo* application of AlBes. The representation of **a-f** is similar to Figure 3. Here 2 AlBes loaded with $1x10^6$ *E. coli* cells/µl expressing *Re*BphP-PCM have been injected *intraperitoneal* in a FoxN1 nude mouse. **(g)** Additional metrics to analyze the performance of the different unmixing methods. Methods shown in bar plots highlighted as bold color. Note that the different metrics cannot be used in total as quality assessment but rather have to be interpreted based on the importance of certain factors, e.g. a low false positive rate.

Supplementary Info 2: Photo-switching based unmixing methods.

As an example, for the utilization of AlBes a number of different unmixing strategies for photo-switching contrast have been compared and the unique reproducible shape and intensity of AlBes has been used as ground truth.

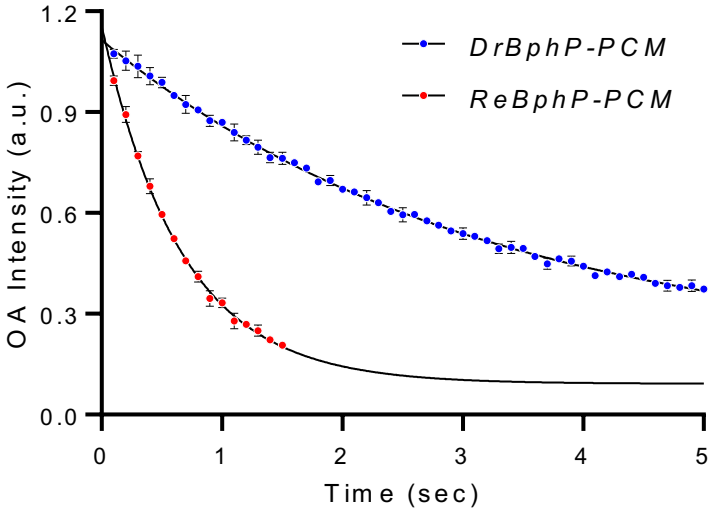
Unmixing of photo-switching data relies on extracting the unique temporal signature of the signal which is dependent on the known illumination schedule. In the experiment this photo-switching is generated by illumination with a repetitive sequence of multiple pulses of OFF-switching and ON-switching light; e.g.: (15 pulses 770 nm, 15 pulses 680 nm) x 20 cycles. With this, the approach is fundamentally different to the conventional spectral unmixing approaches since it is independent from overlapping spectral characteristics of strong endogenous absorbers like blood hemoglobin. Different approaches have been suggested and are exemplarily compare in this work:

1) **Differential method**: This is the simplest method, it relies on the different signal intensities of the ON-and OFF-state label. In the implementation used herein the maxima and minima of the mean OFF-switching cycle at each pixel are extracted. Not photo-switching background shows relatively comparable signals while the photo-switching label shows a strong differential.

2) **Kinetic method**: This method additionally takes the exponential character of the photo-switching induced temporal change of the signal into account. This allows more segmentation strength and additionally allows a classification between different labels if they show sufficiently different switching characteristics manifesting in different time constants for the exponential decay. In the implementation used herein the mean OFF-switching cycle is fitted with a single exponential function and the time-constant are used to separate the photo-switching label from non-switching background.

3) **Manual multi parametric method**: This is an augmented form of the kinetic method where additionally the r-square and directionality of the fit is evaluated (positive exponentials are indicative of artefacts caused by fluence effects).

4) **Machine learning method**: In the complex environment the photo-switching signal of a label might be additionally identified by other indicative parameters. These can be obvious parameters like a signature in frequency space due to the repetitive nature of the photo-switching, but also other metrics like a ratio between signal amplitude and overall signal which is powerful to differentiate false positives associated with strong signal from non-switching absorbers. The rules interconnecting all parameters required for segmentation are trained with machine learning approaches like for example decision trees (random forest). In this work we used 12 parameters and decision-tree based model (i-ii) the coefficient for the exponential fit of the mean kinetic (mean of all cycles), iii) R2 of the fit, iv) the mean intensity over the signal, v) amplitude (max-min) of all the signal, vi-ix) median maximums and minimums of cycles along with standard deviation, x) number of cycles with increasing or decreasing trend, xi) the length of the part of the cycle that shows a trend, i.e. at what point the signal vanishes in the noise, and xii) Fourier coefficient for the expected frequency defined by the photo-control schedule).



Supplementary Figure 6: MSOT Mean of switching kinetic for tubing filed with *E. coli* expressing rsOAPs *Re*BphP-PCM and DrBphP-PCM in agar lipid phantoms.