Supplementary Information: Functional Screening of Antibody-Secreting Cells by Co-Culture with Reporter T Cells Using PicoShells

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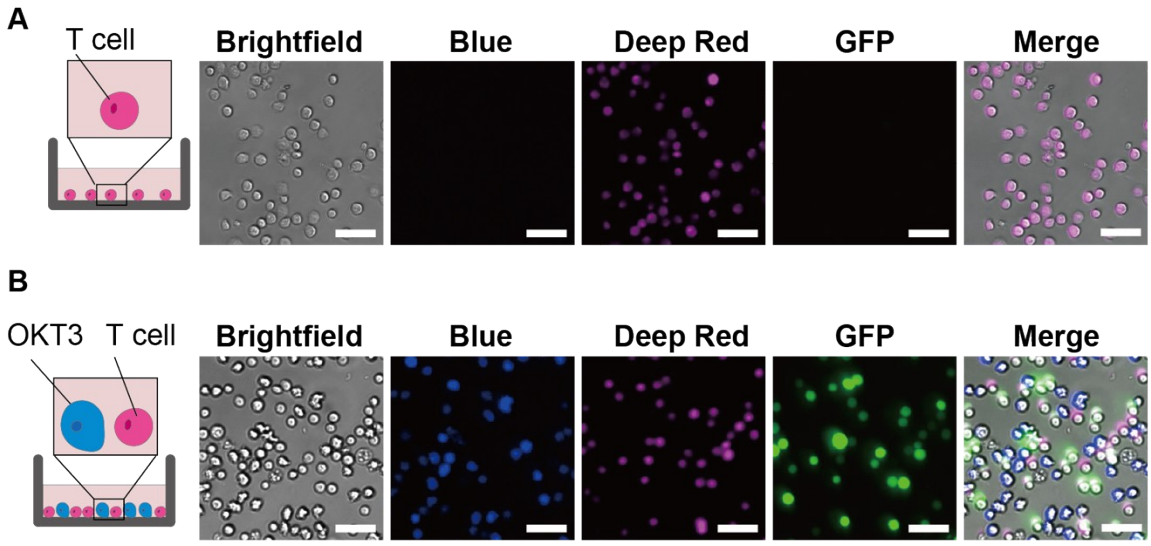

Figure S1. GFP expression in reporter T cells co-cultured with OKT3 for 24 hours. (A) Reporter T cells (CellTracker Deep Red), containing a plasmid with an NFAT activation promoter linked to GFP, do not exhibit GFP expression on their own. Brightfield and separate channels of the fluorescence microscopy images are shown, along with a merged image. (B) In the presence of OKT3 (CellTracker Blue) which secrete anti-CD3 IgG antibody, GFP expression is observed in reporter T cells. Brightfield and separate channels of the fluorescence microscopy images are shown, along with a merged image. Scale bars = 100 μm.

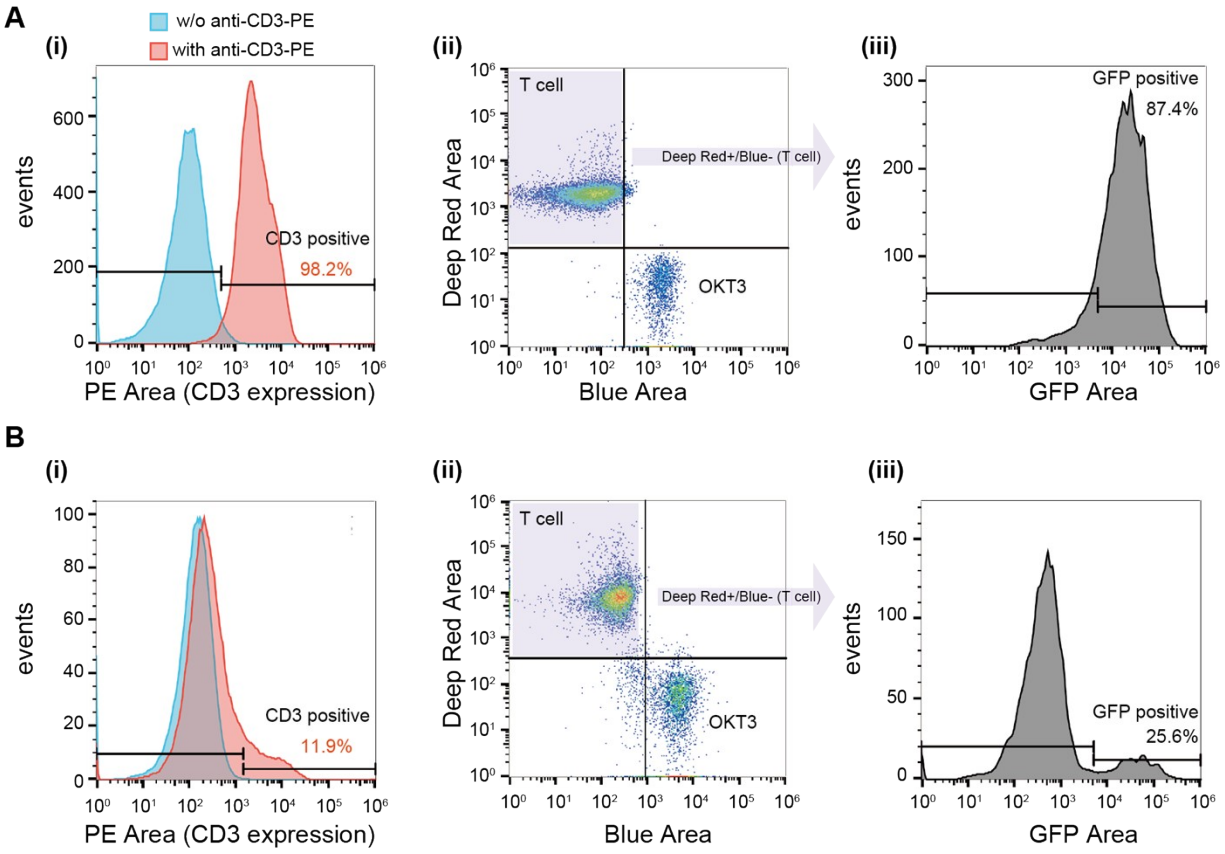

Figure S2. CD3 and GFP expression level comparison between Jurkat-NFAT-F5-TCR GFP reporter T cells and Jurkat-NFAT GFP reporter T cells. (A) Characterization of Jurkat-NFAT-F5-TCR GFP reporter T cells. (i) CD3 expression level analyzed using PE anti-CD3. The CD3 positive ratio was determined to be 98.2% after setting thresholds for CD3 negativity and positivity using the PE Area. (ii) Flow cytometry scatter plot with CellTracker Blue (staining OKT3 cells) and CellTracker Deep Red (staining T cells) intensities of after co-culturing with OKT3 for 24 hours. (iii) Flow cytometry histogram showing GFP expression level. The GFP-positive percentage for Jurkat-NFAT-F5-TCR GFP reporter T cells was 87.4%. (B) Characterization of Jurkat-NFAT GFP reporter T cells. (i) CD3 expression level analyzed using PE anti-CD3. A lower level of CD3 expression compared to that of Jurkat-NFAT-F5-TCR GFP reporter T cells. (ii) Flow cytometry scatter plot with CellTracker Blue and CellTracker Deep Red intensities of after co-cultured with OKT3 for 24 hours. (iii) Flow cytometry histogram showing GFP expression level of reporter T cells. The GFP-positive percentage for Jurkat-NFAT GFP reporter T cells was 25.6%. These results suggest that higher CD3 expression is critical for activation by anti-CD3 antibodies produced by OKT3 leading to more GFP expression, reflecting increased T cell activation.

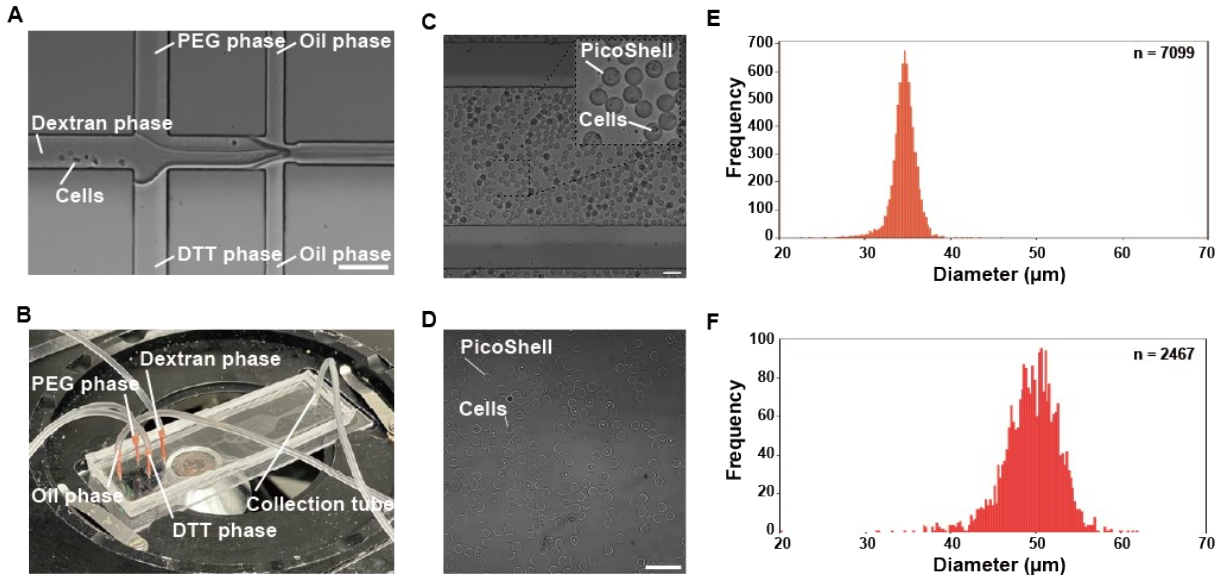

Figure S3. Fabrication of PicoShells encapsulating cells. (A) Image of the flow-focusing junction within the droplet generation chip, producing picoliter-scale droplets containing 4-arm PEG-mal, DTT, and dextran with cells. (B) Image of fabrication setup on a microscope. (C) Image of droplets downstream of the generation site. Following phase separation between dextran and 4-arm PEG-mal with DTT, dextran gathers at the core of each particle, while an outer hydrogel layer forms and is crosslinked through a thiol-maleimide reaction between 4-arm PEG-mal and DTT. (D) Image of PicoShells in culture media. (E) Histogram of PicoShell diameters in oil phase before the removal of the surfactant from the droplet surface (n = 7099). The average diameter was 34.55 μm, with a coefficient of variation (CV) of 0.05. (F) Histogram of PicoShell diameters in medium (n = 2467) after swelling in aqueous phase. The average diameter was 49.46 μm, with a coefficient of variation (CV) of 0.07. Scale bars = 100 μm.

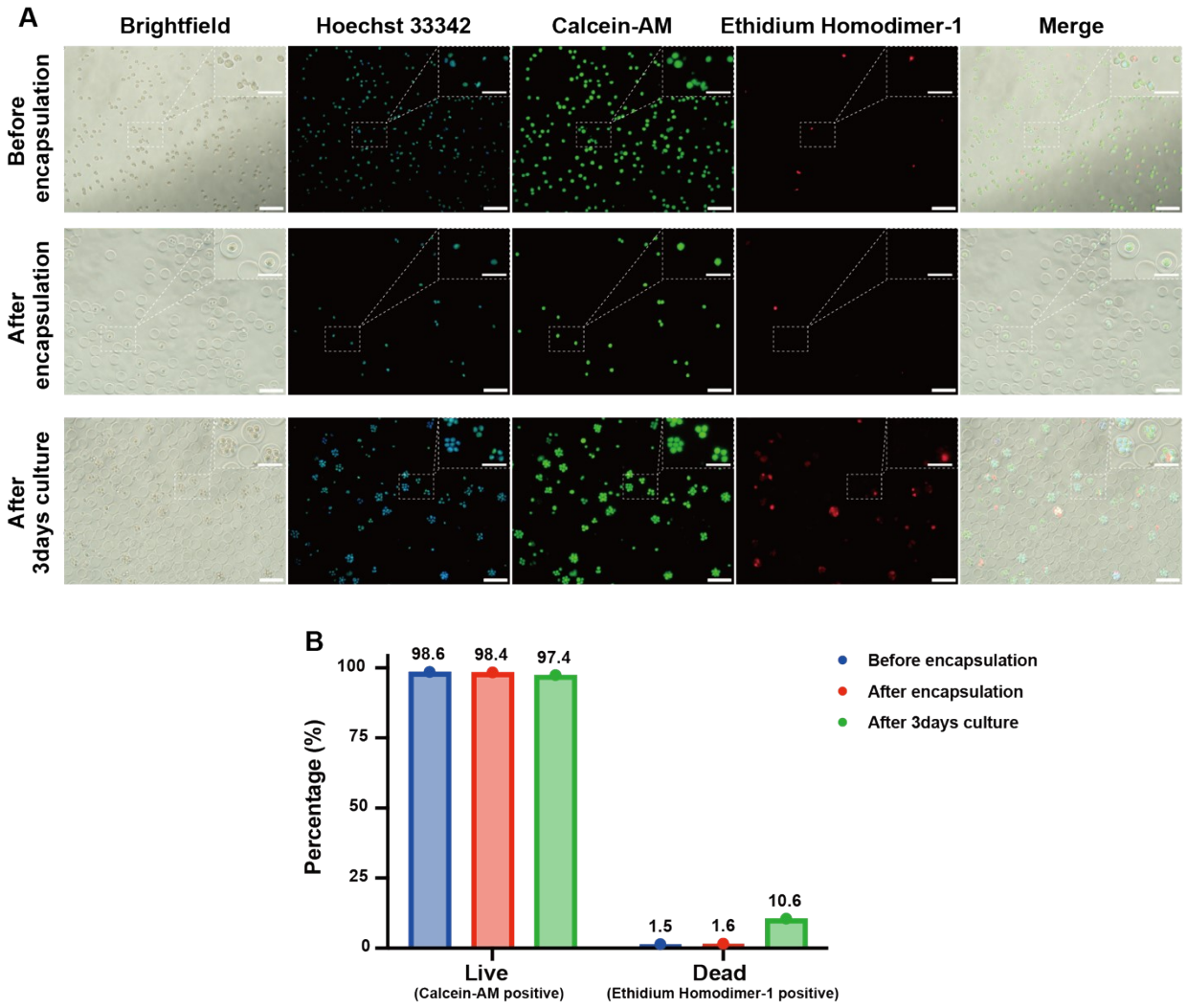

Figure S4. Live/dead assay of MF20 hybridoma cells before and after PicoShell encapsulation and after 3 days of culture. (A) Representative fluorescence and brightfield images of MF20 hybridoma cells stained with Hoechst 33342 (blue, nuclei), Calcein-AM (green, live cells), and Ethidium Homodimer-1 (red, dead cells) under three conditions: before encapsulation, immediately after encapsulation, and after 3 days of culture in PicoShells. Merge panels include brightfield and fluorescence overlays. All dyes were able to diffuse into the hydrogel shell, allowing viability assessment of encapsulated cells. (B) Quantification of the cell viability under each condition. Cells were identified by brightfield images and segmented using Cellpose. The percentages of live (Calcein-AM–positive) and dead (Ethidium Homodimer-1–positive) cells were determined using ImageJ. Double-negative samples were excluded from the analysis, as they were likely cell debris. Only cells confirmed to be inside PicoShells (via brightfield image overlay) were included in the encapsulated and 3-day culture groups. Data represent a single representative experiment (n = 728 cells before encapsulation; 81 cells after encapsulation; 885 cells after 3 days of culture). Scale bars = 100 μm and 50 μm (insets).

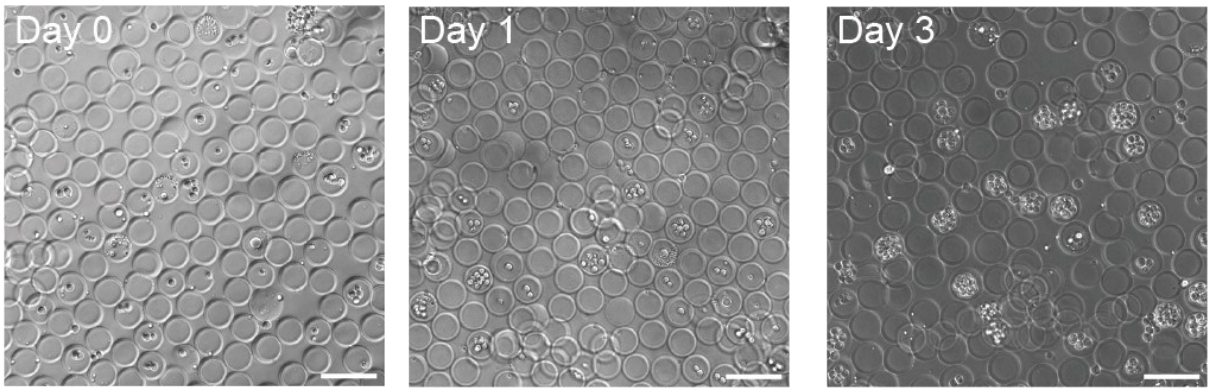

Figure S5. Growth data for long-term culture in PicoShells. After encapsulating Jurkat-NFAT GFP reporter T cells, we cultured the PicoShells in RPMI medium for 3 days. Throughout the culture period, there was an increase in the number of cells within the PicoShells, demonstrating that the encapsulated cells can proliferate effectively in this environment. Scale bars = 100 μm.

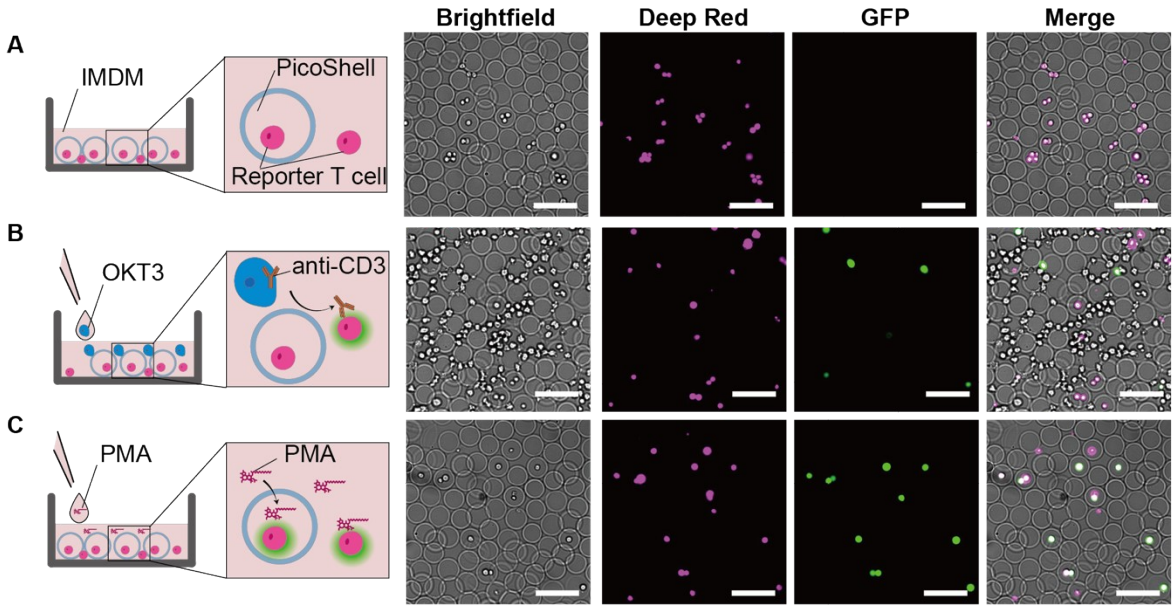

Figure S6. **Permeability of PicoShells to small molecules and IgG antibodies.** (A) Schematic showing the experimental setup, brightfield, and fluorescence microscopy images. Reporter T cells alone, stained with CellTracker Deep Red (magenta), were encapsulated in PicoShells. (B) The addition of OKT3 outside of PicoShells and incubation for one day does not induce GFP expression (green) in encapsulated reporter T cells (magenta), while GFP expression is observed in reporter T cells outside the PicoShells. (C) Phorbol 12-myristate 13-acetate (PMA), a small molecule that activates reporter T cells (magenta) by stimulating calcium signaling pathways, was added to induce GFP expression (green) in reporter T cells encapsulated within PicoShells. Scale bars = 100 μm.

Table S1: Distribution of hybridoma and reporter T cells in PicoShells. Rows indicate the number of Jurkat cells (ranging from 0 to 4), while columns represent the number of hybridoma cells (ranging from 0 to 3) within the PicoShells.

	0 Hybridoma	1 Hybridoma	2 Hybridoma	3 Hybridoma	Total
0 Jurkat	79.4%	2.36%	0.08%	0.00%	81.82%
1 Jurkat	14.8%	1.64%	0.13%	0.00%	16.58%
2 Jurkat	1.01%	0.21%	0.04%	0.00%	1.27%
3 Jurkat	0.21%	0.04%	0.00%	0.04%	0.30%
4 Jurkat	0.00%	0.00%	0.04%	0.00%	0.04%
Total	95.40%	4.26%	0.30%	0.04%	100%

Materials and Methods

Cell Staining, and Cell Preparation

Cells within hydrogel particles were stained using CellTracker Blue and CellTracker Deep Red dyes (Thermo Fisher Scientific, Waltham, MA, USA) to differentiate hybridoma and reporter T cells. The treatment concentrations of CellTracker Blue and CellTracker Deep Red were established at 200 μM, 1 μM, respectively. Following staining, cells were incubated for 30 minutes at 37°C, washed twice with phosphate-buffered saline (PBS; Gibco, Grand Island, NY, USA), resuspended in culture medium. For co-encapsulation, after cell counting with a cellometer (Thermo Fisher Scientific, Waltham, MA, USA) a mixed cell suspension was created, supernatant was removed, and the the dextran phase solution was added. The mixed cell suspension in dextran was subsequently transferred to a syringe for introduction into the microfluidic droplet generator.

PicoShell flow focusing device pretreatment and PicoShell Staining

To minimize nonspecific binding to the channel, the surfaces were treated with 1% Trichloro(1H,1H,2H,2H-perfluorooctyl)silane (Millipore Sigma, Burlington, MA, USA) in Novec™ 7500 (3M, Maplewood, MN, USA). To visualize the particle morphology, we used Alexa Fluor™ 488 C5 Maleimide (Thermo Fisher Scientific, Waltham, MA, USA), which was added to a DTT-containing solution at 1 μg/ml. In this mixture, the maleimide reacts with DTT to generate fluorescently labeled thiol compounds. These fluorescent thiols then covalently bind to PEG-maleimide during PicoShell formation, resulting in the incorporation of fluorescence into the hydrogel structure.

Flow Cytometry Setup

Single-particle fluorescence analysis was conducted using an SH800 Cell Sorter (Sony, Tokyo, Japan), equipped with 405 nm, 488 nm, 561 nm, and 638 nm lasers. Calibration used a 130 μm sorting chip to achieve a droplet throughput of 2000 events per second, while event rates during measurements were kept between 100 and 800 events per second by diluting the sample with PBS. For PicoShell sorting, the event rate was adjusted to ~150 Hz to ensure single-PicoShell sorting. Analysis of sorted particles was performed using FlowJo 10.10.0.

Droplet Diameter Analysis

To evaluate the uniformity of droplet shapes, the inner and outer diameters of particles were measured from images obtained using a Nikon Eclipse Ti microscope (Nikon, Tokyo, Japan). The inner and outer peripheries of the particles were identified using CellPose ver. 3.0.10. The diameter and circularity of each particle were calculated using the following formulas in ImageJ (Java 13.0.6)

Diameter, $L = 2 \times \text{sqrt}(\text{area} / \pi)$

Circularity, $\text{Circularity} = 4\pi(\text{area} / \text{perimeter}^2)$

Data visualization included generating histograms through Python ver. 3.11.5 to analyze the distribution of these measurements.

Statistics and Reproducibility

All statistical analyses were performed using GraphPad Prism software (version 9.0, GraphPad Software, San Diego, CA, USA).

Live/dead assay

To assess cell viability, we used MF20 hybridoma (DSHB; Iowa City, IA, USA) and LIVE/DEAD® Viability/Cytotoxicity Kit (Thermo Fisher Scientific, Waltham, MA, USA). Samples were first collected and resuspended in culture medium, followed by staining with Calcein-AM and Ethidium Homodimer-1 at final concentrations of 1 μM and 2 μM, respectively. To visualize nuclei and identify cell locations, Hoechst 33342 (Thermo Fisher Scientific, Waltham, MA, USA) was added at a final concentration of 5 μg/ml. After dye addition, samples—either in suspension or encapsulated in PicoShell hydrogel particles—were incubated at 37 °C for 20 minutes and washed twice with PBS. Fluorescence images were acquired using a fluorescence microscope. Cell positions were identified using brightfield images and segmented with Cellpose (v3.0.10). Fluorescence intensities of Calcein-AM and Ethidium Homodimer-1 were quantified using ImageJ. Double-negative samples were excluded from the analysis, as they were likely cell debris. The localization of cells inside PicoShells was manually confirmed using the corresponding brightfield images.