nature protocols

https://doi.org/10.1038/s41596-024-00972-6

Protocol

Check for updates

BEHAV3D: a 3D live imaging platform for comprehensive analysis of engineered T cell behavior and tumor response

In the format provided by the authors and unedited

Supplementary Information

Supplementary Methods Supplementary Protocol with Information of generation and culture of CAR19 T-cells.

Supplementary Figure 1 Characterization of photobleaching and phototoxicity effects

Supplementary Figure 2 Random Forest Classifier accuracy rates

Supplementary Video 1. Representative video of an imaging experiment performed on the Zeiss LSM 880. Left panel displays raw imaging data of a co-culture of CD8+ CAR19 cells (blue), CD4+ CAR19 cells (green) and ALL cells (yellow). Dead dye presence is visualized in red. Right panel displays segmented and tracked data CD8+ CAR19 cells (blue), CD4+ CAR19 cells (green) and ALL cells (grey). The track of the CAR19 cells' represents cell path over the last hour.

Supplementary Video 2. Representative video of an imaging experiment performed on the Leica SP8. Left panel displays raw imaging data of a co-culture of CD8+ CAR19 cells (blue), CD4+ CAR19 cells (green) and ALL cells (yellow). Dead dye presence is visualized in red. Right panel displays segmented and tracked data CD8+ CAR19 cells (blue), CD4+ CAR19 cells (green) and ALL cells (grey). The track of the CAR19 cells' represents cell path over the last hour.

Supplementary Video 3. Representative video showing one single TEG cell, displaying morphological plasticity and destroying and entire tumor organoid in over 10 hours. Left panel displays raw imaging data with TEG cells (blue), BC tumor organoid (yellow). Dead dye presence is visualized in red. Right panel displays segmented and tracked TEG cells (blue), BC tumor organoid (grey). Full T cells track is shown in blue.

Supplementary Video 4. Representative video showing one single motile CD8+ CAR19s, performing 5 tumor cell killings in 8 hours. CD8+ CAR19 cells (blue), CD4+ CAR19 cells (green) and ALL cells (yellow). Dead dye presence is visualized in red.

Supplementary Table 1. Table to quantify tracking accuracy metrics.

Supplementary Methods 1

Information of generation and culture of CAR19 T-cells

The CAR19 gene was constructed by linking the FMC-63 single chain variable fragment (GenBank ID: ADM64594.1) to a CD8 hinge (AA 138-206, Ref sequence ID NP_001759.3) and transmembrane domain, 4-1BB (CD137, AA 214-241 UniProt sequence ID Q07011)

transactivation domain, and CD3zeta signaling domain (CD247, AA 52-121, Ref sequence ID: NP_000725.1). The sequence was codon optimized and synthesized (Genscript), after which it was cloned in to the pCCL-cPPT-hPGK-ORF-bPRE4-SIN lentiviral transfer vector[1]. Lentiviral particles were produced using standard calcium phosphate transfection (Clontech, 631312) of HEK-293T-cells with the pMDL-g/pRRE, pMD2-VSVg, and pRSV-Rev plasmids[2,3]. Transducing units per mL of concentrated vector batches were determined using serial dilution on Jurkat cells followed by flow cytometric analysis using a biotin-conjugated CD19 CAR detection Reagent (Miltenyi Biotec, 130-129-550) and an APC-conjugated streptavidin reagent (Biolegend, 405207). Findings were confirmed using a serial dilution on HeLa cells followed by a qPCR to quantify the vector copy number of the construct within the DNA.

PBMCs were separated from blood using Ficoll-Paque PLUS separation (Cytiva, 17-1440-03), after which cells were stained with an anti-CD3 FITC (Biolegend, UCHT1). T-cells were sorted with a Sony SH800S sorter based on CD3 expression. Afterwards, CD3+ T-cells were stimulated and transduced at a multiplicity of infection of 10 according to a previously described protocol[1].

Following transduction, CD4+ and CD8+ cells were enriched separately with a MACS Cell Isolation kit (Miltenyi Biotec 130-045-101 & 130-045-201) according to manufacturer's instructions. After enrichment, purity and transduction efficiency were determined with flow cytometry using a panel with biotin-conjugated CD19 CAR detection Reagent (Miltenyi Biotec, 130-129-550), APC-conjugated streptavidin reagent (Biolegend, 405207), CD3-BUV496 (BD, UCHT1), anti-CD8 APC (APC/Fire810, Biolegend, SK1), anti-CD4 BUV805 (BD, TPA-T4) and eFluor (Thermo Fisher, 65-0866-14). Data was acquired on a Cytek Aurora.

References

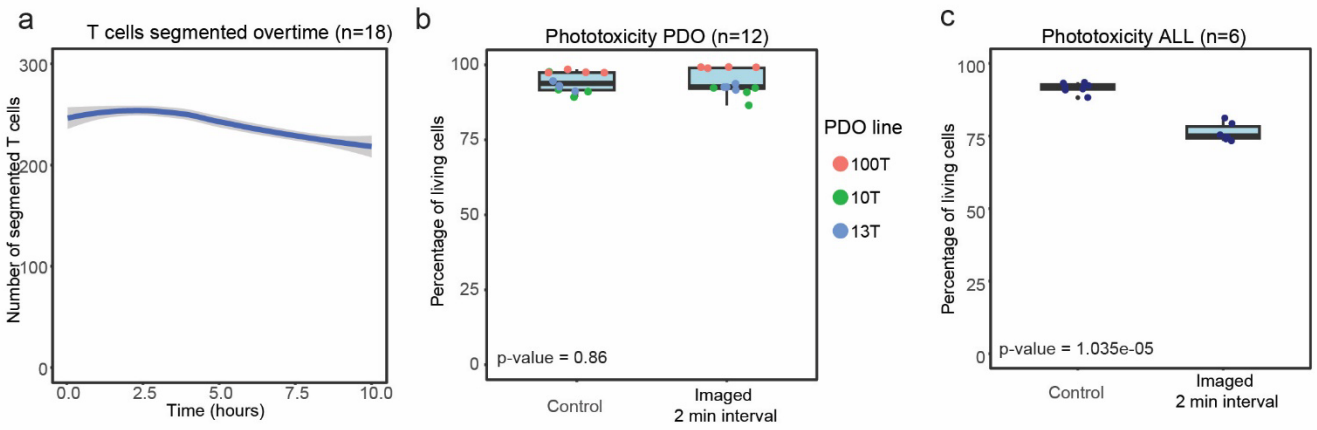
1. Presti, V. L. *et al.* Efficient lentiviral transduction method to gene modify cord blood CD8+ T cells for cancer therapy applications. *Mol. Ther. - Methods Clin. Dev.* 21, 357–368 (2021).

2. Dull, T. *et al.* A Third-Generation Lentivirus Vector with a Conditional Packaging System. *J. Virol.* 72, 8463–8471 (1998).

3. Zufferey, R. *et al.* Self-Inactivating Lentivirus Vector for Safe and Efficient In Vivo Gene Delivery. *J. Virol.* 72, 9873–9880 (1998).

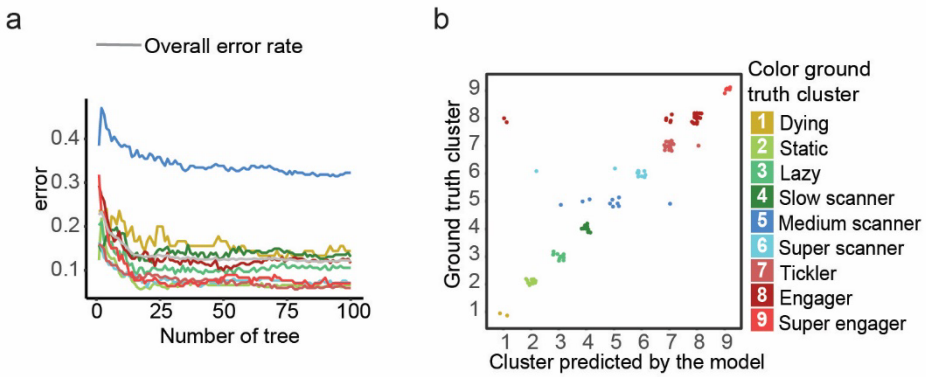
Supplementary Figures

Supplementary Figure 1 Characterization of photobleaching and phototoxicity effects



Supplementary Figure 1 Characterization of photobleaching and phototoxicity effects (a) Quantification of segmented eFluor labelled T cells at all timepoints demonstrates only a minor reduction in segmentation effectiveness due to photobleaching or photoxicity (16.4%). Shown average values of 18 representative experiments. (b-c) Quantification of photoxicity effect on PDOs (b) and ALL tumor cells (c) upon 16 hours of imaging. Number of voxels belonging to living and dead cells (b) were quantified by segmentation after 16 hours of imaging with a 2 minute interval on a LSM880 confocal system. For ALL cells number of single living and dead cells were quantified (c). As a control, wells were imaged only at the last timepoint. Individual points represent number of cells per well. Two-tailed unpaired t test was performed to compare conditions.

Supplementary Figure 2 Random Forest Classifier accuracy rates



Supplementary Figure 2 Random Forest Classifier accuracy rates. Error rate of the training data per cluster and overall for all trees (a) and correlation plot between ground truth cluster classification and predicted cluster classification (b). In b color represents ground truth cluster. Figure is reused from Extended Data Fig 2 d-e of ref 8.