Mapping T cell dynamics to molecular profiles through behavior-guided transcriptomics

Wezenaar, A.K.L.; Pandey, U.; Keramati, F.; Hernandez-Roca, M.; Brazda, P.; Román, M.B.; ... ; Rios, A.C.

nature protocols

https://doi.org/10.1038/s41596-024-01126-4

Protocol extension

Check for updates

Mapping T cell dynamics to molecular profiles through behavior-guided transcriptomics

A. K. L. Wezenaar[1,2,8], U. Pandey[1,2,8], F. Keramati[1,8], M. Hernandez-Roca[3], P. Brazda[1,4], M. Barrera Román[1,2], A. Cleven[4], F. Karaiskaki[4], T. Aarts-Riemens[4], S. de Blank[1,2], P. Hernandez-Lopez[4], S. Heijhuurs[4], A. Alemany[5], J. Kuball[4,6], Z. Sebestyen[4], J. F. Dekkers[1,2,7], H. G. Stunnenberg[1], M. Alieva[3,9] ✉ & A. C. Rios[1,2,9] ✉

Abstract

The rise of cellular immunotherapy for cancer treatment has led to the utilization of immune oncology cocultures to simulate T cell interactions with cancer cells for assessing their antitumor response. Previously, we developed BEHAV3D, a three-dimensional live imaging platform of patient-derived tumor organoid (PDO) and engineered T cell cocultures, that analyzes T cells' dynamics to gain crucial insights into their behavior during tumor targeting. However, live imaging alone cannot determine the molecular drivers behind these behaviors. Conversely, single-cell RNA sequencing (scRNA-seq) allows researchers to analyze the transcriptional profiles of individual cells but lacks spatio-temporal resolution. Here we present an extension to the BEHAV3D protocol, called Behavior-Guided Transcriptomics (BGT), for integration of T cell live imaging data with single-cell transcriptomics, enabling analysis of gene programs linked to dynamic T cell behaviors. BGT uses live imaging data processed by BEHAV3D to guide the experimental setup for cell separation based on their PDO engagement levels subsequently followed by fluorescence-activated cell sorting and scRNA-seq. It then integrates in silico simulations of these experiments to computationally infer T cell behavior on scRNA-seq data, uncovering new biomarkers for both highly functional and ineffective T cells, that could be exploited to enhance therapeutic efficacy. The protocol, designed for users with fundamental cell culture, imaging and programming skills, is readily adaptable to diverse coculture settings and takes one month to perform.

This protocol is an extension to: *Nat. Protoc.* **19**, 2052–2084 (2024): https://doi.org/10.1038/s41596-024-00972-6

[1]Princess Máxima Center for Pediatric Oncology, Utrecht, the Netherlands. [2]Oncode Institute, Utrecht, the Netherlands. [3]Institute for Biomedical Research Sols-Morreale, Spanish National Research Council-Universidad Autónoma de Madrid, Madrid, Spain. [4]Center for Translational Immunology, University Medical Centre Utrecht, Utrecht, the Netherlands. [5]Department of Anatomy and Embryology, Leiden University Medical Center, and the Novo Nordisk Foundation Center for Stem Cell Medicine, Leiden node, Leiden, the Netherlands. [6]Department of Hematology, UMC Utrecht, Utrecht University, Utrecht, the Netherlands. [7]Education Center, University Medical Center Utrecht, Utrecht, the Netherlands. [8]These authors contributed equally: A. K. L. Wezenaar, U. Pandey, F. Keramati. [9]These authors jointly supervised this work: M. Alieva, A. C. Rios. ✉e-mail: malieva@iib.uam.es; a.c.rios@prinsesmaximacentrum.nl

Key points

- Behavior-Guided Transcriptomics (BGT) is an extension to the BEHAV3D protocol that integrates T cell live imaging data with single-cell transcriptomics, linking gene programs to dynamic T cell behaviors.
- BGT overcomes limitations of traditional single-cell RNA sequencing, by matching T cell transcriptomic states to outcomes such as cancer cell targeting, revealing biomarkers that guide more effective immunotherapy strategies.

Key references

Dekkers, J. F. & Alieva M. et al. *Nat. Biotechnol.* **41**, 60–69 (2023): https://doi.org/10.1038/s41587-022-01397-w

Alieva M. et al. *Nat. Protoc.* **19**, 2052–2084 (2024): https://doi.org/10.1038/s41596-024-00972-6

Protocol extension

Introduction

To evaluate the performance of immunotherapy against cancer, coculture systems have emerged as indispensable tools. These experimental models combine T cells and tumor cells to simulate T cell behavior and cancer targeting potential, offering insight into the mode of action of immunotherapy and facilitating the optimization of treatment strategies to enhance efficacy[1–3]. At the same time, live-cell imaging has emerged as a potent method for characterizing the spatial cellular organization and dynamics within these cocultures[4], serving as a potent read-out of the efficacy of different engineered T cell concepts in immuno-tumor cocultures[2,5–11]. For instance, live imaging has proven useful in deciphering early formation of T cell receptor (TCR) microclusters on the T cell surface upon antigen recognition[12] or identifying defects at various stages of lysosome polarization and degranulation that have been implicated in tumor resistance against chimeric antigen receptor (CAR) T cells[8]. Implementing patient-derived organoids (PDOs) in these coculture live-cell imaging settings can, furthermore, replicate patient variability in antitumor responses. We previously developed BEHAV3D, a three-dimensional (3D) live imaging platform tailored to such PDO cocultures, leading to the discovery of a variety of T cells' functional and dysfunctional behaviors (Fig. 1a). Importantly, this included 'super engager' engineered T cells (Fig. 1b), characterized by their capacity to establish prolonged engagement with PDOs and presenting with potent serial killing capabilities[2,13] (Fig. 1c).

However, while live imaging can provide detailed structural and temporal information, it lacks the ability to measure transcriptome-wide gene expression or molecular profiles. By contrast, single-cell RNA sequencing (scRNA-seq) allows researchers to unbiasedly dissect the transcriptional profile of individual cells[14–17], without relying on preselected markers, as is the case with live-cell imaging. However, it only offers a snapshot of T cell biology, whereas the recognition of T cell functionality spanning a continuum of transitioning cell states during cancer targeting emphasizes the necessity for advanced methodologies incorporating time resolution. Such methodologies are essential for elucidating the molecular mechanisms driving the mode of action of dynamic T cells throughout the sequence of events involved in tumor targeting. Therefore, integrating output from 3D live imaging and scRNA-seq holds immense potential by bridging spatial, behavioral, functional and molecular information[4]. It provides a comprehensive understanding of which gene expression programs regulate different engineered T cell functional states, such as those involved in tumor recognition or killing. This could inform the development of more effective cellular therapies. Here, we present an extension to the BEHAV3D protocol, entitled Behavior-Guided Transcriptomics (BGT), which includes an imaging-guided transcriptomic experimental and analytical pipeline tailored to delineate the underlying molecular programs responsible for different behaviors of T cells targeting PDOs (Supplementary Video 1). BGT is implemented in immune oncology cocultures of engineered T cells with PDOs and utilizes T cell behavioral dynamic knowledge generated from the original BEHAV3D pipeline[2,13] to inform a specific T cell enrichment strategy, followed by scRNA-seq and behavioral probability mapping.

The BGT extension has greatly enhanced our ability to identify molecular programs governing diverse behaviors of engineered T cells. Notably, it has added substantial value by uncovering a distinct gene program, with previously undescribed genes for T cell function, exhibited by super engager T cells, characterized by their exceptional ability to sequentially kill multiple cancer cells[2]. The full scope of the protocol, ranging from coculture setup, experimental separation of T cells at different targeting stages, scRNA-seq, data processing, pseudotime trajectory inference and behavioral probability mapping, is illustrated in Fig. 2a–f and in Supplementary Video 1. Moreover, we provide several resources, including a troubleshooting table and a comprehensive GitHub repository, accompanied with software packages conveniently bundled together in an image container to ensure consistency and reproducibility of results. Additionally, we provide two demo videos that illustrate the installation and execution of the computational procedures of BGT (Supplementary Videos 2 and 3). Altogether, we offer a versatile detailed experimental and analysis protocol for matching T cell behavioral and molecular profiles in coculture assays with organoids.

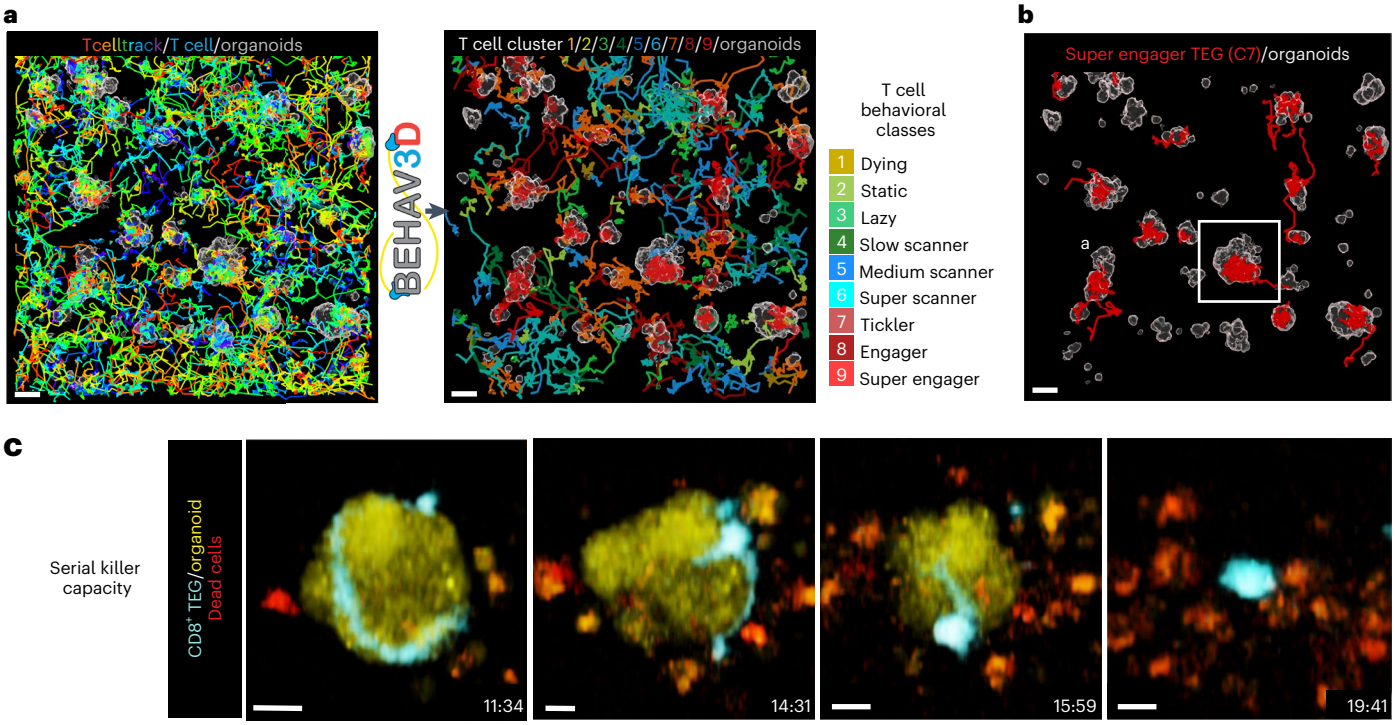


Fig. 1 | Highly variable T cell behavioral subtypes discovered with BEHAV3D imaging platform. a, Single T cells from live 3D imaging data are tracked (left) and classified into different behavioral clusters using BEHAV3D (right). **b**, Imaging data from **a** showing the 'super engager' T cell behavioral cluster displaying prolonged organoid interactions. **c**, Example of a 'super engager' T cell (blue) serial killing an entire tumor organoid (yellow). Scale bars, 50 µm (**a** and **b**), 30 µm (**c**). Panels **a**–**c** reproduced from ref. 2, CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/).

Development of the protocol

The BGT approach operates on the principle that within 3D coculture systems, engineered T cells with different functional behaviors also exhibit different PDO engagement levels (Fig. 1a): 'dying', 'static', 'lazy', 'scanners' not exhibiting engagement to PDOs, 'ticklers' and 'engagers' with short contacts with PDOs and 'super engager' T cells with prolonged PDO engagement that demonstrated the highest killing potency (Fig. 1b). These behaviors were observed across various engineered T cell products (CAR T cells, TCR T cells and αβ T cells engineered to express a γδ TCR (TEGs)) (Extended Data Fig. 1a) when cocultured with different PDO types (breast cancer, head and neck cancer and diffuse midline glioma) (Extended Data Fig. 1b). Based on this concept, in BGT, T cells are cocultured with PDOs and segregated into distinct groups according to their engagement levels with PDOs (Figs. 2b,c and 3a). These experimentally enriched T cell groups undergo fluorescence-activated cell sorting (FACS) followed by unbiased scRNA-seq (Fig. 2d). In parallel, BGT performs the same experiment in silico, segregating the dynamic T cell data extracted by the BEHAV3D imaging pipeline[2,13] according to level of engagement of the T cells with PDOs (Fig. 2a,e). This makes predictions of the frequencies of the diverse behavioral T cell populations across the experimentally segregated groups. These in silico experimental simulation data are then integrated with the scRNA-seq data from the experimentally enriched T cell groups to predict the behavioral T cell phenotype in each sequenced cell (Fig. 2f).

Comparison with other methods

Traditional scRNA-seq provides static snapshots of gene expression at specific endpoints, lacking crucial temporal information on how cell states change over time. Computational methods, such as pseudotime and RNA velocity[18], attempt to infer these temporal dynamics by analyzing gene expression patterns or distinguishing between spliced and unspliced mRNAs. Most recently technologies, such as Zman-seq[19], address the temporal gap by introducing time

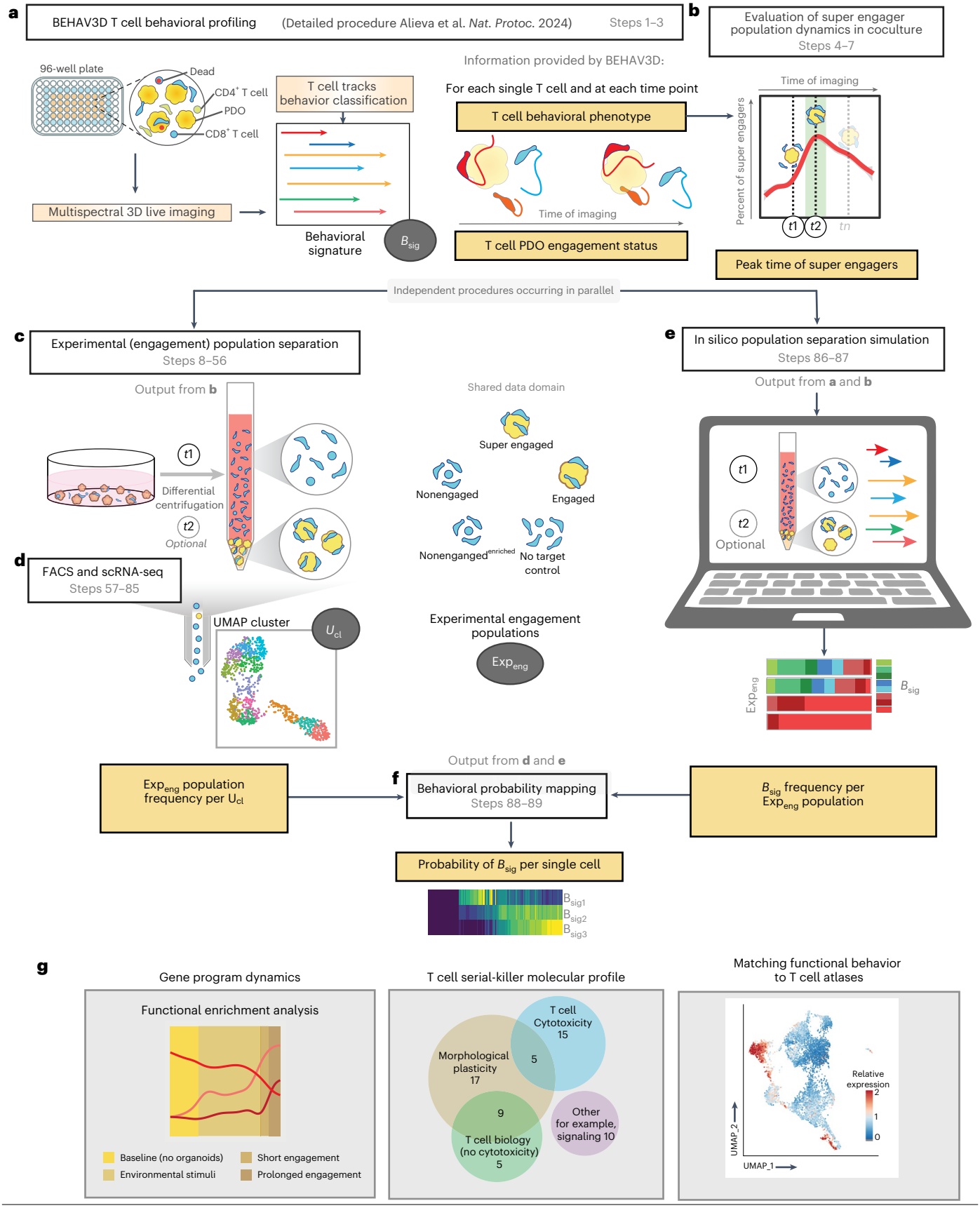

a
BEHAV3D T cell behavioral profiling (Detailed procedure Alieva et al. Nat. Protoc. 2024) Steps 1–3
96-well plate
Dead
CD4+ T cell
PDO
CD8+ T cell
Multispectral 3D live imaging
T cell tracks behavior classification
Behavioral signature
Bsig
b
Evaluation of super engager population dynamics in coculture
Steps 4–7
Information provided by BEHAV3D:
For each single T cell and at each time point
T cell behavioral phenotype
Time of imaging
T cell PDO engagement status
Time of imaging
Percent of super engagers
t1
t2
tn
Peak time of super engagers
Independent procedures occurring in parallel
c
Experimental (engagement) population separation
Steps 8–56
Output from b
t1
Differential centrifugation
t2
Optional
d
FACS and scRNA-seq
Steps 57–85
UMAP cluster
Ucl
Shared data domain
Super engaged
Nonengaged
Engaged
Nonenganged enriched
No target control
Experimental engagement populations
Expeng
e
In silico population separation simulation
Steps 86–87
Output from a and b
t1
t2
Optional
Expeng
Bsig
Output from d and e
f
Behavioral probability mapping
Steps 88–89
Expeng population frequency per Ucl
Bsig frequency per Expeng population
Probability of Bsig per single cell
Bsig1
Bsig2
Bsig3
g
Gene program dynamics
Functional enrichment analysis
Baseline (no organoids)
Short engagement
Environmental stimuli
Prolonged engagement
T cell serial-killer molecular profile
T cell Cytotoxicity 15
Morphological plasticity 17
5
9
Other for example, signaling 10
T cell biology (no cytotoxicity) 5
Matching functional behavior to T cell atlases
Relative expression
2
1
0
UMAP_2
UMAP_1

Fig. 2 | Schematic representation of BGT protocol and applications. **a**–**f**, BGT experimental preparation includes BEHAV3D T cell behavioral profiling (**a**), evaluation of 'super engager' T cell dynamics in coculture (**b**), experimental (engagement) population separation (**c**), FACS and scRNA-seq (**d**) and behavioral probability mapping (**e** and **f**). An overview of BEHAV3D is shown in **a**. The cocultures of engineered T cells labeled with fluorescent dyes and tumor organoids are imaged, followed by single T cell tracking and behavior classification (B_{eng}). The number of T cells in the 'super engager' behavior population identified in **a** are plotted overtime to identify the peak time of 'super engagers' in **b**. Using the peak time of 'super engagers' identified in **b**, tumor engaging ('engaged' and 'super engaged') and nontumor engaging ('nonengaged' and 'nonengaged^{enriched}') T cell populations (Exp_{eng}) are separated using density centrifugation in **c** and sorted by FACS and analyzed with scRNA-seq in **d**. The population separation procedure used in **c** is simulated in silico in **e** to infer the behavioral signature (B_{sig}) for each of the tumor engaging and nontumor engaging T cell populations isolated in **c**. The probability of the behavioral signature of each individual T cell is mapped in the scRNA-seq dataset using transitive property of probability, shown in **f**. **g**, The protocol has a range of applications including gene program dynamics, identification of gene profiles linked to serial killing T cells and linking functional behavior from the cocultures to T cell atlases. Panel **g** adapted from ref. 2, CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/).

stamps into circulating immune cells and then tracking them in tissues for days. While such technical advances now offer valuable insights into how immune cells transition between states upon tumor homing, it does not connect these states with specific outcomes such as interacting and killing cancer cells. To address this gap and fully understand the broader spectrum of T cell actions, integrating single-cell imaging with transcriptomics is essential.

So far, several approaches have been developed to integrate live imaging and single-cell transcriptomic information. Each of these methods has their own specific advantages and disadvantages with respect to scalability, compatibility with complex coculture models and unbiased analysis, which are summarized in Box 3 of our recent review on live-cell imaging[4]. Phototagging approaches use photoactivatable fluorescent molecules to label regions of interest but, up to now, require manual input, introducing bias and limiting single-cell throughput for subsequent transcriptomics analysis[20–22]. Cellular confinement strategies that place individual cells into individual wells for imaging and sequencing[23,24], enabling unbiased selection and characterization of cells exhibiting specific behavioral traits, such as tumor targeting, visualized under the microscope. However, such methodologies are limited to single tumor-immune cell interactions, while BGT operates effectively with complex cocultures. Live-seq[25] allows time-course transcriptomics of individual cells based on the patch-clamp method, while maintaining cellular viability, but similar supervised robotic picking approaches[26] suffer from scalability issues and unsuitability for complex 3D cultures. Contrastingly, BGT is a computational integration strategy based on behavioral inference that circumvents the need for imaging and sequencing the same individual cells, making it compatible with complex cocultures, while increasing scalability.

Applications

BGT represents an innovative single-cell transcriptomic approach precisely designed to capture distinct T cell dynamic states when targeting tumor cells, leveraging their behaviors. Unlike conventional static single-cell transcriptomic analyses, BGT employs live-cell imaging of organoid cocultures with T cells to reveal how T cells dynamically regulate their gene programs during tumor targeting, including the sequential killing of multiple tumor cells referred to as 'serial killing' (Fig. 1c). This enhanced temporal resolution facilitates the identification of genes uniquely associated with time-resolved behaviors, offering a novel approach to pinpoint targets relevant to T cell function and efficacy that are not detected with static methods (Fig. 2g). Additionally, this versatile methodology could pave the way for exploring the underlying molecular mechanisms affecting T cell behaviors with diverse immunomodulatory interventions.

BGT's analytical framework can be adapted to investigate dynamics of other immune cell populations such as macrophages, broadening its utility beyond T cell immunotherapy. With advancements in live-cell imaging to capture multiple cell populations simultaneously, our framework could be integrated with more complex coculture assays, including multiple immune cells cultured with PDOs or tumor fragments[27]. Lastly, by leveraging the widespread availability of PDOs across diverse cancer types[28], this approach can advance our understanding of the molecular pathways dictating immune cell dynamics and how tumor type and patient-specific features influence these mechanisms. Thus, by encompassing a broader spectrum

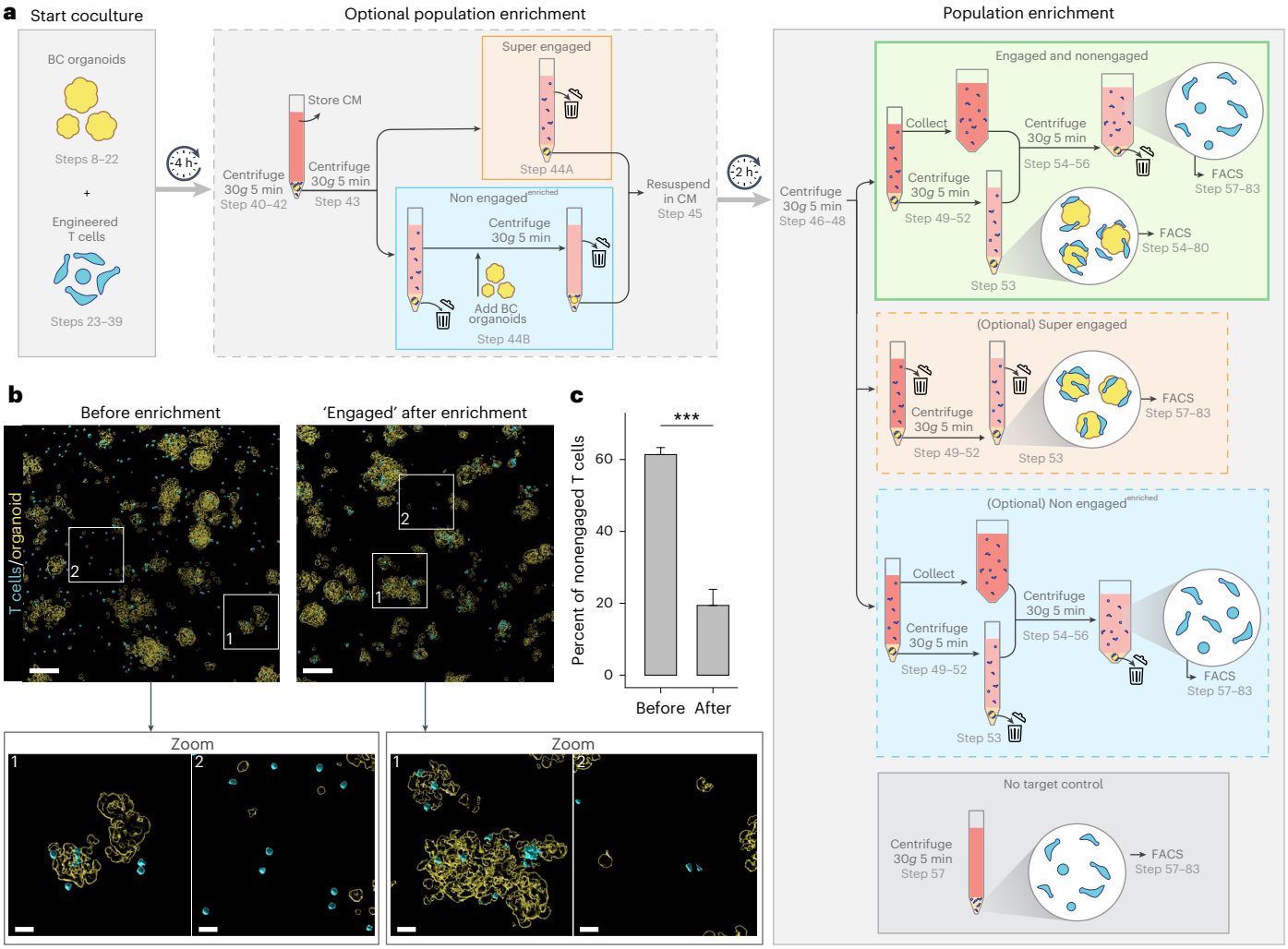


Fig. 3 | Experimental separation of T cell behavioral populations. **a**, A schematic representation of the procedure to separate T cell populations. The procedure consists of the preparation of a T cell–tumor organoid coculture (left), an optional additional enrichment step to obtain 'super engaged' and 'nonengaged^{enriched}' populations (middle) and density centrifugation enrichment of 'engaged' and 'nonengaged' populations and (optional) 'super engaged' and 'nonengaged^{enriched}' populations. BC, breast cancer; CM conditioned medium. **b**, Representative images showing cocultures before and after enrichment for the 'engaged' population. Scale bar, 100 µm, for zoom, 20 µm. **c**, A quantification of the percentage of T cells that are not engaging with a tumor organoid before and after enrichment for the 'engaged' population (mean ± standard error of the mean). Unpaired *t*-test, $P = 0.0009$.

of immune cell interactions and patient-specific responses, BGT has the potential to drive innovations in personalized immunotherapeutic strategies, with the ultimate goal of improving patient outcomes of cancer treatment.

Limitations

While in vitro 3D coculture systems may not fully replicate the complexity of the tumor microenvironment in vivo, we have previously shown that gene signatures retrieved with BGT in these systems correlate with gene profiles of matching tumor-infiltrating T cell populations found in patients[2]. Thus, although lacking the critical influence of an intact tumor microenvironment, T cell gene signatures obtained with BGT carry translational value for in vivo tumor targeting settings. Extending the protocol to in vivo applications in animal models, would require essential adaptations and optimization related to cell separation and intravital imaging data collection. Furthermore, BGT relies on the separation of cells

based on organoid engagement. While our previous findings demonstrated the prevalence of this compartmentalization across various engineered T cell types and PDOs derived from different cancers (Extended Data Fig. 1), it is conceivable that alternative coculture systems may not exhibit this type of 'super engager' behavior, rendering them incompatible with our approach. For instance, cocultures based on tumor cell lines, rather than PDOs, might require an alternative strategy to separate tumor-interacting T cells, for instance, via doublet sorting[29]. Moreover, the BGT approach relies on gradient transcriptomic changes based on the duration of T cell engagement with PDOs. As T cell functionality spans a continuum of transitioning states, BGT is best suited for analyzing cell types with this dynamic progression and may not be applicable to those without it. Finally, our method lacks uniform data resolution across the diverse behavioral populations. T cell subsets categorized as nonengaging can contain different behavioral profiles, such as 'scanner' and 'static/lazy' T cells that cannot be further distinguished with this approach.

Required hardware and expertise for BGT implementation

The BGT pipeline requires the implementation of our previously described BEHAV3D protocol[13]. BEHAV3D[13] demands high-end workstations (random-access memory (RAM) 64GB, central processing unit (CPU) 3.7 HZ or more with at least eight cores, GPU with 16 GB video random-access memory (VRAM) or more) for data processing with Imaris ensuring analysis efficiency. For applying the BGT data analysis pipeline, a general workstation is sufficient. In terms of user expertise, a basic knowledge of R programming is necessary to execute the data analysis pipeline. However, due to the specific demands of the scRNA-seq analysis pipeline, conducting new experiments would probably necessitate familiarity with scRNA-seq analytical procedure. For user convenience, we have included comprehensive instructions on executing the scripts, accompanied by software packages in an image container, as well as self-explanatory Jupyter notebooks and R scripts and two demo videos for BGT installation and execution (Supplementary Videos 2 and 3).

Experimental design

BEHAV3D T cell behavioral profiling (Steps 1–3)

For 'super engager' population dynamics estimation and in silico T cell population separation based on the duration of PDO engagement, BGT requires prior implementation of our previously described BEHAV3D protocol[13]. By employing single-cell tracking of multicolor live 3D imaging T cell data and subsequent behavioral classification, BEHAV3D categorizes T cell tracks based on their behavioral phenotypes (Fig. 1a and Extended Data Fig. 2), including the serial killer 'super engager' behaviors (Fig. 1b,c). Given the inherent experimental variability, it is recommended to conduct the analysis across multiple experimental replicates ($n > 4$) while ensuring data quality (see troubleshooting table of ref. 13). It is important to note that T cell behavioral profiling with BEHAV3D only has to be performed once and can be reused for BGT experiments if the live imaging data used for BEHAV3D is representative of the entire population of T cells. If a new coculture system is used, for example, with a different type of T cell or organoid, T cell behavioral profiling should be performed again.

Evaluation of optimal timing for 'super engaged' enrichment (Steps 4–7)

To enable identification of the optimal separation window for PDO engaging and nonengaging engineered T cells, it is necessary to understand how the T cell 'super engager' population evolves over time in coculture. Utilizing data obtained from BEHAV3D, the dynamic evolution of T cell 'super engager' population frequencies while in coculture with PDOs is quantified. The computed behavioral classification of T cells generated by BEHAV3D is used to infer at every time point the percentage distribution of the different behaviors and used as input data for BGT. These analysis data are employed to define the peak of 'super engager' during the time course of the experiment to identify the optimal time point for enriching for this population of high interest, as it presents with superior killing capabilities than other behaviors and experimentally separate them based on T cell engagement (Steps 4–7) (Fig. 2b). The resulting analysis yields a graphical representation illustrating the progression of 'super engager' frequencies, including

the peak frequency, and defining a time window centered around this peak (Extended Data Fig. 2). This information is utilized to determine the appropriate timing for experimental separation of engaging populations. The computational steps for evaluating optimal timing of 'super engaged' enrichment are implemented in module 1 of our Github repository (https://github.com/imAIgene-Dream3D/BGT/tree/main?tab=readme-ov-file#1-evaluation-of-optimal-timing-for-super-engaged-enrichment).

This procedure only needs to be performed once and can be reused for BGT experiments. It is important to be aware when establishing a coculture with a different organoid type, or even patient donor and/or engineered T cell, tumor 'super engager' frequency evolution might change. Therefore, this analysis should be repeated if the experimental setup changes.

Experimental (engagement) population separation (Steps 8–56)

Enrichment of T cell behavioral populations at different stages of targeting involves the preparation of a coculture assay. Although the setup of the coculture is similar to the coculture prepared for behavioral classification with the original BEHAV3D protocol (refer to ref. 13), there are some critical differences, highlighted below, and explained in the step-by-step procedure. First, it is crucial that the PDO used form 3D structures of ~50 µM in size that can be retained during PDO collection. In other words, the 3D structures should not be easily disrupted by sheer force during resuspension to enable separation of populations using density centrifugation. In our coculture setup with TEGs and PDO donors, the time of optimal tumor engagement ranged between 5 and 6 h, and at these time points, more than 75% of PDOs were still viable, providing a good time frame for population separation (Extended Data Fig. 2). We recommended plating at least 0.8×10^6 T cells per condition in 12-well plates at an effector-to-target ratio of 1:1.5 to ensure a sufficient T cell yield during population separation for transcriptomic analysis.

Besides the differences mentioned above, in general, to minimize variation between experimental replicates and ensure the behavioral populations are similar to the imaging data from BEHAV3D used to map immune cell behavior, we standardize the culture conditions as much as possible, such as organoid culture density, frequency of culture medium refreshing and day of tumor cell and immune cell collection after passaging. While this protocol describes the procedure for breast cancer PDOs, it could be applied to a wide variety of organoids of different origin, as we previously described[13]. Similarly, while we based this protocol on the use of TEGs, of which culture conditions are detailed in ref. 2, this assay can be performed with any type of (engineered) immune cells, as previously described[13].

During PDO T cell cocultures, T cells will go through different stages of tumor targeting. For example, some T cells might never interact or engage with a tumor organoid, while other T cells might shortly engage or even have prolonged interactions with tumor organoids. To enable separation of T cells at these different stages of engagement, we use density centrifugation (Fig. 3a). With this method, the heavier tumor organoids will sediment quicker than the lighter single T cells, allowing for separation of T cells that are engaging (in other words, attached to) a tumor organoid and T cells that are not engaging (and, thus, light single cells) (Fig. 3b,c). In this protocol, we describe the isolation of these engaging T cells (termed 'engaged') as well as the single T cells that are not attached to organoids (termed 'nonengaged') through density centrifugation at the time of peak 'super engager' frequency described in the 'Evaluation of optimal timing for 'super engaged' enrichment' section.

Since at the time of density centrifugation, T cells by chance might be attached to a tumor organoid without having a prolonged engagement, the protocol describes an optional step to remove these cells from the 'engaged' T cell population. To achieve this, after the first density centrifugation step tumor organoids with engaging T cells ('engaged' population) are put back into culture for a few hours, during which T cells that are not engaging with tumor organoids for a longer period of time will detach from tumor organoids. During a second density centrifugation step, the detached T cells (which are now light single cells) can be removed, yielding an enriched population of engaging T cells (termed 'super engaged') (Fig. 3a). Similarly, the fraction of not engaging T cells ('nonengaged') might contain engaging T cells that were momentarily detached from tumor organoids at the time of the first density centrifugation.

Therefore, T cells that are not engaging ('nonengaged') are put back into culture with new tumor organoids for a few hours, allowing any engaging T cells present to attach to tumor organoids. With a second density centrifugation step, the T cells attached to tumor organoids are removed from the not engaged T cells (light single cells), resulting in an enriched population of not engaging T cells (termed 'nonengaged^{enriched}') (Fig. 3a). The plating strategy used to get to these different populations is described in Table 1.

Generally, to achieve optimal behavioral resolution for behavioral-transcriptomics integration, a combination of both techniques is recommended. However, if organoid or immune cell material is limited or performing both one- and two-step enrichment is not possible, we recommend using the simulation tool described in the 'In silico population separation simulation' section (Steps 86 and 87) to predict the behavioral distribution after one- or two-step enrichment and use this as a guide to decide if one- or two-step enrichment is optimal for the specific experimental question. To keep conditions similar to those used for BEHAV3D imaging, the cells need to be kept in conditioned medium after the first enrichment step during the two-step enrichment protocol. Therefore, it is important to consider during experiment design that for two-step enrichment, separate wells need to be seeded for 'super engaged' and 'nonengaged^{enriched}' enrichment, unlike one-step enrichment, which occurs at endpoint and, therefore, allows extraction of both 'engaged' and 'nonengaged' populations from the same well (Table 1).

FACS and scRNA-seq (Steps 57–85)

In this protocol, we describe FACS and preparation of behavior-enriched T cells for the SORT sequencing (SORT-seq) sequencing platform. However, the method described in this protocol is not restricted to SORT-seq but compatible with a wide range of single cell sequencing platforms compatible with low cell numbers[30]. Adjust the procedure as necessary if a different sequencing protocol is used. SORT-seq data undergo processing via the standard analytical workflow for single-cell sequencing, where different subtypes of T cells are identified based on marker expression (such as $CD4^+$ and $CD8^+$), followed by pseudotime trajectory inference, to reconstruct the sequence of T cell evolution based on gene expression (Fig. 4a). It is worth noting that while we offer preconfigured scripts for semiautomated execution, processing a new dataset with distinct characteristics may necessitate modifications to the analytical pipeline. The computational steps for FACS and scRNA-seq analysis are implemented in module 2 of our GitHub repository (https://github.com/imAIgene-Dream3D/BGT/tree/main?tab=readme-ov-file#2-scrnaseq-data-preprocessing). Detailed explanations of key

Table 1 | Example plate layout for experimental population separation

Plate	Well	Used for population	PDO	T cells	Steps
1	1	No target control	None	4.2×10^5 in 2 ml	After 6 h start from Step 57
	2				
	3	'Engaged' and 'nonengaged'	In 1 ml	4.2×10^5 in 1 ml	After 6 h follow Steps 46–56
	4				
	5				
2	1	(Optional) 'Super engaged'	In 1 ml	4.2×10^5 in 1 ml	After 4 h follow Steps 40–45 After 6 h follow Steps 46–56
	2				
	3				
	4	(Optional) 'Nonengaged^{enriched}'	In 1 ml	4.2×10^5 in 1 ml	After 4 h follow Steps 40–45 After 6 h follow Steps 46–56
	5				
	6				
	7	(Optional) 'Nonengaged^{enriched}'	In 2 ml	None	After 4 h follow Step 44B(iii–iv)
	8				
	9				

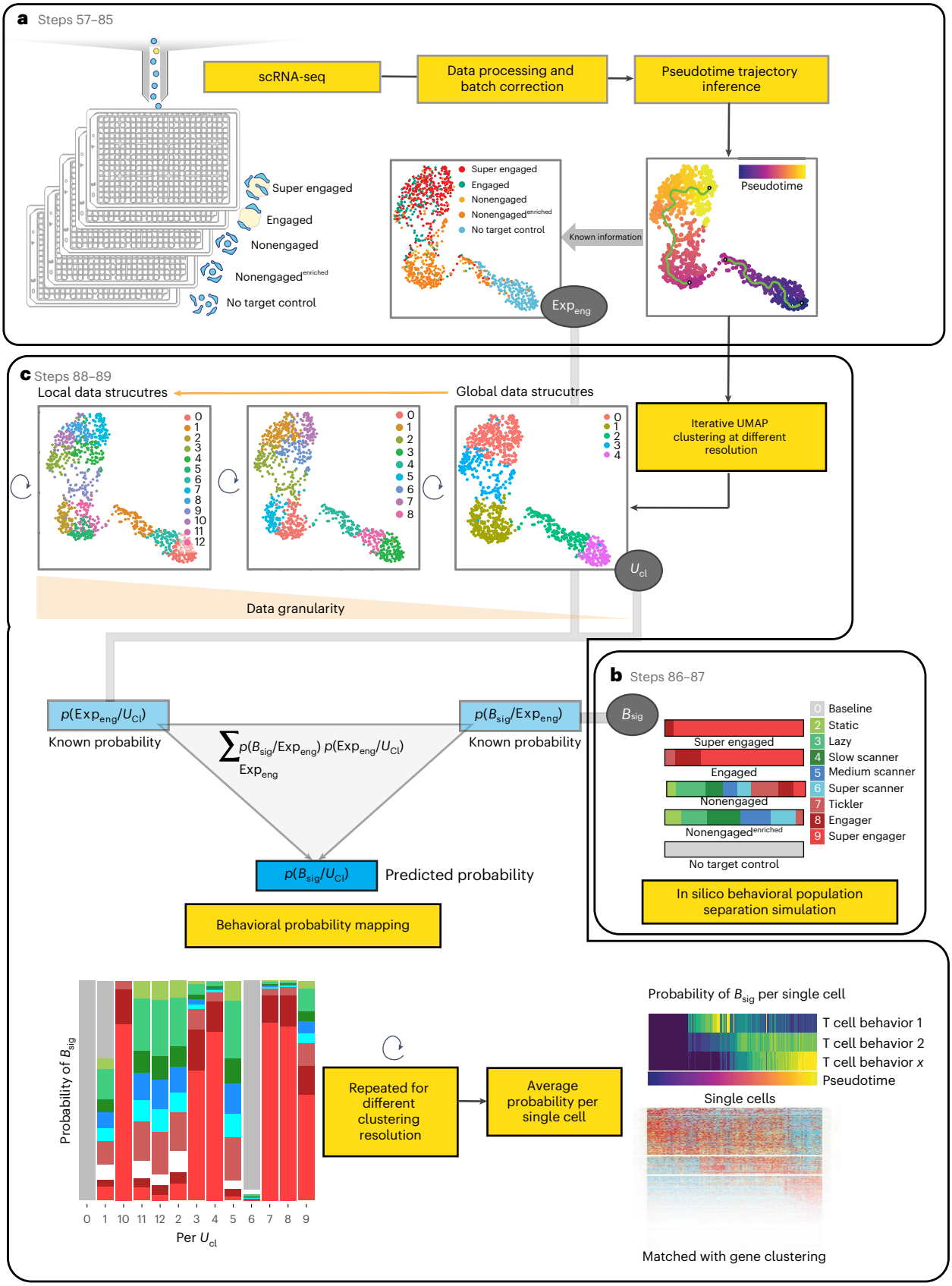

a Steps 57–85
scRNA-seq
Data processing and batch correction
Pseudotime trajectory inference
Super engaged
Engaged
Nonengaged
Nonengaged enriched
No target control
Known information
Pseudotime
Exp eng
c Steps 88–89
Local data strucutres
Global data strucutres
Iterative UMAP clustering at different resolution
U cl
Data granularity
b Steps 86–87
B sig
Baseline
Static
Lazy
Slow scanner
Medium scanner
Super scanner
Tickler
Engager
Super engager
In silico behavioral population separation simulation
p(Exp eng/U Cl)
Known probability
p(B sig/Exp eng)
Known probability
Exp eng
p(B sig/U Cl)
Predicted probability
Behavioral probability mapping
Probability of B sig
Per U cl
Repeated for different clustering resolution
Average probability per single cell
Probability of B sig per single cell
T cell behavior 1
T cell behavior 2
T cell behavior x
Pseudotime
Single cells
Matched with gene clustering

Fig. 4 | BGT integration strategy diagram. a, scRNA-seq of T cells, stratified into PDO engagement populations (Exp_{eng}: 'super engaged', 'engaged', 'nonengaged', 'nonengaged^{enriched}' and no target control) undergoes standard data processing and batch correction followed by pseudotime trajectory inference. **b**, The in silico population separation predicts the frequencies of different behavioral signatures B_{sig} in different engagement populations (Exp_{eng}). **c**, A UMAP clustering is conducted through multiple iterations to generate single-cell clusters at both local and global resolutions (U_{cl}). Behavioral probability mapping is executed across various clustering resolutions, providing, at each iteration, a cell-specific probability of belonging to a particular behavioral class, which is then averaged.

adjustments that may be necessary are provided in our Jupyter notebooks and troubleshooting table. Additionally, while it is suggested to install the latest versions of software dependencies, they can provide a slightly different output. For consistency in our demo results we have provided a container software image[31] (https://zenodo.org/records/10953043).

In silico population separation simulation (Steps 86 and 87)

This protocol is founded on behavioral inference, which entails predicting frequencies of different engineered T cell behavioral populations within various experimentally separated T cell groups. To make these predictions, we utilize the dynamic behavioral data generated by BEHAV3D[13]. This simulation employs time-series data of T cells, collected through the BEHAV3D protocol, which include detailed information on individual T cell behaviors across all imaging time points. For each T cell, BEHAV3D assigns a behavioral class (Fig. 1a) and tracks its PDO engagement status at each time point (Fig. 2a). Our in silico simulation (Fig. 2e) procedure then uses this information to virtually separate populations in accordance with the time point(s) used for the one- or two-steps population separation experimental setup we used, mirroring the experimental procedure outlined in the 'Experimental (engagement) population separation' section. The output allows us to acquire these separated populations virtually and predict within each population the relative frequencies of different T cell behavioral classes (Figs. 1a and 4b). The computational steps for in silico population separation simulation are implemented in module 3 of our GitHub repository (https://github.com/imAIgene-Dream3D/BGT/tree/main?tab=readme-ov-file#3--in-silico-population-separation-simulation). A detailed explanation of this procedure, along with an in-depth breakdown of the code functionality, is provided in Supplementary Methods. Since this procedure is performed in silico, it can run in parallel with the 'Experimental (engagement) population separation' procedure. Note that, as sequencing solely captures living cells, the frequencies of the 'dying' T cell behavioral phenotype are omitted. Moreover, labeling and analyzing different T cell subpopulations within BEHAV3D, such as $CD4^+$ and $CD8^+$ cells, will bring a better resolution to the final outcome, as these cells exhibit distinct behavioral profiles and transcriptomes.

In this simulation, we assume that the behavioral patterns observed in BEHAV3D are consistent and can be extrapolated to predict behaviors in similar experimental conditions. Furthermore, the separation process is assumed not to substantially alter the behavior of T cells beyond what is already captured in the dynamic data. The live imaging data should be representative of the entire population of T cells, for which we recommend using data from at least four to six BEHAV3D replicates.

Behavioral probability mapping (Steps 88 and 89)

To infer behavior from processed scRNA-seq data of T cells, we integrate insights derived from in silico population separation (Fig. 2f). This methodology hinges on the principle of probability transitivity. Our dataset is segmented into three intersecting domains: UMAP_cluster (U_{cl}), Behavioral_signatures (B_{sig}) and Experimental_engagement_state (Exp_{eng}). U_{cl} signifies the cluster to which a sequenced cell is assigned and delineates its position on the pseudotime inference uniform manifold approximation and projection (UMAP), offering insight into its proximity to neighboring cells and the respective U_{cl} of those neighbors (Fig. 4a). This information is extracted from the sequencing data. B_{sig} is derived from live imaging data, processed through in silico simulation, and corresponds to the behavioral class of T cells (Fig. 4b). Exp_{eng} serves as a shared domain between imaging and sequencing procedures, indicating the experimental group to which a T cell belongs. Hence, through in silico simulation, we obtain a known data domain: the probability distribution (p) of each B_{sig} within each Exp_{eng} group (Fig. 4b). Similarly, from scRNA-seq data, we obtain another known data domain:

the probability distribution of each Exp_{eng} group within each U_{cl}. By applying the probability transitivity principle as depicted in the formula below, we are able to estimate an unknown data domain: the distribution of each B_{sig} within each U_{cl} (Fig. 4c). This process effectively bridges the imaging and sequencing dimensions of the data.

$$p(B_{sig}|U_{cl}) = \sum_{Exp_{eng}} p(B_{sig}|Exp_{eng}) \times p(Exp_{eng}|U_{cl})$$

To improve the precision of the data, we execute this procedure iteratively using various UMAP clustering resolutions. The level of granularity in UMAP clustering determines whether we analyze local or broader cell–cell relationships. Thus, we initiate clustering at a low resolution, resulting in fewer clusters and enabling an assessment of the global data structure. Conversely, higher-resolution clustering allows us to examine the immediate neighbors of cells and their Exp_{eng} state, leading to a more detailed comprehension of the data structure. The outcome of this analysis provides for each sequenced cell a probability of belonging to the different behavioral class (B_{sig}). Utilizing this data in conjunction with gene clustering derived from pseudotime trajectory inference, we delineate distinct stages of targeting, encompassing baseline T cells before coculture, exposed T cells and T cells exhibiting short or long PDO engagement. The computational steps for behavioral probability mapping are implemented in module 4 of our GitHub repository (https://github.com/imAIgene-Dream3D/BGT/tree/main?tab=readme-ov-file#4-behavioral-probability-mapping-module).

Materials

Biological materials

- Human breast cancer organoids: obtained from the Hubrecht Organoid Technology (www.huborganoids.nl) biobank (see 'Methods' and 'Supplementary Information' from ref. 2 for more detail)
  ▲ **CAUTION** Any experiments using human material must conform to relevant institutional and national regulations, and informed consent must be obtained. Compliance with the Dutch Medical Research Involving Human Subjects Act was ensured for every material obtained by requesting the necessary authorizations by the ethical committee of the UMC Utrecht (METC UMCU).
- TEG T cells[32]: for generation, peripheral blood of anonymous healthy donors was purchased from the Dutch blood bank (Sanquin). Informed consent was obtained from all donors (see 'Methods' from ref. 2 for more detail)

Reagents

- A83-01 (Tocris Bioscience, cat. no. 2939)
- Advanced Dulbecco's modified Eagle's medium/F12 (adDMEM/F12; Thermo Fisher Scientific, cat. no. 12634-028)
- B27 supplement, pH 6–8 (50×) (Thermo Fisher Scientific, cat. no. 17504-44)
- GlutaMAX, pH 4.7–6 (100×) (Thermo Fisher Scientific, cat. no. 35050-061)
- HEPES 1 M (100×) (Thermo Fisher Scientific, cat. no. 15630-080)
  ▲ **CAUTION** HEPES is hazardous. Avoid contact with skin and eyes. Wear gloves and protective eye goggles.
- Heregulin β1 (Peprotech, cat. no. AF-100-03)
- *N*-acetyl-L-cysteine (Sigma-Aldrich, cat. no. A9165-5g)
  ▲ **CAUTION** *N*-acetyl-L-cysteine is hazardous. Avoid contact with skin and eyes. Wear gloves and protective eye goggles.
- Nicotinamide (Sigma-Aldrich, cat. no. N0636)
  ▲ **CAUTION** Nicotinamide is hazardous. Avoid contact with skin and eyes. Wear gloves and protective eye goggles.

- Noggin-conditioned medium, produced by a Noggin-producing cell line, which can be obtained from the laboratory of Prof. H. Clevers (Hubrecht Institute). Preparation instructions can be found in Box 2 of ref. 1
- Primocin (Invivogen, cat. no. Ant-pm-1)
  ▲ **CAUTION** Primocin is hazardous so manipulate in a biosafety cabinet. Avoid direct contact with skin and avoid breathing fumes. Wear gloves and protective eye goggles.
- Recombinant human epidermal growth factor (EGF) (Peprotech, cat. no. AF-100-15)
- Recombinant human fibroblast growth factor (FGF)-7 (Peprotech, cat. no. AF-100-19)
- Recombinant human FGF-10 (Peprotech, cat. no. AF-100-26)
- R-spondin-1-conditioned medium, produced by R-sponding-1-producing cell line, which can be obtained from the laboratory of Prof. C. Kuno (Stanford University) Preparation instructions can be found in refs. 33–35.
- SB 202190 (Sigma-Aldrich, cat. no. S7067)
- Y-27632 dihydrochloride (ROCK inhibitor) (Abmole Bioscience, cat. no. M1817)
- RPMI medium 1640 + GlutaMAX, pH 7–7.4 (RPMI–GlutaMAX; Thermo Fisher Scientific, cat. no. 61870-010)
- Penicillin–streptomycin (Thermo Fisher Scientific, cat. no. 15140-122)
  ▲ **CAUTION** Penicilin–streptomycin is hazardous. Manipulate in a biosafety cabinet. Avoid direct contact with skin and avoid breathing fumes. Wear gloves and protective eye goggles.
- Fetal bovine serum (FBS), heat-inactivated (Thermo Fisher Scientific, cat. no. 10500064)
- Human serum (Sanquin, cat. no. E8683R00)
- Cultrex reduced growth factor (RGF) basement membrane extract (BME), type 2 (R&D systems, cat. no. 3533-005-02); Matrigel (for example, BD Biosciences, cat. no. 356231) can be used as an alternative with equal performance
- Pamidronate, disodium salt (Sigma Aldrich, cat. no. 506600-10MG)
- Dulbecco's phosphate-buffered saline, no calcium, no magnesium, 1×, pH 7–7.3 (DPBS; Thermo Fisher Scientific, cat. no. 14190-144)
- Bovine serum albumin (BSA), modified fraction V (Sigma Aldrich, cat. no. A9418)
- Dimethyl sulfoxide (DMSO; Sigma Aldrich, cat. no. D2650)
  ▲ **CAUTION** DMSO is highly flammable.
- DMSO anhydrous, pH 6–8 (Thermo Fisher Scientific, cat. no. D12345)
  ▲ **CAUTION** DMSO anhydrous is highly flammable.
- Trypan Blue solution (Sigma Aldrich, cat. no. T8154)
  ▲ **CAUTION** Trypan Blue solution is hazardous. Avoid direct contact with skin. Wear gloves and protective goggles.
- TrypLE Express (1×) (Thermo Fisher Scientific, cat. no. 12605-010)
  ▲ **CAUTION** TrypLE Express is hazardous. Manipulate in a biosafety cabinet. Avoid direct contact with skin. Wear gloves and protective eye goggles.
- Conjugated antibodies, user defined (used antibodies in this setup are listed in Table 2).
- UltraComp eBeads Plus Compensation Beads (Thermo Fisher Scientific, cat. no. 01-3333-42)
- ArC Amine Reactive Compensation Bead Kit (Thermo Fisher Scientific, cat. no. A10346)

Equipment

Cell culture

- 1.5 ml Safe-lock tubes (Eppendorf, cat. no. 0030120086)
- 2.0 ml Safe-lock tubes (Eppendorf, cat. no. 0030120094)
- 2.0 ml Cryovials (Greiner Bio-One, cat. no. 122263)
- Biosafety cabinet (Interflow, model no. MVK 130 IIA)

Table 2 | Example antibody staining mix for FACS

Antibody/ dye	Dilution	Vendor, cat. no.	RRID
Anti-CD3-APC	1:80	BioLegend, 344811	https://scicrunch.org/resolver/RRID:AB_10644010
LIVE/DEAD Fixable Near-IR Dead Cell Stain	1:1,000	ThermoFisher, L34975	N/A

- Cell strainer, 70 µm (Greiner, cat. no. 542070)
- CELLSTAR 96-well plate (Greiner Bio-One, cat. no. 655180)
- CellSTAR flask, TC, PS, 50 ml, 25 cm^2 (Greiner Bio-One cat. no 690175)
- Centrifuge for 15/50 ml Falcon tubes (for example, Eppendorf, cat. no. 5804R)
- Counting chamber (for example, Bürker-Türk, Merck, cat. no. BR719520)
- CO_2 incubator, 37 °C and 5% CO_2 (for example, Thermo Fisher Scientific, cat. no. 4141)
- Falcon tubes, 15 ml, conical bottom (Greiner Bio-One, cat. no. 188271)
- Falcon tubes, 50 ml, conical bottom (Greiner Bio-One, cat. no. 227261)
- Inverted brightfield microscope, objectives 5×, 10×, 20× (for example, Leica, cat. no. DMi1)
- Microcentrifuge (for example, Eppendorf, cat. no. 5424R)
- Multi-well tissue 12-well culture plates (Greiner Bio-One, cat. no. 665180)
- Multi-well 12- or 24-well suspension plates (Greiner Bio-One, cat. nos. 665102, 662102)
- T25 culture flasks, tissue culture treated (Greiner Bio-One, cat. no. C6481)
- T75 culture flasks, tissue culture treated (Greiner Bio-One, cat. no. C7231)
- Pipetman L, P20L, P200L, P1000L (Gilson, cat. nos. FA10003M, FA10005M, FA10006M)
- Pipette tips with filter (for example, Gilson Pipetman, Diamond tips)
- Serological pipettes 5, 10, 25 ml (Greiner Bio-One, cat. nos. 606180, 607160, 760160)

FACS

- Sarstedt 96-well tissue culture plate, round bottom (Sarstedt, cat. no. 83.3925)
- Falcon 5 ml round-bottom tubes (Corning, cat. no. 352063)
- Multichannel pipette P12x200 (Gilson, cat. no. F144073)
- Flow cytometric cell sorter (BD Biosciences, model no. FACSAria II Cell Sorter or equivalent, any flow cytometric cell sorter can be used)

scRNA-seq

- 384-Well plates containing 50 nl of barcoded primer and 10 µl mineral oil per well (provided by Single Cell Discoveries when using their SORT-seq service)
- Multiwell aluminium foil plate sealers (Sigma Aldrich, cat. no. Z617601-100EA)

Imaging

- Zeiss LSM880 (20×/0.8 DRY. Working distance: 550 µm) (Zeiss, cat. no. 420650-9901-000) or equivalent confocal microscope setup, necessary to run the BEHAV3D pipeline related to this protocol

Image analysis

- Dell Precision workstation (64-bit, Windows 10, 1 TB RAM, Intel(R) Xeon(R) Gold 5122 CPU at 3.60 GHz (two processors), NVIDIA Quadro P4000 (8 Gb VRAM), which is necessary to run the BEHAV3D pipeline related to this protocol

Software

- Imaris 9.5 or higher (Oxford Instruments Bitplane). Packages Imaris TrackLineage and Imaris XT are required for cell tracking and for the 'channel arithmetic' function (https://imaris.oxinst.com/) necessary to run the BEHAV3D pipeline related to this protocol
- R (tested on MacOS Big Sur with R version 4.1.1 and on Windows 10 with R version 4.3.2 Specific packages and version required can be found in the BEHAV3D repository (https://github.com/AlievaRios/BEHAV3D) and in the BGT repositories (https://github.com/imAIgene-Dream3D/BGT)

Reagent setup

Reagent setup for breast cancer organoid medium

- adDMEM/F12+++: supplement 500 ml of adDMEM/F12 with 5 ml penicillin–streptomycin (1% vol/vol), 5 ml GlutaMAX (1% vol/vol) and 5 ml HEPES (1% vol/vol). Store at 4 °C for up to 6 months

- DPBS with 0.1% (wt/vol) BSA (DPBS-B): used for diluting specific growth factors. Dissolve 0.1 g BSA (modified fraction V) per 100 ml DPBS (room temperature (RT), 20–22 °C) and filter sterilize over a 0.22 µm filter. Store 1 ml aliquots at −20 °C for up to 2 years
- Breast cancer organoid medium: the medium components and final concentrations are listed in Table 4 of ref. 13. To make 200 ml of type 1 expansion medium, add to 153 ml of adDMEM/F12+++: 20 ml of R-spondin-1- conditioned medium, 20 ml of Noggin-conditioned medium, 4 ml of 50× B27 supplement, 2 ml of nicotinamide (1 M in DPBS), 500 µl *N*-acetyl-L-cysteine (500 mM in H2O), 400 µl Primocin (1 mg/ml), 10 µl Y-27632 (100 mM in DMSO), 100 µl Heregulin β1 (10 µM in DPBS-B), 100 µl human FGF-7 (10 µg/ml in DPBS-B), 40 µl human FGF-10 (100 µg/ml 0.1% DPBS-B), 20 µl A83-01 (5 mM in DMSO), 2 µl human EGF (500 µg/ml 0.1% DPBS-B) and 6.8 µl SB202190 (30 mM in DMSO). This medium can be stored for 1 week at 4 °C
- R-spondin-1-conditioned medium: the procedure of R-spondin-1-conditioned medium production with 293T-HA-RspoI-Fc cells has been described in Box 1 of ref. 35. R-spondin-1-conditioned medium can be stored at 4 °C for 6 months
- Noggin-conditioned medium: the procedure of Noggin-conditioned medium production with HEK293-mNoggin-Fc cells has been described in Box 2 of ref. 1. Noggin-conditioned medium can be stored at 4 °C for 6 months
- A83-01: to prepare a 5 mM stock solution (10,000×), dissolve 10 mg powder in 5 ml DMSO (RT). Store 50 µl aliquots at −20 °C for up to 1 year
- Nicotinamide: o prepare a 1 M stock solution (100×), dissolve 6 mg of powder in 50 ml of DPBS (RT). Store 1 ml aliquots at −20 °C for up to 1 year
- *N*-acetyl-L-cysteine: to prepare a 500 mM stock solution (400×), dissolve 815 mg powder in 10 ml H2O (RT). Store 500 µl aliquots at −20 °C for up to 1 year
- Heregulin β1: to prepare a 10 µM stock solution (2,000×), dissolve 100 µg powder in 1,333 µl DPBS (RT). Store 100 µl aliquots at −20 °C for up to 1 year, avoiding freeze–thaw cycles
- Human EGF: to prepare a 500 µg/ml stock solution (100,000×), dissolve 500 µg powder in 1 ml DPBS-B (RT). Store 100 µl aliquots at −20 °C for up to 1 year, avoiding freeze–thaw cycles
- Human FGF7: to prepare a 100 µg/ml master stock solution (20,000×), dissolve 100 µg powder in 1 ml DPBS-B (RT). Store 100 µl aliquots at −80 °C for up to 1 year. To prepare a 10 µg/ml stock solution (2,000×), dilute the master stock ten times in DPBS-B (add 100 µl master stock to 900 µl DPBS-B). Store 100 µl aliquots at −20 °C for up to 1 year, avoiding freeze–thaw cycles
- Human FGF10: to prepare a 40 µg/ml stock solution (2,000×), dissolve 100 µg powder in 2.5 ml DPBS-B (RT). Store 100 µl aliquots at −20 °C for up to 1 year, avoiding freeze–thaw cycles
- SB 202190: to prepare a 30 mM stock solution (30,000×), dissolve 5 mg powder in 500 µl DMSO (RT). Store 10 µl aliquots at −20 °C for up to 1 year
- Y-27632 dihydrochloride (ROCK inhibitor): to prepare a 100 mM stock solution (20,000×), dissolve 10 mg powder in 312 µl DMSO (RT). Store 100 µl aliquots at −20 °C for up to 1 year

Reagent setup for T cells

- T cell culture: culture T cells in T cell culture medium at a density of 0.7–1 × 10^6 T cells/ ml in a T25 (volumes <10 ml) or T75 (volumes >10 ml) flask in upright position. We recommend resting T cells in T cell culture medium for 2–4 d before use in cocultures if frozen T cells or T cells directly after expansion are used
- T cell assay medium: supplement 500 ml of RPMI–GlutaMAX with 50 ml FBS (10% vol/vol), and 5 ml penicillin–streptomycin (1% vol/vol). Store at 4 °C for up to 6 months
- T cell culture medium: supplement 500 ml of RPMI–GlutaMAX with 25 ml human serum (5% vol/vol) and 5 ml penicillin–streptomycin (1% vol/vol). Store at 4 °C for up to 6 months

Reagent setup for coculture

- Pamidronate, disodium salt: to prepare a 25 mM stock solution (500×), dissolve 10 mg powder in 1,100 µl H_2O (RT). Store 20 µl aliquots at 4 °C for up to 6 months
- Coculture medium: to prepare a 10 ml fresh solution, combine 5 ml of breast cancer organoid medium (50% vol/vol) with 5 ml of T cell assay medium (50% vol/vol) and

supplement with 250 µl BME (2,5% vol/vol) and 20 µl pamidronate (500×). Coculture medium can be stored at 4 °C for up to 1 week

▲ **CRITICAL** When organoids of different origin/type are used (for example, lung cancer), complete culture medium of that particular organoid type should be used instead of breast cancer organoid medium.

▲ **CRITICAL** Once BME has been added, thoroughly resuspend three to five times with a 10 ml serological pipette. This will enable BME to be properly dissolved and avoiding BME clump in the medium that can impact proper imaging of the cocultures assay.

▲ **CRITICAL** Addition of pamidronate is specific to the TEGs and induces the accumulation of the phosphoantigen IPP to stimulate tumor cell recognition[36]. If other immune cells are used, leave pamidronate out of the coculture medium.

Reagent setup on PDO propagation and collection

- PDO culture propagation and collection: PDO culture propagation and collection are performed as described in our previous protocol[13]

Reagent setup for FACS

- FC buffer: supplement 50 ml DPBS (RT) with 1 ml FBS (2% vol/vol), store at −20 °C for up to 4 weeks

Procedure

BEHAV3D T cell behavioral profiling

● **TIMING 5–16 h**

1. Follow Steps 1–31 from the protocol for BEHAV3D for T cell-PDO coculture setup[13].
2. Follow Steps 32–45 from the protocol for BEHAV3D for T cell-PDO imaging setup[13].
3. Follow Steps 44–52 from the protocol for BEHAV3D for T cell behavioral analysis[13]. Instructions on how to install and run the processing tools are described in our GitHub BEHAV3D repository as well as in the protocol for BEHAV3D[13] (https://github.com/AlievaRios/BEHAV3D).

   ▲ **CRITICAL STEP** On successful execution of Step 49 from the protocol for BEHAV3D[13] for T cell behavioral dynamics classification module, the classified_tcell_track_data.rds file is generated among other rds files. It is the only data input required from BEHAV3D for implementing the BGT pipeline.

BGT installation

● **TIMING 5–10 min**

4. Follow the GitHub instructions to install the processing tools as described in our GitHub BGT repository (https://github.com/imAIgene-Dream3D/BGT, Supplementary Video 2).
5. In the provided config_template (https://github.com/imAIgene-Dream3D/BGT/blob/main/config_template.yml), fill in the file-path to the classfied_tcell_track_data.rds derived as an output of Step 3 under the parameter classified_tcell_track_data_filepath_rds.

Evaluation of optimal timing for 'super engaged' enrichment

● **TIMING 5 min**

6. Set up the config_template for module 1, that is, evaluation of optimal timing for 'super engaged' enrichment, by filling in the parameter imaging_time, the time (in hours) between end of Step 1 and start of imaging in Step 2 (Supplementary Video 3).

   ▲ **CRITICAL STEP** Usually coculture imaging experiments (Step 2) start 60–90 min after the coculture setup (Step 1), due to the need of microscope setup. For correct estimation of 'super engager' frequency dynamics in coculture it is necessary to specify the time (in hours) it took to start imaging after the coculture.

7. Follow the GitHub instructions to run module 1 (https://github.com/imAIgene-Dream3D/BGT/tree/main?tab=readme-ov-file#1-evaluation-of-optimal-timing-for-super-engaged-enrichment, Supplementary Video 3). The module generates two key output files:
   - T-cell_engagementVsTime.png: a visualization showing the temporal dynamics of super engager T cell frequencies, including separate trajectories for $CD4^+$ and $CD8^+$ populations
   - T-cell_engagementVsTime.csv: a time-series data of super engager frequencies for downstream analysis

   ◆ **TROUBLESHOOTING**

PDOs for coculture setup

● **TIMING 20–30 min**

▲ **CRITICAL** This section describes the collection of breast cancer PDOs from a 12-well suspension plate grown in 3D in a BME support matrix for coculture with immune cells and subsequent population separation. Depending on the type of organoid culture and culture density upon collection, 1 well of a 12-well plate of organoids is sufficient for 1–2 wells of a 12-well culture plate.

8. Aspirate all expansion medium from a well containing organoids by holding the plate at a 45° angle and placing the aspiration tip to the side of the well, not getting close to the BME drops.
9. To detach the BME drops from the plate, add 1 ml of TrypLE to the well and forcefully pipet up and down six to ten times per well with a p1000 pipette, aiming at the BME drops.
10. To get the organoids out of the detached BME drops, directly after Step 9 hold the plate at a 45° angle and forcefully pipet the BME–TrypLE mixture with organoids up and down ten times with a p1000 pipette. This will dissociate most of the BME, but the organoids will remain intact. Avoid bubble formation as this may accelerate organoid dissociation.

    ◆ **TROUBLESHOOTING**
11. Directly after Step 10, transfer organoids into a 15 ml Falcon tube with 10 ml ice-cold adDMEM/F12+++, using a p1000 pipette.
12. Rinse the well with 1 ml adDMEM/F12+++ to ensure no organoids remain in the well.
13. (Optional) Aspirate the organoid suspension using a 10 ml serological pipette, place a 70 µm cell strainer tilted in a 45° angle on top of the same 15 ml tube, and pipet the organoid suspension through the strainer into the 15 ml tube by placing the tip of the 10 ml pipet containing the organoid suspension in the corner of the strainer.

    ▲ **CRITICAL STEP** This step is advised when the organoid culture contains (>5%) organoids larger than 70 µm (Fig. 2b of ref. 13). This is important to minimize differences in size between individual organoids in the same well.
14. (Optional) To remove single tumor cells and debris, centrifuge the 15 ml Falcon tube with organoids at 30*g* for 5 min, at 4 °C, remove the supernatant by decanting the fluid in a waste bottle, add 10 ml ice-cold adDMEM/F12+++ and bring the organoids into suspension by gently resuspending three times using a 10 ml serological pipette.

    ▲ **CRITICAL STEP** Perform this step when the organoid culture contains many single cells or debris (refer to Extended Data Fig. 1d of ref. 13). Removing single cells and debris will ensure minimal differences in size between organoids in a well.

    ■ **PAUSE POINT** The 15 ml Falcon tube with processed organoids can be stored on ice for 30 min to first collect other cultures before continuing with Step 15.
15. Centrifuge the 15 ml Falcon tube with organoids at 300*g* for 5 min, at 4 °C.
16. Remove the supernatant by decanting the fluid in a waste bottle and add 1–3 ml ice-cold adDMEM/F12+++ for each well collected. Use 1 ml per collected well for organoid cultures with a low density and up to 3 ml per collected well for organoid cultures with a high density (Extended Data Fig. 1c of ref. 13). For example, if three wells of a low-density line were collected add 3 × 1 ml = 3 ml.
17. To bring the organoids to the correct density, bring the organoids into suspension by gently resuspending three times using a p1000 pipette and transfer 100 µl of organoid suspension

into a flat bottom 96-well plate using a p200 pipette. Place the plate at RT for 1–5 min so that the organoids can settle at the bottom. Use an inverted brightfield microscope (5×, 10× or 20× objective) to check the organoid density and afterward discard the 96-well plate (refer to Fig. 2c of ref. 13 for guidance of optimal organoid density).

▲ **CRITICAL STEP** Organoid density is important to ensure consistency between organoid lines and experiments. Because the organoid density influences immune cell behavior, organoid density should be kept as similar as possible between different conditions or experiments.

18. Transfer the desired volume of organoid suspension to a 15 ml Falcon tube as follows. If the organoid density as assessed in the previous step was optimal, transfer 1000 µl of organoid suspension per well of the final 12-well culture plate (Table 1) to a 15 ml Falcon tube. If the organoid density as assessed in the previous step was too sparse or too dense, adjust the volume proportionally. For example, if it was twice as dense, transfer 500 µl of organoid suspension per well. Make sure to take >10% in excess to account for pipetting errors.

▲ **CRITICAL STEP** It is recommended to plate at least 0.8×10^6 T cells per condition to have enough 'engaged' and 'nonengaged' T cells for analysis. This might require multiple wells per condition depending on the T cell seeding density used (Table 1).

▲ **CRITICAL STEP** If the optional extra enrichment step is used in Step 40, plate extra organoid wells to enrich for 'nonengaged^{enriched}' and 'super engaged' T cells. For an example experimental setup refer to Table 1.

19. Centrifuge the 15 ml Falcon tube with organoids at 300*g* for 5 min, at 4 °C.
20. Remove the supernatant by decanting the fluid in a waste bottle and remove the remaining supernatant carefully with a p1000 pipette.
21. Resuspend the pellet in 1 ml coculture medium by resuspending 5× with a p1000 pipette. Fill up to the volume required to achieve the adequate concentration for plating and resuspend again 5× with a p1000 pipette (for volume <2 ml) or 5 ml serological pipette (for volume >2 ml).
22. Transfer 1,000 µl organoid suspension to each well of a 12-well tissue culture plate (Table 1).

▲ **CRITICAL STEP** To ensure optimal distribution of organoids, when plating your replicates, we advise to distribute organoids across experimental conditions before distributing across replicates.

■ **PAUSE POINT** Place the plate in an incubator (5% CO_2, 37 °C) and continue with Step 23. The plate can be stored for up to 8 h.

T cell plating

● **TIMING 20 min**

23. Bring the T cells from a culture flask into suspension by gently resuspending three times using a 10 ml serological pipette and transfer the T cell suspension to a 15 ml tube.

◆ **TROUBLESHOOTING**

24. Fill the tube with T cell assay medium up to 15 ml for optimal washing.
25. Centrifuge the 15 ml Falcon tube with T cell suspension at 300*g* for 5 min, at 4 °C.
26. Remove the supernatant by aspirating the fluid.
27. Add 15 ml T cell assay medium and gently resuspend three times.
28. Centrifuge the 15 ml Falcon tube with T cell suspension at 300*g* for 5 min, at 4 °C.
29. Remove the supernatant by decanting the fluid in a waste bottle. Remove the remaining supernatant carefully with a p1000 pipette.
30. Add 0.5–2 ml of T cell assay medium to the tube with a p1000 pipette and gently resuspend five times.
31. Count the cells by taking 10 µl of cells and mixing it with 10 µl Trypan Blue solution. Count using a counting chamber and an inverted brightfield microscope (10× or 20× objective). One confluent well of a 24-well plate commonly contains 2–4 million cells.

◆ **TROUBLESHOOTING**

32. Transfer 4.2×10^5 T cells per well of a 12 well plate (Table 1) to a 15 ml Falcon tube.
33. Centrifuge the 15 ml tube with T cells at 300*g* for 5 min, at 4 °C.
34. Remove the supernatant by decanting the fluid in a waste bottle. Remove the remaining supernatant carefully with a p1000 pipette.

35. Resuspend the pellet in 1 ml coculture medium by resuspending 5× with a p1000 pipette. Fill up with coculture medium to the desired concentration (Table 1) and resuspend again 5× with a p1000 (for volume up to 2 ml) or 5 ml serological pipette (for volumes >2 ml).
36. Transfer 1,000 µl of T cell suspension to all required wells of the 12-well tissue culture plate previously seeded with organoids (Step 22).
37. Add 1,000 µl coculture medium to the wells containing only organoids or T cells (refer to Table 1 for an example plate layout).
38. Resuspend each well of the coculture gently five times aiming at different directions in the well.
39. Place the plate in an incubator (5% CO_2, 37 °C) for 4–6 h.

Population separation

● **TIMING 6–8 h**

▲ **CRITICAL** For isolation of 'engaged' and 'nonengaged' T cell populations using one density centrifugation step, proceed directly to Step 46. For isolation of 'super engaged' and 'nonengaged^{enriched}' populations using two density centrifugation steps, start at Step 40.

▲ **CRITICAL** For a schematic overview of the population separation procedure refer to Fig. 3a.

(Optional) 'Super engaged' and 'nonengaged^{enriched}' T cell enrichment only

▲ **CRITICAL** Enrichment of 'super engaged' and 'nonengaged^{enriched}' T cells requires that additional wells were set up is Step 18 (Table 1, plate 2, wells 1–9)

40. After incubating the coculture for 4 h (until *t*1 identified in Step 7 (Extended Data Fig. 2), resuspend wells 5× with a p1000 and transfer cells to a 15 ml Falcon tube.
    ■ **PAUSE POINT** The 15 ml Falcon tube with collected cells can be stored on ice for 30 min to first collect other wells before continuing with Step 41.
41. Centrifuge the 15 ml tube with cells at 300*g* for 5 min, at 4 °C.
    ▲ **CRITICAL STEP** Note that for washing steps a centrifugation speed of 300*g* is used while for separation we use a speed of 30*g* (Fig. 3a).
42. Transfer supernatant to a new 15 ml tube. Make sure to keep the conditioned medium retrieved from the wells.
    ▲ **CRITICAL STEP** After density centrifugation separation, cells will be resuspended in the conditioned medium.
43. Resuspend cell pellet in 15 ml T cell assay medium and centrifuge 30*g* for 5 min at 4 °C.
44. For 'super engaged' enrichment follow option A. For 'nonengaged^{enriched}' enrichment, follow option B.
    (A) **'Super engaged' enrichment:**
        (i) Carefully take up the supernatant without disturbing the pellet with a 5 ml serological pipette and discard.
        (ii) Remove the remaining supernatant carefully with a p1000 pipette and discard.
        (iii) Resuspend the pellet in 2 ml per well collected conditioned medium and add 2 ml of cell suspension back to the designated wells of the 12-well tissue culture plate (Fig. 3a and Table 1, plate 2, wells 1–3).
    (B) **'Nonengaged^{enriched}' enrichment:**
        (i) Carefully take up the supernatant without disturbing the pellet with a 5 ml serological pipette and add it to a new 50 ml tube.
        (ii) Remove the remaining supernatant carefully with a p1000 pipette and add it to the 50 ml tube from Step 44B(i), discard the 15 ml tube with the pellet.
        (iii) Resuspend wells containing only organoids (Table 1, plate 2, wells 7–9) 5× with a p1000 and transfer cells to the 50 ml Falcon tube from Step 44B(ii).
        (iv) Rinse the wells with 1 ml T cell assay medium to ensure no cells are left behind.
        (v) Centrifuge the 50 ml tube with cells at 300*g* for 5 min, at 4 °C.
        (vi) Carefully take up the supernatant without disturbing the pellet with a 5 ml serological pipette and discard.
        (vii) Remove the remaining supernatant carefully with a p1000 pipette and discard.

(viii) Resuspend the pellet in 2 ml per well collected conditioned medium from Step 42 and add 2 ml of cell suspension back to the designated wells of the 12-well tissue culture plate (Fig. 3a and Table 1, plate 2, wells 4–6).

45. Place the plate back in an incubator (5% CO_2, 37 °C) for another 2 h to reach a total coculture time of 6 h (*t*2 in Extended Data Fig. 2).

'Engaged', 'nonengaged', 'super engaged' and 'nonengaged^{enriched}' T cell enrichment

46. After incubating the coculture until it reaches the maximal 'super engager' time point, defined in Step 7 (*t*2 in Extended Data Fig. 2, 6 h for data showcased), resuspend all wells 5× with a p1000 and transfer cells to a 15 ml Falcon tube.
    ▲ **CRITICAL STEP** In case multiple wells were plated per condition, replicates can be pooled in one 15 ml Falcon tube in this step.
47. Rinse the wells with 1 ml T cell assay medium to ensure no cells are left behind and fill up the tube to 15 ml with T cell assay medium.
    ■ **PAUSE POINT** The 15 ml Falcon tube with collected cells can be stored on ice for 30 min to first collect other wells before continuing with Step 48.
48. Centrifuge the 15 ml tube with cells at 30*g* for 5 min, at 4 °C.
49. Carefully take up the supernatant without disturbing the pellet with a 5 ml serological pipette and transfer to a 50 ml Falcon tube (Table 1, plate 1 and plate 2, wells 4–6) or, for 'super engaged' enrichment (Table 1, plate 2, wells 1–3), discard, making sure to leave ~500 µl supernatant behind (Fig. 3a).
50. Remove the remaining supernatant carefully with a p1000 pipette and add to the 50 ml Falcon tube from Step 49 (Table 1, plate 1 and plate 2, wells 4–6) or discard for 'super engaged' enrichment (Fig. 3a and Table 1, plate 2, wells 1–3).
51. Resuspend the pellet in 1 ml T cell assay medium by resuspending 5× with a p1000 pipette. Fill up with T cell assay medium to 15 ml and resuspend again 5× with a 10 ml serological pipette.
52. Repeat Steps 48–51 to further separate T cells that are engaging from T cells that are not engaging.
53. Discard the 15 ml tube with pellet from the 'nonengaged^{enriched}' enrichment (Fig. 3a and Table 1, plate 2, wells 4–6).
    - The 15 ml tubes now contain the enriched 'engaged' (Table 1, plate 1, wells 3–5) and (optional) 'super engaged' T cells (Table 1, plate 2, wells 1–3) and can be used for FACS in Step 57 (Fig. 3a).
      ◆ **TROUBLESHOOTING**
      ▲ **CRITICAL STEP** After 'engaged' enrichment T cells should contain <20% nontumor engaging cells (Fig. 3c). Refer to Fig. 3b for an example of how cocultures look before and after enrichment.
54. Centrifuge the 50 ml tube from Step 52 containing the supernatant at 30*g* for 5 min at 4 °C.
55. Carefully take up the supernatant containing the 'nonengaged' and 'nonengaged^{enriched}' T cells without disturbing the pellet with a 10 ml serological pipette and transfer to a new 50 ml Falcon tube, making sure to leave ~500 µl supernatant behind.
56. Remove the remaining supernatant carefully with a p1000 pipette and add to the new 50 ml Falcon tube from Step 55. Discard the old 50 ml tube containing the pellet from Step 55.
    - The 50 ml tubes now contain the enriched 'nonengaged' (Table 1, plate 1, wells 3–5) and (optional) 'nonengaged^{enriched}' T cells (Fig. 3a and Table 1, plate 2, wells 4–6)
      ◆ **TROUBLESHOOTING**

FACS and scRNA-seq sample preparation

● **TIMING 2–3 h**

57. Centrifuge the tubes with enriched cell populations from Step 53 and Step 56 and 'no target' control (Table 1, plate 1, wells 1–2) T cells at 300*g* for 5 min, at RT.
58. Remove the supernatant by decanting the fluid.

Protocol extension

59. Resuspend the cells in 2 ml per tube TrypLE and incubate for 7 min at 37 °C to dissociate organoids and generate a single cell suspension.
    ▲ **CRITICAL STEP** To keep treatment of all conditions consistent, also incubate T cell only control conditions in TrypLE.
    ◆ **TROUBLESHOOTING**
60. After 7 min, add 10 ml T cell assay medium.
61. Centrifuge at 300*g* for 5 min, at 4 °C.
62. Remove the supernatant by decanting the fluid.
63. Remove the remaining supernatant carefully with a p1000 pipette.
64. Resuspend cells in 200 µl FC buffer and transfer to a 96-well round bottom plate for staining.
    ▲ **CRITICAL STEP** For staining multiple conditions simultaneously a 96-well round bottom plate is recommended. The compatibility with multichannel pipettes enables faster staining of multiple samples compared to, for example, FACS tubes. If staining in FACS tubes is preferred, for Steps 64–72 add double the indicated volume.
65. Centrifuge the plate with cells at 300*g* for 5 min, at 4 °C.
66. In the meantime, prepare antibody mix by adding 100 µl FC buffer per sample to a 1.5 ml Eppendorf, add antibodies at the desired concentration (Table 2) and mix well by pipetting up and down 5×.
67. Remove supernatant from each well and add 100 µl antibody mix or 100 µl FC buffer as unstained control to the cells. Resuspend well and incubate 20 min at 4 °C in the dark.
68. Prepare compensation controls in a separate plate:
    - Vortex the UltraComp eBeads and add one drop (~30 µl) per well of a 96-well round bottom plate. One well should be prepared for each antibody tested, along with one well without any antibody as a negative control. For amine-reactive dead dyes, such as the LIVE/DEAD Fixable Near-IR Dead Cell Stain, instead vortex the ArC reactive beads and add one drop (~30 µl) per well
    - Add antibodies to the wells in the same concentration used for staining cells (Table 2)
    - Incubate for 20 min at 4 °C in the dark
69. Add 100 µl FC buffer to the wells with cells and compensation controls.
70. Centrifuge the plates at 300*g* for 5 min, at 4 °C.
71. Remove the supernatant and resuspend in 200 µl FC buffer per well and mix by resuspending 5×.
72. Repeat Steps 70–71.
73. Transfer samples to FACS tubes.
74. Prepare collection plates for scRNA-seq, by thawing 384-well plates containing well-specific barcoded primers (obtained from Single Cell Discoveries) at RT for 5 min.
75. Centrifuge plate for 1 min at 2,000*g* at 4 °C.
76. Store plates at 4 °C until used for sorting.
77. Right before sorting, vortex the ArC negative beads and add 1 drop to the well containing stained ArC reactive beads.
78. Transport the cells and the 384-well plates on ice covered from light to the FACS.
    ■ **PAUSE POINT** The plate with stained cells can be stored on ice for 30 min to set up the FACS machine and run compensation samples.
    ◆ **TROUBLESHOOTING**
79. Sort the live $CD3^+$ T cells on a FACS Aria Cell Sorter (or equivalent, see 'Equipment' section) into the 384-well plates, make sure that each well contains 1 cell. Refer to Extended Data Fig. 3 for an example gating strategy.
    ◆ **TROUBLESHOOTING**
80. Once all wells on a plate are filled, immediately seal the plate with multiwell aluminium foil plate sealers.
81. Centrifuge plate for 1 min at 2,000*g* at 4 °C to bring the sorted cells in contact with the well-specific barcoded primers.
82. Place the plate on dry ice to snap freeze the sorted cells.
    ■ **PAUSE POINT** The 384-well plates can be stored at −80 °C for up to 3 months.

83. Submit the 384-well plates to a scRNA-seq service for sequencing. For example, we have submitted 384-well plates to Single Cell Discoveries (https://www.scdiscoveries.com/), a single-cell sequencing service provider in the Netherlands, where scRNA-seq was performed according to an adapted version of the SORT-seq protocol[37] with primers described in ref. 38. In brief, heat-lyse cells at 65 °C, followed by cDNA synthesis. After second-strand cDNA synthesis, pool all the barcoded material from one plate into one library and amplify using in vitro transcription. Following amplification, perform library preparation following the CEL-Seq2 protocol[39] to prepare a cDNA library for sequencing using TruSeq small RNA primers (Illumina). Sequence the DNA library with paired-end sequencing on an Illumina Nextseq 500, high output, with a 1 × 75 bp Illumina kit (read 1: 26 cycles, index read: 6 cycles, read 2: 60 cycles).

scRNA-seq data preprocessing

● **TIMING ranges from 1 h to 10 h depending upon scRNA-seq dataset complexity and user experience**

84. Set up the working directory path for module 2, that is, scRNA-seq data preprocessing, by modifying the home parameter in the initial script, Part1_SORTseq_QC.rmd (https://github.com/imAIgene-Dream3D/BGT/tree/main?tab=readme-ov-file#2-scrnaseq-data-preprocessing).
85. Follow the GitHub instructions to run module 2 scripts in sequence:
    - Script 1: performs quality control and preprocessing of raw SORTseq data, generating normalized expression matrices
    - Script 2: identifies and annotates T cell subsets, producing subset classification files
    - Script 3: constructs pseudotime trajectory for TEG subsets, outputting trajectory plots and ordering data
    - Script 4: performs dynamic gene clustering along the trajectory, generating gene cluster assignments and expression patterns
    - Script 5: analyzes TEG enrichment against in vivo tumor-reactive T cell markers, producing comparative analysis reports

    ▲ **CRITICAL STEP** Please ensure that you follow the scripts on GitHub in the specified order, from the first to the fifth. Deviating from this sequence will result in the subsequent steps not functioning correctly.

    ◆ **TROUBLESHOOTING**

In silico population separation simulation

● **TIMING 5 min**

86. Set up the config_template from Step 5, for module 3 (Supplementary Video 3), that is, in silico simulation population separation, by filling in the parameter: n_timepoints for the number of separation procedures to include in the simulation. (1 if only 'engaged' and 'nonengaged' populations were extracted (starting at Step 46), 2 if also 'super engaged' and 'never-engaged' populations were extracted (starting at Step 40)).
87. Follow the GitHub instructions to run module 3 (https://github.com/imAIgene-Dream3D/BGT/blob/main/README.md#3--in-silico-population-separation-simulation). The module generates the following outputs:
    - Time-specific engagement plots (plot_cd8_timepoint_X.png, plot_cd4_timepoint_X.png) showing cluster distribution at each separation interval
    - Quantitative engagement frequency data (CD8_engagement_behavior_freq.csv, CD4_engagement_behavior_freq.csv) documenting T cell engagement dynamics over time

    ▲ **CRITICAL STEP** The n_timepoints parameter in the config_template is crucial for accurately modeling the experimental separation steps and must correspond to the actual number of separation steps to be conducted in the experiments.

    ◆ **TROUBLESHOOTING**

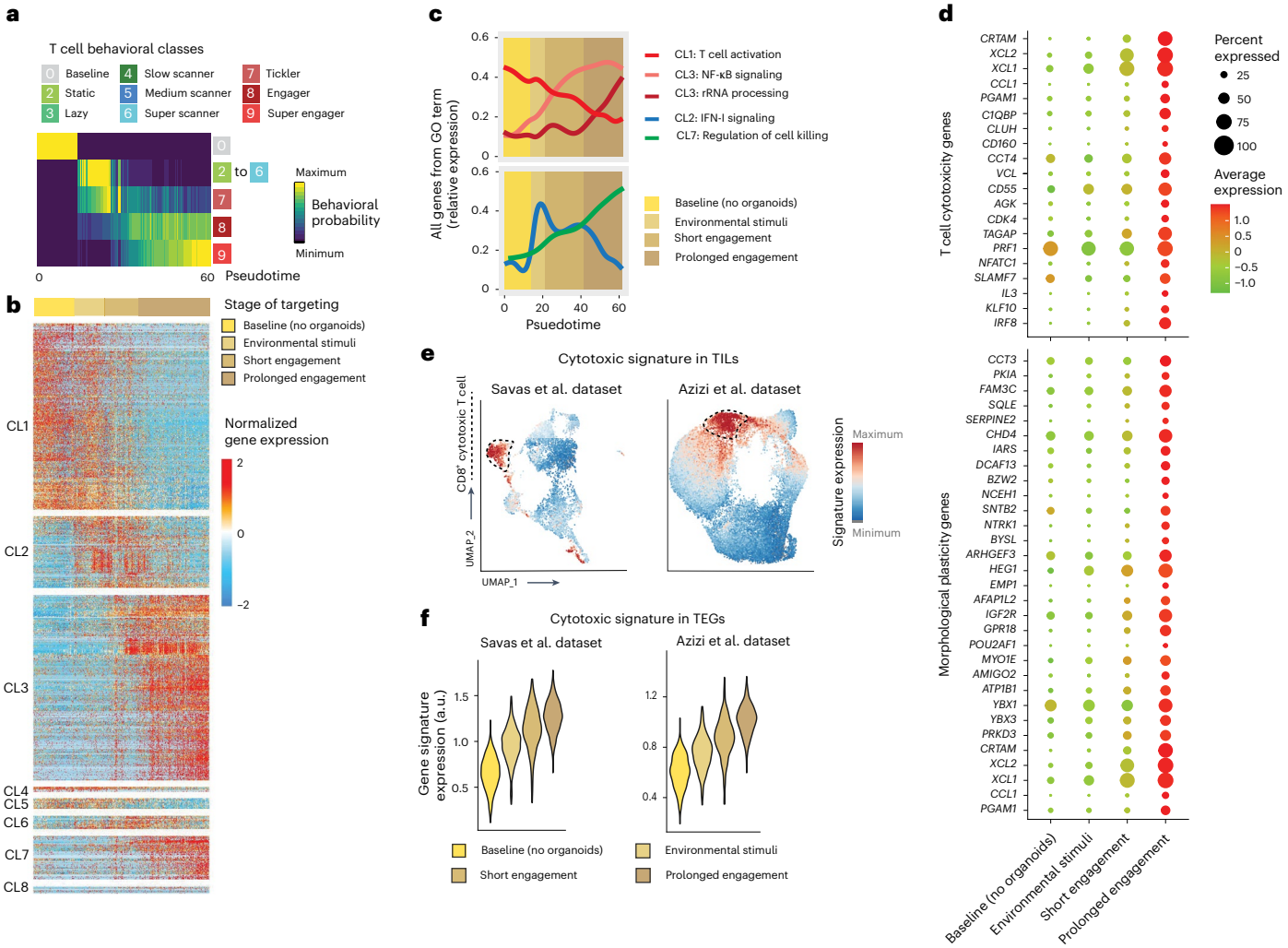


Fig. 5 | BGT integrates single cell transcriptome and functional behavior. **a**, A heat map representing the probability distribution of different behavioral signatures and no target control over pseudotime of CD8$^+$ TEGs. The colors represent the scaled probability for each behavioral group. **b**, A heat map showing normalized gene expression dynamics of CD8$^+$ TEGs following exposure to and engagement with PDOs. The columns represent T cells ordered in pseudotime, and the rows represent gene expression grouped based on similarity, resulting in eight gene clusters. Top: the horizontal color bar represents the corresponding stage of targeting based on data in **a**. **c**, An averaged gene expression for all genes belonging to specified GO terms, enriched in the associated gene clusters, over pseudotime. The background color shading denotes the targeted stage, while line colors signify the GO terms. rRNA, ribosomal RNA; NF-κB, nuclear factor-κB; IFN-I, type I interferon. **d**, A gene expression dot plot of selected genes composing the (serial) killer gene program separated by function. The rows depict genes, while columns depict stage of targeting. The dot color gradient indicates average expression, while size reflects the proportion of cells expressing a particular gene. **e**, A UMAP embeddings of tumor-infiltrating lymphocytes (TILs) isolated from human breast cancer tumor samples from the Savas et al.[40] and Azizi et al.[41] studies, color-coded for relative expression of a cytotoxic gene signature identified in each dataset. The dashed line delineates the CD8$^+$ cytotoxic T cell populations identified in each study. **f**, Violin plots for CD8$^+$ TEG showing expression of the cytotoxic gene signature identified in Savas et al. (left) or Azizi et al. (right). The colors indicate different stages of targeting. Panels **a**–**c** and **e** reproduced from ref. 2, CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/).

Behavioral probability mapping

● TIMING ranges from 5 to 20 min depending upon the size of the scRNA-seq dataset and the computational resources allocated for the analysis

88. Set up the config_template for module 4, that is, behavioral probability mapping, by filling in the parameter: scRNA_seq_dataset to specify the file-path for scRNA_seq_dataset.rds derived as an output of the Step 85.

89. Follow the GitHub instructions to run module 4 (https://github.com/imAIgene-Dream3D/BGT/tree/main?tab=readme-ov-file#4-behavioral-probability-mapping-module). The module generates the following outputs:
    - Behavioral signature visualization files
        - Probability distribution heat maps of behavioral signatures over pseudotime (as shown in Fig. 5a)
        - Gene expression dynamics heat maps showing T cell evolution post-PDO exposure (as shown in Fig. 5b)
    - Behavioral signature analysis files
        - Behavioral signature probability maps for each cluster
        - CSV files containing behavioral composition data
        - Pseudotime trajectory analysis of T cell state transitions

    ▲ **CRITICAL STEP** This module utilizes the scRNA_seq_dataset.rds data file derived from module 2 in Step 85 and the CD8_engagement_behavior_freq.csv file generated in Step 87.

    ◆ **TROUBLESHOOTING**

Troubleshooting

Troubleshooting advice can be found in Table 3.

Table 3 | Troubleshooting table

Step	Problem	Possible Reason	Solution
7, 87, 89	No such file or directory	Trying to read a file (such as a Seurat object) from a folder that does not exist or the file itself is not generated yet	Ensure the file exists and the file-path is correctly mentioned in the config_template
			Ensure the pipeline has been followed part by part or step by step to generate all derived files needed for downstream analysis steps
10	Organoids are broken up/ dissociated	Specific to behavior of that particular type of organoid culture	At Step 10, reduce resuspension time in TrypLE to keep organoids intact
23	The culture contains many dead cells and/or debris	Confluency of culture was too high when collecting	Collect culture at confluency of ~70% to avoid cellular stress due to nutrient shortage or pH variability that can cause cell death
			Wash cells one additional time by repeating Steps 24–26 to remove debris
31	T cells appear in clumps	The cells were not well resuspended	Gently resuspend five to ten times in Step 30
53	High amount of nonengager T cells present in enriched engager/super engager fractions	Not enough supernatant removed during density centrifugation steps	At Steps 44A(ii) and 50, make sure to remove as much medium as possible with the p1000 pipette without touching the pellet
56	High amount of engager T cells present in nonengager/never engager fractions	Part of cell pellet removed during density centrifugation steps	At Steps 44B(ii), 50 and 55, make sure to not disturb the pellet when taking up the supernatant
59	Organoids do not dissociate	Incubation in TrypLE too short	At Step 59, increase incubation time in TrypLE, regularly resuspend cells and check status of dissociation
	Clumps of cells	Incubation in TrypLE too long	At Step 59, reduce incubation time in TrypLE to reduce cell death
78	No compensation beads detected	Compensation beads not properly resuspended in the tube	Vortex the compensation beads for 30 s at Step 68
79	No $CD3^+$ T cells detected for engager/super engager conditions	PDOs not dissociated well	Increase incubation time in TrypLE in Step 59
	Low viability of T cells	T cells incubated too long in TrypLE	Decrease incubation time in TrypLE in Step 59

Table 3 (continued) | Troubleshooting table

Step	Problem	Possible Reason	Solution
85	Error message 'There is no package called …' appears	The required package has not been installed, and loading was tried without prior installation	Make sure the required package is installed before loading it into the namespace. Please, consult each corresponding package's installation manual to learn how to install it
	Error message 'No such file or directory'	Attempt made to read a file (such as a Seurat object) from a folder that does not exist or the file itself is not generated yet	Ensure the file exists at the destination you are trying to read the file in
			Make sure the pipeline has been followed part by part or step by step to generate all intermediate files needed for downstream analysis steps
			Download and put the corresponding file from provided Zenodo depository
	Error message 'Could not find function'	The corresponding library was not imported before running the command	Make sure 'Load Required Libraries' was run at the beginning of running each part of the analysis pipeline
	Generated figures are too small or not at a proper size	The height and width of the figure was not set before plotting it	In Jupyter notebook, before running the plotting command, use the following command and set the height and width of the figure (change values as you desire) options(repr.plot.width=11, repr.plot.height=5)
	Several cell types are absent	It is possible that only one cell type was sequenced (for example, only $CD8^+$ T cells)	In the case of profiling only one cell type/state, there is no need to follow subsetting pipeline of the analysis workflow. Only read in the raw data and remove low quality cells and perform batch correction (if needed)
	Error message 'root_pr_nodes or root_cells must be provided' appears during pseudotime trajectory analysis (Step 1.3 of part 3 of the analysis pipeline)	The pseudotime analysis requires starting points (root cells) to order cells along the trajectory. These root cells can be selected either through manual selection in an interactive plotting session or by direct specification in the code. The error occurs when neither method is available	Run the analysis in RStudio or VS Code where interactive selection is supported, or directly specify root cells in the code using the root_cells parameter (for example, root_cells=c('A17_10','E19_8'))
	Generating heat map in part 4 of the analysis pipeline takes too long	There are either too many cells sequenced or too many dynamic genes identified in pseudotime trajectory	Use a system with more resources, such as high-performance computing system
			Try to down sample the Seurat object using the following code and visualize only the smaller object with fewer cells downsampled.obj = large.obj[, sample(colnames(large.obj), size = ncol(small.obj), replace=F)]
	Vector memory exhausted (limit reached?)	Run out of RAM	Increase system RAM resources or perform the analysis on a high-performance computing system
			Down sample your dataset and analyze it in batches (with the above code)
89	Cannot open file and/or no such file or directory	R cannot locate or access the file, CD8_engagement_behavior_freq.csv specified by the file path	Manually enter the file path to the variable CD8_behav in the script, Behavioral-guided_transcriptomics.R. Open the script in the text editor, go to line 55, and modify the code to directly include the correct file path to CD8_engagement_behavior_freq.csv

Timing

The times stated below are estimated based on experienced users carrying out the procedures. Researchers new to the procedures might find that they take longer to complete.
Steps 1–3, BEHAV3D T cell behavioral profiling per imaging session: 1 week
Steps 4 and 5, BGT installation: 5–10 min
Steps 6 and 7, evaluation of optimal timing for 'super engaged' enrichment: 5 min
Steps 8–22, plating PDOs for coculture setup: 20–30 min
Steps 23–39, T cell plating: 20 min
Steps 40–56, population separation: 6–8 h
Steps 57–83, FACS and scRNA-seq sample preparation: 2–3 h
(At this stage, the samples are sent to a scRNA-seq service, with a turnaround time of 1–3 weeks to obtain the raw scRNA-seq files.)
Steps 84 and 85, scRNA-seq data preprocessing: 1–10 h, depending upon scRNA-seq dataset complexity and user experience

Steps 86 and 87, in silico population separation simulation: 5 min
Steps 88 and 89, behavioral probability mapping: 5–20 min, depending upon the size of the scRNA-seq dataset and the computational resources allocated for the analysis.

Anticipated results

This protocol describes the application of BGT, an integrative method designed to link T cell dynamic behaviors in coculture with PDOs to their transcriptomic profiles. This multimodal approach gives invaluable insights into the underlying molecular mechanisms governing imaging-based defined behavioral clusters. We present the use of BGT to analyze TEG behavioral-transcriptomic profiles in 3D coculture with breast cancer PDOs.

The output of BGT provides scRNA-seq data along with a probability indicating the likelihood of each cell belonging to a particular behavioral class (Fig. 5a,b). Based on this behavioral probability map, the cells ordered along the pseudotime can be grouped into different stages of targeting, encompassing baseline TEGs (cell predicted to have a high probability of no target control baseline TEGs), TEGs exposed to PDOs without active interaction (cells with high probability of 'static' and 'scanning' populations), TEGs with short engagement time to PDOs (high 'tickler' and 'engager' probability but low 'super-engager' probability) and TEGs with long engagement time to PDO (high 'super-engager' probability). BGT analysis of $CD8^+$ TEGs led to the identification of dynamic transcriptional programs linked to the different stages of tumor targeting (from no engagement to prolonged engagement) (Fig. 5c). Importantly, dynamic gene clustering uncovered specific genes induced in highly cytotoxic $CD8^+$ cancer-targeting TEGs ('super engager' TEGs) upon prolonged engagement with organoids, termed the 'serial killing gene program' (Fig. 5d). While several genes in this program are known to be associated with T cell cytotoxicity (for example, PRF1 and CRTAM), we also identified genes not previously linked to the cytotoxic phenotype of T cells. Instead, these genes are involved in morphological plasticity processes such as motility and cell adhesion (for example, AFAP1L2, BYSL and EMP1) (Fig. 5d). This matches the highly dynamic profile observed with imaging, where TEGs anchor on one site of the organoid and send out long protrusions while serially killing the entire organoid (Fig. 1c). This underscores the potential of the BGT approach in discovering new underlying genetic programs. Finally, to validate both our experimental model and scRNA-seq approaches, we used two publicly available datasets of in vivo T cells isolated from the breast cancer tumor microenvironment[40,41]. In these datasets, we identified marker genes of the most potent cancer-targeting in vivo T cells and validated that those genes are highest expressed in the cluster of 'super engager' TEGs which show the highest potential to kill PDOs in imaging data (Fig. 5e,f). Together, this illustrates the application of BGT in uncovering gene signatures driving the diverse and highly dynamic tumor targeting behaviors that we observe with live imaging and, importantly, the relevance of these gene signatures in tumor-targeting of in vivo T cells.

Data availability

All data described in this protocol are available from the corresponding author upon reasonable request. Representative raw imaging datasets used for PDO behavioral map generation can be found on BioImage Achive under accession no. S-BIAD448 (https://www.ebi.ac.uk/biostudies/studies/s-biad448) and representative processed imaging datasets can be found on Dryad (https://doi.org/10.5061/dryad.3n5tb2rp6). Single cell RNA-seq data of this study can be found on Gene Expression Omnibus under accession no. GSE172325. Processed demo data are made publicly available through GitHub (https://github.com/imAIgene-Dream3D/BGT/tree/main/Demo) and can be accessed through an online data browser (https://cells.ucsc.edu/?ds=behavior-guided-tx).

Code availability

All source codes of BEHAV3D are publicly available through GitHub (https://github.com/imAIgene-Dream3D/BEHAV3D). All source codes of BGT are publicly available through GitHub (https://github.com/imAIgene-Dream3D/BGT/). Singularity image container for installation reproducibility is provided on Zenodo via https://zenodo.org/records/10953043 (ref. 31).

Received: 26 April 2024; Accepted: 3 December 2024;
Published online: 10 February 2025

References

1. Cattaneo, C. M. et al. Tumor organoid–T-cell coculture systems. *Nat. Protoc.* **15**, 15–39 (2020).
2. Dekkers, J. F. et al. Uncovering the mode of action of engineered T cells in patient cancer organoids. *Nat. Biotechnol.* https://doi.org/10.1038/s41587-022-01397-w (2022).
3. Schnalzger, T. E. et al. 3D model for CAR-mediated cytotoxicity using patient-derived colorectal cancer organoids. *EMBO J.* **38**, e100928 (2019).
4. Alieva, M., Wezenaar, A. K. L., Wehrens, E. J. & Rios, A. C. Bridging live-cell imaging and next-generation cancer treatment. *Nat. Rev. Cancer* **23**, 731–745 (2023).
5. Romain, G. et al. Multidimensional single-cell analysis identifies a role for CD2–CD58 interactions in clinical antitumor T cell responses. *J. Clin. Invest.* **132**, e159402 (2022).
6. Khazen, R. et al. Functional heterogeneity of cytotoxic T cells and tumor resistance to cytotoxic hits limit anti-tumor activity in vivo. *EMBO J.* **40**, e106658 (2021).
7. Weigelin, B. et al. Cytotoxic T cells are able to efficiently eliminate cancer cells by additive cytotoxicity. *Nat. Commun.* **12**, 5217 (2021).
8. Bandey, I. N. et al. Designed improvement to T-cell immunotherapy by multidimensional single cell profiling. *J. Immunother. Cancer* **9**, e001877 (2021).
9. Davenport, A. J. et al. Chimeric antigen receptor T cells form nonclassical and potent immune synapses driving rapid cytotoxicity. *Proc. Natl Acad. Sci. USA* **115**, E2068–E2076 (2018).
10. Hamieh, M. et al. CAR T cell trogocytosis and cooperative killing regulate tumour antigen escape. *Nature* **568**, 112–116 (2019).
11. Yan, C. et al. Single-cell imaging of T cell immunotherapy responses in vivo. *J. Exp. Med.* **218**, e20210314 (2021).
12. Yi, J., Balagopalan, L., Nguyen, T., McIntire, K. M. & Samelson, L. E. TCR microclusters form spatially segregated domains and sequentially assemble in calcium-dependent kinetic steps. *Nat. Commun.* **10**, 277 (2019).
13. Alieva, M. et al. BEHAV3D: a 3D live imaging platform for comprehensive analysis of engineered T cell behavior and tumor response. *Nat. Protoc.* https://doi.org/10.1038/s41596-024-00972-6 (2024).
14. Chu, Y. et al. Pan-cancer T cell atlas links a cellular stress response state to immunotherapy resistance. *Nat. Med.* **29**, 1550–1562 (2023).
15. Bai, Z. et al. Single-cell antigen-specific landscape of CAR T infusion product identifies determinants of CD19-positive relapse in patients with ALL. *Sci. Adv.* **8**, eabj2820 (2022).
16. Majzner, R. G. et al. GD2-CAR T cell therapy for H3K27M-mutated diffuse midline gliomas. *Nature* **603**, 934–941 (2022).
17. Papalexi, E. & Satija, R. Single-cell RNA sequencing to explore immune cell heterogeneity. *Nat. Rev. Immunol.* **18**, 35–45 (2018).
18. Ding, J., Sharon, N. & Bar-Joseph, Z. Temporal modelling using single-cell transcriptomics. *Nat. Rev. Genet.* **23**, 355–368 (2022).
19. Kirschenbaum, D. et al. Time-resolved single-cell transcriptomics defines immune trajectories in glioblastoma. *Cell* **187**, 149–165.e23 (2024).
20. Genshaft, A. S. et al. Live cell tagging tracking and isolation for spatial transcriptomics using photoactivatable cell dyes. *Nat. Commun.* **12**, 4995 (2021).
21. Leun, A. Mvander et al. Single-cell analysis of regions of interest (SCARI) using a photosensitive tag. *Nat. Chem. Biol.* **17**, 1139–1147 (2021).
22. Hu, K. H. et al. ZipSeq: barcoding for real-time mapping of single cell transcriptomes. *Nat. Methods* **17**, 833–843 (2020).
23. Lane, K. et al. Measuring signaling and RNA-seq in the same cell links gene expression to dynamic patterns of NF-κB activation. *Cell Syst.* **4**, 458–469.e5 (2017).
24. Liu, Z. et al. Integrating single-cell RNA-seq and imaging with SCOPE-seq2. *Sci. Rep.* **10**, 19482 (2020).
25. Chen, W. et al. Live-seq enables temporal transcriptomic recording of single cells. *Nature* **608**, 733–740 (2022).
26. Wehling, A. et al. Combined single-cell tracking and omics improves blood stem cell fate regulator identification. *Blood* **140**, 1482–1495 (2022).
27. Ding, S. et al. Patient-derived micro-organospheres enable clinical precision oncology. *Cell Stem Cell* **29**, 905–917.e6 (2022).
28. Drost, J. & Clevers, H. Organoids in cancer research. *Nat. Rev. Cancer* **18**, 407–418 (2018).
29. García-Guerrero, E. et al. Selection of tumor-specific cytotoxic T lymphocytes in acute myeloid leukemia patients through the identification of T-cells capable to establish stable interactions with the leukemic cells: "doublet technology". *Front. Immunol.* **9**, 1971 (2018).
30. Haque, A., Engel, J., Teichmann, S. A. & Lönnberg, T. A practical guide to single-cell RNA-sequencing for biomedical research and clinical applications. *Genome Med.* **9**, 75 (2017).
31. Hernandez-Roca, M. et al. Singularity image for behavioral guided transcriptomics (BGT) pipeline (1.0). *Zenodo* https://zenodo.org/records/10953043 (2024).
32. Gründer, C. et al. γ9 and δ2CDR3 domains regulate functional avidity of T cells harboring γ9δ2TCRs. *Blood* **120**, 5153–5162 (2012).
33. Fujii, M., Matano, M., Nanki, K. & Sato, T. Efficient genetic engineering of human intestinal organoids using electroporation. *Nat. Protoc.* **10**, 1474–1485 (2015).
34. Broutier, L. et al. Culture and establishment of self-renewing human and mouse adult liver and pancreas 3D organoids and their genetic manipulation. *Nat. Protoc.* **11**, 1724–1743 (2016).
35. Drost, J. et al. Organoid culture systems for prostate epithelial and cancer tissue. *Nat. Protoc.* **11**, 347–358 (2016).
36. Marcu-Malina, V. et al. Redirecting αβT cells against cancer cells by transfer of a broadly tumor-reactive γδT-cell receptor. *Blood* **118**, 50–59 (2011).
37. Muraro, M. J. et al. A single-cell transcriptome atlas of the human pancreas. *Cell Syst.* **3**, 385–394.e3 (2016).
38. Brink, S. Cvanden et al. Single-cell sequencing reveals dissociation-induced gene expression in tissue subpopulations. *Nat. Methods* **14**, 935–936 (2017).
39. Hashimshony, T. et al. CEL-Seq2: sensitive highly-multiplexed single-cell RNA-seq. *Genome Biol.* **17**, 77 (2016).
40. Savas, P. et al. Single-cell profiling of breast cancer T cells reveals a tissue-resident memory subset associated with improved prognosis. *Nat. Med.* **24**, 986–993 (2018).
41. Azizi, E. et al. Single-cell map of diverse immune phenotypes in the breast tumor microenvironment. *Cell* **174**, 1293–1308.e36 (2018).

Acknowledgements

We thank the Princess Máxima Center for Pediatric Oncology for technical support and the Hubrecht Institute and Zeiss for imaging support and collaboration. All imaging was performed at the Princess Máxima Imaging Center. We thank the HUB for providing breast cancer PDOs and the Princess Máxima Center Organoid Facility for organoid culture support. We thank Single Cell Discoveries for their help with single-cell sequencing services. We thank D. van Vuurden (Princess Máxima Center for Pediatric Oncology) for providing DMG cultures, M. Koomen (Hubrecht Institute) for support with head and neck cancer PDO culture and members of the Dream3DLAB (Rios group) for offering critical feedback on the project. This work was financially supported by the Princess Máxima Center for pediatric oncology. M.A. and M.H.-R. were supported by the Comunidad de Madrid (2022-T1/BMD-24021). U.P. was supported by Horizon Europe/Marie Skłodowska-Curie Action cofund project number 101081481.

Author contributions

A.K.L.W. and J.F.D. performed scRNA-seq experiments. A.C., T.A.-R., P.H.-L., S.H. and F. Karaiskaki produced TEGs. F. Keramati and P.B. analyzed scRNA-seq data, and M.H.-R. further adapted the computational workflow. M.A. designed and performed the behavioral inference analysis, with assistance of A.A. U.P. further developed the workflow with assistance of S.d.B. M.B.R. made the video summary. Z.S. and J.K. provided the TEG cells and supervised work with these cells. M.A. and A.C.R. designed the study. A.K.L.W. and M.A. wrote the manuscript, with support from A.C.R., F. Keramati and U.P. All authors critically revised the manuscript.

Competing interests

A.C.R. and J.F.D. (along with H. Clevers) are named as inventors on (pending) patents related to the organoid technology. Z.S. and J.K. are inventors on different patents for γδ TCR sequences, recognition mechanisms and isolation strategies. J.K. is scientific cofounder and shareholder of Gadeta. The remaining authors declare no competing interests.

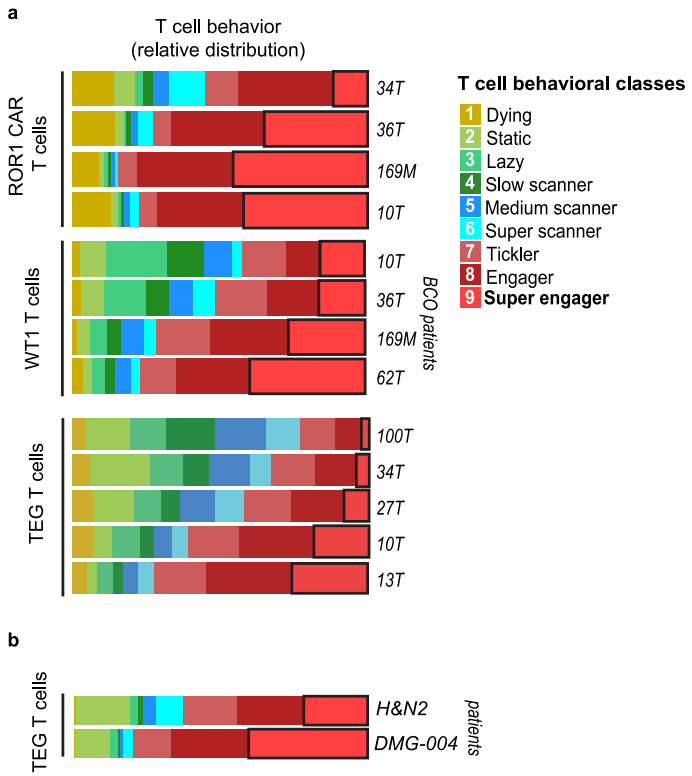
Additional information

Extended data is available for this paper at https://doi.org/10.1038/s41596-024-01126-4.

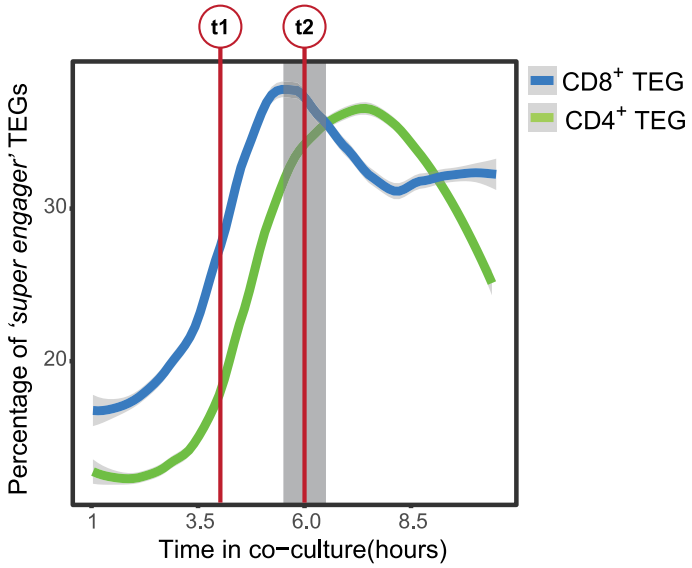
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41596-024-01126-4.

Correspondence and requests for materials should be addressed to M. Alieva or A. C. Rios.

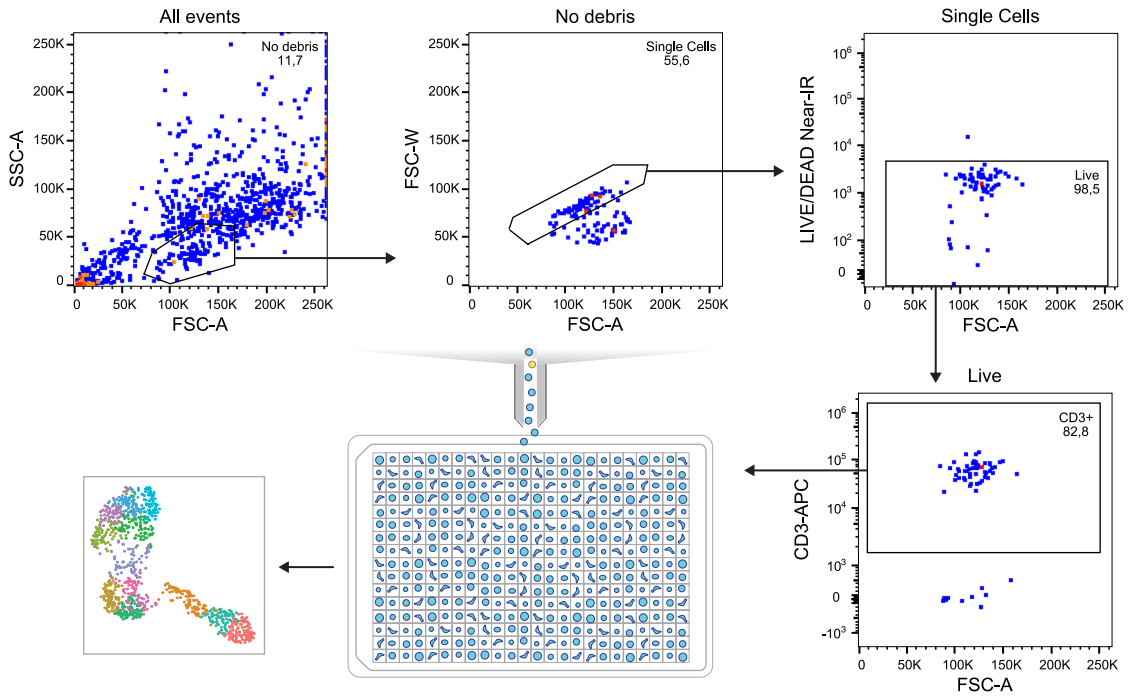
Peer review information *Nature Protocols* thanks Dirk Loeffler and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.



Extended Data Fig. 1 | *'Super engager'* behavior detected across T cell immunotherapy concepts and tumor cocultures. (**a**) Relative behavioral cluster distribution of different types of engineered T cells cocultured with breast cancer (BC) PDOs. (**b**) Relative behavioral cluster distribution of TEGs cocultured with PDOs derived from different tumor types. Differences in *'super engager'* population size are highlighted.



Extended Data Fig. 2 | TEG *'super engager'* behavior dynamics in coculture. Dynamic change of the percentage of TEGs cocultured with 13T PDOs exhibiting *'super engager'* behavior over time in coculture. Line color denotes CD4+ (green) or CD8+ (blue) TEGs. Grey area indicates the window with highest 'super engager' behavior. Red lines indicate optimal timepoint for population enrichment (t2 for 1-step enrichment and t1 and t2 for 2-step enrichment). Panel is adapted from ref. 2.



Extended Data Fig. 3 | FACS and scRNA-seq of population-enriched T cells. Schematic of the FACS and scRNA-seq procedure. Population-enriched T cells are FACSed according to the gating strategy shown into 384 well plates and subsequently sequenced.