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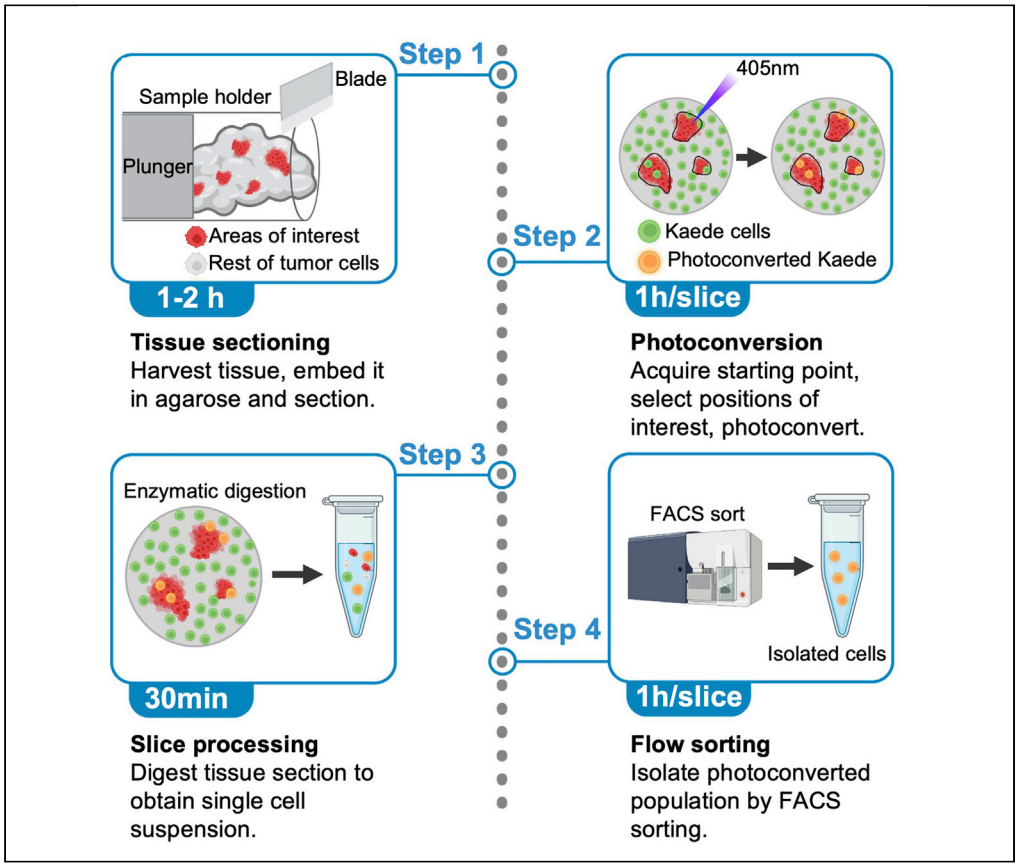
Protocol to isolate live single cells while retaining spatial information by combining cell photolabeling and FACS



Single-cell techniques have revolutionized biology; however, the required sample processing inherently implies the loss of spatial localization. Here, using an approach called photoconversion of areas to dissect micro-environments (PADME), we detail steps to isolate live single cells from a primary breast tumor while retaining spatial information by combining cell photolabeling and FACS (fluorescence-activated cell sorting). These live cells can be subsequently used for myriad techniques, from flow cytometry to single-cell RNA sequencing or other single cell "omics" approach.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Obtention of single-cell suspension for sc-omics keeping the original spatial resolution

Spatial transcriptomics technique using photoconvertible protein and tissue sectioning

Detailed protocol to obtain live tissue sections using a compresstome machine

Photoconversion of Kaede protein from tissue using a point scanner confocal microscope

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Protocol to isolate live single cells while retaining spatial information by combining cell photolabeling and FACS

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SUMMARY

Single-cell techniques have revolutionized biology; however, the required sample processing inherently implies the loss of spatial localization. Here, using an approach called photoconversion of areas to dissect micro-environments (PADME), we detail steps to isolate live single cells from a primary breast tumor while retaining spatial information by combining cell photolabeling and FACS (fluorescence-activated cell sorting). These live cells can be subsequently used for myriad techniques, from flow cytometry to single-cell RNA sequencing or other single cell "omics" approach.

For complete details on the use and execution of this protocol, please refer to Baldominos et al. (2022).

BEFORE YOU BEGIN

PADME is a versatile microscopy-based technique that enables the characterization of specific cell populations within a living tissue at the single cell level that retains information of their intra-tissue location. As an example, we developed this technique to study the transcriptional profile of cells that infiltrate specific niches within a tumor mass. This is achieved by localized photo-labeling of cells in a discrete region of interest that express a photoconvertible protein. In this specific tumor setting, the regions of interest can be defined as those enriched in a tumor sub-population with a distinctive phenotype. Visualization of such regions can be achieved, for example, by transducing tumor cells with fluorescent reporters that are uniquely expressed in the tumor sub-population of interest. Once the infiltrating cells are photolabeled by direct illumination using an optimal light source, they can be FACS-sorted and processed for scRNA-seq.

PADME can be used in a large variety of tissular contexts as long as there is a reporter or tracer to label the specific areas of interest to guide photoconversion. Furthermore, PADME *per se* can be combined with subsequent techniques such as flow cytometry, biochemical and metabolic

approaches, or other "omics" high-throughput analyses (metabolomics, epigenomics or transcriptomics to name a few).

Here we show isolation of specific intra-tumor infiltrating cells in murine primary breast cancer. Such infiltrating cells reside in micro-regions of abundant quiescent cancer cells (QCCs). In this experimental setting, tumor cells were engineered to express a reporter to visualize quiescent cells (i.e., a construct expressing tdTomato-p27K). Photo-labeled immune and stromal populations within regions enriched in QCCs could then be flow-sorted and used for scRNA-seq.

Institutional permissions

All animal procedures were approved by Dana-Farber Cancer Institute IACUC and the Harvard Medical School IACUC and performed according to DFCI protocol #17-017 and HMS protocol # IS00002540. The study is compliant with all relevant ethical regulations regarding animal research.

> *Note:* All experiments must be compliant with the specific regulations of animal care and receive the approval from the relevant institutional review board.

Mice

PADME requires the use of transgenic animals ubiquitously expressing a photoconvertible protein such as Kaede (Tomura et al., 2008), Kikume (KikGR (Nowotschin and Hadjantonakis, 2009)), Dendra2 (Miller et al., 2021), EosFP (Wacker et al., 2007) or PS-CFP2 (Nowotschin and Hadjantonakis, 2009). Alternatively, the use of photoactivatable proteins such as PA-GFP (Victora et al.,2010) is also feasible. However, photoconversion is advantageous since it allows for cell detection before and after photolabeling, while cells of interest are not readily visible before photoactivation. In our model, we make extensive use of Kaede (Ando et al., 2002), a fluorescent protein that emits in the green spectrum but shifts its emission to red upon exposure to violet light. We used 10-week females from an F1 cross of Kaede mice (C57BL/6 background) with Balb/c to avoid rejection of the tumor cell line models we used. Balb/c mice are commercially available, and we obtained them from Jackson laboratories. Kaede mice were obtained from Dr. Michio Tomura upon MTA approval.

Cell lines

4T07 cells were a gift from Dr. Robert Weinberg and were cultured in Iscove's Modified Dulbecco's Medium (IMDM), 10% heat inactivated FBS (Gibco), 1% Penicillin-Streptomycin (Gibco) and 1% Glutamax (Gibco). Protocol was optimized to work with cellular reporters to label regions of interest inside the tumor but it can be easily modified to use antibody staining among other experimental designs.

> ⚠ CRITICAL: Take into account excitation, emission, and photoconversion wavelengths needed in your specific mouse model when adapting the experimental design.

Cells were engineered to express a modified quiescent reporter from Dr. Kitamura (Oki et al., 2014), tdTomato-p27k using lentiviral system. Although tdTomato and photoconverted Kaede fluorescent emissions overlap, they are expressed in different cell types. Kaede-expressing cells are either green (non photoconverted) or double-positive (green, red - photoconverted), thus cannot be confused with tdTomato-p27k single positive tumor cells.

> ⚠ CRITICAL: Transduced cells were selected by FACS sorting at confluency to maximize expression of the quiescent reporter.

Tumor injection

> Timing: 2 weeks

Tumors were induced by intra-mammary fat pad injection.

1. Prepare cell suspension for injection.
   a. Obtain a single-cell suspension of the cells in culture following standard cell culture passaging technique.
   b. Wash cells 2× in sterile PBS.
   c. Count cells and prepare 250,000 cells in 50 μL sterile PBS per mouse.
2. Prepare mice for surgery.
   a. Anesthetize mice with 4% isoflurane in an induction chamber.
   b. Shave hair around the 4th nipple.
   c. Apply eye ointment to prevent corneal drying.
   d. Place the mouse in the nose cone to maintain the delivery of 2% isofluorane. Assess the depth of anesthesia by pinching the mouse hind paw with tweezers to check for absence of pedal reflex.
   e. Clean the area with 70% ethanol wipes and iodine to keep aseptic the area.
3. Mammary fat pad injection.
   a. Make a 2 mm incision next to the nipple area.
   b. Localize mammary gland.
   c. Inject 50 μL of the cell suspension into the mammary fat pad.

   ⚠ CRITICAL: Keep needle parallel to the skin to avoid injecting in the intraperitoneal cavity.

   d. Drop a couple of drops of lidocaine for topical analgesia in the incision.
   e. Close the incision using tissue glue (VetBond®).

Place the chilling block on the freezer

Timing: 1 h

During the embedding process of the tumor, we will use a chilling block (provided with the compresstome) to cool and solidify agarose faster, minimizing the waiting time and reducing heat damage to the tissue.

4. Place the chilling block at -20 for at least 1 h before use to let it cool down.

Prepare 12 well plate

Timing: 10 min

5. Fill all wells from a 12 well plate with 1 mL of non-supplemented IMDM medium to store the tumor and the slices obtained during the process.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Iscove's Modified Dulbecco's Medium (IMDM)	Thermo Fisher Scientific	Cat#31980030
USDA FBS	Life Technologies	Cat#10437028
Penicillin-Streptomycin	Life Technologies	Cat#15140122
Glutamax	Life Technologies	Cat#35050061
Isofluorane	Pivetal	Cat# 21295097

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Puralube Vet ointment	Dechra	Cat#17033-211-38
70% Ethanol wipes	Fisherbrand	Cat#22-363-750
Lidocaine HCL Injectable 2%	DFCI Animal Research Facility Pharmacy	N/A
EZ pack AgaroseLE	Benchmark	Cat#A2501
Hyaluronidase	STEMCELL Technologies	Cat#07461
Collagenase IV	STEMCELL Technologies	Cat#17104019
DNaseI	Sigma-Aldrich	Cat#10104159001
RBC lysis buffer	BioLegend	Cat#420301
BSA	Cell Signaling Technology	Cat#9998S
DAPI	BioLegend	Cat#422801
UltraPure 0.5 M EDTA, pH 8.0	Life Technologies	Cat#15575020
PBS pH 7.4 (1×)	Gibco	Cat#10010-023
HBSS (1×)	Gibco	Cat#14025-092
Super Glue	Gorilla Glue®	N/A
Acetone	Fisher Chemical	Cat#A18-500
Tissue Glue - VetBond®	VetBond®	N/A
Critical commercial assays		
APC Annexin V apoptosis detection kit with PI	BioLegend	Cat#640932
Experimental models: Cell lines		
4T07	Dr. Robert Weinberg, MIT	N/A
Experimental models: Organisms/strains		
Mouse: Balb/c:BALB/cJ (8–10 week, females)	The Jackson Laboratory	Strain#:000651
Mouse:Kaede:Kaede (8 weeks, male)	Dr. Michio Tomura, Osaka Ohtani Univeristy	N/A
Mouse: Kaede-Balb/c F1 (8–10 weeks, females)	N/A	N/A
Recombinant DNA		
3rd Generation Lentiviral packaging vectors	Dr. Brian Brown, Mount Sinai	N/A
mVenus-p27k reporter	Dr. Toshio Kitamura, University of Tokyo	N/A
H2B-tdTomato	Addgene	Cat#58101
tdTomato-p27k reporter	N/A	N/A
Software and algorithms		
FlowJo 10	BD	https://www.flowjo.com
FiJi® (v2.0.0-rc-69/1.52i)	ImageJ	https://imagej.net/software/fiji/
Biorender	BioRender	https://biorender.com/
Other		
Compresstome®	Precisionary	VF-310-0Z
Tissue path superfrost Plus Gold microscope slides	Fisherbrand	Cat#15-188-48
Microscope cover glass #1.5	Fisherbrand	Cat#12544D
Microscope: Zeiss LSM710 point scanner	ZEISS	N/A
FACS: AriaIII	BD	N/A

MATERIALS AND EQUIPMENT

Compresstome

The VF-310-0Z compresstome from Precisionary Instruments LLC was used to obtained live tissue sections.

Point scanner confocal microscope

A Zeiss LSM710 single photon point-scanning confocal unit with galvanometer mirrors attached to a fully motorized Axio-Observer Z1 inverted microscope equipped with a Zeiss motorized stage and a Zeiss Plan Apochromat 20×/0.8 NA DIC air objective was used. A 405 nm 30 mW diode AOTF

modulated line was used for photoconversion. An argon multi-line 488 nm 25 mW Argon AOTF modulated line and a 561 nm 20 mW DPSS AOTF modulated line were used for image acquisition. The emission wavelength range for the green and red fluorescence signals was set to 499–560 nm and 571–735, respectively, and collected by multi-alkali PMTs.

⚠ CRITICAL: Imaging and photoconversion require the use of laser lines that are hazardous for eyes and skin irradiation. Avoid exposure to laser beams and use eye protection when required.

Alternatives: Any single point-scanning confocal microscope or spinning disk confocal microscope equipped with a DMD (Digital Mirror Device) endowed with the capability of drawing irregular polygonal regions of interest (ROI) can be used for this photoconversion-based method. If the microscope software only allows the use of standard ROIs (squared, rectangular, circular or ellipsoid regions), the user will not be able to accurately photoconvert individual cell clusters ("niches") with irregular shapes. This point might be critical or not depending on the scientific question to be addressed. If "irregular" illumination is not required, alternative equipments such as a widefield microscope can be used to image and photoconvert areas of interest.

STEP-BY-STEP METHOD DETAILS

Tumor sectioning

Timing: 1–2 h

After tumor growth and harvest, tumors were processed with a compresstome in order to obtain live tumor slices whose infiltrates can be photoconverted using a point scanner confocal microscope. The use of slices directly exposes deep tumoral areas, avoiding the need of a multiphoton microscope for photoconversion across the whole tumor. Besides, the use of slices also allows for the enrichment of the population/s of interest, increasing recovery yield in the subsequent flow-sorting step.

1. Collect tumor.
   a. Harvest the tumor with the help of scissors and a blade to maintain tissue integrity.
   b. Trim the surrounding fat tissue.
   c. Place the tumor in one well of the 12-well cell culture plate with medium prepared before starting.

   Note: When possible, protect tumor from light to avoid photobleaching or undesired photoconversion.

2. Embed tissue in low melting agarose.
   a. Mounting tumor into the specimen tube.
      i. Cut with a blade the edge of the tumor to generate a flat surface.
      ii. Place one drop of Gorilla Glue® into the tissue holder of the specimen tube.
      iii. Glue the flat surface of the tumor to the tissue holder (Figure 1A).

   ⚠ CRITICAL: Make sure the tissue is located in the center to obtain a block with homogenous agarose thickness around the tissue when embedding.

   b. Prepare the agarose solution.
      i. Dissolve 0.5 g of agarose in 25 mL of PBS to get a 2% agarose solution.

   ⚠ CRITICAL: Agarose concentration in this protocol is optimized for our model of mammary tumors. It is crucial that the agarose density is similar to that of your tissue to obtain clean slices.

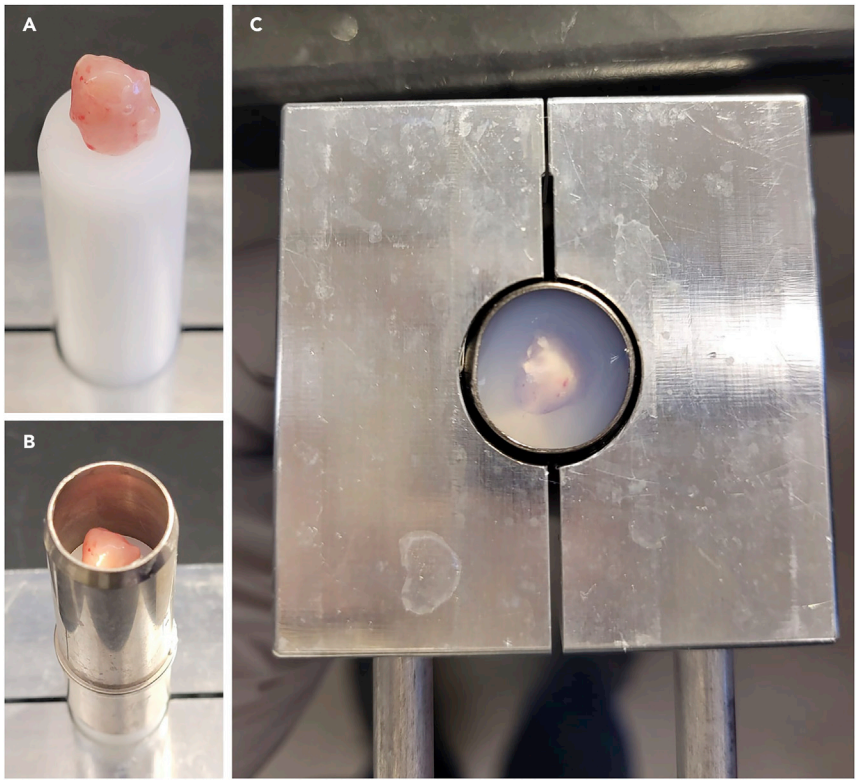

Figure 1. Mounting tumor into specimen tube

(A) Glue tissue to tissue holder (white plunger).

(B) Assemble tissue holder with the metallic tube to create embedding cavity.

(C) Place sample holder into the chilling block to solidify the agarose faster.

ii. Heat the solution in a microwave until the solution gets clear (20–30″ aprox).

iii. Cool down the solution by washing the flask under cold running water to reach 37C.

iv. Use right away to prevent undesired polymerization prior to tissue embedding.

Note: The flask can be kept in a water bath at 37C to avoid solidification.

c. Embed tissue with agarose.

i. Assemble the specimen tube by introducing the sample holder (white plunger) into the metal tube (Figure 1B).

ii. Cut the tip of a 1,000 μL pipette tip to facilitate the pipetting of a dense substance such as agarose.

iii. Pour agarose around the tissue into the sample holder.

⚠ CRITICAL: Avoid bubble formation during this process. Additionally, when embedding the tissue in agarose, be sure to leave approximately 0.2–0.5 cm between the sample and the edge of the tube to set up compresstome settings with the free agarose before reaching the sample.

iv. Introduce the specimen tube into the chilling block until agarose solidifies (Figure 1C).

3. Slice samples with the compresstome.

a. Assemble the sample holder into the compresstome following manufacturer instructions (compresstome VF-310-0Z user manual).

⚠ CRITICAL: Make sure the blade is placed in the correct position and the buffer tray is secured in place with the screw tightly attached. (Figures 2A and 2B).

b. Fill buffer tray with PBS until it covers half of the sample (specimen tube).
c. Sectioning of the tissue.
   i. Push the tissue advancement plunger until the agarose is seen out of the tube.
   ii. Set machine in single cutting mode, speed 1–3 and oscillation 5–6. Set slice thickness to 300 μm.

Note: Settings are optimized for the breast tumors we normally process, but they need to be adjusted for each specific tissue and also throughout the slicing process, as the tumor surface increases and becomes less cohesive. See problem 1 in troubleshooting section for more details.

   iii. Slice through the agarose using the manual fast forward to trim the tissue-free agarose gel using thicker slicing until the tissue appears.

Note: The speed of cutting can also be increased during the trimming.

Note: Do not cut slices thicker than ~2 mm to obtain slices with even surfaces.

Note: During trimming, you can set continuous mode and a higher slice thickness. However, make sure you start reducing the slice thickness gradually before reaching the tissue, since the equipment does not readjust the thickness immediately (it might take a couple of slices to get the desired slice thickness).

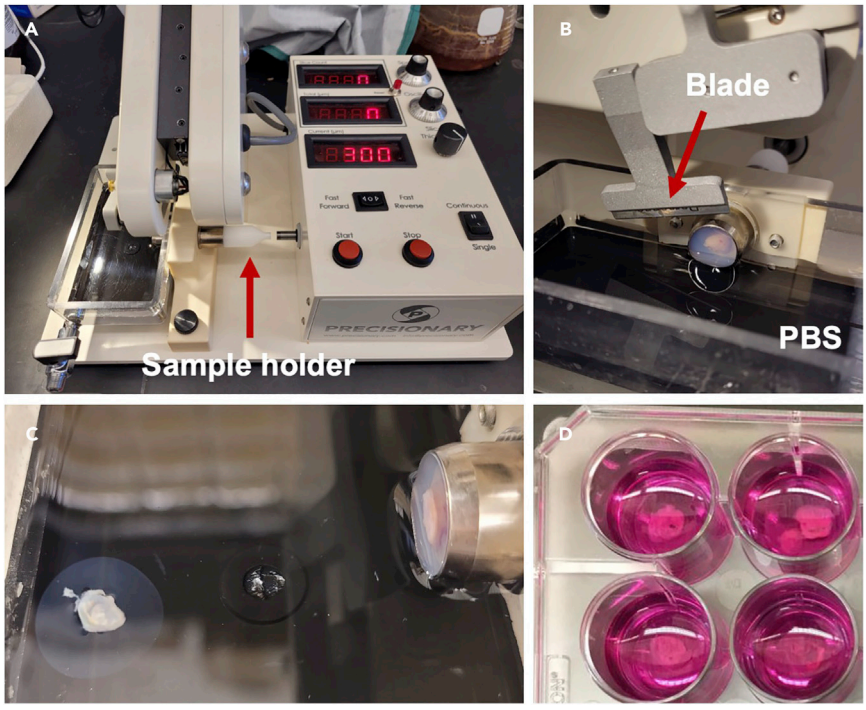

Figure 2. Tissue sectioning with compresstome
(A and B) Compresstome assembly.
(C) Successful floating tissue section.
(D) Sections stored in non-supplemented medium to keep track of sequential slices after sectioning until microscope processing.

iv. Once the tissue is visible, change to single mode and keep adjusting the settings to the tissue needs (Figure 2C).

Note: Generally a speed 1–3 and oscillation 5–6 is suitable for most tissues.

v. Place sequential slices in pairs in one well of the 12-well plate with medium to store them until image acquisition. (Figure 2D).

Note: Keep the 12-well plate on ice and covered from light. Slices should be protected from light to avoid photobleaching or undesired photoconversion.

vi. After finished slicing the tumor, make sure to clean the buffer tray, the blade and the sample holder. Remove the gorilla glue with acetone if needed.

Photoconversion in the microscope

Timing: 1–1.5 h/slice

Live tissue slices are mounted on a slide and photoconverted using a point-scanning confocal microscope that can perform selective illumination of irregular polygonal ROIs to maximize the spatial resolution of the areas of interest.

4. Mount tumor slice on a microscope slide:
   a. Place a few drops of medium on the slide before mounting the slice to facilitate straightening the tissue.
   b. Set the slice on the slide and carefully extend it using tweezers, without inflicting damage to the tissue.
   c. Add a moisturizing agent such as lubricant eye gel to decrease evaporation and maintain/keep slices hydrated during the whole imaging process.

   Note: The use of lubricant instead of alternative physiological aqueous buffers helps avoiding desiccation. Lubricant eye gel might possess slightly different refractive index as compared to water-based buffers impacting in the quality of imaging. However, the aim of this imaging step is the specific photoconversion of cellular niches and not the generation of high-resolution images. As the tissue needs further processing to obtain a viable single-cell suspension, we prioritize the wellbeing of the tissue over the quality of imaging at this step.

   d. Place a coverslip and seal the edges using paraffin or nail polish to fix the sample.

   Note: Use nail polish with caution since it could be toxic for the sample in case of undesired contact.

5. Scan the whole slice by tiling fields of view (FOV) to generate a full-size image of the tissue that helps to identify regions of interest:
   a. Acquire a tile scan using fast scan rate (short pixel dwell time) and low resolution to generate a full-size image of the whole slice:
      i. Collect green (non-photoconverted Kaede) and red (from tdTomato-p27k reporter in quiescent cancer cells in our experimental model) fluorescence by using a Zeiss Plan Apochromat 20×/0.8 NA DIC air objective.

   Note: The acquisition settings used in this experimental setting were as follows: the sample was illuminated with an argon multi-line 488 nm 25 mW Argon AOTF modulated line set to

0.2% transmittance and a 561 nm 20 mW DPSS AOTF modulated line set to 0.2% transmittance, using a multi-bandpass dichroic mirror with 488/561 cut off wavelengths (MBS 488/561). The emission wavelength ranges for the green and red fluorescence signals were set to 499–560 nm and 571–735, respectively, and collected using multi-alkali PMTs. No offset was applied. The microscope was controlled by ZEN Black SP2 acquisition software, scanned unidirectionally with a pixel dwell time of 1.58 μs/pixel, no averaging, a 12-bit digitizer, zoom 1.0×, a pixel size of 0.83 μm and pinhole set to 1 A.U. for 488 nm wavelength. Images were saved with the .czi file format.

Note: This acquisition settings were optimized for Kaede and tdTomato-p27k reporter, they will need modifications for the use of alternative photoconvertible/photoactivatable proteins as well as different reporters.

b. Once a mesoscopic image of the tissue is obtained, mark positions of interest per slice.

Note: In our experimental model, positions were placed in tdTomato-p27k^{High} areas in the first slice and in tdTomato-p27k^{Negative} areas in the consecutive paired slice (Figure 3A).

6. Photoconversion of the identified regions of interest:
   a. Acquire a single confocal image (snapshot) for each position marked in the slice using same microscope settings as in 5.a.i., except for the pinhole aperture that should be set to max.

Note: In confocal microscopy, fluorophore excitation is not restricted to the focal plane.

⚠ CRITICAL: Opening the pinhole allows to recover also the out of focus signal above and below the focal plane. This is critical to assess that the volume to be photoconverted only contains cells of interest (either tdTomato-p27k^{High} or tdTomato-p27k^{Negative} clusters).

   b. Draw a defined region of interest (ROI) in the snapshot using the freehand shape tool of the microscope (Figures 3B and 3C).

Note: If the aim is to compare the approximate number of infiltrating cells or the relative abundance of a cell type among different conditions, equivalent areas should be photoconverted. For this purpose, a ROI can be initially defined and saved, then applied to every position of interest throughout the set of slices from the same experiment.

   c. Photoconvert the Kaede protein specifically within the ROI.

Note: Photoconversion of Kaede in this experimental setting was performed using a Zeiss Plan Apochromat 20×/0.8 NA DIC air objective, a 405 nm 30 mW diode AOTF modulated line set to 0.2% transmittance laser, and a 405 long-pass dichroic mirror. A time series of 325 cycles at a 1.58 μs/pixel (pixel dwell time) was used during photoconversion. This settings might need to be modified for other photoconvertible/photoactivatable proteins.

   d. Assess photoconversion by a single confocal micrograph with dual green-red fluorescence.

Note: Acquisition settings in this case were acquired using the settings from 5.a.i. (Figure 3C). They might need to get modified accordingly to the experimental setting used.

Note: If not successful, check problem 2 in troubleshooting section.

7. Scan the whole slice to check the overall photoconversion using same setting as 5.a.i. (Figure 3B).

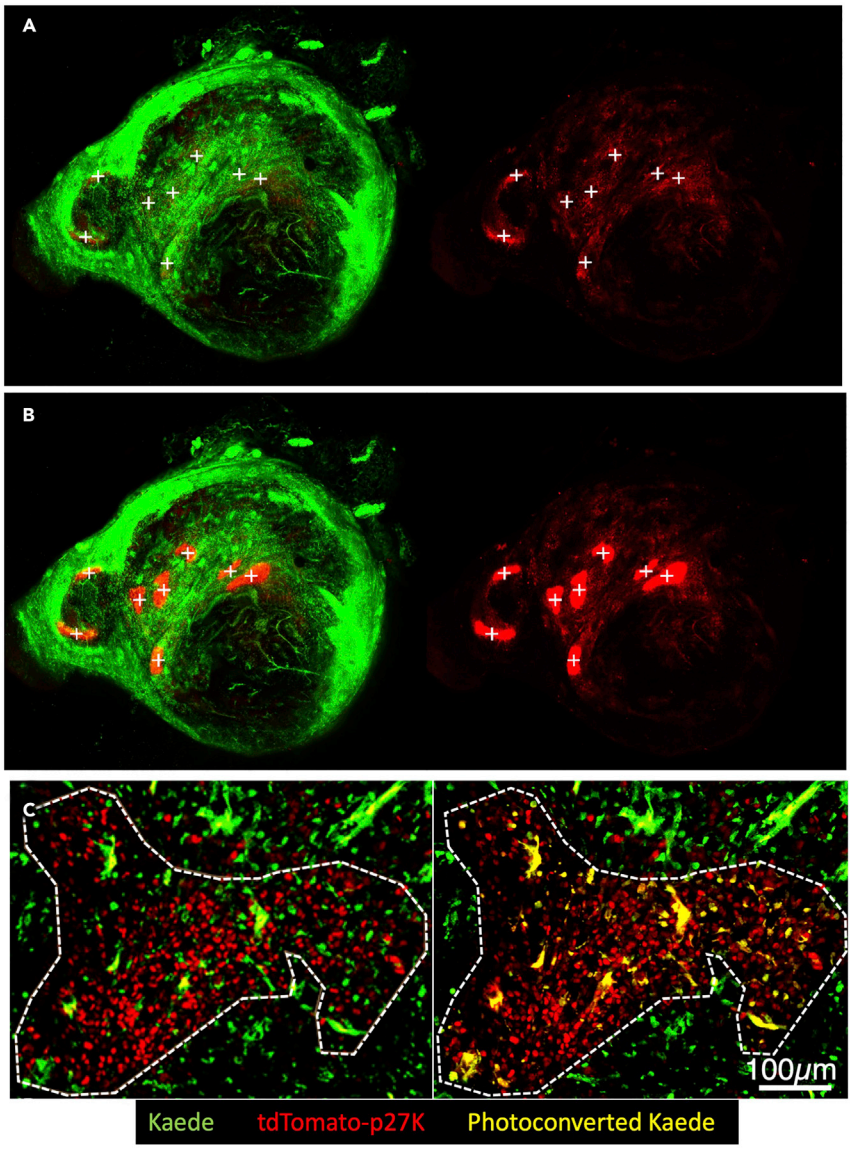

Figure 3. Photoconversion of Kaede protein in tumor slices

(A) Full-size image of a tumor slice showing the distribution of the expression of the non-photoconverted Kaede protein in host cells (green channel) and the expression of the quiescence reporter in specific tumor cells (red channel). The data from the red channel was used to select positions of interest (white crosses) based on the tdTomato-p27k reporter (red).

(B) Full-size image of the same tumor slice acquired after photoconversion to corroborate successful photoconversion in all selected positions. Enhanced red fluorescence corresponds to photoconverted areas.

(C) Representative region of interest drawn based on tdTomato-p27k reporter expression (white dash-dotted line) before and after photoconversion (left and right, respectively). Overlay of green and red channels is shown. Green: non-photoconverted Kaede-expressing cells, red: tdTomato-p27k^{+} tumor cells, yellow: photoconverted Kaede-expressing cells. Scale bar (white) is 100μm. This panel was originally published in *Cell* (Baldominos et al., 2022).

Slice processing and flow sorting populations

Timing: 30–45 min processing + 1 h sorting per slice

Photoconverted slices are digested to obtain single-cell suspensions that are flow-sorted to isolate individual photoconverted cells from the specific tumor region of interest.

8. Tissue digestion to obtain a single-cell suspension:
   a. Prepare digestion buffer as follows:

Digestion buffer	
Reagent	Final concentration
Hyaluronidase	6.8 U/mL
Collagenase IV	400 U/mL
DNaseI	20 μg/mL
FBS	10%
HBSS	Up to 500 μL/slice

   b. Digest slices:
      i. Place 500 μL of the digestion buffer in a 12-well cell culture plate to digest each slice separately.
      ii. Put tissue slice in the solution and disrupt tissue with two needles.
      iii. Incubate for 15–20 min at 37C.
      iv. Pipette up and down with a 1,000 μL pipette to further disrupt tissue.
      v. Filter digestion with a 100 μm (pore size) filter, washing the well and the filter with flow buffer (2 mM EDTA, 0.1% BSA in PBS).
      vi. Spin down at 800 g for 5 min.
   c. Prepare a single-cell suspension for sorting:
      i. Resuspend in 500 μL of red blood cell lysis buffer (BioLegend) and incubate for 5 min at RT.
      ii. Wash with flow buffer.
      iii. Spin down at 800 g for 5 min.

Optional: Stain with antibodies to analyze specific cell populations, such as exhaustion markers in our particular case (Baldominos et al., 2022).

⚠ CRITICAL: As quality control, it is recommended to stain a single-cell suspension from one slice that has gone through the whole procedure, combining an apoptosis marker such as AnnexinV and a viability dye (DAPI, BioLegend). Viable cells will be negative for both markers. The percentage of cell viability in photoconverted samples should be comparable to non-photoconverted ones.

Note: We did not experience major phototoxicity issues with the conditions used for photoconversion.

      iv. Resuspend in flow buffer + DAPI (dilution 1:10,000 from stock, BioLegend).

9. Sort photoconverted population:
   a. Set gates using a single-cell suspension from a non-photoconverted slice (negative control) and another from a photoconverted slice with a big photoconverted area as a positive control (Figure 4A).
   b. Check cell viability using the single-cell suspension stained with AnnexinV and DAPI, as a quality control before proceeding further with the protocol (Figure 4B).

Note: If cell viability is low, check problem 3 in troubleshooting section.

   c. Sort enough cells to comply with the requirements of your subsequent technique.

Note: In our experimental model, the target amount for scRNA-sequencing using 10× genomics platform was 10,000 cells per condition (See problem 4 in troubleshooting section).

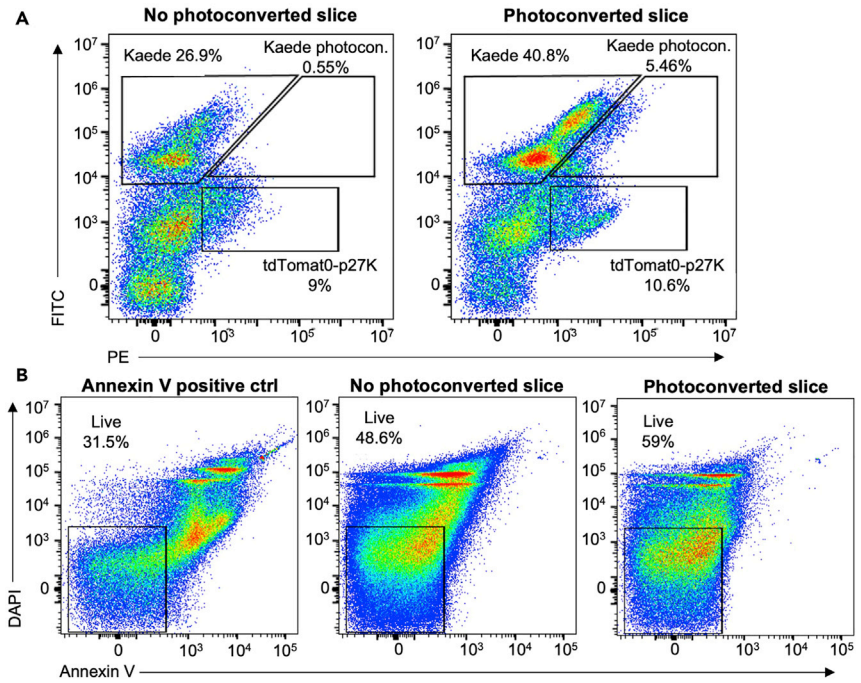

Figure 4. Representative FACS plots of photoconverted infiltrates

(A) Set up gate strategy using the DAPI negative cell population gated from a non-photoconverted sample (left) and from a photoconverted one (right).

(B) Assessment of sample viability after photoconversion using viability dye (DAPI) and annexinV staining (to control for apoptosis).

Note: Sorting in serum-supplemented cell culture medium increases cell viability but requires extra washing steps that might reduce the cell recovery yield.

⚠ CRITICAL: If sorting in serum-supplemented culture medium, use 1.5 mL Eppendorf tubes and a swing centrifuge to minimize cell loss during repeated washes.

d. Samples are ready for standard single-cell RNA-sequencing (such as 10× genomics, the procedure used for our experimental model) or any other analysis dependent on the scientific question to be addressed.

Note: If comparing two different regions inside of a tumor, it is recommended to use hashtags to eliminate technical variability when sequencing paired samples.

EXPECTED OUTCOMES

After performing this protocol, infiltrates in the areas of interest inside of a tumor will be labeled by photoconversion. This protocol will allow to flow-sort single-cell populations from a known spatial location, enabling scRNA-Seq with detailed spatial resolution (Figure 4).

QUANTIFICATION AND STATISTICAL ANALYSIS

Photoconverted infiltrates from different areas were submitted to the Brigham and Women's Hospital Single-Cell Genomics Core for 10× genomics sequencing and analyzed following well-established pipelines in Seurat package (Hao et al., 2021; Stuart et al., 2019; Butler et al., 2018; Satija el at., 2015), as explained in Baldominos et al. (2022).

LIMITATIONS

Overall time from photoconversion to cell recovery is critical for cell viability. For that reason, we limit photoconversion to a maximum of 10 different regions per tissue section, with an area < 50% of the FOV/region. All the microclusters in the tissue section are not photoconverted, but this is not a problem, since we only analyzed photoconverted cells (being 10 regions/slice optimal to obtain enough cell recovery yield with acceptable viability).

PADME might not be the most accurate or quantitative technique to fairly compare, e.g., number of infiltrating cells or the relative abundance of a cell type among different conditions, since photodamage and tissue processing might affect differently specific cell populations.

The consistency of the tissue to be analyzed might not allow the use of tissue sections. This scenario might be overcome by using fresh tissue (such as a whole tumor) and a multiphoton microscope for the photoconversion step. Take into account that light penetrance will continue to be a limiting factor, even using this equipment that allows for deep tissue imaging.

Processing of the tissue (e.g., agarose embedding, sectioning) might partially affect the metabolic or activation status of the cells of interest, inducing subtle changes in their transcriptional profiles, as compared to fresh tissue.

TROUBLESHOOTING

Problem 1

Slices are not cut evenly in thickness or tissue gets disrupted during the cutting process.

During tissue slicing, it is necessary to have the same density across the tissue surface to maintain fix settings. However, tumors are not homogeneous, and the core has usually necrotic areas, that dramatically change the tissue consistency affecting the integrity of the slices.

Potential solution

Adjust speed and oscillation as you cut through the tissue.

The speed of the blade can be adjusted during the cutting process. You can lower the speed when the blade is approaching difficult areas (usually the center of the tumor) to improve cutting. You can also increase or decrease the oscillation to help with tissue integrity. Higher oscillation will improve the cutting, but if the tissue consistency is very soft tissue integrity can be compromised. It is necessary to balance both settings to get an even and consistent tissue slice.

If problems persist, multiphoton microscopy could be used to penetrate deeper in the tissue and allow for accurate photoconversion of thicker slices (easier to generate) or even of a whole-mount tissue preparation. However, fluorophore excitation in a multiphoton microscope is restricted to the focal plane, as opposed to confocal microscopy. This dramatically reduces the amount of photoconverted cells obtained per cycle. Therefore, using this type of equipment increases the overall microscopy time due to the need of photoconversion in several focal planes across the sample, which can be a limiting factor depending on the downstream application.

Problem 2

Suboptimal photoconversion.

Potential solution

If photoconversion is not optimal, you will need to change your microscopy settings by increasing time of fluorescence excitation or laser power, but always being aware of the concomitant increase

in phototoxicity. We recommend increasing the number of cycles of fluorescence excitation, instead of the continuous illumination of the sample.

Problem 3

High phototoxicity and low viability observed in the combined annexin V and viability staining.

The photoconversion process requires violet light exposure of live tissues that can be cytotoxic and induce cell death. Using Annexin V combined with a viability dye as quality control helps to define the microscope settings that are optimal to minimize cell damage.

Potential solution

Adjust microscope settings for photoconversion to reduce photodamage.

Kaede protein is photoconverted from green to red with ultraviolet light. However, increasing the wavelength to the violet spectrum reduces phototoxicity, since a higher lambda radiation carries less energy and will improve the overall quality of the sample. Prolonged exposure also increases the temperature of the sample, contributing to tissue damage. In order to minimize these issues, the laser scanning dwelling time can be reduced. Hence, scanning faster but performing several cycles of laser exposure using the time series setting allows to achieve an optimal photoconversion with less harmful conditions. Another plausible solution to overcome phototoxicity is the use of multiphoton microscopy that relies on infrared wavelengths with lower frequency, lower energy level and higher tissue penetrance than those in the violet spectrum. However, multiphoton technology might not be broadly available as compared to the widespread use of conventional single-photon excitation confocal laser scanning microscopes. Alternatively, the use of a DMD (Digital Mirror Device) or similar spatial light modulators that use LED-based illumination, therefore producing lower irradiation than a point scanning confocal microscope, may be used to reduce phototoxicity.

Problem 4

Poor recovery yield after cell sorting.

If the viability of the samples is acceptable but the cell recovery yield after cell sorting is insufficient to run a scRNA-Seq experiment or another alternative downstream analysis, the number of photoconverted areas should be increased.

Potential solution

Increase the number of slices to be pooled in a single-cell sequencing experiment instead of increasing the amount of photoconverted ROIs/sample will better preserve the cell viability, while increasing the cell recovery yield.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Judith Agudo (judith_agudo@dfci.harvard.edu).

Materials availability

Plasmids and cell lines newly generated in this work are available upon request to the lead contact.

Data and code availability

This paper does not report original sequencing data. Original microscopy images will be shared by the lead contact upon request. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contacts upon request.

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AUTHOR CONTRIBUTIONS

P.B. and O.B. designed and performed experiments, analyzed data, and wrote the manuscript. P.M.L. provided microscopy support and wrote the manuscript. U.H.v-A. and R.S. discussed results and manuscript. J.A. designed and supervised the research and wrote the manuscript.

DECLARATION OF INTERESTS

P.B., O.B., R.S., P.M.L., U.H.v-A., and J.A. have no conflicts of interest to report.

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