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Coupling live-cell imaging and in situ isolation of the same single cell to profile the transient states of predicted drug-tolerant cells

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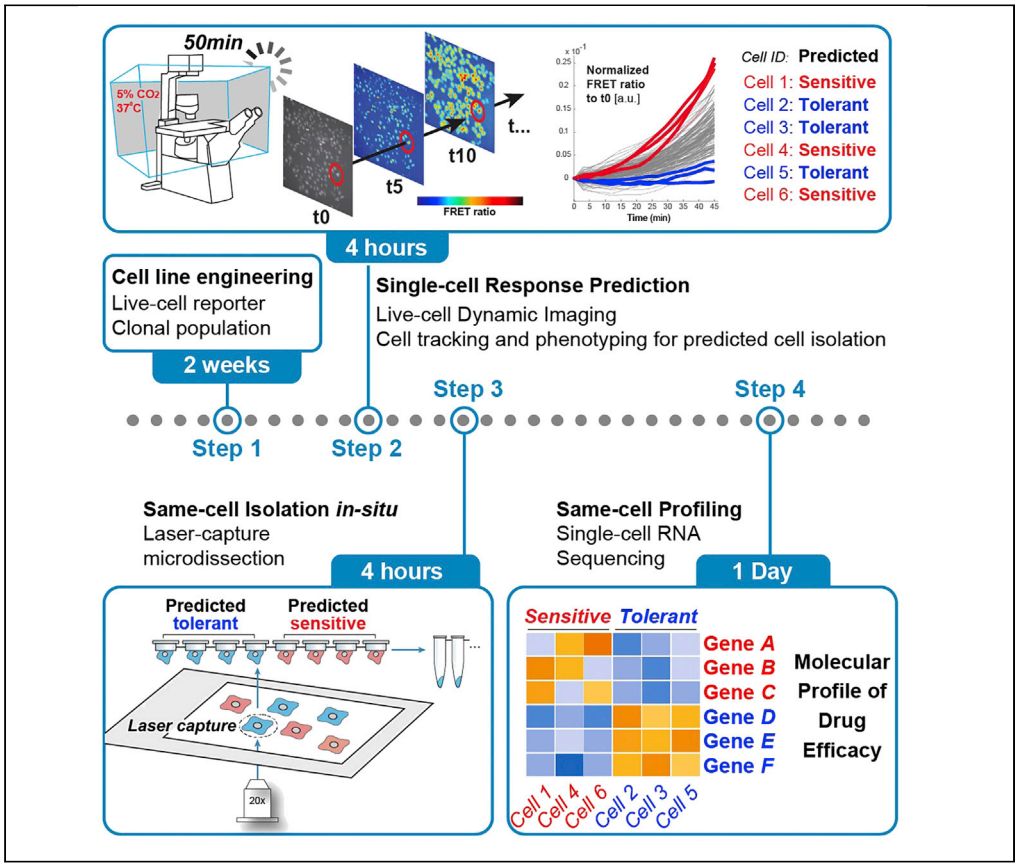
Coupling live-cell imaging and *in situ* isolation of the same single cell to profile the transient states of predicted drug-tolerant cells



Cell response variability is a starting point in cancer drug resistance that has been difficult to analyze because the tolerant cell states are short lived. Here, we present fate-seq, an approach to isolate single cells in their transient states of drug sensitivity or tolerance before profiling. The drug response is predicted in live cells, which are laser-captured by microdissection before any drug-induced change can alter their states. This framework enables the identification of the cell-state signatures causing differential cell decisions upon treatment.

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

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Highlights

Fate-seq provides gene sets of transient cell states

Fate-seq can profile the differential cell fates upon therapeutic treatment

It produces a molecular signature of cell sensitivity at a given drug dose

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Coupling live-cell imaging and *in situ* isolation of the same single cell to profile the transient states of predicted drug-tolerant cells

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SUMMARY

Cell response variability is a starting point in cancer drug resistance that has been difficult to analyze because the tolerant cell states are short lived. Here, we present fate-seq, an approach to isolate single cells in their transient states of drug sensitivity or tolerance before profiling. The drug response is predicted in live cells, which are laser-captured by microdissection before any drug-induced change can alter their states. This framework enables the identification of the cell-state signatures causing differential cell decisions upon treatment.
For complete details on the use and execution of this protocol, please refer to Meyer et al. (2020).

BEFORE YOU BEGIN

In this protocol, we focused on HeLa cells stably expressing the FRET-based Initiator Caspase Reporter Protein (IC-RP,(Albeck et al., 2008)) by viral transduction and FACS selection. This biosensor is composed of Förster resonance energy transfer (FRET) donor-acceptor pair (eCFP -enhanced cyan fluorescent protein, and Venus -yellow fluorescent protein) connected together by an IETD peptide linker that is efficiently cleaved by caspase-8 (Luo et al., 2003). Upon activation of Caspase 8, the linker is cleaved, energy transfer is lost and the eCFP signal increases overtime, while the Venus signal can be used for normalization. However, we also studied the caspase 8 activation dynamics in other cell types such as lung carcinoma cells A549 and colon carcinoma cells HCT116. The cell culture media designated in the following protocol must therefore be adjusted to the cell type of interest.

First, the cells were stably infected with a retrovirus and sorted by FACS for their higher IC-RP expression (of both fluorescent proteins of the FRET pair). They were finally sub-cloned to get a homogeneous expression of the fluorescent reporter IC-RP as described below.

Retrovirus production and infection

Timing: 6 days

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Note: To produce recombinant retroviruses for target cell infections, retroviral transfer vectors must be transfected into retroviral packaging cells that provide the gag and pol genes from the Moloney murine leukemia virus (MMLV) as stated in Takara's infection protocol (http://www.takara.co.kr/file/manual/pdf/632508.pdf). We used here the Retro-X Universal Packaging System featuring the GP2-293 packaging cell line (derived from HEK-293 cells). All envelopes are supplied on separate vectors that are co-transfected with the transfer vector. (Envelope choices include VSV-G, eco, ampho, or 10A1 to allow the selection of the tropism that is most appropriate for the target cells of interest.) We used in this study the pCMV-VSV-G envelope coding vector.

1. Day 1: Seed five million GP2-293 RetroX cells in 10 mL of complete DMEM (supplemented with 10% FBS, 4.5 g/L glucose, 4 mM L-glutamine, 3.7 g/L sodium bicarbonate, 1 mM sodium pyruvate) in a 100 mm cell culture dish. The cells must reach 90% confluency on the day of transfection (Day 2).

2. Day 2: The transfection step is achieved with Lipofectamine 3000 reagent. Please refer to the supplier protocol (https://www.thermofisher.com/fr/fr/home/references/protocols/cell-culture/transfection-protocol/transfecting-plasmid-dna-into-hek-293-cells-using-lipofectamine-ltx-reagent.html). For each 100 mm dish (or each condition):
   a. Thirty minutes before transfection, replace growth medium with 10 mL of fresh complete DMEM.

   ⚠ CRITICAL: Always handle the 293 RetroX cells with gentleness to avoid resuspension.

   b. In a 1.5 mL Eppendorf tube, add 625 μL of Opti-MEM medium and then add 25 μL Lipofectamine 3000 reagent to the Opti-MEM medium with a micropipet.
   c. In another 1.5 mL Eppendorf, add 625 μL of Opti-MEM and then add 10 μg of reporter plasmid (pMSCV-IETD2X-Venus) + 5 μg of envelope plasmid (pCMV-VSVG) + 3 μL of p3000 enhancer reagent.
   d. Transfer the diluted DNA (from step c) to the diluted Lipofectamine (from step b) with a micropipet and then invert the tube three to five times gently.
   e. Quick spin the vial to recover all the sample.
   f. Let the mixture stand for 15 min at room temperature.
   g. Add the mixture drop-wise directly on the 100 mm culture dish.
   h. Incubate overnight at 37°C, 5% CO_2 in a biosafety level 2 laboratory.
3. Day 3:
   a. Aspirate the transfection medium from the 100 mm culture dish and discard it.
   b. Add 5 mL of fresh complete DMEM to the 100 mm culture dish and put it back in the incubator for 48 h (until Day 5). Five mL of fresh medium is sufficient to cover the totality of the 100 mm petri dish surface and it allows to get a higher concentration of viral particles.
4. Day 4: Seed the target cells to infect in 6-well plates (here the HeLa cells) at the optimal density (1.10^5 cells per well) to reach 30% confluency on day 5.
5. Day 5: Infecting target cells.
   a. Take off the 5 mL media from the GP2-293 RetroX cells (containing viral particles) carefully titling the plate, and filter the 5 mL supernatant with a 0.45 μm filter using a 10 mL syringe and plunger into a 50 mL tube that contains 3 mL of fresh DMEM and polybrene (final concentration: 8 μg/mL. Invert the tube once for mixing).

   Pause point: Viral particles can be stored at −80°C up to several months, in the Opti-MEM medium.

b. Aspirate the media on the target cells and discard it.
c. Add 1.5 mL of virus containing supernatant (from step a) to each well in the 6-well plates containing the target cells to infect.
d. Twelve to sixteen hours later, aspirate the media of the target cells and discard it.
e. Add fresh complete DMEM (2 mL per well of a 6-well plate).

6. Day 6: Transfer by trypsinization the infected cells in 100 mm culture dish from one 6-well and let grow 2–3 days prior to the FACS sorting step.

See troubleshooting problem 1.

FACS to obtain clonal cell lines

Timing: 4 h

To obtain a clonal population of infected cells with homogeneous levels of the IC-RP biosensor, cells must be sorted by FACS and selected on their high IC-RP expression levels. Then the cells must be subcloned by limiting dilution or by dispensing single-cells by FACS, to obtain a clonal population expressing the IC-RP, required for the screen. Prepare simultaneously parental cells with no fluorescent protein as gating controls.

7. Culture cells in 100 mm dish until they reach near 80% confluency.

Remark: at this step, freeze a backup mix.

8. Prepare collecting tubes (5 mL polystyrene round bottom 12 × 75 mm test tubes) by adding 2 mL of complete culture medium (or 2 mL of FBS) to coat tubes and place them into the incubator for at least 1 h.
9. Remove culture medium from cells.
10. Wash each plate twice in 1× PBS.
11. Trypsinize cells by adding 1 mL trypsin and immediately aspirate 90% of the trypsin volume. Incubate between 1–3 min and neutralize trypsin and collect cells with 8 mL of complete fresh medium.
12. Count final cell number.
13. Reseed 1 mL (1:8) of the cells in a new 100 mm dish = "safe mix" (It can be discarded afterward if the experiment has completed successfully).
14. Centrifugate the remaining 7 mL at 1000 rpm for 5 min at room temperature, then remove the supernatant, resuspend the pellet in the suitable volume of FACS buffer (1× PBS, 5% FBS, 2.5 mM EDTA) and place it on ice. One milliliter of FACS buffer is required for 10^6 cells. This buffer must be stored at 4°C for up to one month.
15. Transfer the suspension in FACS collecting tubes (filter top FACS 5 mL tube).

Optional: If the cell line used tends to grow in clusters, please use cell straining nylon filter (100 μm) prior to sort cells.

16. Repeat the steps 9–15 with the parental cells (without fluorescent protein), to be used as gating controls.
17. In the ARIA III cell sorter (BD Biosciences), sort cell using the CFP and YFP fluorescence (in the case of IC-RP) and gate the higher double-positive cells (Figure 1). The HeLa cells are sorted using a 100 μm nozzle size, and the double-positive cells are directly platted into wells of a 96-well plate, with one single cell per well. The goal is to obtain 5 to 10 double positive clones after expansion (3–4 weeks), from one 96-well plate.

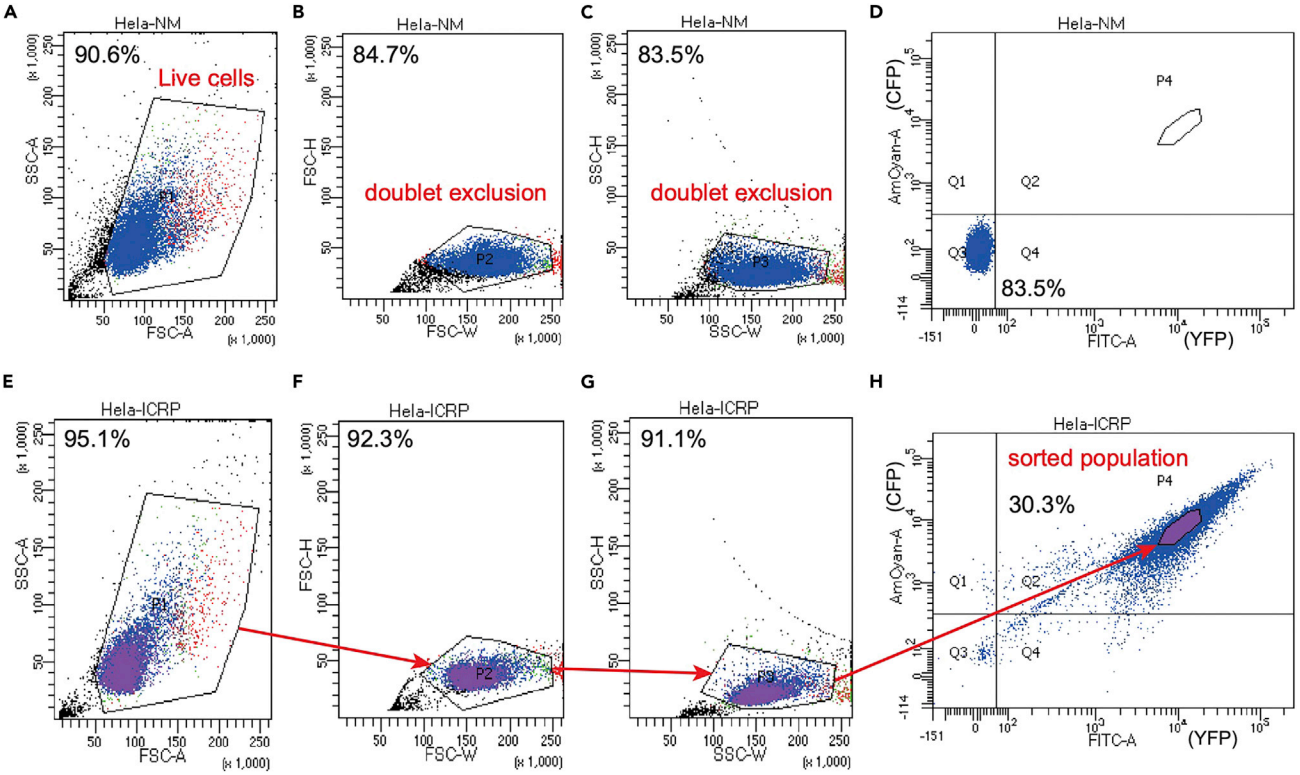

Figure 1. FACS gating strategy for double fluorescent cells (CFP-YFP) selection

(A–D) Parental HeLa cells (HeLa-NM), not containing any fluorescent protein, were used to quantify background levels for each channel (CFP and YFP). In (A–C), Forward and Side Scatter (FSC, SSC) gating were used to identify cell of interest based on size and granularity and to exclude debris and doublet cells. Panel (D) shows the basal fluorescence levels for YFP in x-axis and CFP in y-axis. Those levels were used to identify double negative cells (Q3 quartile, left bottom in D panel).

(E–H) The same gates were applied to the fluorescent population expressing the FRET reporter (HeLa-ICRP). Only 30.3% of double positives cells were selected (P4 gate – panel H) for single cell seeding in 96-well plate for clonal expansion.

18. Locate wells with a single cell colony, check CFP and YFP fluorescence, and expand selected colonies gradually from 96-well plate to 100 mm dish.

Pause point: Selected clonal cells can be freeze up to one year in liquid nitrogen.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Betaïne	Thermo Fisher Scientific	15651859
Dimethyformamide	Sigma	227056
DMEM	Gibco	31966-021
DMEM without phenol red	Gibco	21063-029
dNTP	Promega	U1511
DTT	Promega	P1171
Dynabeads® MyOne™ Streptavidin C1 beads	Life Technologies	65001
EDTA	Invitrogen	15575-038

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
EtOH	Sigma	51976
FACS tube	Falcon	352235
FBS	PAN-Biotech	P30-3306
Fluorobright^{TM} DMEM	Gibco	A18967-01
GlutaMAX	Gibco	35050-061
MgCl2	Sigma	M8266
Nuclease free water	Thermo Fisher Scientific	4387936
OptiMEM	Gibco	51985-026
PBS with Ca++/Mg++	Gibco	14040-133
Penicillin/streptomycin	Gibco	15140-22
Phosphate Buffer Saline (PBS) w/o Ca++/Mg++	Gibco	14190-094
Polybrene	Sigma	TR-1003-G
Rat tail collagen I	Sigma	C3867
Recombinant human (rh) TRAIL	R&D Systems Europe	375-TEC/CF
RNAsin plus	Promega	N26-11
Sodium Dodecyl Sulfate (SDS)	Sigma	436143
SPRIselect	Beckman Coulter	B23317
TAPS	Sigma	T5130
Trypsin	Gibco	25300-054
Tween20	Thermo Fisher Scientific	85113
Critical commercial assays		
High-Capacity cDNA Reverse Transcription Kit	Thermo Fisher Scientific	4368813
KAPA HiFi Hotstart ready Mix	Roche	KR0370
Lipofectamine 3000 Kit	Thermo Fisher Scientific	L3000008
NextSeq500/550 High Output V2 kit	Illumina	FC-404-2005
Deposited data		
Fate-seq differential analysis results (edgeR)	(Meyer et al., 2020)	Table S2 in Meyer et al. 2020
Fate-seq RNA sequencing raw data GEO, GSE138832	(Meyer et al., 2020)	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE138832
Experimental models: Cell lines		
GP2-293 Packaging Cell Line	Takara Bio	631458
HeLa IC-RP (human)	(Roux et al., 2015)	N/A
Oligonucleotides		
Cell ID barcodes	This manuscript	Table 1
Isolated single cell library primers	This manuscript	Table 2
Recombinant DNA		
pMSCV-IETD2x-Venus	(Meyer et al., 2020)	N/A
pCMV-VSVG	Takara Bio	631530
Software and algorithms		
Drop-seq tools	(Macosko et al., 2015)	v1.0
EdgeR	https://bioconductor.org/packages/release/bioc/html/edgeR.html	v3.13
Fiji	https://fiji.sc/	v2
ImageJ	https://imagej.nih.gov/ij/	v1.5.2
Matlab	mathworks.com	r2017a
Pheatmap	https://www.rdocumentation.org/packages/pheatmap/versions/1.0.12/topics/pheatmap	v1.0.12
R	https://www.r-project.org/	v3.6.1
R code for RNA sequencing data analyses	(Meyer et al., 2020)	GitHub repository: https://github.com/jrxlab/fate-seq_proc
Scran	(Lun et al., 2016)	
Seurat	https://satijalab.org/seurat/	v3
Python	https://www.python.org	v3.8

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Python code for cell phenotyping and cell identification for laser capture	This manuscript	GitHub repository: https://github.com/jrxlab/live_cell_phenotyping_capture_proc Release v1.0: https://doi.org/10.5281/zenodo.6671339
STAR	(Dobin et al., 2013)	v2.4.0a
Other		
Frameslide PET	Carl Zeiss	415190-9051-000
PALM MicroBeam	Carl Zeiss	494800-0012-000
DeltaVision™ Elite Imaging System	GE Healthcare	NA

MATERIALS AND EQUIPMENT

Reagents

Growth medium

The growth medium depends on the cell type and origin. In this context we use Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (5%). The details of the recipe can be found below. The FBS must be filtered under sterile conditions. Before use, the complete growth medium must be warmed up in a bottle bath (37°C). The bottle must be stored at 4°C for up to 6-months.

Growth Medium		
Reagent	Final concentration (vol %)	Amount
DMEM	≃ 90% (v/v)	445 mL
FBS	≃ 10% (v/v)	50 mL
Penicillin	100 units/mL	5 mL
Streptomycin	100 μg/mL	5 mL

Imaging medium

As it is the case for the growth medium, the imaging medium must be chosen according to the cell type. In this study we use the DMEM based Fluorobright™ from Gibco. Fluorobright™ must be supplemented with 10% FBS, GlutaMAX™ (100×, 200 mM L-alanyl-L-glutamine in 0.85% NaCl). The FBS must be filtered under sterile conditions. Before use, the complete imaging medium must be warmed up in a bottle bath (37°C). The bottle must be stored at 4°C for up to 6-months.

Imaging Medium		
Reagent	Final concentration (vol %)	Amount
Fluorobright™ DMEM	≃ 90% (v/v)	440 mL
FBS	≃ 10% (v/v)	50 mL
GlutaMAX™	2 mM final	5 mL
Penicillin	100 units/mL	5 mL
Streptomycin	100 μg/mL	5 mL

Rat tail collagen I coating solution

Rat tail collagen I coating solution		
Reagent	Final concentration (μg/mL)	Amount
Collagen, Type I solution from rat tail	40	11 μL
PBS	N/A	989 μL

Note: Rat tail collagen I coating solution must be stored at 4°C for up to one year.

FACS buffer

FACS buffer		
Reagent	Final concentration	Amount
FBS	5% (v/v)	2.5 mL
EDTA (500 mM)	2.5 mM	250 μL
PBS	N/A	50 mL

Note: FACS buffer must be stored at 4°C for up to one month.

Cell Capture Lysis Buffer

Cell capture lysis buffer		
Reagent	Final concentration	Amount
Tween20	0.2% w/v	81 μL
RNAsIN Plus	1.5 U/mL	5 μL

Note: Cell Capture Lysis Buffer must be prepared fresh before use.

STEP-BY-STEP METHOD DETAILS

Coating and seeding cells in FrameSlides PET for laser capture microdissection

Timing: 2–3 h

This part of the protocol describes the coating and seeding of the IC-RP expressing cells on FrameSlides polyethylene terephthalate (PET) (Carl Zeiss, France) for video microscopy followed by laser capture microdissection of the phenotyped cells of interest. In this protocol we use only one FrameSlide per experiment, but the number of conditions can be increased. The SlideHolder can be loaded with up to three FrameSlides.

1. FrameSlide preparation.
   a. Add 1 mL of rat tail collagen coating solution (40 μg/mL) on the FrameSlide membrane. Aliquots must be stored at 4°C for up to 12 months.
   b. Immediately aspirate back 400 μL and let incubate with 600 μL on the FrameSlide.
   c. Incubate 2 h at 37°C, 5% CO_2.
   d. Quick wash membrane twice (30 s) with 600 μL fresh 1× PBS.
2. Take a 100 mm dish from section "FACS to obtain clonal cell lines" step 18 and Figure 1.
3. Count cells.
4. Resuspend cells to reach a density of 6000 cells in 700 μL (≃8500 cells/mL) of imaging medium.
5. Add 700 μL of cells suspension to the coated FrameSlides PET.
6. Let the FrameSlide under the tissue culture hood for 30 min to allow a homogeneous cell seeding.
7. Place the FrameSlides PET on the SlideHolder and incubate for 14 h/overnight at 37°C, 5% CO_2.

CRITICAL: Do not add more than 800 μL of medium on the FrameSlide, so as avoid distorting the membrane. The FrameSlide in step 6 could be placed on a clean bench for 30 min before going in the incubator, if an homogeneous cell seeding cannot be obtained under the tissue culture hood.

Live-cell treatment and video microscopy

Timing: 2 days

This step focuses on the setup and optimization of live-cell time-lapse fluorescence acquisition. In this study, we use the DeltaVision Elite microscope system (GE Healthcare Life Science, Velizy-Villacoubray, France). This system is equipped with an environmental chamber coupled with a CO_2 controller unit Bold Line from Okolab and heat controller/humidity monitor from World Precision instrument. The objective used is a 20× Super-Plan APO objective (NA=0.75). Other types of fluorescent microscopes equipped with environmental chambers can be used but the temperature, humidity, airflow, and CO_2 levels cited below, would have to be optimized by doing a 24-h live-cell experiment confirming proper cell growth. After TRAIL treatment, the cells were imaged in transmitted light every 3 min for 50 min and using the following InsightSSI solid state illumination unit (Applied Precision) with excitation (Ex.) wavelengths and single bandpass emission (Em.) filters: for CFP (Ex. 438/24 nm - Em. 475/24 nm), YFP (Ex. 513/17 nm - Em. 548/22 nm). (The excitation power measured at the sample location should be 9 mW for CFP and 3.5 mW for YFP or less, while maintaining a signal to noise ratio above 1.5; see note step 18 below.).

8. Day 1: Environmental chamber setup for live imaging.
   a. Turn on microscope and workstation.
   b. Turn on the Temperature Controller using the power switch on the back of the unit.
   c. Press the **Temp** button on the front of the controller to display the current temperature set point.
   d. Use the **Up** and **Down** buttons to change temperature to 37°C and press **Display** to save changes.
   e. To adjust the air flow set point press the **Config** button until "Fan Air Flow Set Point" reach 30% and press **Display** to save changes.

   Note: Fan speed below 20% cannot maintain 37°C. A fan speed of 30%–50% is recommended during normal operation.

   Note: Check the temperature inside the environmental chamber, as the temperature setpoint and the actual reading the chamber may vary: modify the setpoint if necessary.

   f. Allow ample time (minimum 6 h) for the system to reach a stable temperature/CO_2 environment.

   Note: Air inside the chamber will come to temperature quickly but system components (stage, objectives, etc.) will take much longer and may cause important focus drift for at least 3 h. To minimize drift within an experiment, allow system to equilibrate for 6 h minimum before imaging.

   g. Verify that humidifier is between $^1/_2$ and $^3/_4$ full of deionized water.
   h. Turn on the premixed gas and set regulator between 5–10 PSI.
   i. Switch the flowmeter to the "ON" position and slowly turn the knob to adjust flow rate between 40–50 (160–207 mL/min).
   j. Bubbles will appear in the humidifier as gas flows into the water. If the humidifier is ≈ $^3/_4$ full, allow 10–15 min for CO_2 saturation.

9. Day 2: Just before imaging, mount the FrameSlides PET on the stage (Zeiss logo on the left). The SlideHolder must be inserted with the red pine on the left (Figure 2).
10. Place the cell cover over the samples and verify that the gasket on the cover surround the outer edge of the SlideHolder.

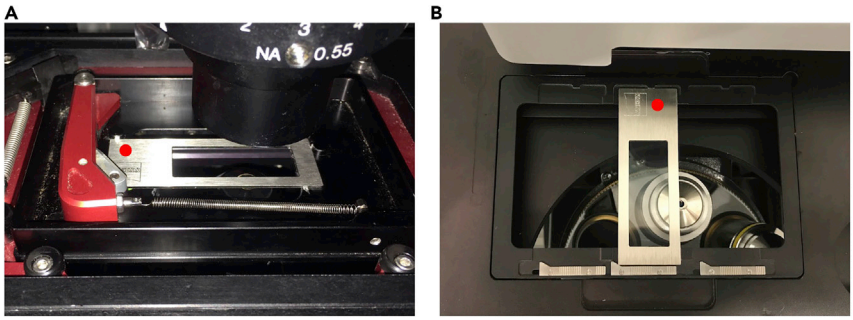


Figure 2. FrameSlide handling for live-cell microscopy and for laser-capture microdissection
(A) The picture shows how to insert the FrameSlide in the Delta Vision's SlideHolder. The Zeiss Logo (red dot) must be placed on the left side directly in contact to the red notch.
(B) The picture shows how to place the FrameSlide in the Zeiss PALM setup for laser microdissection. The FrameSlide must be inserted 90° clockwise in order to have the Zeiss Logo (red dot) on the top of the SlideHolder.

⚠ CRITICAL: Check imaging medium on the slide. Never let the slide dry up when at 37°C.

Note: Although accessible transparent surfaces in the microscope light path should be clean, the FrameSlide PET membrane cannot be cleaned.

11. Click on the Start SoftWoRx™ icon (Figure 3).

Note: The software packages provided with the live-cell microscope may differ but operating the acquisition and the experimental design will include the following steps.

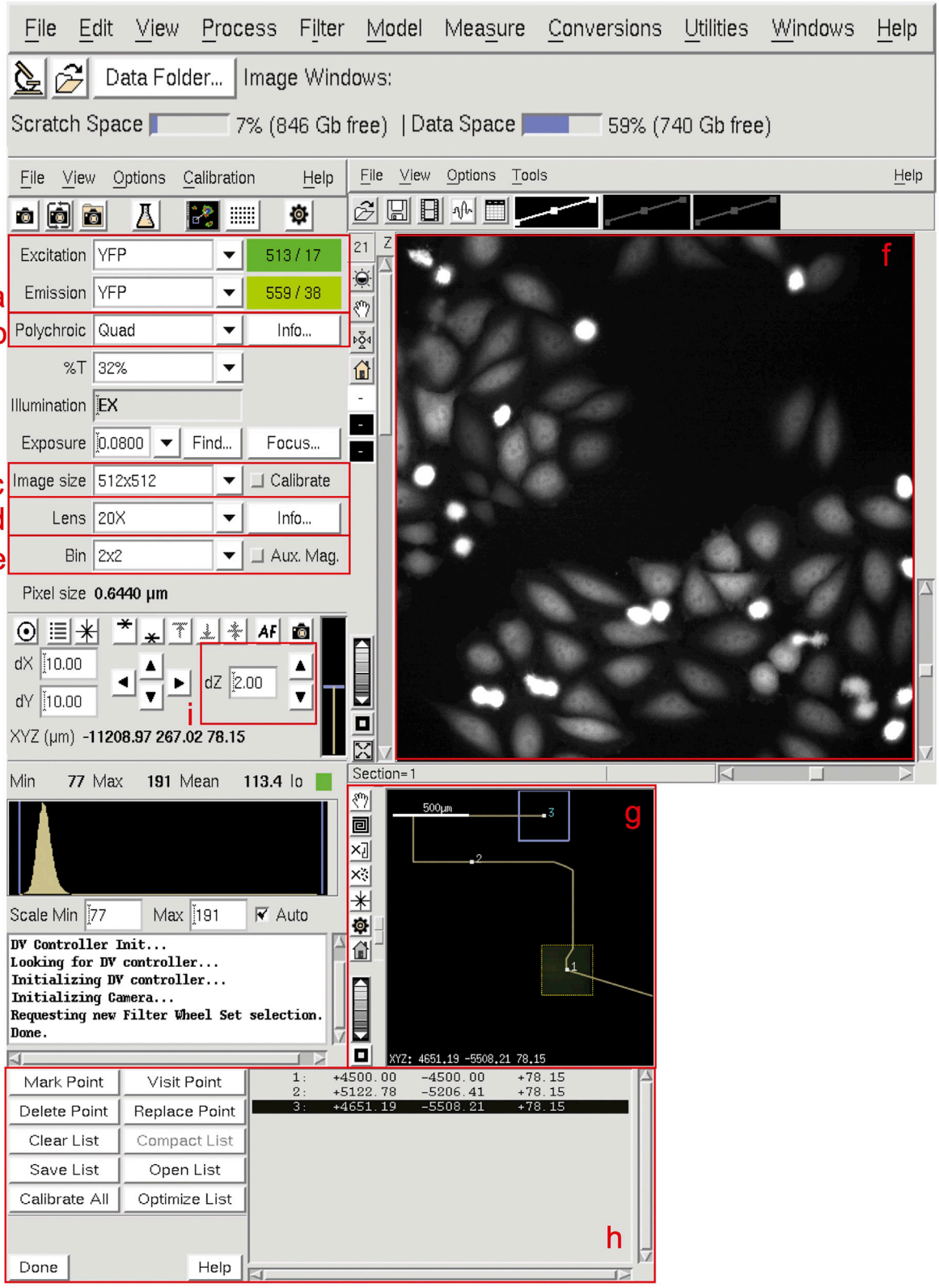
12. From the main menu, chose File and Acquire (Resolve3D) to initialize the system.
13. Rotate objective turret until 20× objective is in the active position. Update selection in the Lens drop down menu (Figure 3D).

Note: The DeltaVision system used here has a 1.59× auxiliary magnification (final magnification 31.8×).

14. Use the Polychroic drop down menu and select Quad (Figure 3B). The filters will be set in the Experiment setup window, in step 18d.
15. Set the Image size to 512 × 512 (Figure 3C).
16. Set the Binning drop down menu to 2 × 2 (Figure 3E).

Note: Using the Edge/sCMOS_5.5, the pixel size ends up at 0.4 μM here. Since only an average pixel intensity of the whole cell is going to be measured here (no sub-cellular feature), the main goal in defining magnification, image size and binning parameters is to frame a sufficient number of cells for the next steps (5–10 cells per field).

17. Focus on sample and find a region of interest.
    a. Check **Filter Monitor** windows to ensure Ex and Em filters match for every channel (CFP, YFP and POL) (Figure 3A).
    b. Open the YFP channel and click on the Continuous Acquisition button to focus on the regions of interest with up to 20 well-separated cells. Stop Continuous Acquisition.
18. Designing the experiment.
    a. Select the Experiment button in the Resolve3D window.
    b. Uncheck the Use fast acquisition button (Figure 4A).
    c. In the sectioning tab uncheck the Z-sectioning button (Figure 4A).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Data Folder...
Image Windows:
Scratch Space 7% (846 Gb free) | Data Space 59% (740 Gb free)
File View Options Calibration Help
File View Options Tools Help
Excitation YFP 513 / 17
Emission YFP 559 / 38
Polychroic Quad Info...
%T 32%
Illumination EX
Exposure 0.0800 Find... Focus...
Image size 512x512 Calibrate
Lens 20X Info...
Bin 2x2 Aux. Mag.
Pixel size 0.6440 µm
dX 10.00
dY 10.00
dZ 2.00
XYZ (µm) -11208.97 267.02 78.15
Min 77 Max 191 Mean 113.4 Io
Scale Min 77 Max 191 Auto
DV Controller Init...
Looking for DV controller...
Initializing DV controller...
Initializing Camera...
Requesting new Filter Wheel Set selection.
Done.
Section=1
500µm
XYZ: 4651.19 -5508.21 78.15
Mark Point
Visit Point
Delete Point
Replace Point
Clear List
Compact List
Save List
Open List
Calibrate All
Optimize List
1: +4500.00 -4500.00 +78.15
2: +5122.78 -5206.41 +78.15
3: +4651.19 -5508.21 +78.15
Done
Help
a
b
c
d
e
f
g
h
i

Figure 3. SoftWoRx desktop display

The SoftwoRx software is presented in several adjustable windows.

(A–E and I) The Resolve3D window (A,B,C,D and E) contains parameters for image acquisition, filter selection and table position control (I).

(F) In (F), the image capture window shows the images as they are captured live.

(G and H) The selection of the different fields is shown in (G) and the point list of their respective coordinates (x,y,z) in (H). Make sure that the turret lens is in the 20× position according to the lens parameters in (D).

d. In the Channels tab (Figure 4B), activate the appropriate number of channels (CFP, YFP and POL) and respective Ex and Em wavelengths for each channel. Select 32% transmittance and 80 ms exposure for both CFP and YFP channels and 10% transmittance and 5 ms exposure for the polarized channel.

Note: Exposure and transmittance parameters should be determined to achieve a signal to noise ratio greater than 1.5 for both CFP and YFP. This ratio can simply be estimated by measuring the pixel intensity within a cell (signal) divided by the pixel intensity measured outside the cell (noise).

See troubleshooting problem 2.

e. Activate the Time-lapse tab and set the Time-lapse (time between points) to 3 min and total time to up to 50 min (Figure 4C).
f. Mark points of interest (Figure 5B) with the Mark Point button in Resolve3D (Figure 5C).
g. Repeat for approximatively 20 regions of interest (points), to be adjusted in order to monitor ≃100 cells in the whole experiment.
h. Save the point list (Figure 5C, red box).
i. Replace the cell cover and let it stabilize on the z-axis during a minimum of 20 min.

⚠ CRITICAL: Do not go beyond the last 5 mm on the right of the FrameSlide PET because no microdissection is possible there and choose fields with more than three cells well separated.

j. From the Resolve3D window, open the point list (Figure 3H).
k. Instead of adjusting the microscope with focus knob, visit each point using the camera and refine the focus position (z axis) using the dZ button in the resolve 3D window (Figure 3I) and if desired the x/y position.
l. Click the Replace Point (Figure 5C, blue Box) button in the point list to save the update position.
m. Activate the Point tab and list all the point you want to visit.
n. Save the experiment.
o. Click the Run button and start acquiring for up to 30 min.

⚠ CRITICAL: This step can be important before treatment, in order to skip the decrease in fluorescence ratio that can be observed at the beginning of the acquisition, when an autofluorescence bleaching is occurring in the CFP channel.

p. Stop the acquisition and proceed to the experiment.

19. Applying recombinant human TRAIL treatment.
    a. Prepare 800 μL of imaging medium containing 25 ng/mL rhTRAIL.
    b. Open the environmental chamber.
    c. Aspirate 700 μL of the imaging media and quickly 800 μL of imaging media containing rhTRAIL (5s max to swap medium).
    d. Click the Run button and start acquiring for up to 50 min.

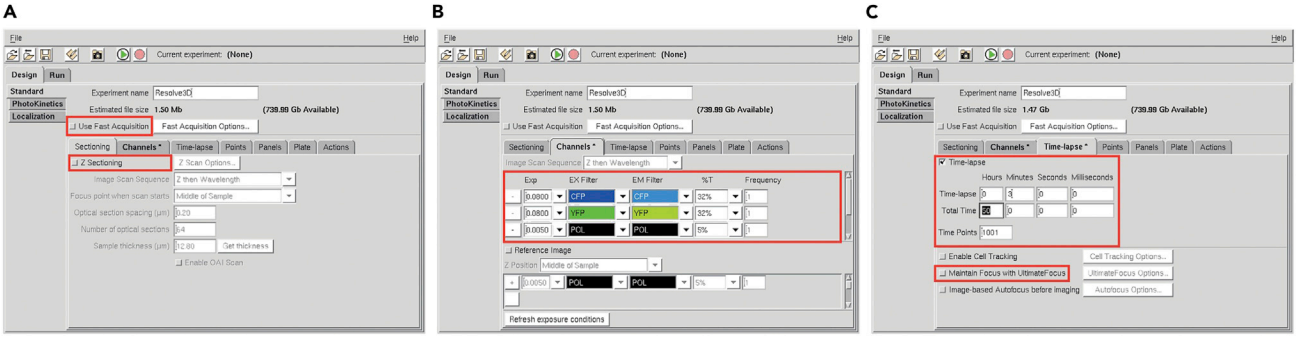

Figure 4. Experiment setup window

(A) Uncheck the checkboxes "Use fast acquisition" and "Z Sectioning" in the sectioning panel. Ensure that the exposure in the channels is set between 25 and 80 ms depending on the fluorescence intensity.

(B) Select 32% transmittance for both fluorescence channels and 5% transmittance for the POL channel.

(C) Set the time-lapse parameters and activate the option "Maintain Focus with UltimateFocus" option.

See troubleshooting problem 3.

20. At the end of the experiment, cool cells by gently plunging the FrameSlide PET into a 50 mL tube filled with PBS containing Ca^{2+}/Mg^{2+}, placed on ice.
21. Get DV files (.dv) and the point list (text format) to move on the Zeiss Palm Microdissection system.

⚠ CRITICAL: Point list coordinates can be incompatible between DeltaVision and Zeiss systems. A conversion of the point lists must be performed to locate the field of interest. To perform the conversion, coordinates must be obtained from both microscopes using the same FrameSlide marked with 4 points. (The points can be simply drawn with a fine marker.) The FrameSlide is mounted on each microscope as described above (with a clockwise 90° rotation between the DeltaVision and the Zeiss (see step 1 on Figure 6). The coordinates from both microscopes are recorded on a point list as described. The Zeiss microscope will flip the coordinates for display, according to a horizontal axe (see Figure 6, step 2). The point lists will be used to determine the coefficients of a linear regression for both x and y coordinates of each point, which can then be applied to convert a point list from the DV to the PALM Zeiss setup. With the manual rotation of the FrameSlide and the Zeiss display flip, y_{Zeiss} only depends on x_{DV}, and x_{Zeiss} only depends on y_{DV} (Figure 6, step3). A second round of this procedure can be repeated using cells instead of marker points in order to increase the accuracy of the conversion.

The python code "DVtoZeiss.py" to compute the conversion between DV and Zeiss (Data S1) is accessible on https://github.com/jrxlab/live_cell_phenotyping_capture_proc (https://doi.org/10.5281/zenodo.6671339), with more information about the 3 steps in the Readme section.

To execute "DVtoZeiss.py" to obtain the coefficients of linear regression and perform a conversion of a point list (with a file with DV coordinates for the 20 fields (the function takes into account the path toward the file (`path_to_dv_pts_file`) and the name of the .pts file (`name_dv_pts_file`)) + a numpy array with the DV coordinates of the marker points (DV_init) + the numpy array of the corresponding coordinates in the Zeiss (Zeiss_init):

```
>>> Import DvtoZeiss

>>> DvtoZeiss.allConversion(Zeiss_init, DV_init, path_to_dv_pts_file, name_dv_pts_file,
path_saved_zeiss_file, name_saved_zeiss_file)
```

To execute "DvtoZeiss.py" to simply perform the conversion of a point list, the function will use our marker point coordinates to compute the conversion DeltaVision to Zeiss:

```
>>> Import DvtoZeiss

>>> DvtoZeiss.allConversion(path_to_dv_pts_file, name_dv_pts_file, path_saved_zeiss_file,
name_saved_zeiss_file)
```

To execute "DvtoZeiss.py" to simply perform the conversion of a point list (once you have obtained the coefficients of linear regression and created the conversion matrix A and the conversion vector B such that $X_{Zeiss=}A.X_{DV}+B$):

```
>>> Import DvtoZeiss

>>> DvtoZeiss.allConversion(A,B, path_to_dv_pts_file, name_dv_pts_file, path_saved_zeiss_
file, name_saved_zeiss_file)
```

The function will automatically save a .csv in the `path_saved_zeiss_file` folder under the name `name_saved_zeiss_file`, which contains the DeltaVision coordinates of each field and the converted coordinates for the Zeiss. The function also returns a pandas dataframe with the DeltaVision coordinates of each field and the converted coordinates for the Zeiss, the conversion matrix A and the conversion vector B.

Image's processing and cell phenotyping

Timing: 30 min to 1 h

In this section we detail the processing of images and their analysis for further capture microdissection of selected cells (predicted sensitive and predicted resistant). The main steps are to separate the images hyperstack into three different substacks (one for each channel CFP, YFP and POL). The polarized channel is only used to survey the morphology of cells during the time-lapse. The CFP and YFP channels are used to establish the CFP/YFP fluorescence intensity ratio overtime. (This CFP/YFP ratio increases in time as the IC-RP is cleaved by active caspase-8.) The ratio is tracked for each cell in the field on the entire time-lapse duration.

Note: The following steps describe the analysis of single cell trajectories using ImageJ and publicly available plugins (https://imagej.nih.gov/ij/plugins/) for most users. However, in-house plugins and other analyses routines can be used here to track single-cell ratio intensities overtime.

The following steps are illustrated in short tutorial (Methods videos S1, S2, and S3).

22. Open the raw image stack sequences in ImageJ software.
23. In the main menu, select Image – Stacks – Tools – Make Substack (Figure 7A, Methods video S1).
24. In the Substack Maker tool, specify the range and the increment corresponding to each channel to have three different substacks, one for CFP, YFP and polarized channels respectively (Figure 7B).
25. Repeat step 23 for each channel.
26. Select the CFP substack, draw a single ROI in a background region.

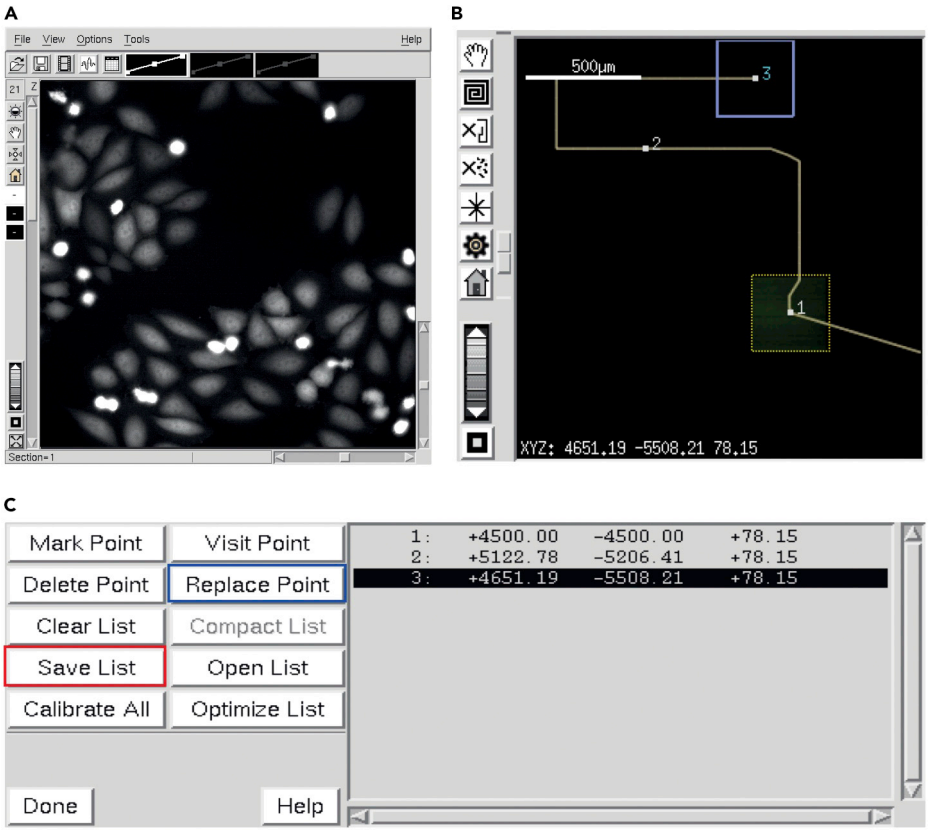

Figure 5. Selection of the different fields of interest

(A) By moving around the different areas of the well with the joystick, select a maximum of 3 fields that do not overlap and in which there are more than three cells.

(B) It is recommended to place the fields more to the center of the wells.

(C) Click "Mark Point". You will see the coordinates of each point in the point list. Save the list. It is possible to replace a point by clicking on "Replace Point".

27. Measure the pixel mean intensity (Analyze Menu and then Measure or ctrl+m) for stack 1 (CFP) (Figure 8A, Methods video S2).
28. Copy the ROI to the YFP substack (Menu Edit/Selection/Restore selection or ctrl+shift+E).
29. Measure the pixel mean intensity (ctrl+m) for substack 2 (YFP).
30. Open the Ratio Plus plugin (https://imagej.nih.gov/ij/plugins/) (Figure 8B). In stack 1, select the CFP substack and the YFP substack in stack 2. Enter the background intensity value for CFP channel and 50% of the intensity value for the YFP channel obtained from steps 26 and 28. The output is a ratiometric stack in 32-bits format showing the CFP/YFP ratio for each object (cell) as a function of time (Figure 8C).
31. In the Analyze menu, select "Set Measurement", and check only the "Grey Mean Values" and "Display Labels" (Figure 9A, Methods video S3).
32. In the Analyze menu, select Tools and ROI manager. Draw one ROI for each cell in the ratiometric stack. (The ROI should fit within the cell. The ROIs can be of different sizes.) The polarized substack is used to control the position of the ROI on each cell of interest. Select all the ROI. Check the "Show All" and "Labels boxes" and then click on "More" option and then "Multimeasures". Click on "measure all slices" and "one row per slice" (Figure 9B). Save the values of ratio intensities in a .csv spreadsheet for further analysis.

Note: The position of the ROI within the cell, does not change significantly the measured trajectory.

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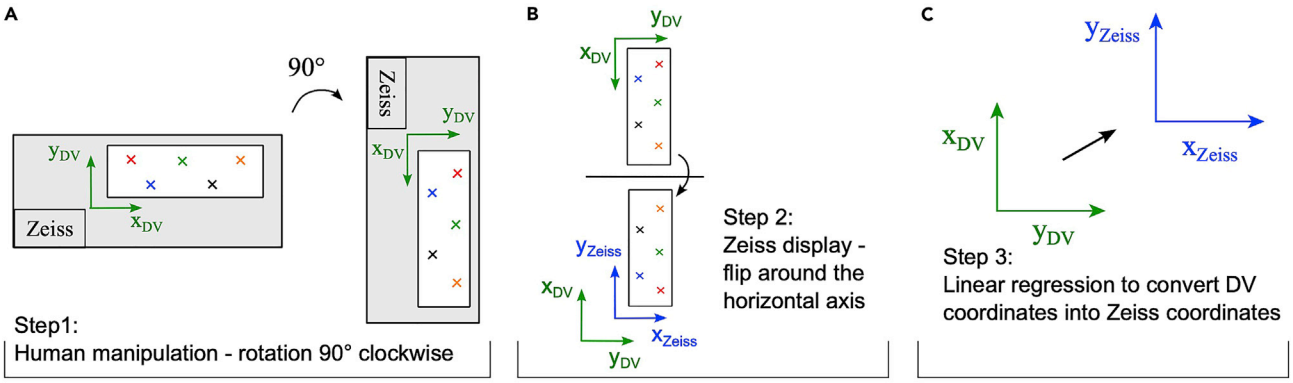

Figure 6. Coordinates conversion from a DeltaVision to a Zeiss AxioObserver microscope

(A) Manual rotation of the FrameSlide: the FrameSlide is rotated 90° clockwise from the DeltaVision to place it in the Zeiss slideholder.

(B) Zeiss software rotation for display: Zeiss software operates a flip of the frame slide for display around the horizontal axis.

(C) Linear regression to convert DeltaVision coordinates into Zeiss coordinates: The rotation and the flip result in an inversion of the x and y axes between the DeltaVision and the Zeiss. In addition, the origins of the reference frame are not the same in the two microscopes. Hence a linear regression is performed to find the mathematical relationships (linear here) that link x_{DV} to y_{Zeiss} and y_{DV} to x_{Zeiss}.

> *Note:* The next steps can be performed using a Python procedure ("Plot_AllTraj_FrameSlide_Cell_Field_Label.py" (Data S1), available on https://github.com/jrxlab/live_cell_phenotyping_capture_proc (https://doi.org/10.5281/zenodo.6671339). This code creates a single label for each cell in the experiment, it ranks the cells depending on the mean time derivative values. This ranking allows to select cells of interest: the 7 Top High Responders and the 7 Bottom Low Responders that would be microdissected for downstream analysis and that can be identified on the images by their unique labels: field#.cell#.

It takes as input a directory containing the .csv files where each file corresponds to a field in the frame slide. The file name must be field#.csv. The path to this directory is to indicate in *DIR_PATH* global variable.

To execute the code, run the Traj_Proc() function. It is the main function that calls all the other functions in the script.

```
>>> def Traj_Proc () :

     print("\nRead Files ...\n")

     experiment, time_vec, labels = Read_csv_files ()

     print("\nProcess Trajectories ...\n")

     experiment = Process_trajectories (experiment)

     print("Compute Mean Time Derivative ...\n")

     mean_derivative = Compute_FRET_Derivative (experiment["process. Frame slide"])

     print("Sort Cells ...\n")

     sorted_mean_deriv,sorted_labels = SortandSave_FRET_deriv (mean_derivative, labels)

     print ("Plot FRET Trajectories ...\n")

     Plot_Top_Bottom_cells (experiment["process. Frame slide"],time_vec, labels,sorted_
labels)

     print("Done")

     return experiment, time_vec,labels, mean_derivative
```

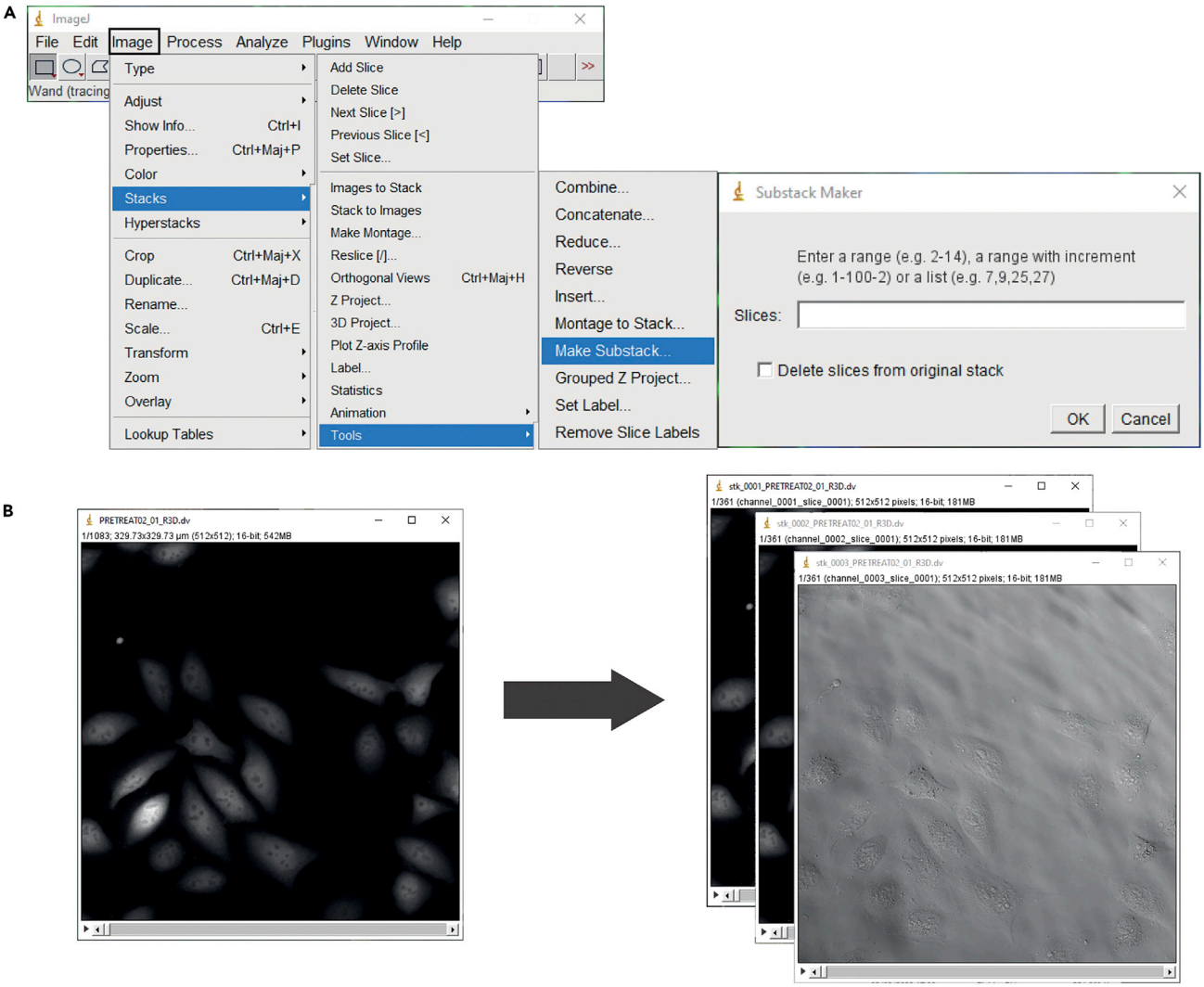

Figure 7. Image substack processing

The original stack consists of a sequence of CFP, YFP or polarized images.

(A) Click on the "Image" menu, "Stacks", "Tools", then "Make Substacks".

(B) The substacks must be reordered into tree sub-stacks for each channel individually. The result of the process is shown in (B).

It returns a dictionary of the raw and the processed FRET trajectories, the time vector, the single-cell labels vector and the mean FRET time derivative vector.

33. Each column corresponds to the time trajectory of one cell FRET ratio. For each trajectory, the minimal value of the FRET ratio is subtracted from the trajectory.
34. FRET cleavage rate (dFRET) is approximated by the time derivative of the FRET ratio, computed using finite differences.
35. Cell trajectories are then ranked by decreasing dFRET. From the ~100 cells tracked, top 7 cells (High Responders) and bottom 7 cells (Low Responders) are selected from the micro-dissection steps.

Note: Only 8 cells will be laser captured per session in total, however it is recommended to select a total of 14 cells to anticipate possible loss of cells during the transition between microscope or during the following micro-dissection.

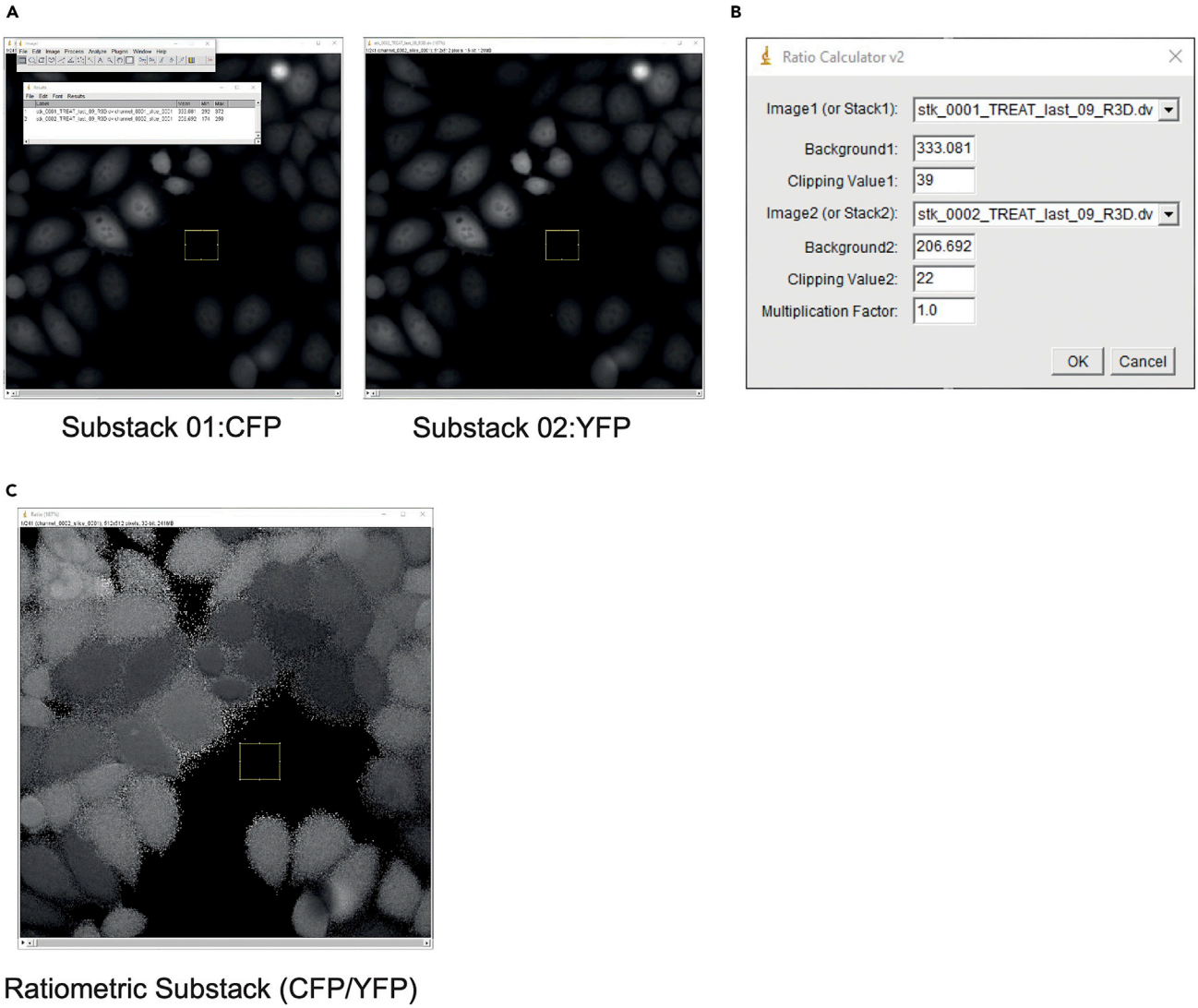

Figure 8. Ratiometric image stack generation

(A) To create the ratiometric image (CFP/YFP), select a region of interest in a background area. Click ctrl+M to measure the average intensity of the pixels and copy the same zone of interest to the second channel by clicking ctrl+E. Then measure the average intensity of the pixels of the background of the second channel. These parameters are important to create the ratiometric image by subtracting the background intensity.

(B) Then open the Ratio Image plugin and enter the settings for the background.

(C) The ratiometric image is then displayed.

> *Note:* If using another cell line (different from the HeLa cells used here), the ratio of high and low responders per 100 cells may vary. In that case a live-cell experiment must be performed first to determine the dFRET threshold above which a cell is considered high responder and the dFRET threshold below which a cell is considered low responder (Meyer et al., 2020).

Zeiss PALM setup

Timing: 30 min

36. Turn on the laser and then the microscope. Turn on the control box and finally turn on the computer.
37. Start MTB2004Configuration.

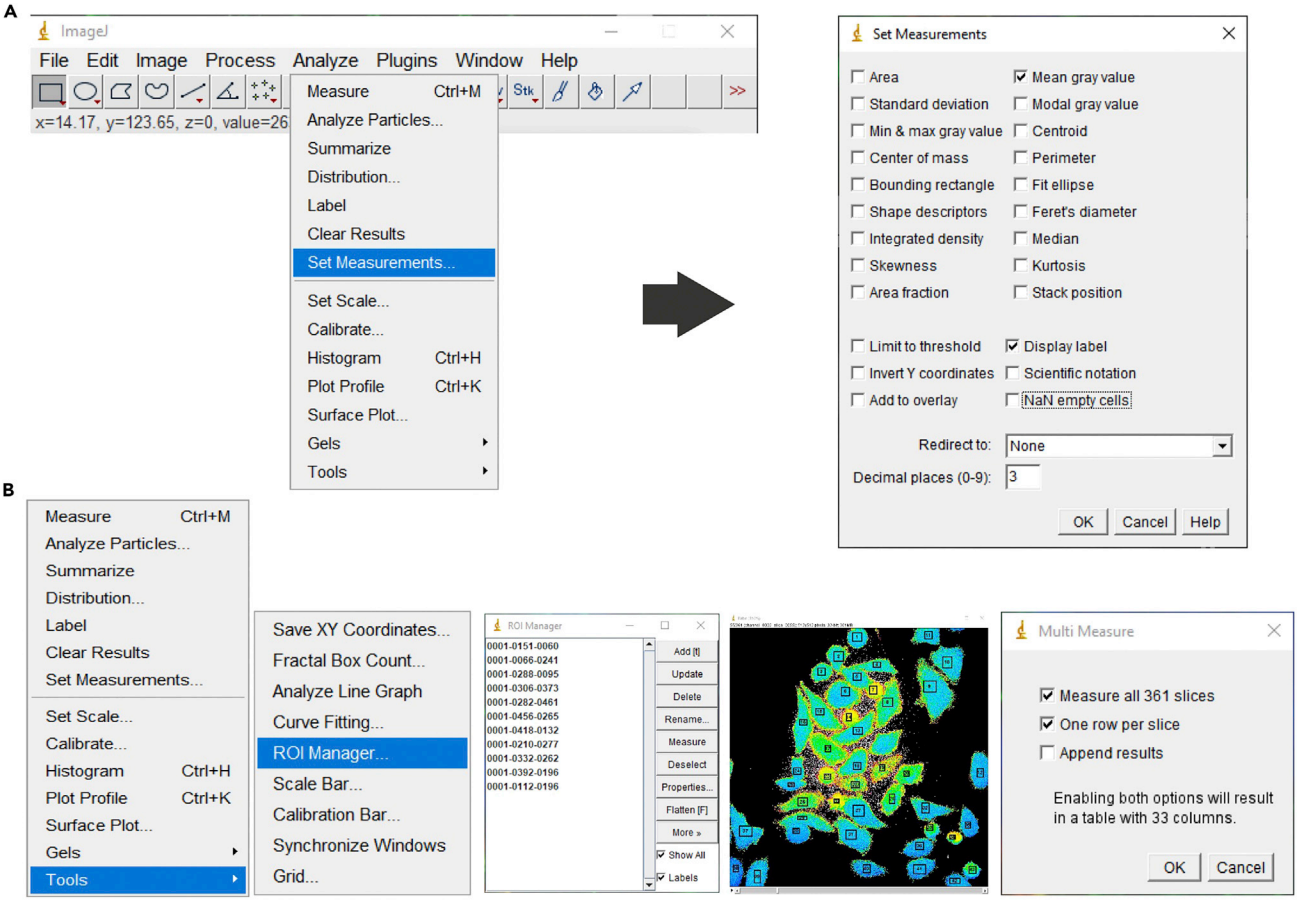

Figure 9. Manual tracking of all cells in a field of interest to get IC-RP trajectories

The principle of this section is to track manually the ratio intensities (on the ratiometric images) during the entire time lapse for all the cells in the field using ImageJ.

(A) Click on "Set measurements" in the "Analyze" menu and select only the "Mean gray values" option.

(B) In the "Analyze" menu, click on "Tools" and then on "ROI manager". Then select a region of interest in the middle of each cell in the field. Then click on "More" and then on "Multi Measures". Finally, activate the option "Measure all slices" and "One Row per Slice."

a. With settings selected, click Write configuration.

38. Start PALMRobo v4.2.
39. Start LPM and write configuration.
40. Start Robot Controller – Open PALM Robo Software and click on view default Bar config.
41. Apply a collar correction to adjust the working distance for a FrameSlide: check on objective lens 20×, between 0 and dot (0.17 mm).
42. Define "collector type".
43. Click on "Autoconfig" button on the camera display panel (Figure 10A).
44. Check the laser capture setup and check RoboMover. Note the initial resting value of the Fine Adjust Z-Axis. Then adjust the Z value to determine the laser capture position: the cap must come as close to the FrameSlide's membrane as possible without touching it. Note the Z value and return to its initial resting value.

⚠ CRITICAL: You may setup the Köhler illumination as shown in the Zeiss tutorial video (https://www.youtube.com/watch?v=4Wj7uFh_YLA), as the focus will be crucial for the cut during the following laser capture step.

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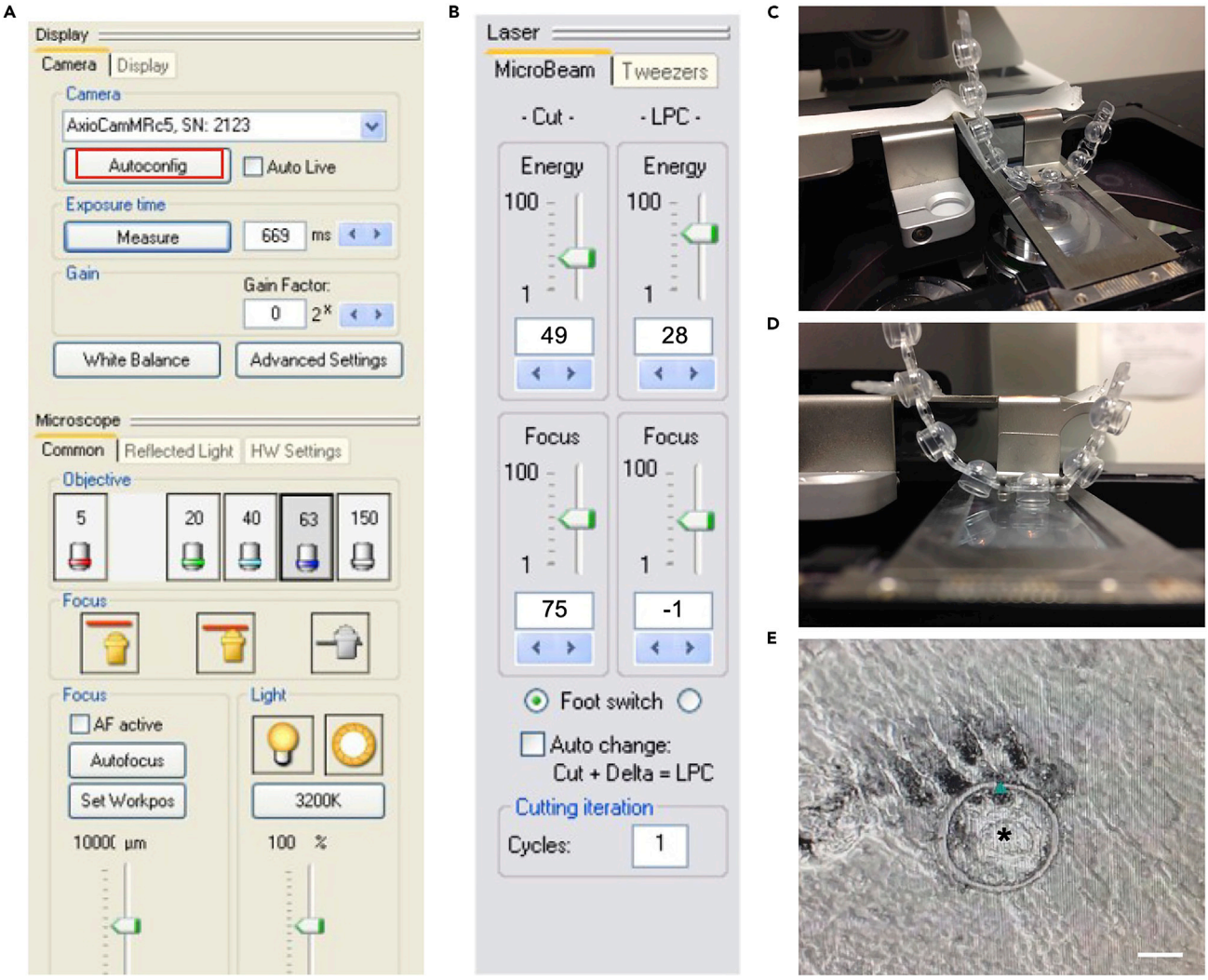

Figure 10. Zeiss PALM setup and laser capture microdissection

The software used in this study is PALMRobo v4.2.

(A) Click on "Autoconfiguration" in the "Camera" area.

(B) The laser intensity parameters for microdissection are displayed. The micro-dissected single cells are catapulted into PCR tube caps, one cell per cap.

(C) To avoid caps to get in contact with the cells or the support membrane, it is important to arrange the strip as shown.

(D) For efficient cell catapulting, the caps are lowered in position.

(E) An example of laser cut around a cell. The asterisk shows the laser-cut cell. Scale bar, 30µm.

Locate phenotyped cells and laser capture by microdissection

Timing: 2 h

45. Choose a fluorescent channel, for example FITC. (This will be used to visualize the cells of interest in the next steps.).
46. Choose the Microbeam Laser.
47. Set up the following parameters: cut energy to 49, LPC (Laser Pressure Catapulting) energy to 28, cut focus to 75 and LPC focus on -1 (Figure 10B).

Note: The parameters can be adjusted incrementally from the parameter set above, using a test FrameSlide prior to micro-dissection.

48. Reduce cutting speed to test the parameters of the laser.

⚠ CRITICAL: Check PBS every 10 min for evaporation.

49. Make sure to capture 8 cells in one strip: draw a list of minimum 14 cells (7 high responders and 7 complete non-responders).
50. Get the Zeiss point list set as "Dot" and save as text file to import in the Palm List.
51. Take the FrameSlide PET out gently.
52. Turn the FrameSlide 90 degrees clockwise Figure 2B (Zeiss logo to the rear).

Note: It is important to keep the change in slide orientation constant, to facilitate the conversion of the point list. The conversion of point list from the live-cell microscope will give the regions of interest to locate the selected cells for the laser-capture.

53. Add 800 μL fresh cold PBS.
54. Locate cells of interest and hit "Freeze."
55. Circle the cell to add to the list, rename cell.
56. Go the next field (region of interest containing the selected cells).
57. When the list containing a mix of high responders vs. non-responders is done (to get ideally 4 of each class per 8-tubes strip):
    a. Pipet 4.3 μL of cell lysis buffer in each tube from a PCR strip tubes. This buffer must be prepared extemporaneously.
    b. Highlight cell #1.
    c. Remove medium with p1000 Pipet.
    d. Place the cap, lower the cap (cap handler set Z-Axis to the cutting position noted in step 44).
    e. Adjust the focus using the phase-contrast.

⚠ CRITICAL: Make sure cap strip is in position as shown in Figure 10C, and that it is lowered to the slide as shown in Figure 10D.

    f. Turn on FITC (or another available fluorescent channel) to visualize the cells during the cut.
    g. Hit laser cut (Figure 10E).
    h. Put cap in higher position (cap handler) and move the cap strip.
    i. Move to next cell.

Note: Cells are captured into PCR tube caps containing 4.3 μL capture lysis buffer.

    j. When 8 cells (8 caps) are captured, the caps are placed on empty labeled tubes.
    k. Spin tubes immediately.
    l. Place tubes on dry ice.

Note: Tubes are kept on ice and frozen in dry ice directly after collection to minimize RNA degradation during sample collection and transport.

Pause point: The cell lysates that have been snap-frozen in dry ice on the last above, can be stored in a freezer at −80°C for up to 1 month.

Laser-captured single-cell sequencing

Timing: 2 h for step 58

Timing: 3 h for steps 59 and 60

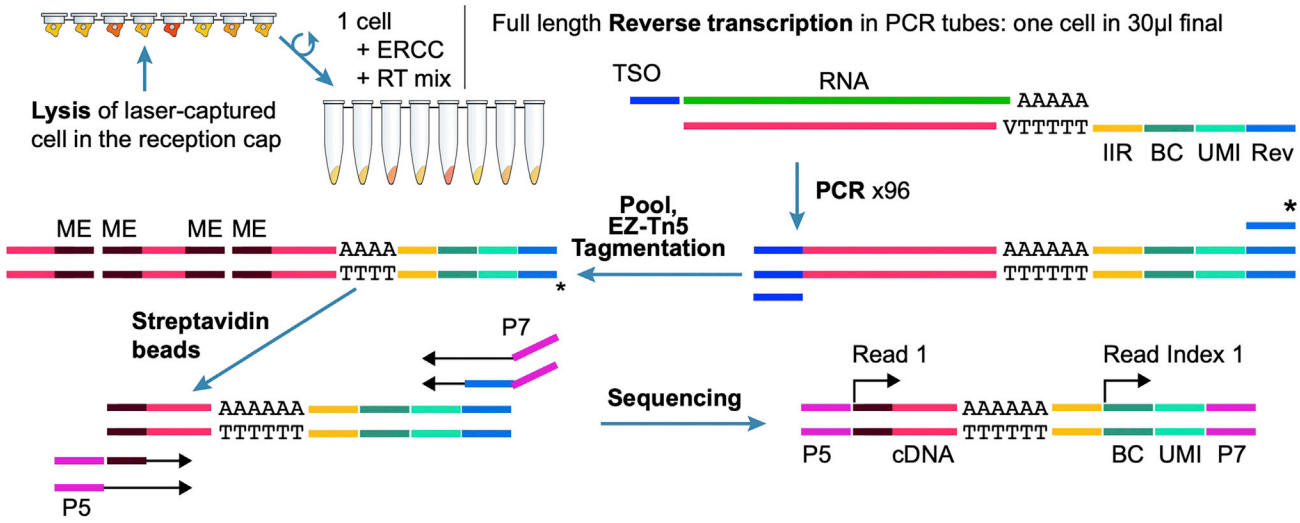

Figure 11. Preparation of isolated single-cell library for RNA sequencing

Laser captured single cells with predicted cell response were collected and lysed in domed caps of 0.2 mL PCR tubes and stored frozen. The lysed cells were spun into PCR tubes with an RT mix, ERCC spike-ins, and reverse transcribed with an RT primer that contained a priming site for amplification (Rev), a random sequence for RNA molecule identification (unique molecular identifier, UMI), a cell barcode (BC) and an Illumina Index Read (IIR). (The template switching oligonucleotide (TSO) adds a second PCR priming site during RT.) After PCR amplification the 3′ biotinylated (*) full-length cDNA was fragmented by tagmentation with EZ-Tn5 transposase (end-joined with mosaic end adaptors, ME) and the 3′ terminal fragments were isolated on Streptavidin beads. Sequences required for Illumina sequencing (P5, P7) were introduced by PCR prior to sequencing. See methods section for details, primer sequences in Tables 1 and 2. Figure from Meyer et al. ((Meyer et al., 2020), Figure S2).

This section focuses on the generation of retrotranscribed cDNA samples and sequencing libraries prior to RNAseq. Technically, any single-cell RNA sequencing strategy suited to isolated cells (in tubes) can be applied here. In the following paragraph, we described the original strategy that was used in Meyer M., Paquet A. et al. (Meyer et al., 2020). The cell lysates were used to perform retrotranscription in 0.2 mL PCR tubes with an RT mix containing ERCC (External RNA Controls Consortium) spike-ins, primers that introduce a priming site (Rev), a unique molecular identifier (UMI) sequence, a cell barcode sequence (BC) and an Illumina Index Read (IIR). After amplification, the 3′biotinylated full-length cDNA were tagmented with the EZ-Tn5 transposase. The 3′terminal fragments were then isolated on streptavidin beads. The P5 and P7 sequences required for Illumina sequencing were added by traditional PCR (see schematic in Figure 11). Primers sequences are provided in Tables 1 and 2.

58. cDNA preparation.
    a. Prepare 0.2 mL PCR tubes with the following pre-mix. Thaw the reagents on ice. Add 2.43 µL of a pre-mix containing 10,000 molecules Life Technology ERCC-spike-In Mix, 2.5 mM dNTPs and 3.7 µM single barcoded reverse transcription primer. Pre-mix containing stripes can be stored at −80°C until sample microdissection.
    b. Add only one cell in each tube containing this pre-mix by microdissection and brief centrifugation. Single-cell in pre-mix containing stripes can be stored at −80°C or be send in dry ice until further RT reaction.
    c. Add 8.27 µL 1.8× reverse transcription buffer (Invitrogen), 4.53 mM DTT, 1.8 M Betaïne, 10.8 mM $MgCl_2$, 3.62 µM template switching oligonucleotide, 0.6 U/µL Promega RNAse IN Plus, 7.5 U/µL Invitrogen SuperScript II reverse transcriptase.
    d. Reverse transcribes each tube in thermocycler 10 min at 25°C, 90 min at 42°C, 15 min at 70°C and kept at 10°C until PCR amplification.
    e. Amplify samples by PCR with KAPA HiFi Hotstart ready Mix for 20 cycles with 0.8 µM forward PCR primer, 0.8 µM biotinylated reverse PCR primer.

Table 1. Cell barcode adaptor sequences

Cell Barcode	Adaptor sequence with barcode
CGACACGC	TTAGCAGGTCGTGAGGTGTCATVNNNNNNNNNN{Cell_Barcode_1_90}GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTTTTTTTTTTTTTTTTTTTTTTTVN
ACGCGCTC	
TCGACACA	
CATAGTAC	
CACTCGTC	
ATATATGT	
GCGCTGTG	
CTGCGACG	
TATATGTA	
GCTAGCTG	
TCGTATAG	
CTGCAGAG	
ACATACTA	
GATATACA	
TATGCTAC	
GCAGCTGC	
CATGCTCT	
TGCAGACT	
TCACGTAC	
CTGAGTGC	
CGACTCTA	
ATCTGTGC	
TACGAGTA	
TCATCTCG	
AGAGCTGT	
TATACACT	
GTCGTGTG	
GCTATATC	
GCGCTCAT	
TGTCTGAG	
TCTCTATG	
GCACACTC	
ACACACAG	
GCTGTCAC	
ACGCGTGT	
CTCTGATC	
CGCAGCAC	
TGTGCTCG	
TCTCTGCA	
ATGCTCTG	
GACGCAGC	
ATCGTCGA	
CACGACTC	
AGCGTATG	
CTACTCGT	
TGCTGATG	
CTGTAGTA	
TGCGTGCT	
TGTGAGAT	
ATGCTGAT	
TACATAGC	

(*Continued on next page*)

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Table 1. *Continued*

Cell Barcode	Adaptor sequence with barcode
TCACATCA	
TATCGCTA	
GCAGCACA	
TCGCAGTC	
ACTCTCGA	
CATCGTCA	
CTAGCGCG	
ACTATCAG	
CACACTGT	
CGCGTGTA	
TGCTCTGA	
TGCATGAC	
TACACTCG	
GACGCTAG	
CTACAGCA	
CTCTATCG	
TGTAGTGT	
TCTGCATA	
AGCGCACA	
TGAGAGTG	
GTACTACA	
TCGAGATC	
GTACTGTC	
GATAGCGA	
ATATAGAC	
AGCAGATA	
TCGACTAC	
TGATAGCA	
TCACACGT	
TACTGACA	
GAGACTAT	
CGCGTCAG	
GCGCGAGC	
CTACGAGC	
AGACGCGT	
TACTGTGT	
TGTATACG	
GATACATG	
TAGACGAG	

f. Incubate samples 3 min at 98°C followed by 20 cycles at 98°C for 20 s, 64°C for 15 s, 72°C for 6 min followed by a final extension for 5 min at 72°C.
g. Pool the samples and purified with 0.8× Beckman Coulter SPRIselect beads to remove primers and small DNA fragments.
h. Place on the magnet until the solution clears, remove the supernatant and wash with 200 μL of 85% EtOH (freshly prepared), quick spin 5 s, put back on the magnet and remove any remaining EtOH by pipetting, dry 2 min at RT, and take off the magnet.
i. Elute in 30 μL nuclease free water by pipetting up and down 15 times and leave 2 min at RT. Put the tube back on the magnet.
j. When the solution clears, aspirate the supernatant, and save it for the next step at RT if used immediately. If not, the supernatant (cDNA) can be saved at 4°C for 72 h or at −20°C for up to 4 weeks.

Table 2. Primer sequences for isolated single-cell cDNA library preparation

Name	5′–> 3′ sequence	IDT ref number
Primer Fwd	ACAGAAGGTTGCCGTTCAGGTA	72493645
Primer Rev-biotin	/5Biosg/TTAGCAGGTCGTGAGGTGTCAT	72493642
TSO-96	ACAGAAGGTTGCCGTTCAGGTArGrGrG	72493644
P5-PE adapter	AATGATACGGCGACCACCGAGA TCTACACTCGTCGGCAGCGTCA GATGTGTATAAGAGACAG	72543148
P7 adapter	CAAGCAGAAGACGGCATACGAGA TTAGCAGGTCGTGAGGTGTCAT	72543149
P5 primer	AATGATACGGCGACCACCGAGAT	69287361
P7 primer	CAAGCAGAAGACGGCATACGAGAT	69287362
Tn5 Lower strand	/5Phos/CTGTCTCTTATACACATCT	67986456
Tn5 Upper strand	AGATGTGTATAAGAGACAG	67986455
read1	ACACTCTTTCCCTACACGACGCTCTTCCGATCT	
readindex1	GATCGGAAGAGCACACGTCTGAACTCCAGTCAC	

Note: Do not dry the pellet more than 2 min to avoid over drying. If that is the case, the resuspension can be difficult to achieve.

59. Library preparation.
    a. Batch of barcoded pools of cDNAs (typically between 10–20 ng of cDNA) can be purified one more time to concentrate final amount: group all pools in the same tube to a final volume of 100 μL completed with nuclease-free water, purify a second time with SPRIselect beads (0.8×), as performed above in steps 57g to 57j, and elute in 12 μL nuclease free water.
    b. Perform a quality and quantity control with a BioAnalyzer High Sensitivity DNA kit (ref 5067-4626, Agilent) on 1 μL of purified pooled cDNA. The cDNAs should be distributed between 200 bp to 9000 bp with a most at around 1500 bp as shown in Figure 12A.

    See troubleshooting problem 4.

    c. At room temperature, pipet 9 μL (typically 10–20 ng of cDNA) and add 4 μL TAPS buffer (50 mM TAPS-NaOH, pH8.5, 25 mM $MgCl_2$), 2 μL dimethylformamide and 5 μL of transposon.
    d. Incubate at 55°C for 7 min (as suggested by the manufacturer for cDNA longer than 2500 bp) and cool the samples to 10°C for 5 min.
    e. Add 4 μL of 0.1% SDS and incubate 10 min at 65°C to detach the transposase.
    f. Cool samples to 4°C.

    Alternatives: A commercial tagmentation kit (Illumina) can be used for library preparation.

    Tagmentation can alternatively be done with reagents from the commercial Nextera®XT Sample Prep Kit (ref 15032350, Illumina).

    Note: Tagmentation is the initial step in library prep where unfragmented cDNA is cleaved and tagged for analysis.

    g. Pipet 24 μL Dynabeads® MyOne™ Streptavidin C1 beads (Ref 65001, Invitrogen) in a 1.5 mL Eppendorf tube.
    h. Prepare Dynabeads® MyOne™ Streptavidin C1 beads according to the manufacturer's instructions.
    i. Resuspend beads in 10.5 μL nuclease free water to capture the biotinylated 3′terminal fragments in the following steps.

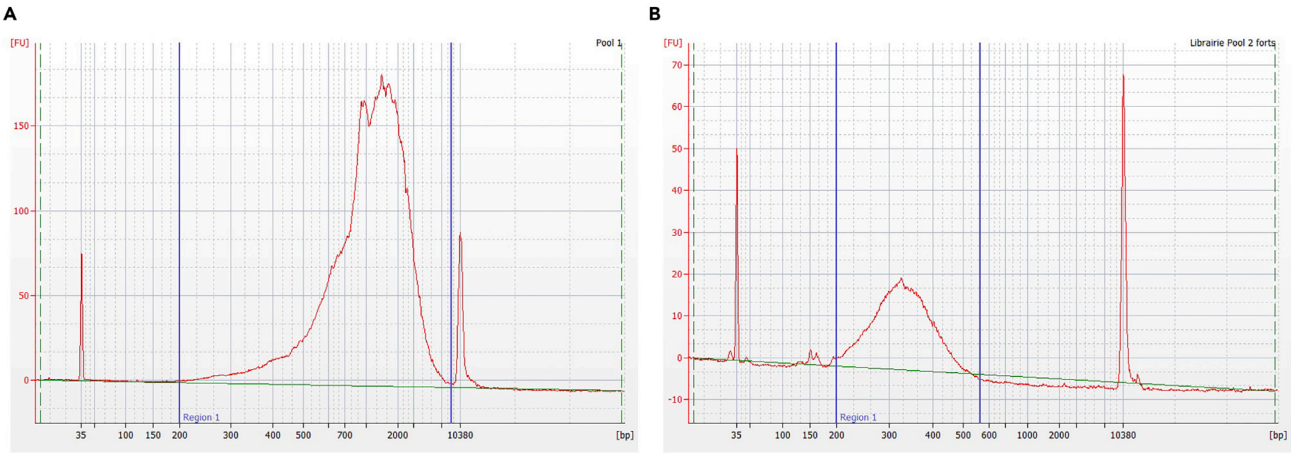


Figure 12. Representative BioAnalyzer cDNA profile

(A) Electropherogram view of 1 μL of cDNA sample run on an Agilent BioAnalyzer High Sensitivity DNA Chip. A region comprised between 200 bp and 9000 bp is represented by blue lines on x-axis. This frame indicates cDNA accurate concentration in the corresponding bioanalyzer region table.

(B) Electropherogram view of 1 μL of final library on an Agilent BioAnalyzer High Sensitivity DNA chip. The region comprised between 200 bp and 550 bp (blue lines on x-axis) is used for library quantification in the Bioanalyzer corresponding region table. Minor peaks below 200 bp may be observed and correspond to adapter dimer peaks. If peak below 200 bp is more prominent, add nuclease-free water to a final volume of 100 μL and repeat SPRI-Select purification a second time (step 59l) and then repeat Bioanalyzer quantification.

j. Amplify 5′ terminal fragments in 25 μL with KAPA HiFi HotStart Ready Mix and 0.5 μM reverse library primer, 0.5 μM forward library primers and 0.125 μM extended forward primers.
k. Proceed to a gap filling step by incubating at 75°C for 3 min followed by 98°C for 30 s, 15 cycles of 98°C for 10 s, 55°C for 30 s, and 72°C for 30 s and 72°C for 2 min.
l. Fill the volume of Illumina library obtained to 100 μL, add 90 μL SPRIselect beads for size selection of fragments > 200 bp. Pipet mix 15 times with pipet at 180 μL, incubate 5 min at RT.
m. Place on the magnet. Remove the supernatant.
n. Wash beads with 200 μL 85% EtOH. Place on the magnet and remove the residual EtOH by pipetting.
o. Dry 2 min at RT.
p. Elute the library by pipetting 15 times with 10 μL nuclease free water. Leave the solution 2 min at RT (off the magnet), then put back on the magnet until the solution clears, and pipet out the supernatant (Library).
q. Perform a quality and quantity control with a BioAnalyzer High Sensitivity DNA kit (ref 5067-4626, Agilent) on 1 μL of purified libraries. The final library should be distributed between 200 bp to 550 bp with a most at around 350 bp as shown in Figure 12B.

See troubleshooting problem 5.

60. Sequencing: Libraries were sequenced with a NextSeq500/550 High Output V2 kit (75 cycles) that allows up to 91 cycles with an index1 read of 20 nucleotides (8 bp cell barcode and 12 bp Unique Molecular Identifier, UMI; Table 1) and a read1 of 71 nucleotides for cDNA sequencing with custom sequencing primers (Table 2).

Bioinformatics analysis

61. cDNA alignment: after Fastq file production using Illumina bcl2fastq software, the first step is to align the read1 against human build reference using a splice-aware aligner. In the following example, cDNA reads mapping was performed using STAR (Dobin et al., 2013) against human hg19 following Encode RNA-seq recommendations. We obtain a BAM file composed of SAM records describing how/where cDNA reads aligned to the reference genome.

Note: Encode RNA-seq recommandations (https://www.encodeproject.org/documents/cede0cbe-d324-4ce7-ace4-f0c3eddf5972/@@download/attachment/ENCODE%20Best%20Practices%20for%20RNA_v2.pdf.).

Cell barcodes and UMI processing: *TagBamWithReadSequenceExtended* pipeline from Drop-seq toolkit (Macosko et al., 2015) was then used to tag the SAM records with the cell barcode of the cell from which the cDNA originated (named "XC") and the UMI (named "XM") extracted from index1 reads (cell barcode: 1–8 nt, UMI: 9–20 nt).

Note: Drop-seq toolkit (Macosko et al., 2015) is accessible at https://github.com/broadinstitute/Drop-seq.

Note: The BAM file of our example fate-seq are accessible at GEO accession number GSE138832 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE138832).

62. Digital Gene Expression count matrix production: *TagReadWithGeneExon* pipeline from *Drop-seq.jar* toolkit is then used to tag SAM records of cDNAs falling inside gene locus with a gene name tag (GE in our example). Then *DigitalExpression* pipeline using edit distance of 1 is used for production of sc-RNAseq count table (counts of UMI).

Note: The sc-RNAseq count table (Digital Gene Expression count matrix) of our example is also accessible at GEO accession number GSE138832 (link in step 61 and the second Note).

Single-cell data analysis

For the single-cell data analysis, the cell response phenotypes and the scRNAseq count table will be required.

63. The cell response phenotypes are the ones that were obtained previously from the live-cell experiments (high and low responders from step 35).
64. The count table is generated from the bioinformatics analysis above.
65. Standard single cell data analysis workflows can be used, such as workflows based on R packages Seurat, scran or edgeR. As an example, the single-cell data analyses performed for the fate-seq of the HeLa cells described in this protocol treated with TRAIL, are available here: https://github.com/jrxlab/fate-seq_proc, and the expected results are shown in Figure 13.

EXPECTED OUTCOMES

Coupling signaling dynamics and gene expression in the same cell is a long-standing pursuit in multimodal single-cell analyses, to link functional response phenotypes to molecular profiles. To achieve this goal, we developed fate-seq, a workflow coupling three single-cell approaches: a predictive measure of single-cell drug response by *live-cell microscopy*, in order to isolate predicted sensitive and predicted resistant cells by *laser-capture microdissection*, and then profile each captured cell by *single-cell RNA sequencing* directly (with no cell dissociation, sorting, nor microfluidics handling).

We illustrate this protocol with an example showing how it is possible to isolate single-cell based on their predicted response phenotypes (Figure 14). We were able to separate sensitive cells from resistant cells during a treatment that induce fractional killing (e.g., TRAIL treatment) and isolate cell individually, before they commit to a definitive response that alters their integrity or their molecular context (transcriptome and proteome).

In this example, we used a FRET biosensor (IC-RP) to visualize the caspase 8 activity in tumor cells within 50 min of TRAIL treatment at a concentration of 25 ng/mL that is close to its IC_{50} in this cell

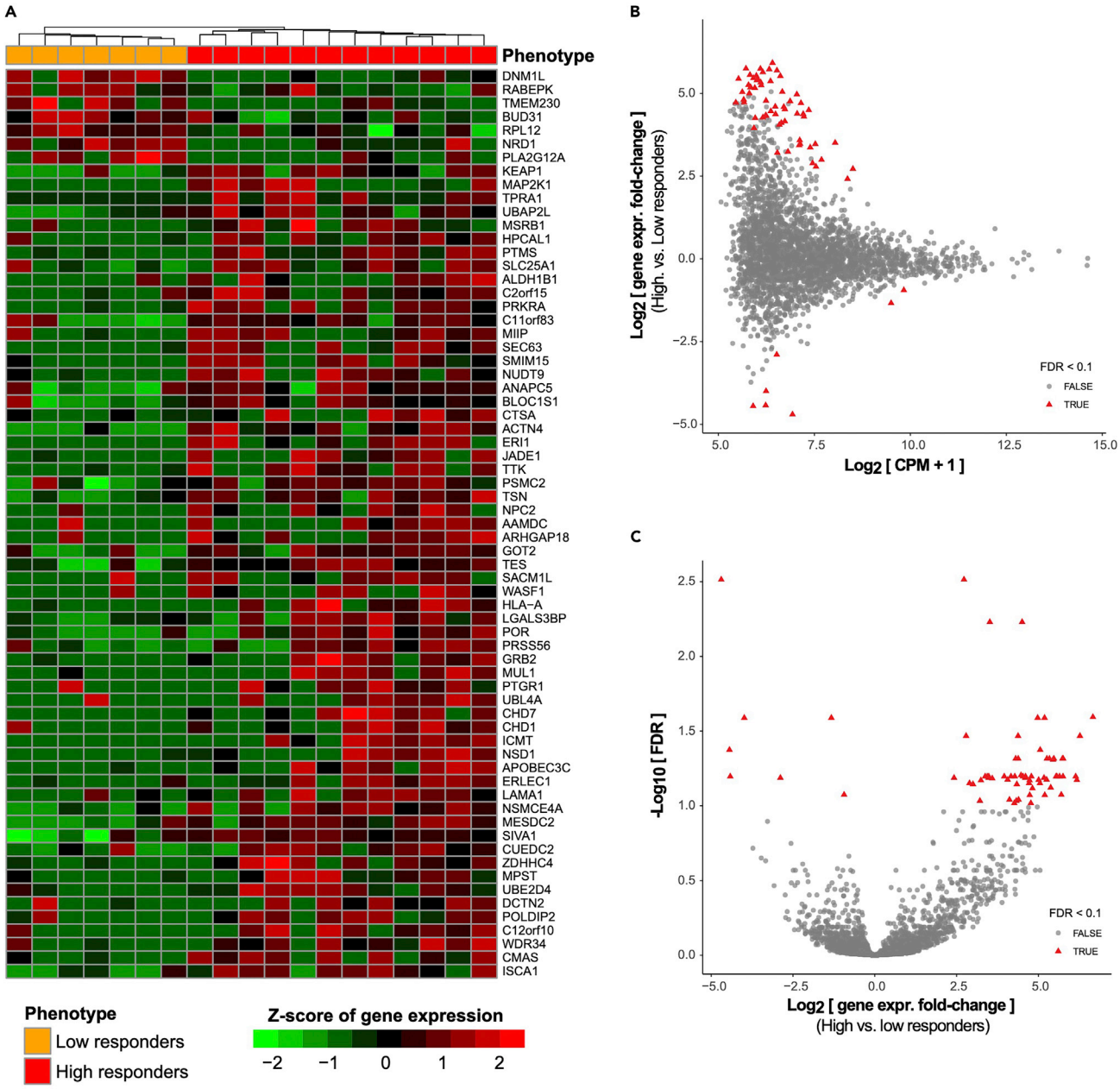


Figure 13. Bioinformatics analyses producing a drug-efficacy gene set from fate-seq data

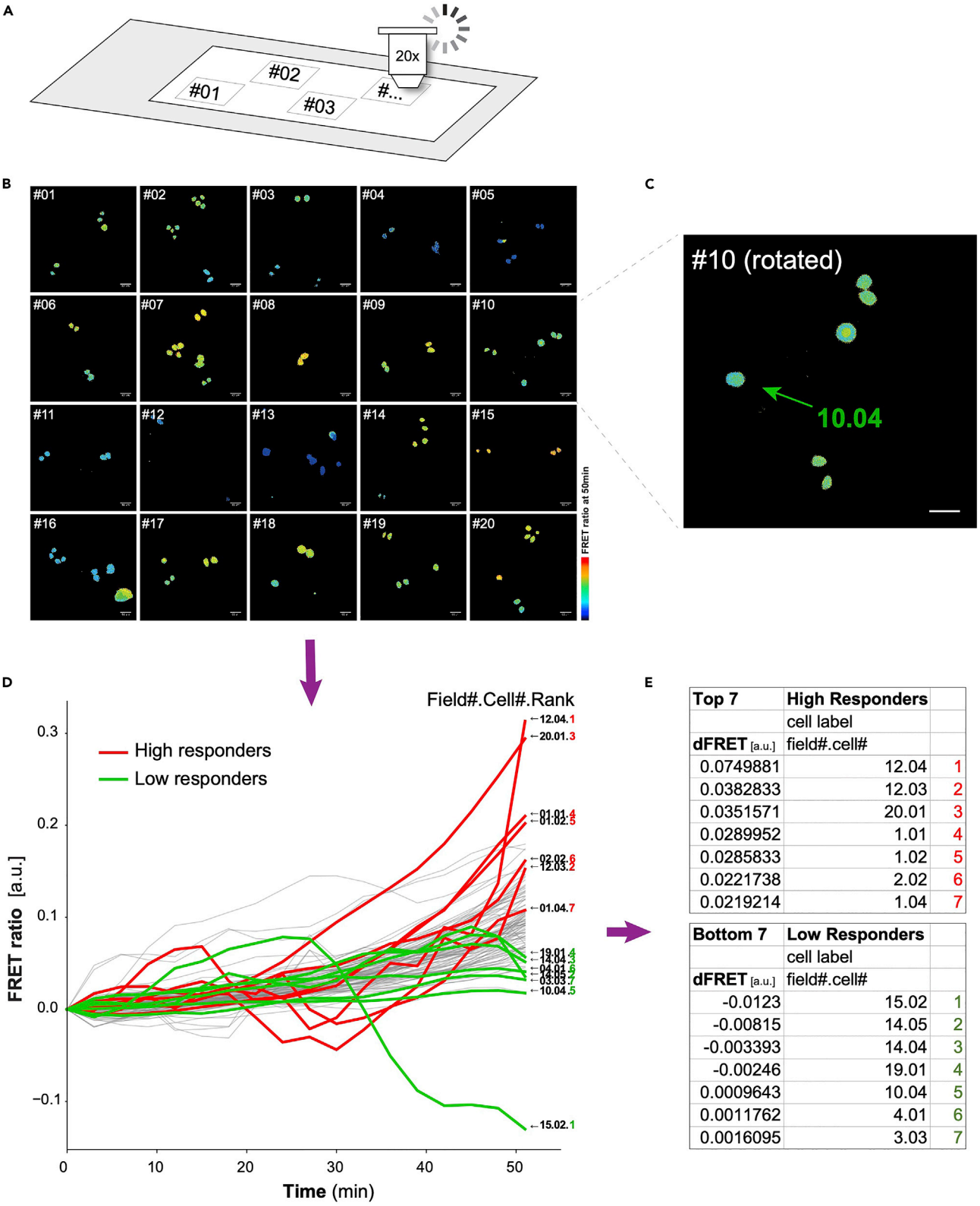
The cell response (Phenotype) obtained from live-cell imaging is used to define high and low responder cells in a differential analysis of the matched single-cell RNAseq data, to find marker genes of drug resistance. Here, we have represented the results of such an analysis based on the data from (Meyer et al., 2020), where candidate differentially expressed genes are defined as genes with FDR < 0.1.

(A) Heatmap of gene expression data for genes with FDR < 0.1, with High/Low Responder phenotype labels displayed in the color bar at the top.

(B) MA-plot: x-axis represents the average gene expression level for each gene on $\log_2$ scale; y-axis represents the difference in gene expression levels between high versus low responders (cell phenotypes) on $\log_2$ scale. Red triangles represent the differentially expressed genes with FDR < 0.1.

(C) Volcano-plot: x-axis represents the difference in gene expression levels between high versus low responders (cell phenotypes) on $\log_2$ scale; y-axis represents the FDR (- $\log_{10}$ FDR). Red triangles represent the differentially expressed genes with FDR < 0.1.

line. In this time interval, some cells exhibit a clear commitment to TRAIL, depending on the caspase 8 activation rate. This single-cell method enables the selection of the predicted tolerant versus sensitive cells at the drug IC_{50}, and the differential gene expression analyses of both subclasses, to



Top 7	High Responders	
	cell label	
dFRET [a.u.]	field#.cell#	
0.0749881	12.04	1
0.0382833	12.03	2
0.0351571	20.01	3
0.0289952	1.01	4
0.0285833	1.02	5
0.0221738	2.02	6
0.0219214	1.04	7

Bottom 7	Low Responders	
	cell label	
dFRET [a.u.]	field#.cell#	
-0.0123	15.02	1
-0.00815	14.05	2
-0.003393	14.04	3
-0.00246	19.01	4
0.0009643	10.04	5
0.0011762	4.01	6
0.0016095	3.03	7

Figure 14. Example fate-seq experiment coupling live-cell microscopy with laser-capture of the same cells, using predictive metrics of drug response
(A) Schematic of a FrameSlide showing 20 fields of interest.
(B) Example live-cell microscopy experiment for IC-RP fluorescence measurements in each identified cell. Fifty-minute live-cell microscopy experiment was performed for each cell cultured on a membrane ready for micro-dissection. About 20 fields of cells are monitored, and all cells are tracked for their FRET signal in time. Scale bars, 68μm.
(C) Field #10 showing the cells number 4 that will be selected for laser-capture micro-dissection (cells are labeled in numerical order from left to right then top to bottom). Scale bar, 68μm.
(D) Single-cell trajectories of FRET ratio from the IC-RP are reported for each cell tracked to perform the fate prediction.
(E) The caspase-8 activation rate (dFRET, time derivative of FRET ratio (Roux et al., 2015)) is calculated 50 min post TRAIL treatment (25 ng/mL) for each single-cell, as an accurate predictor of cell response over time (Meyer et al., 2020). The top 7 predicted cells of each group (High and Low Responders) can be laser-captured and further processed. In this process, one same cell is phenotyped in live-cell microscopy, isolated by laser-capture micro-dissection and profiled by single-cell RNA-seq.

determine the TRAIL efficacy molecular signature. This approach can be transferred to different treatments inducing caspase-8 activation (e.g., FAS ligand, TNF-alpha and DR4/5 agonists), or any cell signaling that is predictive of cell response, to assess the molecular profiles of drug-tolerant cells.

Here, we propose a multimodal profiling approach to couple a predictive live-cell assay to single-cell isolation *in situ*, that enables the omics analyses of each same cell that has been individually phenotyped. As an example, we identified the molecular factors causing the incomplete response of sensitive cell types at the IC_{50} of a cancer therapeutic, TRAIL (Figure 13). We show that our framework allows the identification of molecular factors of cell decision upon treatment, rather than induced factors by the treatment.

QUANTIFICATION AND STATISTICAL ANALYSIS

Live-cell imaging analyses

For the analysis of the FRET signal, the background-subtracted CFP and YFP images were divided to get a ratiometric image (CFP/YFP) using ImageJ and custom plug-ins to track cells. Signals were normalized by subtracting the minimum value across all time points from each single-cell time course and the average trajectory of the corresponding drug vehicle-treated cells. Cell death assessment was performed by visual inspection in transmitted light of the cell morphology change, caspase-8 activation rates were determined from CFP/YFP ratio trajectories (Roux et al., 2015).

Statistical analyses on live-cell experiments

Statistical significance of the difference in maximal activation rates should be assess using Wilcoxon rank sum test, statistical significance of the surviving fractions in experiments repeats should be assessed using two-sample t-test, and Cell Survival probabilities (Empirical Survivor Function, eCDF) should be compared with a two-sample Kolmogorov-Smirnov test.

LIMITATIONS

In this protocol, we use a FRET biosensor. This type of analyses requires the use of a fluorescence microscope with the appropriate fluorescent channels and an environmental chamber that carefully monitors the appropriate temperature and CO_2 levels. The other limitation is the nature of the biosensor itself (target sequence, FRET pairs). In this study, we focus on the activation of caspase 8 which is a marker of commitment to apoptosis (signaling of death receptors). However, this workflow enables the separation of cells based on their precited response phenotype, which is not limited to caspase 8, and can be used to study any cellular dynamic signaling, which heterogenous behavior can inform on the molecular determinants of cell decision.

TROUBLESHOOTING

Problem 1

Low viral transduction efficiency.

Potential solution

Depending on the cell type of interest, the infection step can be less effective, and virus titer should be adjusted. We recommend concentrating the viral particles by ultracentrifugation (225,000 g for 2 h at 4°C).

Problem 2

The fluorescence excitation used can be phototoxic for cells.

Potential solution

Decrease exposure time for CFP (and possibly YFP) below 80 ms (Depending on the setup the exposure time for CFP could be as short as 25 ms and should not exceed 200 ms.) Signal/Noise ratio should be greater than 1.5.

Problem 3

The focus is loss during experiment.

Potential solution

Depending on the microscope setup, focus control can be used to avoid drift in focus. In addition, careful handling, such as adding the treatment very slowly and dropwise, may be required. After treatment, it is advised to wait for the plate to settle in the plate holder in the environmental chamber (~20 min) before adjusting the focus prior to running the experiment.

Problem 4

If recovered cDNA amount is less than 10 ng.

Potential solution

A minimum of 3 reactions containing one cell is needed to quantitate cDNA with a BioAnalyzer High Sensitivity kit (Agilent), 8 or more pooled reactions is needed to recover around 10 ng of cDNA. Increase cDNA PCR cycle number to 22 instead of 20 if fewer reactions are pooled.

Problem 5

If fragments after tagmentation are too small and library quantity obtained is therefore too low after size selection of fragment above 200 bp.

Potential solution

Decrease tagmentation incubation to 5 min instead of 7 min if less than 10 ng is obtained. Transposase will cut cDNA in smaller fragments than attempted if starting material is not sufficient.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jérémie Roux (jeremie.roux@univ-cotedazur.fr).

Materials availability

Plasmids and cell lines generated in this study are available from the lead contact upon request, with an MTA when required.

Data and code availability

Fate-seq RNA sequencing raw data can be accessed on GEO, accession # GSE138832: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE138832, Python code for Live-cell phenotyping and capture is available at: https://github.com/jrxlab/live_cell_phenotyping_capture_proc (https://doi.org/10.5281/zenodo.6671339).

R code for RNA sequencing data analyses is available at: https://github.com/jrxlab/fate-seq_proc.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101600.

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AUTHOR CONTRIBUTIONS

Methodology, B.B., A.P., M.-J.A., M.M., K.L., L.P., A.C., M.P., R.W., and J.R.; software, A.C., M.P., and J.R.; resources, P.B., P.H., and J.R.; writing – original draft, B.B. and J.R.; writing – review & editing, B.B., A.P., M.-J.A., K.L., R.W., and J.R.; visualization, B.B. and J.R.; funding acquisition, J.R.; supervision, J.R.

DECLARATION OF INTERESTS

Methods and results presented here are part of a patent application (J.R. and A.P.).

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