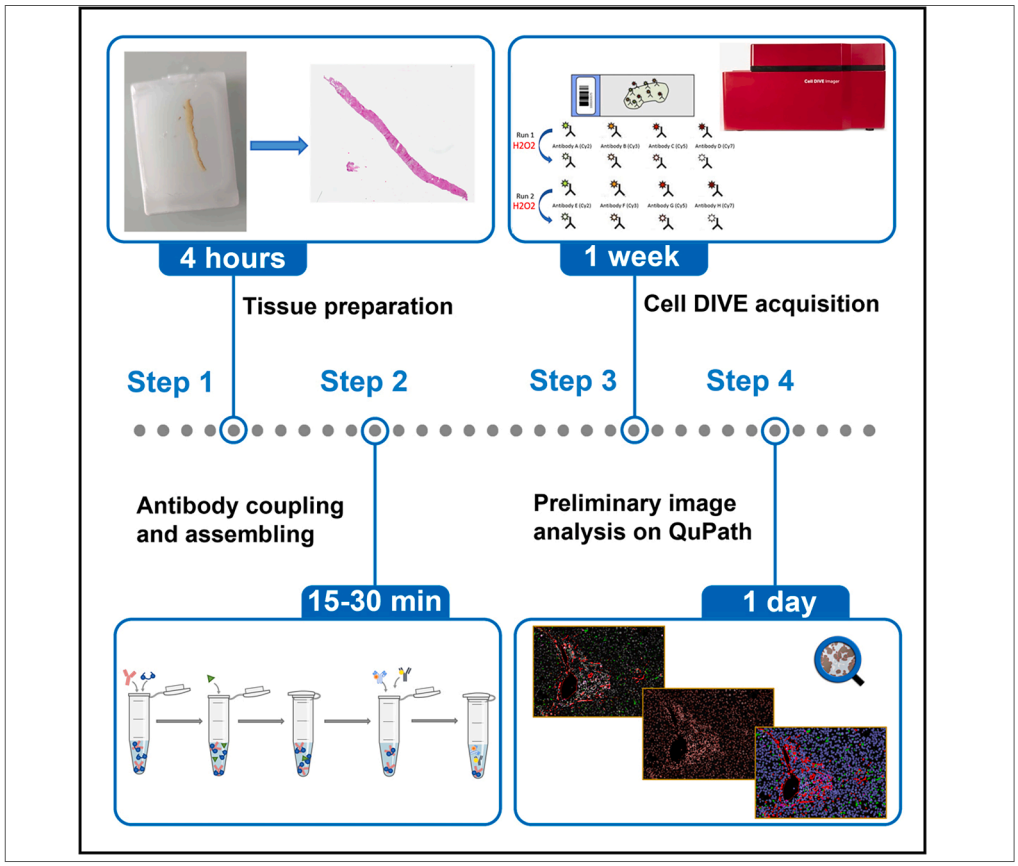
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Protocol

Protocol for studying the immune microenvironment of human hepatocellular carcinoma by Cell DIVE multiplex immunofluorescence imaging



Understanding tumor heterogeneity by examining how cells are organized in tissues is essential to better decipher the biological processes involved in cancer and to improve therapeutic management. Here, we present a protocol for a multiplex immunofluorescence (mIF) technique that enables the detection of an array of protein markers and allows immunophenotyping on a single section of human hepatocellular carcinoma. We describe the procedures for antibody labeling, mIF image acquisition using Cell DIVE, and preliminary analysis using QuPath software.

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

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Highlights

Step-by-step procedure for tissue and antibody preparation

Instructions for Cell DIVE image acquisition

Image assembly, cell segmentation, and phenotyping in QuPath

Adaptable to different tissues and pathological contexts

Delaporte et al., STAR Protocols 6, 103946 September 19, 2025 © 2025 The Authors. Published by Elsevier Inc.
https://doi.org/10.1016/j.xpro.2025.103946

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Protocol for studying the immune microenvironment of human hepatocellular carcinoma by Cell DIVE multiplex immunofluorescence imaging

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https://doi.org/10.1016/j.xpro.2025.103946

SUMMARY

Understanding tumor heterogeneity by examining how cells are organized in tissues is essential to better decipher the biological processes involved in cancer and to improve therapeutic management. Here, we present a protocol for a multiplex immunofluorescence (mIF) technique that enables the detection of an array of protein markers and allows immunophenotyping on a single section of human hepatocellular carcinoma. We describe the procedures for antibody labeling, mIF image acquisition using Cell DIVE, and preliminary analysis using QuPath software.

BEFORE YOU BEGIN

Hepatocellular carcinoma (HCC) is the sixth leading cause of cancer-related death worldwide, mainly due to the limited options for targeted therapy and both intra- and inter-tumor heterogeneity.[1] The immune microenvironment plays a key role in the pathogenesis of the disease and the response to treatment.[2] It is therefore necessary to characterize the immune cell subsets, their functional status and their spatial arrangement in the tumor microenvironment to better understand this heterogeneity and to tailor the therapeutic strategy to each individual.

The protocol below uses a specific immunophenotyping panel applied to slides of human livers from patients with hepatocellular carcinoma. This panel is also suitable for studying immune infiltration in the human liver in other contexts and can be adapted to tissues other than the liver.

The following protocol describes the specific steps for multiplex immunofluorescence imaging using the Cell DIVE[3] technology on human liver biopsies (Figure 1). A key requirement for this protocol is the availability of the Cell DIVE Imager instrument and the Mx Workflow and Cell DIVE Acquisition software.

Users will need to modify and optimize the necessary antibody panels and custom markers as needed for their studies.

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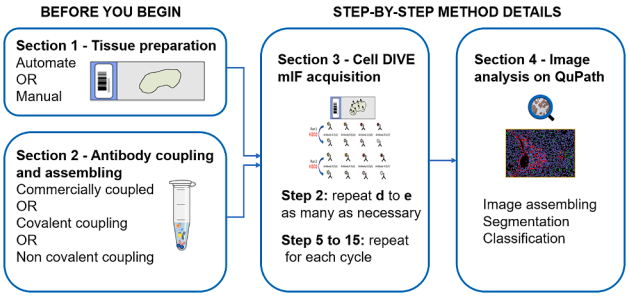


Figure 1. Schematic summary of the workflow for tissue processing, antibody preparation, image acquisition, and treatment

Institutional permissions

This protocol uses human tissue. All experiments were approved by the personal protection committee under the French Ministry of Higher Education and Research. Please note that ethical permissions should be obtained by ethical committee to perform this protocol.

Tissue preparation

Timing: 4 h

This section is for the deparaffinization, antigen retrieval and non-specific blocking steps of FFPE tissue slides using the Roche Ventana Discovery Ultra automated system. Another automated system may be used depending on user convenience as long as antigen retrieval is performed at pH 6 and 9. An alternate manual protocol is also proposed.

Note: The Roche Reaction Buffer can be substituted by 1X PBS solution.

1. Fix your tissue in formol and prepare the embedding into paraffine blocks according to standard histology procedures.[4]
2. Cut a 4 µm tissue slide from a histological block of formalin-fixed, paraffin-embedded tissue using a microtome.
   a. Pre-heat the oven at 60°C.
   b. Collect the tissue on a SuperFrost slide.
   c. Place the slide in an oven at 60°C for 1 h to allow the tissue to adhere as much as possible to the glass. It is also possible to leave the slide for 16 h at 37°C.

   Note: Because Cell DIVE technology allows the entire slide to be imaged, it is possible to place multiple tissue sections on the same slide. For example, a tumor sample and a non-tumor sample from the same patient.

3. Perform the deparaffinization and antigen retrieval steps using the automated slide staining system.

   Note: For deparaffinization, program 4 min of Roche Ez Prep solution at 75°C and for antigen retrieval, program two consecutive 16-min cycles at 100°C in Roche Cell Conditioning Solution CC1 buffer at pH 9, then in Roche Cell Conditioning Solution CC2 buffer at pH 6.

4. Wash the slide in a staining jar filled with the Roche reaction buffer performing smooth back and forth movements for 5 min.

   Note: The automated system leaves an oily film on the slide. It is therefore important to rinse the slide well at this stage to allow the subsequent solutions to penetrate the tissue.

Alternate method for deparaffinization and antigen retrieval

The Cell DIVE imaging is compatible with any automated system for deparaffinization and antigen retrieval. If you don't have access to an automated system, you may perform a manual protocol.[5]

- Incubate the slide at 37°C for 30 min.
- Deparaffinize sections by immersing the slides in 3 successive xylene baths for 10 min each.
- Rehydrate by passing slides through two changes of 100% ethanol for 5 min each and 95, 70, and 50% ethanol for 2 min each.
- Rinse in tap water for 5 min.

Note: Antigen retrieval is performed using two steps with Citrate Buffer Antigen Retrieval Solution (pH 6.0) then Tris–EDTA Buffer Antigen Retrieval Solution (pH 9.0).

- Warm a water bath until 80°C.
- Place the slides in a staining jar filled with Citrate Buffer Antigen Retrieval Solution (pH 6.0) and cover with a plastic film.
- Incubate the staining jar for 20 min in the water bath.
- Place the slides in a staining jar filled with pre-warmed Tris–EDTA Buffer Antigen Retrieval Solution (pH 9.0) and cover with a plastic film.
- Incubate the staining jar for 20 min in the water bath.
- Allow the slides to cool (18-22°C) in the staining jar filled with retrieval buffer for 20 min.
- Wash in 1X PBS before continuing with the immunostaining protocol.

5. Permeabilize the tissue section.
   a. Add 150 µL (or enough to entirely cover the tissue section) of the permeabilization solution (as described in the "Material and equipment" section).
   b. Incubate the slide for 10 min.
   c. Wash in Roche reaction buffer with slight agitation (60 to 100 rpm) of the slide for 2 min.
6. Saturate the tissue section to avoid non-specific binding.
   a. Add 150 µL (or enough to entirely cover the tissue section) of the blocking solution (as described in the "Material and equipment" section).
   b. Incubate the slide at (18-22°C) for 60 min.
   c. Wash in a staining jar in reaction buffer (50-100 mL) with slight agitation of the slide for 2 min.
7. Label the nuclei with DAPI by applying 150 µL (or enough to entirely cover the tissue section) of a solution 10 µg/mL for 10 min then wash the slide in reaction buffer (50-100 mL) with slight agitation for 2 min.
8. Add a few drops (2-3) of DAPI-mounting medium to the slide and position the coverslip.
   a. Wait 2 min for the mounting medium to dry.
   b. Clean the slide meticulously to remove all micro-bubbles and any traces of dirt.

Note: We recommend to use DAPI-containing mounting medium in addition to DAPI staining, in order to obtain a strong nuclear signal that will facilitate the nuclei-based image registration step (see section acquisition of mIF image).

Note: Use a minimum volume of mounting medium to facilitate subsequent removal of the coverslip (see section acquisition of mIF image).

Note: You can pause here and keep the slides horizontally in the dark for up to two weeks at 4°C.

Antibody coupling

Timing: 30 min (for step 9) or

Timing: 15 min (for step 10)

Many fluorescent-labeled antibodies are present in the market that fit with Cell Dive imaging. If your choice of antibody is not available with the appropriate fluorochrome, we propose two different coupling methods. The first method is based on covalent coupling and removal of unbound dye using desalting columns. This method is applicable with a large variety of fluorophore from different suppliers but requires an amine-free buffer and a concentrated antibody (0.5–1 mg/mL). It is more suitable for large quantities of antibodies. The second method is quicker and compatible with most primary antibodies regardless of their buffer composition. Based on a specific interaction between the dye and the constant immunoglobulin fragment, only the isotype of the primary antibody and the host species need to be taken into account. Small amount of diluted antibody may be used.

⚠ CRITICAL: The Cell DIVE dye inactivation process is only compatible with cyanine-based dyes. Make sure you are using one of the following dyes in each channel: Green (Cyanine 2, C 450); Orange (Cyanine 3, AF 555, CF 555); Far-Red (Cyanine 5, AF 647) or NIR (Cyanine 7, AF 750, CF 770). The blue channel is reserved for DAPI staining and is mandatory for image alignment.

Note: These two methods are mutually exclusive, perform either step 9 OR step 10.

9. Covalent coupling to a synthetic fluorochrome.
   This method is performed with an amine-reactive probe and a follow up is enabled by microvolume spectrophotometry (NanoDrop). Suggested providers are Biotum Mix-n-Stain Antibody Labeling Kits or Invitrogen Standard Amine Antibody Labeling Kits.
   a. Assess the antibody concentration, provided by the supplier or by nanodrop microvolume spectrophotometry at 280 nm.

   Note: If the antibody is devoid of carrier protein (albumin, gelatin, …) use: concentration of antibody (mg/mL) = A280 × 0.74.[6]

   Note: If the solution contains other proteins than antibody, approximate the total protein concentration by 1 unit A280 = 1 mg/mL. Example: anti-Glypican3 antibody provided in TRIS containing 1% BSA. Total A_{280} = 0.762 UA, equivalent to 0.76 mg/mL of total protein.

   b. Check whether the antibody concentration and buffer are compatible with the coupling, in particular the buffer should contain no free amine (eg TRIS) and the concentration of the antibody should be 0.5 to 1 mg/mL. Refer to the dye provider.
   c. If the antibody meets these criteria, skip to g. If not, perform a desalting and concentration of the antibody is achieved on an ultracentrifugation column (step d. to f.).
   d. Place an appropriate volume of the antibody (up to 400 µL) in the Amicon ultra concentrator and centrifuge at 14,000 g for 1 min, or until all of the liquid has filtered into the lower chamber.
   e. Add 400 µL of PBS to the Amicon ultra concentrator and centrifuge at 14,000 g for 1 min., or until all of the liquid has filtered into the lower chamber.
   f. Add an appropriate volume of PBS to the Amicon ultra concentrator to obtain a final antibody concentration of 0.5–1 mg/mL. Resuspend the antibody by carefully pipetting up and down and recover it from the device.
   g. For each 10 µL of antibody, add 1 µL of 1 M sodium bicarbonate pH 8.3 (final bicarbonate concentration 0.1 M), or dye reaction buffer provided by the supplier.
   h. Mix antibody with amine-reactive dye in a 3-fold molar excess, or follow dye supplier ratio. Incubate for 15 to 30 min at (18-22°C) in the dark.
   i. Coupling efficiency may be assessed by nanodrop spectrophotometry. An example of spectra before and after coupling is presented Figure 2.

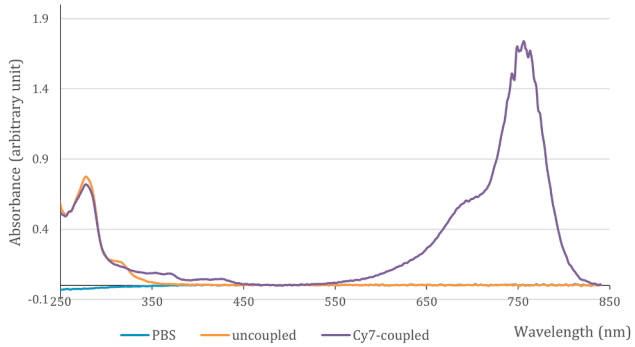


Figure 2. Example of absorption spectra for the covalent coupling method

Absorption spectra were recorded on NanoDrop for PBS buffer (blue), desalted antibody before coupling (orange) and Cy7-coupled antibody. Absorbance at 280 nm allows protein concentration assessment. Peak at 750 nm correspond to the cyanine 7.

10. Non covalent coupling (alternative method).
    This method is performed with the FlexAble antibody labeling kit from Proteintech.

⚠ CRITICAL: Make sure to use the correct kit for the species and isotype of the antibody.

a. Make sure you've equilibrated all reagents to (18-22°C) before carrying the assay.
b. For 0.5 µg of the primary antibody to be coupled, add 1 µL of FlexLinker. If needed, bring up volume to 8 µL with FlexBuffer.
c. Mix gently and incubate for 5 min at (18-22°C) in the dark.
   EXAMPLE: anti-PD-L1 antibody provided at 874 µg/mL in an HEPES buffer containing 100 µg/mL BSA. Set up has determined that 0.9 µg were required for one slide. Use 1 µL antibody, 2 µL FlexLinker and 5 µL of FlexBuffer.
d. Add 2 µL of FlexQuencher for 1 µL of FlexLinker.
e. Mix again and incubate for another 5 min at (18-22°C) in the dark. The antibody is now ready to be used.

Note: Primary antibodies of the same species must be labeled individually. Primary antibodies of different species can be labeled simultaneously using the appropriate species-specific linker. Once the primary antibodies are labeled, they can be combined and used as desired in your staining panel. In particular, it is possible to combine several labeled antibodies of the same species within the same round or in successive rounds. Our panel for this protocol is given in Table 1.

Note: Both monoclonal and polyclonal antibodies can be used in the Cell DIVE process, but keep in mind that polyclonal antibodies provide a stronger signal but often have a higher background.

Note: When choosing the dye/antibody combination to use, it is essential to consider antigen abundance, tissue autofluorescence and fluorochrome brightness. As Cyanine 5 is brighter than other cyanines, the far-red channel is a highly efficient detection channel, recommended for low-abundance targets. Furthermore, tissues are often autofluorescent in the green channel, so choose to put a target that stands out strongly in this channel. If an antigen is low abundance consider conjugation to dyes in the far-red channel, as cyanine 5 has a higher brightness. If an antigen is high abundance consider conjugation to dyes in the green and near infrared channels, as the staining will exceed background.

Table 1. Panel of 20 antibodies for Cell DIVE imaging of human hepatocellular carcinoma

Run 1	CD15 [SSEA-1] (1/50) Alexa Fluor 488	CTLA4 [CAL49] (1/50) Alexa Fluor 555	PD1 [EPR4877] (1/100) Alexa Fluor 647	PD-L1 [E1L3N] (1/25) XP 750
Run 2	CD11c [EP1347Y] (1/100) Alexa Fluor 488	CD16 [EPR16784] (1/100) Alexa Fluor 555	CD11b [D6X1N] (1/80) Alexa Fluor 647	CD69 [FN50] (1/80) Alexa Fluor 750
Run 3	CD64 [10.1] (1/50) Alexa Fluor 488	CD68 [KP1] (1/50) CF 555	CD8a [C8/144B] (1/100) Alexa Fluor 647	CD56 [123A8] (1/20) Alexa Fluor 750
Run 4	CD20 [EP459Y] (1/100) Alexa Fluor 488	CD31 [EPR3094] (1/20) Alexa Fluor 555	CD4 [EPR6855] (1/20) Alexa Fluor 647	CD3ε [C3e/1308] (1/20) DyLight 755
Run 5	Pan-CytoKeratin [C11] (1/100) Alexa Fluor 488	LAG3 [D2G4O] (1/25) XP AF 555	Wheat Germ Agglutinin (WGA) (1/50) CF 640R	Glypican-3 [1G12] (1/20) CoraLite Plus 750

The antibody clone has been placed in square brackets and its dilution in parentheses. In the panel below, Glypican-3 and PD-L1 antibodies were coupled by the covalent and non-covalent methods respectively. The other antibodies were purchased pre-coupled.

Note: For each antibody of the panel, it is important to test first for the optimal dilution on its own and then to validate the dilution into the labeling cycle, as the optimal dilution can vary in the mixture.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Alexa Fluor 488 anti-mouse/human CD15 (SSEA-1) (1/50)	BioLegend	Cat# 125610; RRID:AB_1089189
Alexa Fluor 555 Anti-CTLA4 antibody (CAL49) (1/50)	Abcam	Cat# ab283489; RRID:AB_2943202
Alexa Fluor 647 Anti-PD1 antibody (EPR4877) (1/100)	Abcam	Cat# AB201825; RRID:AB_2728811
PD-L1 (E1L3N) XP rabbit antibody (1/25)	Cell Signaling Technology	Cat# 13684; RRID:AB_2687655
Alexa Fluor 488 anti-CD11c antibody (EP1347Y) (1/100)	Abcam	Cat# ab275738; RRID:AB_2943188
Alexa Fluor 555 anti-CD16 antibody (1/100)	Abcam	Cat# ab281764, RRID:AB_3532177
ITGAM/CD11B (D6X1N) rabbit mAb (1/80)	Cell Signaling Technology	Cat# 49420; RRID:AB_2799357
Alexa Fluor 750 CD69 antibody (FN50) (1/80)	Novus Biologicals	Cat# NBP1-43387AF750; RRID:AB_3532183
Alexa Fluor 647 CD64 (X54-5/7.1) (1/50)	BioLegend	Cat# 139322 RRID: AB_2566561
CD68 monoclonal mouse antibody (KP1) (1/50)	Biotium	Cat# BNC550512-100 RRID: AB_3677390
Alexa Fluor 647 anti-human CD8a (1/100)	BioLegend	Cat# 372906; RRID:AB_2650712
NCAM-1/CD56 antibody (123A8) Alexa Fluor 750 (1/20)	Novus Biologicals	Cat# NBP2-34518AF750; RRID:AB_3532188
Alexa Fluor 488 anti-CD20 antibody (1/100)	Abcam	Cat# ab198941; RRID:AB_3532176
Alexa Fluor 555 anti-CD31 antibody (EPR3094) (1/20)	Abcam	Cat# ab279331; RRID:AB_2943193
Alexa Fluor 647 anti-CD4 antibody (EPR6855) (1/20)	Abcam	Cat# ab196147; RRID:AB_2923526
CD3 epsilon antibody (C3e/1308) DyLight 755 (1/20)	Novus Biologicals	Cat# NBP2-54392IR; RRID:AB_3532184
Pan-Keratin (C11) mouse mAb Alexa Fluor 488 conjugate (1/100)	Cell Signaling Technology	Cat# 4523; RRID:AB_836889
LAG3 (D2G4O) XP rabbit mAb Alexa Fluor 555 conjugate (1/25)	Cell Signaling Technology	Cat# 56141; RRID:AB_3532186
Wheat Germ Agglutinin (WGA) conjugates CF 640R (1/50)	Biotium	Cat# 29026; RRID:AB_3532187
Glypican-3 (1G12) unconjugated mouse monoclonal antibody (1/20)	Cell Marque	Cat# 261M-95; RRID:AB_1159661
Biological samples		
Human liver blocks	CHU Angers	N/A
Chemicals, peptides, and recombinant proteins		
Roche cell conditioning solution CC1 and CC2	Roche	Cat#950-500 and 950-223
Xylene	Ward's Science	Cat#470045-580
Ethanol absolute	Fisher Scientific	Cat#BP2818500
1X PBS solution (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4)	Sigma-Aldrich	Cat#P7059

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Citric acid anhydrous	Sigma-Aldrich	Cat#C2404
Trizma-base	Sigma-Aldrich	Cat#T1503
EDTA	Sigma-Aldrich	Cat#E6758
Tween 20	Sigma-Aldrich	Cat#P2287
DAPI	Sigma-Aldrich	Cat#D9564-10MG
Roche DISCOVERY Ab Diluent	Roche	Cat#760-10B
Hydrogen peroxide solution 30% (w/w) in H_2O	Sigma-Aldrich	Cat#H1009-100mL
Mounting medium: DAPI Fluoromount-G	SouthernBiotech	Cat#010-20
Roche Reaction buffer concentrate (10X)	Roche	Cat#950-300
Software and algorithms		
QuPath	Queen's University Belfast, Northern Ireland	https://qupath.github.io/
Aivia community	Leica Microsystems	https://www.aivia-software.com/aivia-community
Mx workflow	Leica Microsystems	https://www.leica-microsystems.com/products/light-microscopes/p/cell-dive/
Cell DIVE Acquisition	Leica Microsystems	https://www.leica-microsystems.com/products/light-microscopes/p/cell-dive/
Stardist	Martin Weigert and Uwe Schmidt	https://stardist.net
Warpy	Bio-Image Analysis Platform	https://github.com/BIOP/qupath-extension-warpy/
Other		
VWR SuperFrost Plus Microscope Slides	Avantor	Cat#631-0108
Water bath precision general purpose	Fisher Scientific	Cat#15305867
Orbital shaker	Dutscher	Cat#022057B
Epredia coverslips thickness 1 (0.16 mm) 24 × 50 mm,	Fisher Scientific	Cat#10318963
Axygen screw cap tubes	Thermo Fisher Scientific	Cat#12559107
Eppendorf Safe-Lock tubes	Eppendorf	Cat#0030121023
Incubation chamber: HybEZ II Hybridization System	Bio-Techne	Cat#PN 321710/321720
KIMTECH Science precision wipes	Thermo Fisher Scientific	Cat#10623111
Microtome	Leica	Cat#RM 2145
Label printer	Brady	Cat#IP300
Memmert CO_2 incubator	Fisher Scientific	Cat# 15799639
Cell DIVE Imager	Leica	https://www.leica-microsystems.com/products/light-microscopes/p/cell-dive/
NanoDrop eight spectrophotometer Thermo Fisher	OZYME	Cat# 912A1099
FlexAble kit	Proteintech	Cat# KFA524
Amicon Ultra centrifugal filter 10 kDa	Millipore	Cat#UFC501024
Mix-n-stain kit	Biotum	Cat# 92264
Standard Amine Reactive Antibody Labeling Kits	Invitrogen	N/A

MATERIALS AND EQUIPMENT

Citrate buffer antigen retrieval solution pH 6

Reagent	Final concentration	Amount
Anhydrous citric acid	0.01 M	1.92 g
dH_2O	–	Qsp 1 L
Adjust to pH 6.0 with NaOH	–	–
Tween-20	0.05%	0.5 mL
Total	N/A	1 L

Store at (18°C–22°C) for up to 3 months.

Tris–EDTA buffer antigen retrieval solution pH 9		
Reagent	Final concentration	Amount
Tris	10 mM	1.21 g
EDTA	1 mM	0.37 g
dH_2O	–	Qsp 1 L
Adjust to pH 9.0 with NaOH	–	–
Tween-20	0.05%	0.5 mL
Total	N/A	1 L
Store at (18°C–22°C) for up to 3 months.		

Blocking solution		
Reagent	Final concentration	Amount
PBS 1X	N/A	180 mL
BSA 1%	3%	6 g
Donkey Serum 1X	10%	20 mL
Total	N/A	200 mL
Store at 4°C for up to 1 month.		

Permeabilization solution		
Reagent	Final concentration	Amount
Triton 10%	0.3%	15 mL
PBS 1X	N/A	485 mL
Total	N/A	500 mL
Store at 4°C for up to 6 months.		

DAPI solution		
Reagent	Final concentration	Amount
DAPI	10 mg/mL	10 mg
Roche Ab diluent OR 1 X PBS	N/A	1 mL
Total	N/A	1 mL
Store at 4°C for up to 1 week.		

Inactivation solution		
Reagent	Final concentration	Amount
Milli-Q Water	N/A	3.5 mL
NaHCO3 0.5 M	–	1 mL
H_2O_2 30%	3%	0.5 mL
Total	N/A	5 mL
Store at 4°C for up to 1 week.		

⚠ CRITICAL: Hydrogen peroxide is corrosive and sensitive to oxidation. Handle with care, and avoid contact with air as much as possible.

STEP-BY-STEP METHOD DETAILS

Acquisition of mIF image using Cell DIVE imager

In this part, you will need to use sequentially Mx Workflow and Cell DIVE acquisition softwares. Open both of them before starting the following protocol.

Note: The Roche Reaction Buffer can be substituted by 1X PBS solution.

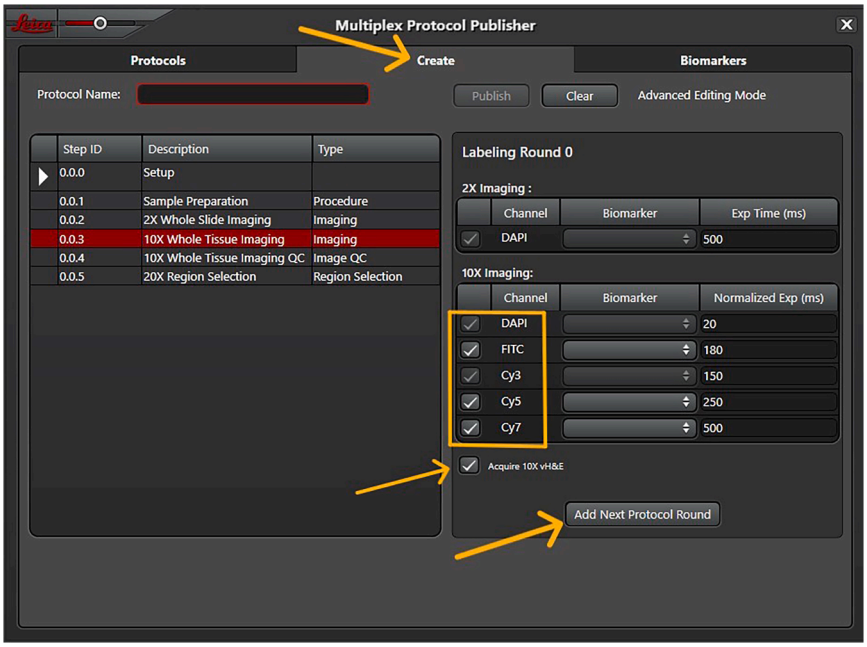


Figure 3. Screenshot of the protocol creation process
After clicking on "Create" tab (top orange arrow), choose fluorophore, select "acquire 10X vH&E, then "Add next protocol round".

1. Print a label for slide recognition with the slide ID that does not contain a space in the name.

Protocol and slide setup

Timing: 10 min

2. Create a protocol in Mx Workflow software (Figure 3).
   a. Click on "Tools" on the top menu bar – "Protocol publisher".
   b. Click on "Create", write the protocol name, then click on "create set-up round".
   c. Designer Round: for Round 0 to acquire the first auto-fluorescence image of the tissue, click on all the fluorochromes that will subsequently be used in the protocol.
   d. Click on "Acquire 10X vH&E" to acquire a virtual hematoxylin and eosin image which will be used for subsequent image analysis.
   e. Click on "Add next protocol Round".
   f. On the "20X Imaging" column in labeling Round 1 tab, select the same required channel (FITC, Cy3, Cy5, Cy7) and associate it with a preexisting biomarker name or create a new one in the "Biomarkers" tab. We recommend to leave exposure time in the "Normalized Exp (ms)" tab set by default.
   g. Repeat step e. and f. as much as required.
   h. Click on "publish" and close the "protocol publisher" window when your protocol is ready.
3. Add a slide to the protocol.
   a. Click on "Slide" in the top menu bar - "Create New Batch".
   b. Choose the name of the protocol you've just created and the Slide ID then click on "Add Slide" then "Create batch". Your slide will then appear on the software's main page.
   c. Double click on the slide ID to display slide advancement in the protocol.

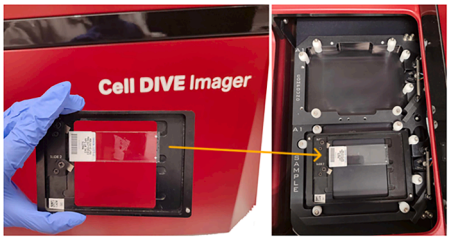


Figure 4. Dual slide cassette for step 4-a and further
The Cell Dive imager is provided with a sample carrier fitted for 2 slides that would be imaged one after the other.

ROI selection and autofluorescence recording

Timing: 20 min to 2 h depending on the size of the tissue and the number of fluorescent channels to be imaged

4. Acquire the slide prior antibodies in order to record the intrinsic autofluorescence of the tissue.
   a. Place the mounted and cleaned slide in the dual slide cassette (Figure 4), then in the imager cassette chamber.
   b. On Mx Workflow, click on "complete" in the section corresponding to the sample preparation to indicate that you are ready for scan.
   c. On Cell DIVE acquisition, "Load" the slide. The imager will scan the barcodes and capture images with 2X, and show a preview of the whole slide.
   d. In the "Tools" tab in Cell Dive Acquisition, click on "Draw Region" and draw your Field of View (FOV). Click on "Save Regions" then "Eject".
5. Click on "Load" for the 10X scan.
   a. Once completed, go on Mx Workflow. When stitching is complete, click on "Viewer QC" button to open the quality check window.
   b. Check on the different channels and especially on the DAPI that the image is correctly scanned. To do so, click on the open eye icon and on "set as A" to view the different channels. If satisfied, click on "Submit QC" \ "Pass and reload slide" to access to the ROI selection window.
   c. On the 10X DAPI tab, click on "ADD ROI" to draw a more precise scan region then click on "Send to lab" twice to access to the 20X scan (Figure 5). This zone must be defined carefully as it will correspond to the scan region for all future marking cycles.
   d. "Click on "Eject" in Cell DIVE acquisition.
6. Click on "Load" in Cell DIVE acquisition for the 20X scan.
   a. Once completed, go on Mx Workflow and when stitching is complete, click on "Viewer QC" button to open the quality check window.
   b. Check on the different channels and especially on the DAPI that the image is correctly scanned. If satisfied, click on "Submit QC" \ "Pass and exit", if not on "Fail and exit" and it will take you back to the previous step to redefine the ROI.
   c. Close the Cell DIVE Viewer window. Tissue autofluorescence measurement step is complete.
   d. Click on "Eject" in Cell DIVE acquisition to recover the slide and prepare it for hybridization with antibodies.

Note: At steps 5b and 6b, you verify that DAPI intensity is sufficient and that no traces of dust or bubbles interfere with imaging.

Antibody hybridization and Cell DIVE acquisition

Timing: 90 min to 3 h depending on the size of the tissue and the number of fluorescent channels to be imaged

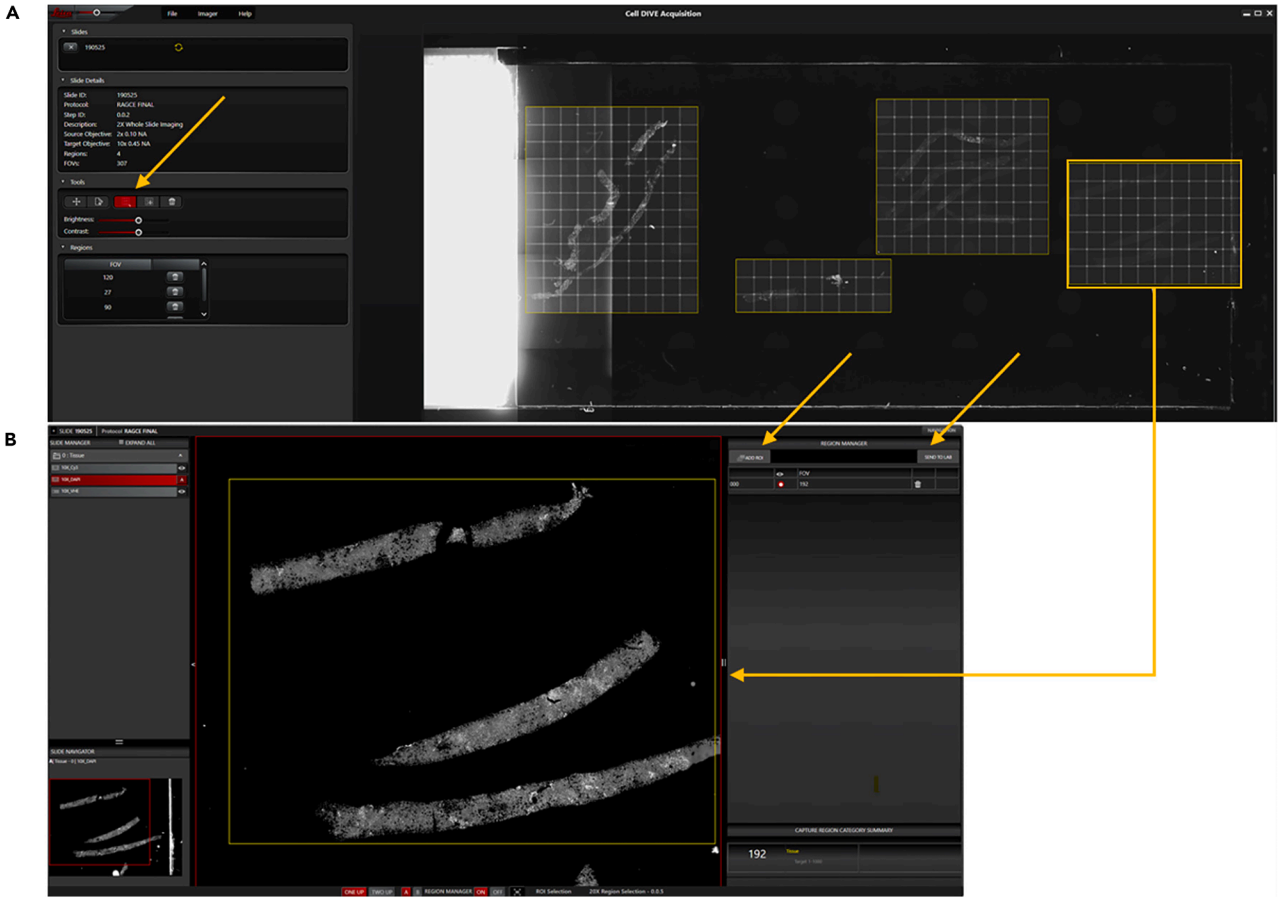


Figure 5. Screenshot of the ROI selection process

(A) After loading the slide on the acquisition software, select the "draw region" tool indicated by the arrow on the left (step 4-d). Draw and save your Fields of view (FOV). This example displays 4 FOVs corresponding to tumoral and not tumoral samples.

(B) After 10x scan, click on Add ROI (step 5-c) to precise your region of interest then "send to lab".

Repeat as many times as there are marking cycles and at the end obtain a final image compiling all the biomarkers. An example of timing is given in Table 2.

7. Prepare the slide for hybridization.
   a. Remove the slide from the cassette and immerse it in Roche reaction buffer or PBS in a vertical staining jar to allow the coverslip to detach gently (Figure 6).

   Note: If coverslip does not detach spontaneously within 5 min, place the place the staining jar on an orbital shaker set to 60 RPM for 5 min.

   b. Wash the slide with small back and forth movements for 5 min.

   Note: It is important for tissue to stay hydrated. Keep slides in 1X PBS until the next solution.

8. Prepare a final volume of 150 µL in Roche Ab diluent (or 1X PBS) per slide containing up to 4 antibodies coupled to a fluorochrome in channel cyanine 2, cyanine 3, cyanine 5 or cyanine 7.

   Example: Volumes used with the antibodies in the last cycle of our panel. The dilution was made in Roche Ab diluent for a final volume of 150 µL. Antibodies in cyanine 2 and 7 channels were both mouse monoclonal antibodies.

Fluorochrome	Cyanine 2	Cyanine 3	Cyanine 5	Cyanine 7
Antibody	Pan-CytoKeratin (1/100) Alexa Fluor 488	LAG3 (1/25) XP AF 555	WGA (1/50) CF 640R	Glypican-3 (1/20) CoraLite Plus 750
Volume	1.5 μL	6 μL	3 μL	7.5 μL

a. Centrifuge the mixture at 2,000 g for 60 sec to remove the antibody aggregates.
b. Place the mixture on the tissue slide. To prevent evaporation, we recommend to cover with a coverslip or a parafilm cut slightly larger than the tissue and to place the slide in the tray of the incubation chamber with humidified paper.
c. Incubate for 1 h at 37°C or for 16 h at 4°C.
d. Remove the coverslip as in step 7, wash the slide in Roche Reaction Buffer.

9. Label the nuclei with DAPI by applying 150 μL of a solution 10 μg/mL for 5 min.
   a. Wash the slide in reaction buffer with small back and forth movements.
   b. Add a few drops of DAPI-containing mounting medium to the slide and position the coverslip.
   c. Clean the slide meticulously with distilled water or ethanol to remove all micro-bubbles and any traces of dirt.
10. Put the slide in the imager cassette.
    a. On Mx Workflow, click on "Complete" to indicate that the hybridization step is finished.
    b. In the Cell DIVE acquisition software, click on "Load". The imager will then recognize the barcode on the slide, acquire it at 20X and superimpose the images obtained on the previous image by re-aligning the nuclei.
11. On Mx Workflow, when stitching is complete, click on "Viewer QC" button to open the quality check window.
    a. If satisfied of slide cleanness and DAPI intensity, click on "Submit QC" \ "Pass and exit", if not on "Fail and exit" and it will take you back to the previous step 10.
    b. Close the Cell DIVE Viewer window.
12. Click on "Eject" to recover the slide and place it in a reaction buffer tank for 5 min to remove the coverslip (same as step 7).
13. Inactivate dyes.
    a. Add 200 μL of inactivation solution (as described in the "Material and equipment" section) and incubate in the dark for 15 min.

Table 2. Example of acquisition and handling times for the Cell Dive acquisition

Cycle	Steps in the cell DIVE imager	Steps out of the cell DIVE imager	Approx. Timing (h)
	Auto-fluorescence recording		01:00 - 02:00
Cycle 1		Incubation cycle 1 antibodies	01:00
	Cycle 1 image acquisition		01:00 - 02:00
	Nuclei-based registration		00:30
		Dye inactivation	00:30
	Image acquisition		01:00 - 02:00
	Nuclei-based registration		00:30
Cycle 2		Incubation cycle 2 antibodies	01:00
	Cycle 2 image acquisition		01:00 - 02:00
	Nuclei-based registration		00:30
		Dye inactivation	00:30
	Image acquisition		02:00
	Nuclei-based registration		00:30
Cycle n	…		

Scanning in the imager takes a variable amount of time, depending on the size of the tissue to be imaged. The example is given for a 10 mm x 1 mm liver biopsy slide. If the Cell Dive imager is provided without automated staining system, the antibody solution and inactivation solution deposition stages require the intervention of the experimenter, and have a fixed duration whatever the size of the tissue

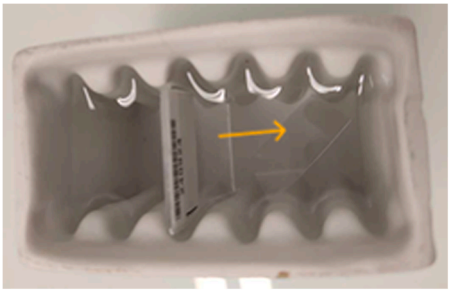


Figure 6. Coverslip removal in a vertical staining jar
The slide is inserted vertically, and the coverslip comes off spontaneously in the reaction buffer. The coverslip is indicated by the arrow.

b. Discard excess of solution and repeat application of inactivation solution.
c. Wash the slide in Reaction Buffer for 5 min.

14. Label the nuclei with DAPI by applying 150 µL of a solution 10 µg/mL for 5 min.
    a. Wash the slide in reaction buffer with small back and forth movements.
    b. Add a few drops of DAPI-containing mounting medium to the slide and position the coverslip.
    c. Clean the slide meticulously with distilled water or ethanol to remove all micro-bubbles and any traces of dirt.
    d. Place the slide in the cassette for imaging.
15. On Mx Workflow, click on "complete" and on Cell DIVE acquisition, "Load".
16. Return to step 7 for the next hybridization cycle.

Note: We recommend to use DAPI-containing mounting medium in addition to DAPI staining, in order to obtain a strong nuclear signal that will facilitate the nuclei-based image registration step.

Note: Depending on the number of marking cycles and the size of the tissue, the image size can be very large and exceed 1 To. Make sure you have enough space on the internal storage of the computer connected to the machine, to avoid computer crashes.

Preliminary image analysis on QuPath

Timing: 30 min

This section is for image and statistical analysis using an open-source software designed for large histological images. Here is one example of an analysis workflow for the identification and localization of different immune cells. The analysis workflow consists of the following steps: assembling of the final image, cell segmentation with Stardist and cell classification.

QuPath software is available for download at https://qupath.github.io/.

Assembling the final image in QuPath using the Warpy extension

Compiled Cell DIVE images in TIFF format are very large (several hundred gigabytes) and cannot be read as such by the QuPath analysis software. Here are the steps involved in assembling the images from each color channel to obtain a single pyramidal image of the entire slide, combining all markings.

Warpy documentation: https://imagej.net/plugins/bdv/warpy/warpy.

17. Download and install Warpy.
    a. Download the latest Warpy version with this link: https://github.com/BIOP/qupath-extension-warpy/releases/.

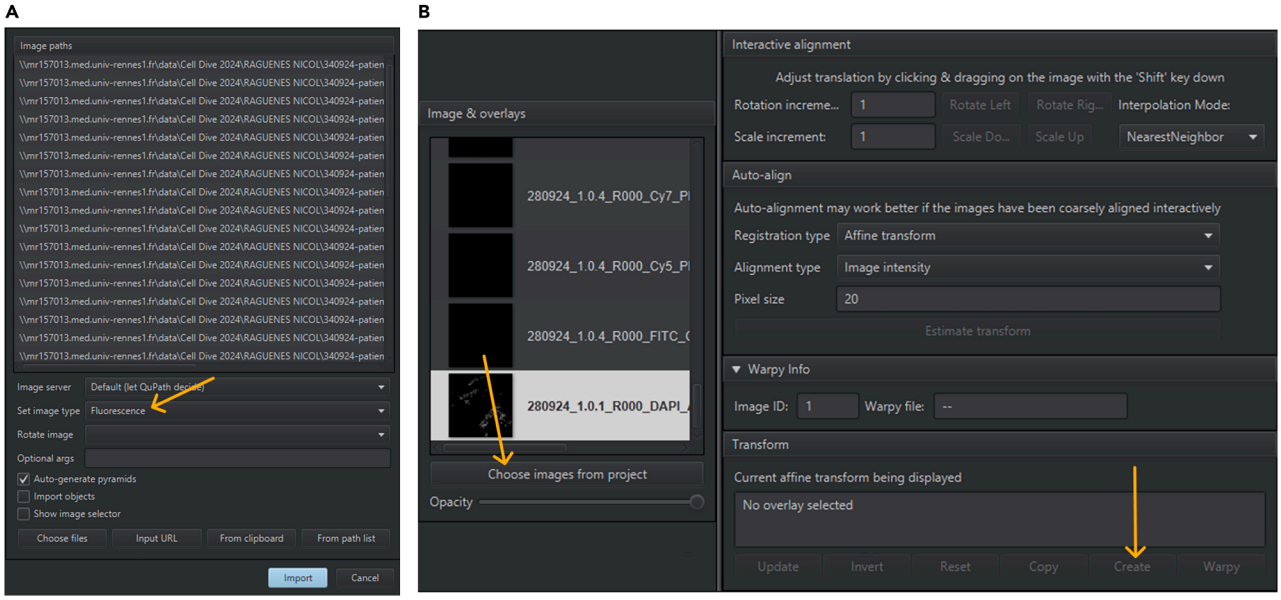


Figure 7. Screenshot of Warpy image assembling

(A) After creating a QuPath project, drag and drop your final TIF images in the project (step 18-b) and check the files for importation. Select "fluorescence" as image type (left arrow).

(B) After selecting "interactive image combiner Warpy" (step 18-d), click "choose images from project" (middle arrow) then "create".

b. Drag and drop qupath-extension-warpy-0.3.1.zip file (or any new release) into the QuPath window.

18. Combine your multiplex image.
    a. Open QuPath and create a project.
    b. Drag and drop all the final TIF images corresponding to the same Region of Interest (ROI) in the Qupath project and set image type as "fluorescence", then import (Figure 7A).
    c. Display the image corresponding to the first DAPI which serves as a reference image on which the other images have been aligned by clicking on the "*YourSlide*_1.0.1_*YourROI*_DAPI_AF_F.ome.tif" image.
    d. Click on "analyze" \ "Interactive image combiner Warpy" \ "choose images from project"\ "create" (Figure 7B).

Note: You will then have an image called "overlay" which combines all the markings and that the software will display all in white by default.

19. Use code 1 to automatize the assignation of your biomarkers to a chosen color and improve visualization of the final image. Launch by clicking "Automate" \ "Script editor" \ "Run".

Code 1 : modify with your biomarker names.

```
def server = getCurrentServer()

def n = server.nChannels()

def new_names = []

def new_colors = []

map = [DAPI: getColorRGB( 204, 204, 204 ),
```

```
  Glypican: getColorRGB( 255, 0, 255 ),
  WGA: getColorRGB( 255, 255, 153 ),
  PanCK: getColorRGB( 0, 255, 0 ),
  CD20: getColorRGB( 0, 255, 255 ),
  CD4: getColorRGB( 51, 102, 51 ),
  CD31: getColorRGB( 255, 0, 0 ),
  CD3: getColorRGB( 241, 125, 246 ),
  CD64: getColorRGB( 0, 0, 255 ),
  CD56: getColorRGB( 128, 0, 128 ),
  CD68: getColorRGB( 0, 0, 255 ),
  CD8a: getColorRGB( 255, 102, 102 ),
  CD11b: getColorRGB( 22, 20, 255 ),
  CD11c: getColorRGB( 201, 15, 24 ),
  CD69: getColorRGB( 112, 60, 255 ),
  CD16: getColorRGB( 221, 129, 146 ),
  PDL1: getColorRGB( 0, 0, 255 ),
  PD1: getColorRGB( 41, 159, 176 ),
  CD15: getColorRGB( 50, 97, 25 ),
  CTLA4: getColorRGB( 52, 94, 14 ),
  Va72: getColorRGB( 128, 102, 204 ),
  FoxP3: getColorRGB( 128, 0, 0 ),
  CD161: getColorRGB( 153, 153, 51 )
  ]
for (int i = 0;i < n; i++) {
  name = server.getChannel(i).toString()
  namesplit = name.split("_")
  if (namesplit.size() > 1) {
    if (namesplit[2] == "Channel") {
      name = getProjectEntry().getImageName()
      namesplit = name.split("_")[2] // à changer
      new_name = namesplit.split("-")[0]
      }
    else {
      new_name = namesplit[4].split("-")[0]
    }
    print(name + " has been renamed " + new_name)
    name = new_name
    color = map[name]
```

```
    if (color == null){
      color = getColorRGB( 150, 150, 150 )
    }
  }
  else {
    name = namesplit
    color = server.getChannel(i).getColor() // current color
  }
  new_colors += color
  new_names += name
}
print(new_names)
setChannelNames(*new_names)
setChannelColors( *new_colors )
```

Image verification

Timing: 20 min

Prior to beginning data analysis, each combined image undergoes quality control through a visual inspection of the entire slide using QuPath. Bubbles, tissue folding or other artifacts are manually annotated and excluded from the analyzed ROI.

Nuclear and cellular segmentation using Stardist

Timing: 2 h, may vary with image size and computer performance

20. Download and install Stardist.
    a. Download the qupath-extension-stardist-0.5.0.jar on QuPath with this link: https://github.com/qupath/qupath-extension-stardist/releases.
    b. Drag and drop the QuPath-extension-stadist.jar file into the QuPath window.
    c. Download the cell segmentation model with this link https://github.com/qupath/models and save them in the corresponding QuPath project file so that everything you need for the analysis is in a unique folder.
21. Annotate the area of tissue whose nuclei you wish to segment.

    Note: This step can be carried out by either by drawing a zone, using the magic wand tool, or using a pixel classifier that detects the presence of tissue.

22. Segment your annotation.
    a. Click on "Extensions" \"StarDist"\ "StarDist fluorescence cell detection script".
    b. Modify the model you wish to use on the 21st row of the script *(def modelPath = "/path/to/model.pb")* and enter the correct path.
    c. Save your script then "Run". You will visualize the segmentation result directly, nuclei will appear circled in red (Figure 8).

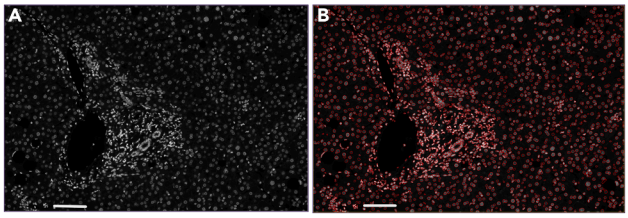


Figure 8. Example of segmentation with Stardist model
(A) Original image used for segmentation showing DAPI-labeling of nuclei.
(B) Overlay of DAPI-staining and result of segmentation in red (step 22-c). The "Cell display" parameter selected here is "nuclei only" (step 22-d). Scale bar represents 200 μm.

d. Click "View" \"Cell display" and select between "Cell boundaries only", "Nuclei only" and "Nuclei and cell boundaries".

Note: You can optimize your model by modifying the detection probability threshold and the pixel size.

Cell phenotyping using object classifier

Timing: 3 h, may vary with image size and computer performance

23. Create the different cellular labels or classes that you need in the "Annotations" window (highlighted in blue Figure 9).
24. Train a classifier with positive and negative examples, repeat for each class you have defined.
    a. Select small segmented regions for training of the classifier.
    b. Click on "Classify" \ "Object classification"\ "Train object classifier".
    c. Select the « Points » tool (highlighted in orange Figure 9). Click on "Add" and choose the label or class you want to assign the point tool to.
    d. Select positive cells with the Point Tool. You can follow and correct the classification as you train the model by clicking on "Live update".
    e. Name and save the classification model.

Note: Try to give a similar number of examples for all categories so as not to create an imbalance.

25. Validate your model on a different small region, before launching it on the whole slide.
    a. Draw a small region of annotation.
    b. Launch "Classify" \ "Object classification" \ "Load object classifiers" \ "Apply classifier".

An example is given in Figure 10 for staining with CD3 (pink), CD31 (red), CD68 (green) and DAPI (gray).

EXPECTED OUTCOMES

The scan of all marking cycles results in an image of the whole slide, compiling all 20 markers of the protein panel at single-cell resolution (Figure 11). This project comprises a panel of cell positioning markers (DAPI, WGA), structural markers of normal or pathological liver (Glypican-3 and Pan-CK), vascular markers (CD31), markers of myeloid (CD15, CD16, CD11b, CD11c, CD64) and general lymphoid (CD3, CD4, CD8, CD20, CD56) populations. It also enables to search for subpopulations of T lymphocytes or innate-lymphoid cells. Lymphocyte activation (CD69) and exhaustion (CTLA4, LAG-3) markers, as well as immune checkpoints (PD-1, PD-L1) were added in order to investigate the functional status of these cells. QuPath allows to segment the stained cells, based on the

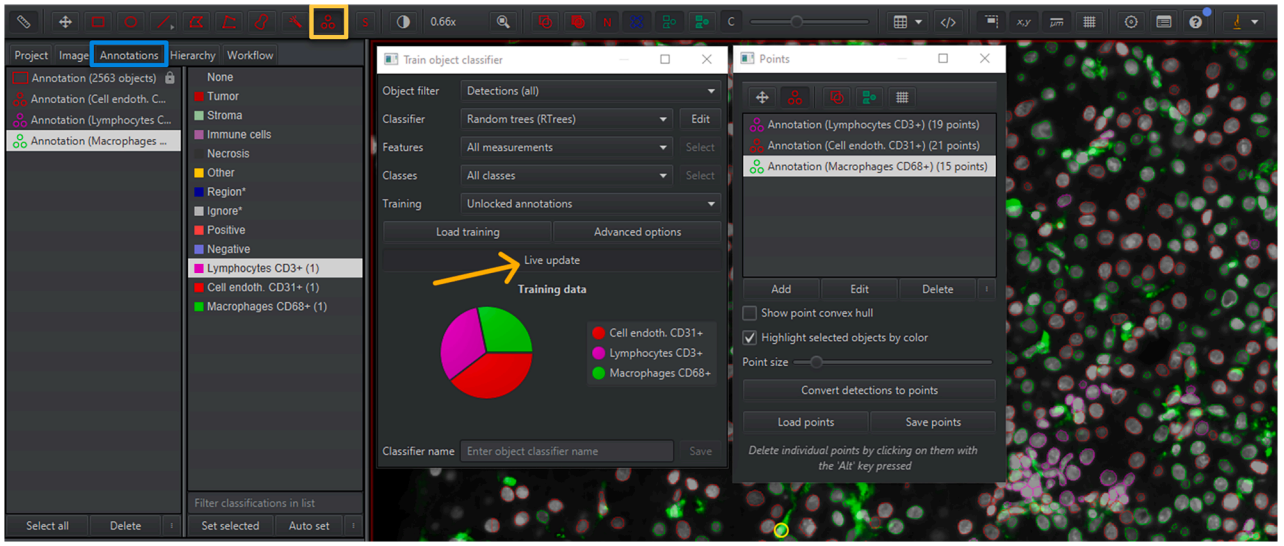


Figure 9. Screenshot of train object classifier process steps 23 and 24

Blue box highlights annotation tab for creating as many classes as you need. The "point" tool, framed in orange at the top, is used to provide examples of the classes already created (step 24-d). Click "live update" (orange arrow) to view the results of the classification model live. A pie chart appears, showing the number of examples given for each class.

detection of nuclei performed through a pre-trained deep-learning model. Those cells are classified into positive or negative for each class of marker thanks to a supervised object classifier. Phenotypes of interest and specific immunological subpopulations are determined based on combination of these classes. More complex unsupervised classifications are beyond the scope of this protocol.

Subsequent statistical analysis of the quantitative data derived from the classification is not presented here. It will provide information such as phenotype counts, counts within specific regions to quantify the presence and location of immune cells in different groups of HCC patients. By enabling the simultaneous detection of numerous markers on a single tissue section, this innovative direct immunofluorescence tool is one of the emerging technologies developed to overcome the constraints of conventional histology.

This methodology is applicable to the study of immune infiltration in the human liver in contexts other than HCC, but also in tissues other than the liver.

This multiplexed imaging technology has been validated in the human liver. It allows detailed mapping of the immune infiltrate of each biopsy examined.

LIMITATIONS

The major limitation for this protocol is that it requires a Cell DIVE imaging system. Image acquisition procedures will have to be reviewed if different mIF imager is used, but image analysis methodology may remain similar.

This protocol can be applied to the liver, but may be inadequate to more delicate organs where there is a risk of tissue detachment as marking cycles progress. If such a phenomenon occurs, cycle alignment based on DAPI will not be processed correctly, compromising further analysis.

Regarding the image analysis, The Stardist cellular segmentation method is not ideal for cells that are not round. The model is very efficient for the nuclei but the cell expansion tool does not

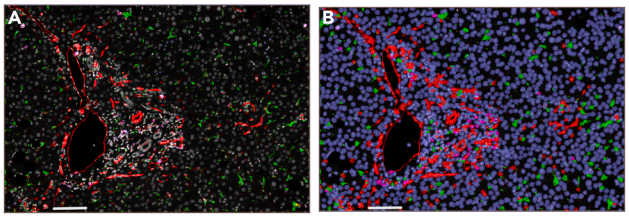


Figure 10. Example of application of the classification model step 25
(A) mIF image showing CD3 (pink), CD31 (red) and CD68 (green) and DAPI (gray) staining.
(B) Objects created by the segmentation and assigned to Lymphocytes CD3+ (pink), Cell endoth. CD31+ (red) or Macrophages CD68+ (green) classes by the classification model on image A. Scale bar represents 200 μm.

sometimes reflect biological reality. The release of new segmentation methods that take into account membrane markers and not just DAPI should help improve this constraint.

TROUBLESHOOTING

Problem 1

The tissue may move or come off the slide completely during acquisition cycles.

Potential solution

The bake time or temperature may need to be increased to improve adherence of the tissue to the charged microscope slide. Different tissue types may require different baking times.

Problem 2

At quality check after 10x scan (step-by-step method step 5) you may realize that DAPI intensity is weak.

Potential solution

As image registration is based on nuclei-staining, a strong DAPI intensity is important. Use DAPI-containing mounting medium or perform another 5 min step of DAPI staining.

Problem 3

Certain fluorochromes may be more difficult to quench with the mild H2O2 inactivation method (step-by-step method step 13). Some residual signal remains in the autofluorescence acquisition and may accumulate with the fluorescence of subsequent cycles.

Potential solution

Verify that the chemical structure of the fluorochrome is compatible with oxidative inactivation and then perform a soft antigen retrieval in the Roche Discovery Ultra.

Problem 4

The scan was interrupted during the acquisition (step-by-step method step 10 to 15).

Potential solution

The management and storage of the hundreds of gigabytes of images generated by the Cell DIVE can be problematic. Always check that there is enough space on the computer hard disk before starting an experiment, otherwise the Cell DIVE may interrupt the acquisition. It's important to work on a computer with plenty of storage capacity and to make regular copies to an external server or NAS.

Problem 5

Less abundant phenotypes are overlooked by classification (step-by-step method step 24).

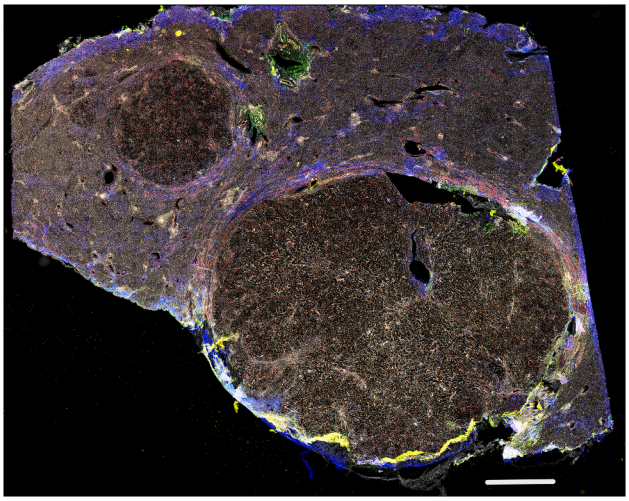


Figure 11. Example of final mIF image of human hepatocellular carcinoma whole slide

For visualization purposes, only 5 markers are displayed: WGA (yellow), CD4 (orange), CD20 (green), CD31 (red), CD68 (blue) and DAPI (gray). Scale bar represents 2 mm.

Potential solution

It's important to give positive and negative examples in similar number for each of the classes otherwise machine-learning may be biased.

Problem 6

Red blood cells may have a lot of antibodies bound to them. They may be false positives.

Potential solution

These cells have no nuclei. Therefore, they can be excluded from the segmentation process and not counted in the analysis.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Céline Raguénès-Nicol (celine.raguenes-nicol@univ-rennes.fr).

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Margaux Delaporte (margaux.delaporte.1@univ-rennes.fr).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The code generated during this study is available using this DOI on Zenodo: https://doi.org/10.5281/zenodo.15641761. https://zenodo.org/records/15641761?preview=1&token=eyJhbGciOiJIUzUxMiJ9.eyJpZCI6ImZhN2U1MzhlLTY4YjAtNDIxZC05OGEyLWMzNWI4YjM2NjMyMyIsImRhdGEiOnt9LCJyYW5kb20iOiJkYzU1ZGYwMWFkMjkyNTc5MDNlZmViYTVlM2QyZGM4NSJ9.sXh0D0neGKsYzAqUXcfPoVlMogJd-9OKgiq895gDOT5ATtC_UVcgweFERu_j1pfSzkDsIyTzTUUb7KlJlYOzXQ.

ACKNOWLEDGMENTS

This project was financed by Allocations de Recherche Doctorale Région Bretagne - ARED Inserm, Association Française pour l'étude du Foie (AFEF), Ligue contre le cancer (CD22 and 72), Fédération Hospitalo-Universitaire GO-NASH, and Fondation pour la Recherche Médicale.

AUTHOR CONTRIBUTIONS

Conceptualization, C.R.-N. and M.D.; technical support, P.B., M.G., T.P., B.T., and A.S.; experimentation, M.D. and P.B.; writing – original draft, M.D.; writing – review and editing, C.R.-N.; funding acquisition, C.R.-N. and M.S.; supervision, C.R.-N.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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