Journal of Visualized Experiments

Semi-Automatic PD-L1 Characterization and Enumeration on Circulating Tumor Cells from Non-Small Cell Lung Cancer Patients by Immunofluorescence

--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59873R3
Full Title:	Semi-Automatic PD-L1 Characterization and Enumeration on Circulating Tumor Cells from Non-Small Cell Lung Cancer Patients by Immunofluorescence
Keywords:	Lung cancer, circulating tumor cells, circulating free DNA, immunofluorescence assay, CTC, ClearCell FX-1, PD-L1
Corresponding Author:	Julie PAYEN-GAY Centre de Recherche en Cancerologie de Lyon Caluire, FRANCE
Corresponding Author's Institution:	Centre de Recherche en Cancerologie de Lyon
Corresponding Author E-Mail:	lea.payen-gay@univ-lyon1.fr
Order of Authors:	Jessica Garcia
	David Barthelemy
	Florence Geiguer
	Julie Ballandier
	Chassidy Jonhson
	Jean-Phillippe Aurel
	Frédérique Lebreton
	Claire Rodriguez-Lafrasse
	Brigitte Manship
	Sébastien Couraud
	Julie PAYEN-GAY
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	LYON (France)

TITLE:

Semi-Automatic PD-L1 Characterization and Enumeration of Circulating Tumor Cells from Non-Small Cell Lung Cancer Patients by Immunofluorescence

AUTHORS AND AFFILIATIONS:

Jessica Garcia[1,2,3], David Barthelemy[1,3], Florence Geiguer[1,3], Julie Ballandier[1,3], Kathryn W. Li[4], Jean-Philippe Aurel[4], Frédérique Le Breton[5], Claire Rodriguez-Lafrasse[1], Brigitte Manship[2], Sébastien Couraud[3,6,7], Léa Payen[1,2,3]

[1]Laboratoire de Biochimie et Biologie Moléculaire, Groupe Hospitalier Sud, Hospices Civils de Lyon, Lyon, France
[2]University of Lyon, Claude Bernard University, Cancer Research Center of Lyon, Lyon, France
[3]Hospices Civils de Lyon Cancer Institute, Circulating Cancer (CIRCAN) Program, Pierre Bénite, France
[4]Biolidics Limited, Singapore
[5]Institut de Pathologie Multisites des HCL-Site Sud, Hospices Civils de Lyon, Lyon, France
[6]Acute Respiratory Disease and Thoracic Oncology Department, Lyon Sud Hospital, Cancer Institute of Hospices Civils de Lyon, Pierre-Bénite, Lyon, France
[7]EMR-3738 Therapeutic Targeting in Oncology, Lyon Sud Medical Faculty, Lyon 1 University, Oullins, France

Corresponding author:
Léa Payen (lea.payen-gay@univ-lyon1.fr)

Email addresses of co-authors:
Jessica Garcia (jessica.garcia02@chu-lyon.fr)
David Barthelemy (david.barthelemy@chu-lyon.fr)
Florence Geiguer (florence.geiguer@chu-lyon.fr)
Julie Ballandier (julie.ballandier@chu-lyon.fr)
Kathryn W. Li (Kathryn.wqli@gmail.com)
Jean-Phillippe Aurel (jp@biolidics.com)
Frédérique Le Breton (frederique.lebreton@chu-lyon.fr)
Claire Rodriguez-Lafrasse (claire.rodriguez-lafrasse@chu-lyon.fr)
Brigitte Manship (brigitte.manschip@lyon-unicancer.fr)
Sébastien Couraud (sebastien.couraud@chu-lyon.fr)

KEYWORDS:

lung cancer, circulating tumor cells, circulating free DNA, immunofluorescence assay, CTC, spiral microfluidic device, PD-L1

SUMMARY:

The characterization of circulating tumor cells (CTCs) is a popular topic in translational research. This protocol describes a semi-automatic immunofluorescence (IF) assay for PD-L1 characterization and enumeration of CTCs in non-small cell lung cancer (NSCLC) patient samples.

ABSTRACT:

Circulating tumor cells (CTCs) derived from the primary tumor are shed into the bloodstream or lymphatic system. These rare cells (1−10 cells per mL of blood) warrant a poor prognosis and are correlated with shorter overall survival in several cancers (e.g., breast, prostate and colorectal). Currently, the anti-EpCAM-coated magnetic bead-based CTC capturing system is the gold standard test approved by the U.S. Food and Drug Administration (FDA) for enumerating CTCs in the bloodstream. This test is based on the use of magnetic beads coated with anti-EpCAM markers, which specifically target epithelial cancer cells. Many studies have illustrated that EpCAM is not the optimal marker for CTC detection. Indeed, CTCs are a heterogeneous subpopulation of cancer cells and are able to undergo an epithelial-to-mesenchymal transition (EMT) associated with metastatic proliferation and invasion. These CTCs are able to reduce the expression of cell surface epithelial marker EpCAM, while increasing mesenchymal markers such as vimentin. To address this technical hurdle, other isolation methods based on physical properties of CTCs have been developed. Microfluidic technologies enable a label-free approach to CTC enrichment from whole blood samples. The spiral microfluidic technology uses the inertial and Dean drag forces with continuous flow in curved channels generated within a spiral microfluidic chip. The cells are separated based on the differences in size and plasticity between normal blood cells and tumoral cells. This protocol details the different steps to characterize the programmed death-ligand 1 (PD-L1) expression of CTCs, combining a spiral microfluidic device with customizable immunofluorescence (IF) marker set.

INTRODUCTION:

Tumor antigen-specific cytotoxic T-lymphocytes (CTLs) play a crucial role in the response to cancers through a process known as cancer “immune surveillance”. Their anti-tumor functions are enhanced by immune checkpoint blockade antibodies such as CTLA-4 inhibitors and PD-1/PD-L1 inhibitors. In non-small cell lung cancer (NSCLC), anti-PD-1/PD-L1 therapies result in response rates ranging from 0%–17% in patients with PD-L1-negative tumors and 36%–100% in those expressing PD-L1. The robust responses to PD-1/PD-L1 blockade observed in melanoma and NSCLC are shown by evidence of improved overall response rate (RR), durable clinical benefits, and progression-free survival (PFS). Currently, anti-PD1 treatments are the standard of care in second-line NSCLC treatment with nivolumab regardless of PD-L1 expression and with pembrolizumab in patients expressing PD-L1 ≥1%. In first-line treatment, standard of care is pembrolizumab alone in patients with NSCLC expressing PD-L1 ≥50% and can be potentially enhanced with chemotherapy (platin and doublet drug depending on histologic subtype)[1,2].

However, such an approach to patient management is debatable[3], since PD-L1 expression in tumor cells by immunohistochemistry (IHC) is probably not the most ideal companion biomarker. Others such as tumor mutation burden[4] (TMB), microsatellite instability (MSI), and/or microbiota are possibly interesting in this setting either alone or in combination. NSCLC are known to be heterogeneous tumors, either spatially (from a tumor site to another one) or temporally (from diagnosis to recurrence). Patients with NSCLC are usually fragile, and iterative invasive tissue biopsies may be an issue. Indeed, re-biopsy rate at first progression ranges from 46%–84% depending on series, and successful re-biopsy (meaning with histological and full molecular

analysis) ranges from 33%–75%. This means that 25%–67% of patients cannot receive a comprehensive re-biopsy analysis during first progression[5,6,7,8].

The advent of "liquid biopsies" has thus generated considerable enthusiasm in this particular setting, as it enables crucial reassessment of molecular alterations during disease progression by examining circulating free DNA (cfDNA) derived from circulating tumor cells (CTCs). These live cells are released from the tumor into the bloodstream, where they circulate freely. Although not routinely used, the analysis of CTCs appears to be highly promising in the case of molecular and phenotypic characterization, prognosis, and predictive significance in lung cancer (*via* DNAseq, RNAseq, miRNA and protein analysis). Indeed, CTCs likely harbor phenotypic characteristics of the active disease rather than the initial markers (detected on tissue biopsies at diagnosis). Furthermore, CTCs bypass the problem of spatial heterogeneity of the tumor tissue, which may be a crucial issue in small biopsies. Consequently, PD-L1 expression on CTCs may potentially shed light on the discrepancies derived from its use as a predictive biomarker using tumor tissue.

Recently, PD-L1 expression has been tested in CTCs of NSCLC. Almost all of the patients tested[9] were PD-L1 positive, complicating the interpretation of the result and its clinical use. Overall, PD-L1-positive CTCs were detected in 69.4% of samples from an average of 4.5 cells/mL[10]. After initiation of radiation therapy, the proportion of PD-L1-positive CTCs increased significantly, indicating upregulation of PD-L1 expression in response to radiation[11]. Hence, PD-L1 CTCs analysis may be used to monitor dynamic changes of the tumor and immune response, which may reflect the response to chemotherapy, radiation, and likely immunotherapy (IT) treatments.

To date, CTCs isolation and PD-L1 characterization rely on various methods such as anti-EpCAM-coated magnetic bead-based CTC capturing, enrichment-free based assay, and size-based[12,13] CTC capture assays. However, CTCs were only detected in 45%–65% of patients with metastatic NSCLC, thus limiting their ability to provide any information for more than half of metastatic NSCLC patients. In addition, CTC count was low in most of these studies using size-based approach[10]. Furthermore, this method has led to discrepancies such as the detection of CD45(-)/DAPI(+) cells with "cytomorphological patterns of malignancy" in the bloodstream of healthy donors. These concerns highlight the need for a highly sensitive method of CTC collection associated with immune-phenotyping of atypical CD45(-) cells from healthy whole blood using additional cancer biomarkers (i.e., TTF1, Vimentin, EpCAM, and CD44) in NSCLC.

Consequently, we evaluated a spiral microfluidic device that uses inertial and Dean drag forces to separate cells based on size and plasticity through a microfluidic chip. The formation of Dean vortex flows present in the microfluidic chip results in larger CTCs located along the inner wall and smaller immune cells along the outer wall of the chip. The enrichment process is completed by siphoning the larger cells into the collection outlet as the enriched CTC fraction. This method is particularly sensitive and specific (detection of around 1 CTC/mL of whole blood)[14] and can be associated with customized immunofluorescence (IF) analyses. These tools will enable setting up of a positive threshold for clinical interpretation. A workflow is thus described that enables biologists to isolate and immunophenotype CTCs with a high rate of recovery and specificity. The

protocol describes optimal use of the spiral microfluidic device to collect CTCs, the optimized IF assays that can be customized according to cancer type, and use of free open-source software for measuring and analyzing cell images to perform a semi-automatic numeration of the cells according to fluorescent staining. In addition, microscope multiplexing can be carried out depending on the number of fluorescent filters/markers available.

PROTOCOL:

Samples were prospectively collected within the framework of the CIRCAN ("CIRculating CANcer") cohort based at the Lyon University Hospital following patient written consent. This study was integrated into the CIRCAN_ALL cohort. The study CIRCAN_ALL was recognized as non-interventional by the CPP South-East IV dated 04/11/2015 under the reference L15-188. An amended version was recognized as non-interventional on 20/09/2016 under reference L16-160. The CIRCAN_ALL study was declared to the IT and freedom correspondent of the Hospices Civils de Lyon on 01/12/2015, under the reference 15-131. Blood collection was performed when physicians observed the earliest indication of tumor progression.

NOTE: Use all the reagents and materials outlined in **Table of Materials** with the respective storage conditions for pre-analytical sample preparation and immunofluorescence assay. Substituting reagents and/or modifying storage conditions could result in suboptimal assay performance.

1. Decontamination of spiral microfluidic device

NOTE: Decontamination of the spiral microfluidic device is a requirement to remove all immunofluorescence background generated from bacteria contamination, explore the cytomorphology of CTCs, and be able to differentiate them from normal immune cells. The protocol is optimized for blood samples collected in K_2EDTA tubes within 6 h after blood sampling and enriched using the spiral microfluidic device in clean conditions. Using this assay for other types of samples (other biological fluids) may require additional optimization. This decontamination protocol should be done once per week.

1.1. **Preparation of reagents**

1.1.1. Preparation of the diluent buffer

1.1.1.1. Sterilize 20 mL of diluent additive reagent using a 0.22 µm syringe filter and add directly to 1 L of 1x phosphate buffer saline (PBS) ultra-pure grade (**Table of Materials**).

1.1.2. Sterilization of reagents and the input straw

1.1.2.1. Sterilize the RBC lysis buffer and resuspension buffer (RSB; **Table of Materials**) using a 0.22 µm syringe filter and stock in a new 50 mL polypropylene conical tube for each solution.

1.1.2.2. Sterilize the input straw by incubating at room temperature (RT) for 1 h in the all-purpose cleaning reagent (**Table of Materials**). Transfer the straw to the bleach-based cleaning agent (**Table of Materials**) and incubate at RT for 1 h.

1.1.2.3. Rinse the input straw twice with sterile PBS for 1 h each and store the sterilized input straw in a surgically sterile bag (**Table of Materials**).

1.2. **Decontaminating the spiral microfluidic device**

1.2.1. Disinfection using the all-purpose cleaning reagent

1.2.1.1. Disconnect the diluent bottle cap from the diluent port of the spiral microfluidic device by unscrewing the brown screw. Under a microbiological safety cabinet, transfer up to 250 mL of all-purpose cleaning reagent (**Table of Materials**) into a new empty bottle.

1.2.1.2. Screw the diluent bottle cap and straw to the bottle of 250 mL all-purpose cleaning reagent under a microbiological safety cabinet. Attach this bottle to the diluent port of the spiral microfluidic device by screwing back the brown screw.

1.2.1.3. Transfer up to 100 mL of bleach (1% final concentration; **Table of Materials**) to the waste container supplied in the run kit (**Figure 1A**).

1.2.1.4. Load a new sterile input straw onto the spiral microfluidic device (**Table of Materials**) in the input port. Load a new 50 mL centrifuge tube in the input port. Load a new 50 mL centrifuge tube in the output port.

1.2.1.5. Proceed to prime the spiral microfluidic device by clicking on **Prime** on the spiral microfluidic device (3 min). Remove the input tube after the prime is completed.

1.2.1.6. Transfer up to 15 mL of all-purpose cleaning reagent to a new 50 mL centrifuge tube with a serological pipette under a microbiological safety cabinet and attach the tube to the input port of the spiral microfluidic device.

1.2.1.7. Before starting the run, check that the solution is free of excessive bubbles. If bubbles are present, remove them by slow aspiration with a pipette.

1.2.1.8. Load a decontamination microfluidic chip in the spiral microfluidic device. Run a Program 3 on the spiral microfluidic device by clicking on **Run** and selecting the **Program 3 (31 min)**.

NOTE: The Program 3 of the spiral microfluidic device enables a rapid enrichment of CTCs in 31 min.

1.2.1.9. Continue on with the spiral microfluidic device's cleaning step using the remaining volume of the all-purpose cleaning reagent in the input tube.

1.2.1.10. Discard the input tube after cleaning step is completed, leaving behind the input straw.

1.2.2. Decontamination using the bleach-based cleaning agent

1.2.2.1. Disconnect the all-purpose cleaning reagent bottle cap from the diluent port of the spiral microfluidic device by unscrewing the brown screw. Under a microbiological safety cabinet, transfer up to 250 mL of bleach-based cleaning agent (**Table of Materials**) in a new empty bottle (**Figure 1A**).

1.2.2.2. Screw the all-purpose cleaning reagent bottle cap and straw to the bottle containing the Bleach-based cleaning agent under a microbiological safety cabinet. Attach this bottle to the diluent port of the spiral microfluidic device by screwing back the brown screw.

1.2.2.3. Transfer up to 15 mL of bleach-based cleaning agent to a new 50 mL centrifuge tube input tube using a serological pipette under a microbiological safety cabinet. Load the 50 mL centrifuge tube input position. Load an empty tube in output position.

1.2.2.4. Before processing the run, check that the sample is free of excessive bubbles and if any are present, remove the bubbles by aspirating them slowly with a pipette.

1.2.2.5. Run Program 3 by clicking on **Run** and selecting the **Program 3 (31 min)**. After the run, proceed directly to the cleaning step using the remaining volume of bleach-based cleaning agent in the input tube.

1.2.2.6. Discard the input and output tubes.

1.2.3. Rinse the spiral microfluidic device.

1.2.3.1. Disconnect the bleach-based cleaning agent bottle cap from the diluent port of the spiral microfluidic device by unscrewing the brown screw. Under a microbiological safety cabinet, transfer the straw from the bottle containing the bleach-based cleaning agent to the new bottle containing the diluent buffer. Screw the bottle to the spiral microfluidic device.

1.2.3.2. Transfer up to 15 mL of sterilized water (**Table of Materials**) to a new 50 mL centrifuge tube input tube using a serological pipette under a microbiological safety cabinet. Load the 50 mL centrifuge tube input position. Load an empty tube in output position.

1.2.3.3. Before processing the run, check that the sample is free of excessive bubbles and if any are present, remove the bubbles by aspirating them slowly with a pipette.

1.2.3.4. Run Program 3 by clicking on **Run** and selecting the **Program 3 (31 min)**. After the run, proceed directly to the cleaning step using the remaining volume of sterilized water in the input tube.

1.2.3.5. Discard the input and output tubes.

2. Maintenance to keep the spiral microfluidic device bacteria-free

NOTE: The routine maintenance should be done at the end of the day during the last cleaning step.

2.1. Transfer up to 7 mL of bleach-based cleaning agent into the new 50 mL centrifuge tube using a serological pipette under a microbiological safety cabinet. Screw the bleach-based cleaning tube in input port of the spiral microfluidic device.

2.2. Before processing the clean run, check that the input sample is free of excessive bubbles and if any are present, remove the bubbles by aspirating them slowly with a pipette.

2.3. Run the clean on the spiral microfluidic device.

3. Pre-analytical enrichment of CTC from patient blood samples

3.1. Collect 7.5 mL of blood in the K_2EDTA tube and keep under gentle agitation to avoid cell sedimentation and clotting. Process within 6 h.

NOTE: If the blood is collected in a cell-free DNA blood collection tube containing preservative, store at 4 °C until processing. Ensure that the blood sample, RBC lysis buffer, and RBS buffer are at RT before proceeding with the enrichment step.

3.2. Transfer up to 7.5 mL of whole blood to a new 50 mL centrifuge tube input tube using a serological pipette under a microbiological safety cabinet.

3.3. Centrifuge at 1,600 x *g* for 10 min at RT. Collect the plasma fraction with a pipette without disturbing the buffy coat. Replace the plasma fraction by adding directly equivalent volume of PBS up to 7.5 mL.

3.4. Gently add RBC lysis buffer (**Table of Materials**) to blood sample to a final volume of 30 mL (for a K_2EDTA tube) or 37.5 mL (for a cell-free DNA blood collection tube). Gently invert the blood collection tube 10x and incubate for 10 min at RT.

NOTE: The blood sample turns darker red during RBC lysis. If no change (from dark red and opaque) is observed after 10 min, gently invert the tube 3x and leave to stand for another 5 min maximum. Do not leave the sample in RBC lysis buffer for more than 15 min because it can compromise the sample quality and assay performance.

3.5. Centrifuge the lysed blood sample at 500 x *g* for 10 min at RT, with centrifuge brakes on (or highest deceleration speed). Use a Pasteur pipette or serological pipette to gently remove the

supernatant until the volume reaches the 4–5 mL mark. Then, use filtered micropipette tips to remove the remaining supernatant.

3.6. Using a P1000 micropipette with a filtered tip, add 1.0 mL of RSB to the wall of the 50 mL centrifuge tube input tube. To avoid introducing bubbles into the mix, resuspend the cell pellet by gently pipetting up and down until the sample is homogeneous.

3.7. Add an additional 3 mL of RSB to the wall of the 50 mL centrifuge tube input tube (total volume 4 mL). Avoid introducing bubbles into the mix. Gently mix the cell suspension by gently pipetting up and down.

NOTE: In the unlikely event that regular pipetting is unable to break down cell clumps (defined by being visible or blocking the pipette tip), filter the sample through a 40 µm cell strainer to remove any clumps. Add 150 µL of RSB to the sample to make up for volume loss from filtering. Note that this method is to be used sparingly and only when large clumps are observed.

3.8. Before proceeding to the enrichment step, check that the sample is free of excessive bubbles and if any are present, remove the bubbles and take care not to discard any sample. If tiny bubbles are present, their removal is not required.

3.9. Process the sample on the spiral microfluidic device.

4. Enrichment of CTCs from patient whole blood with the spiral microfluidic device

4.1. Load a new spiral microfluidic chip. Load two empty 50 mL centrifuge tubes in input and output ports.

4.2. Run a prime by clicking on **Prime** on the spiral microfluidic device (3 min). Remove the input and output tubes and load the sample to be processed in input port.

4.3. Load a clear 15 mL conical tube in output port to collect enriched CTCs. Run **Program 3** by clicking on **Run** and selecting the **Program 3 (31 min)**.

4.4. Unload the output tube and centrifuge at 500 x *g* for 10 min (acceleration: 9; deceleration: 5). With a 5 mL serological pipette, remove supernatant stopping at the 2 mL mark on the conical 15 mL tube. With a micropipette, remove supernatant stopping at 100 µL mark on the conical 15 mL tube. Process the enriched sample directly for immunofluorescence staining.

5. Immunofluorescence staining

5.1. Enumerate on a chambered slide with a hemocytometer-type grid the number of cells per mL. Dilute the enriched sample with 0.2% anti-binding solution (**Table of Materials**) to a concentration reaching 100,000 cells/100 µL per cytospin.

5.2. Moisten the contour of the sample chamber using cotton (**Table of Materials**) with 50 µL of 0.2% anti-binding solution. Place a polylysine glass-slide in the sample chamber and close.

5.3. Coat a tip with 0.2% anti-binding solution by pipetting up and down 3x. Resuspend the enriched sample and transfer the cell solution into the sample chamber. Centrifuge with a dedicated centrifuge (**Table of Materials**) at 400 rpm for 4 min (acceleration low).

5.4. Place a silicon isolator around the area of deposition. Let dry the glass-slide under a microbiological safety cabinet for 2 min.

5.5. Prepare the fixation solution by diluting 1 mL of 16% paraformaldehyde (PFA) with 3 mL of sterile PBS. Add 100 µL of fixation solution (4% PFA) per sample and incubate at RT for 10 min. Remove fixation solution and perform three washes with 200 µL of PBS and incubate at RT each for 2 min.

CAUTION: Use PFA under a chemical safety cabinet to prevent inhalation.

5.6. Prepare the saturation solution by diluting the fetal bovine serum (FBS) at 5%, Fc receptor (FcR) blocking reagent at 5%, and bovine serum albumin (BSA) at 1% in sterile PBS (**Table of Materials**). Add 100 µL of saturation solution per sample and incubate for 30 min at RT. Remove saturation solution.

5.7. Add 100 µL of antibody solution per sample (CD45 antibody 1/20; PanCK antibody 1/500; PD-L1 antibody 1/200; Qsp saturation solution 100 µL) (**Table of Materials**). Place the polylysine glass-slide in a 100 mm x 15 mm Petri dish. Moisten an absorbent paper with 2 mL of sterile water and close the Petri dish with the lid. Place at 4 °C overnight and protect from the light.

5.8. Remove the antibody mix and perform 3 washes with 200 µL of PBS incubating each wash for 2 min. Let the sample dry for 5 min and protect it from the light. Place 10 µL of mounting solution (**Table of Materials**) in the area of deposition and cover with a microscope coverslip without making a bubble. Seal the coverslip with nail polish.

6. Acquisition of immunofluorescent images with straight fluorescent microscope and associated software

6.1. Use a straight fluorescent microscope with an X/Y motorized platform. Use a 20x objective to take 8-bit RGB tiff images in four channels corresponding to DNA dye (4',6-diamidino-2-phénylindole [DAPI]), PanCK dye (fluorescein isothiocyanate [FITC]), PD-L1 dye (CY3), and CD45 dye (CY5). Turn on the mercury lamp 15 min before use, and adapt the microscope and associated software to semi-automatized shoot.

6.2. Place the glass-slide on the platform.

6.3. In acquisition menu, define the four channels and set up the exposure time (DAPI: 15 ms, FITC [PanCK]: 500 ms; CY3 [PD-L1]: 800 ms; CY5 [CD45]: 1000 ms). Define the tiles to scan. Click **Tiles**. In **Advanced experiment**, define the area to scan.

6.4. Adjust the focus on the screen. Click **Start experiment**.

6.5. Export TIF files of each channel and specifically name the image file with this information: Sample_NumberofTilesRegion_dye_NumberOfSubtiles.tif (e.g., Sample1_TR1_c1m01). Name dye as follows: the DAPI channel is c1, FITC channel is c2, CY3 channel is c3, and CY5 channel is c4.

7. Analysis of immunofluorescent images with image analysis software

7.1. Download and install the free image analysis software from the Broad Institute website. Accept all default during installation. Open the image analysis software and click **File** | **Pipeline from file** | **Analysis_4channels_CTC.cppipe**.

NOTE: The pipeline converts RGB color images into grayscale, removes artifacts by smoothing images with a median filter, identifies nuclei and cytoplasm, quantifies fluorescence intensities of each channel, and exports them into an excel file.

7.2. Drop files in the **file list**. Update the metadata to group the files by tiles.

NOTE: All the instructions to group images are specified in the software. Name files appeared in **NamesAndTypes** module and files are grouped according to the number of the tile and channel per samples.

7.3. Click **View Output settings** and specify a correct default output. Click **Analyze Images**. Open the spreadsheet file corresponding to **measure_intensity** parameters.

REPRESENTATIVE RESULTS:

The first pre-requisite was to obtain uncontaminated (infectious agent-free) collections of CTCs for tissue culture and avoid IF background generated. The decontamination protocol enabled cleaning of all the pipes and pumps, and it resulted in the collection of CTCs with a good recovery rate without bacterial contamination. The enriched samples were compared without and with the decontamination protocol workflow of the spiral microfluidic device. To validate the decontamination protocol, the A549 cell line was used in absence of whole blood and enriched directly using the spiral microfluidic device. Without the optimized decontamination protocol, high bacterial contamination was observed in the tissue culture of enriched A549 cell line after only 24 h, which caused death and cytomorphological changes in eukaryotic cells (**Figure 1B**).

In contrast, after the cleaning protocol, living A549 cells were obtained by growing in 2D culture after 10 h of tissue culture and media removal and in 3D conditions (**Figure 1B**), as well as patient samples (**Figure 1C**). The potential CTC are identified with a red cross (**Figure 1C**).

Figure 2 recapitulates the complete workflow for immunofluorescence phenotyping of enriched CTCs from whole blood. It is composed of four major steps: whole blood sampling, CTC enrichment, immunofluorescence (IF) assay, and image analysis using software. Previously, the recovery rate of the spiral microfluidic device has been addressed[14]. Using fluorescent mimicking CTCs (mCTC), this recovery rate was established at 1.3 CTCs/mL whole blood[14].

The present work focused on setting up the optimal conditions for IF analysis of enriched CTCs and downstream visualization (**Figure 2**). First, to test the specificity of the PD-L1 antibody, two cell lines were used: (1) PC3 high-positive-PD-L1 cell line and (2) SW620 low-positive-PD-L1 cell line. The cells were then enriched with the spiral microfluidic device and analyzed by IF. All cells were stained with the tumor anti-PanCK marker, white blood cell anti-CD45 marker, anti-PD-L1 (useful in lung cancer), and DAPI (nuclear dye). White blood cells were identified as positive for DAPI and CD45, while cancer cells were identified as positive for DAPI and PanCK and negative for CD45. The PC3 high-PD-L1-positive cell line was positively stained for PD-L1, while a lower PD-L1 expression was detected in the SW620 low-PD-L1-negative cell line.

Then, the following were compared: the (i) liquid IF staining assay, (ii) staining of CTCs directly deposited onto polylysine-coated slides, and (iii) IF staining of CTCs after cytospin on polylysine-coated slides. It was clearly observed that the recovery rate of CTCs depended on the type of protocol used (**Figure 3A**). In the liquid IF staining assay, the recovery rate was only 10% for the number of spiked mCTC was the lowest. This low recovery rate presents an issue for most patients with metastatic NSCLC, as it significantly limits the ability of these tests to isolate the few CTCs and provide phenotypic information. The second and third sections described (direct deposition of mCTC or CTC onto polylysine-coated slides without and with cytopsin) systematically had recovery rates exceeding 60% (**Figure 3A**).

Figure 3B shows representative images of these IF assays using whole blood samples from the same patient, either using the liquid IF staining assay or IF staining assay on polylysine-coated slides with cytospin. The enumeration of nuclei was clearly different between the two assays (**Figure 3B**). The nuclear DAPI staining provided the enumeration of total cells in the sample, and the biomarker staining enabled highlighting of the green the PanCK-positive cells, orange in the PD-L1-positive cells, and red in the CD45 residual white cells (**Figure 3B**).

Next, to cytologically differentiate white blood cells from tumoral cells, the shape of the nucleus has to be visualized, since it is characteristic of the cell type. **Figure 3C** demonstrates the outlines of the nuclei that are blurry and morphology that is unusual in the absence of the cytospin step. The optimized protocol thus included cytospinning of enriched CTCs on polylysine-coated slides followed by 4% paraformaldehyde (PFA) fixation, for preserving the slides before IF staining. This optimized protocol had similar recovery rates as the deposition of mCTC directly onto polylysine-coated slides (**Figure 3A**), even when very few cells were added. Since this additional step enable preservation of nuclear morphology (**Figure 3C**), granulocytes were identified with their multi-lobed nuclei, as well as tumoral cells (labelled with a red cross in the nucleus) with their nuclear abnormalities, malignancy patterns, and larger size compared to white blood cells.

After optimization of the IF protocol, a proof-of-concept was conducted using whole blood from metastatic patients. Samples were prospectively collected within the framework of the CIRCAN routine cohort based at the Lyon University Hospital. Blood collection was usually performed when physicians observed the earliest indication of tumor progression. All tumor cases were histologically or cytologically confirmed on FFPE biopsy specimens during the initial diagnosis. Here, CTCs analyses at progression were performed by investigators who did not have access to or prior knowledge of clinical data. Detailed pre-analytical considerations have been previously published[15].

In **Figure 4**, **Table 1**, and **Table 2**, different findings are presented from patient samples. The CD45(+), PanCK(-), PD-L1(-) profile represents the immune cells. The residual count of white blood cells was shown to be strongly variable and dependent on the whole blood sample. The range in this small pilot cohort was 648–11,000 white CD45(+) cells (**Figure 5A**). Consequently, immediately after CTC enrichment, an enumeration of the collected cells was included to adjust the cellular density on the cytospin area at a density of 100,000 cells/cytospin (see section 6). This enabled performance of several cytospins per patient and optimization of the microscopic observation for manual enumeration and use of image analysis software pipeline.

In **Figure 4A-C**, **Table 1**, and **Table 2**, typical cases are reported in which residual white blood cell counts were highly different:

(i) The first profile is the CD45(-), PanCK(+), and PD-L1(+) presented in **Figure 4A** . Often the size of the cells is superior to 13 µm in diameter and the nucleus morphology is irregular, representing a cytomorphological pattern of malignancy. This population is likely composed of CTC.
(ii) The second profile is the CD45(-), PanCK(-), and PD-L1(+). As already reported, not all CTCs express the PanCK biomarker (**Figure 4A**).
(iii) The third profile is the CD45(-), PanCK(+), and PD-L1(-). As already reported, not all CTCs express the PD-L1 biomarker.
(iv) The fourth profile is the CD45(+), PanCK(+), and PD-L1(+) presented in **Figure 4B**. It represents the atypical activated immune cells in patient whole blood. This population has been described in several publications[16,17,18] and represents approximately 5% of the total cells following enrichment. The presence of this population may increase the rate of false positive CTCs in a sample if the intensity of the CD45 signal is too low and morphology of the nucleus is not well-conserved. This strongly highlights the need for carrying out complementary tumoral biomarker staining, such as Vimentin and/or Epcam in this immunofluorescence assay.
(v) Finally, the last profile includes the unlabeled cells CD45(-), PanCK(-), and PD-L1(-), highlighted in **Figure 4C**. The nucleus in this population often shows cytomorphological patterns of malignancy, and the size is over 13 µm in diameter. The percentage of these cells in the samples is highly variable according to the patient whole blood. This highlights the need to use complementary tumoral biomarkers to confirm the tumoral pattern of this cell sub-population.

In **Table 1** and **Table 2,** the cell count of 16 samples was reported from advanced metastatic NSCLC patients. The cells were classified according to the expression of the biomarkers. High

variability was observed in the sub-populations obtained. As already reported in independent studies, CD45(-), PanCK(+), and PD-L1(+) profiles were found in most samples. Nevertheless, as the CTC population is highly heterogeneous, patient samples also contained CD45(-), PanCK(-), and PD-L1(+) sub-populations, the CD45(-), PanCK(+), and PD-L1(-) sub-populations and unlabeled cells CD45(-), PanCK(-), and PD-L1(-) sub-populations. The level of residual white blood cells was highly variable among analyzed samples.

To facilitate cell enumeration, a pilot pipeline was set up using the image analysis software for an automated analysis of the immunofluorescence images. The workflow is described in **Figure 5**. In this case, it is important to acquire high quality immunofluorescence images in terms of contrast and fluorescence intensity. Depending on the capacity of cluster calculation of the hardware, the image analysis pipeline can be applied to the complete merged image of the cytospin or on a representative area of the cytospin.

Here, based on the microscope, a semi-automated scan of the cytospin (X/Y; the Z focus is not included) area generated 150–200 merged images. These images can be merged together and directly analyzed using the image analysis pipeline. Nevertheless, this procedure is time- and calculation cluster resource-consuming, an important limitation for its routine use in laboratories. Therefore, based on prior experience in the field of cellular hematology, it was decided to analyze representative areas of each sample after verifying under a microscope that the distribution of cells was homogenous on the entire area of the cytospin. Then, 25% of the total area of the cytospin (around 40 tiles) was scanned with the florescence microscope to generate 40 x 4 independent images. The merged file was split by channels, and image files were automatically generated with the microscope software (see section 7; **Figure 5A**). These files were imported into an image analysis pipeline for analysis according to the parameters described (see section 8; **Figure 5A**).

In **Figure 5B**, we manually identified a representative image displaying four CTCs [CD45(-), PanCK(+), and PD-L1(+)] among 77 immune cells [CD45(+), PanCK(-), and PD-L1(-)]. **Figure 5B** illustrates how the image analysis software identified and enumerated the number of cells based on the DAPI staining. It also illustrates how the image analysis software counted the secondary objects. Finally, fluorescence intensities for each fluorescent channels were reported for all objects reported in the images.

The background was calculated and represented by the negative cells present in the sample. For example, the non-activated immune cells have low fluorescence intensity and enabled measuring of the background of the PanCK and PD-L1 staining. The fluorescent signal was deemed positive if the fluorescence intensity exceeded that of the background by two-fold (based on the analysis of four independent patient samples). Concerning CD45 staining, as the expression level of CD45 is highly variable in the white blood cells sub-populations, the threshold of positivity was set as low as possible. It was based on the analysis of images of 10 healthy whole blood stained with the CD45 antibody. The pilot analysis (n = 4) showed concordance between manual enumeration and image analysis software enumeration (**Table 2**). Each cell on the cytospin is identified by

image analysis software and enables biologists to track the cell and manually confirm the results, if needed.

FIGURE AND TABLE LEGENDS:

Figure 1: Overview of the workflow for decontamination of spiral microfluidic device instrument. (**A**) Three major steps included in the decontamination process (see protocol), illustrating the localization of the input and outputs of the instrument. (**B**) Representative images of A549 cell line enrichment before and after decontamination of the instrument. The impact of the presence of an infectious agent on the viability and morphology of collected cells is shown. In the presence of bacteria, cell morphology and viability was modified. Scale bar = 20 µm. (**C**) 3D cell culture of enriched patient samples of lung, prostate and breast cancer. The red cross corresponds to atypical cells. Scale bar = 20 µm.

Figure 2: Overview of the workflow of immunofluorescence analysis from whole blood sampling to analysis of the fluorescence images. The major steps are shown as follows: blood collection for whole blood, CTC collection with spiral microfluidic device, immunofluorescence assay, and image analysis software. The choice of biomarker was driven by better identification of the various populations of cells observable on the cytospin slide (CD45 for immune cells, PanCK and PD-L1 for lung cancer cells).

Figure 3: Recovery rate of three independent staining protocols. (**A**) Comparison of the recovery rate of mCTCs from liquid staining, direct staining of cell deposited onto polylysine-coated slides, and cell staining after cytospin on polylysine-coated slides. (**B**) Representative images of patient samples processed by liquid IF protocol and direct immunostaining protocol with the cytospinstep. Cells were stained with CD45 monoclonal antibody (clone HI30) Alexa Fluor 647; PanCK monoclonal antibody (clone AE1/AE3) Alexa Fluor 488; 4',6-diamidino-2-phénylindole (DAPI). Scale bar = 20 µm. (**C**) Representative images of DAPI staining of cell enrichment with and without the cytospin step. The morphology and size of the nuclei were shown using DAPI staining. Red cross highlights cells with abnormalities in the nucleus in the right-hand image. In the left-hand image, the image is fuzzy, since cells are not at the same level (x-, y-, and z-axes). Scale bar = 10 µm.

Figure 4: Identification of cell profiles. (**A**) Representative images of patients with different CTC profiles. The fluorescence channels are presented separately. The merged images are shown on the left. They are stained with CD45 monoclonal antibody (clone HI30) Alexa Fluor 647; PanCK monoclonal antibody (clone AE1/AE3) Alexa Fluor 488; PDL-1 monoclonal antibody (clone 29E2A3) phycoerythrin; 4',6-diamidino-2-phénylindole (DAPI); arrows point to atypical cells. (**B**) Representative images of immunostaining of two patient samples with atypical white blood cells profiles. Cells were stained with CD45 monoclonal antibody (clone HI30) Alexa Fluor 647; PanCK monoclonal antibody (clone AE1/AE3) Alexa Fluor 488; PDL-1 monoclonal antibody (clone 29E2A3) phycoerythrin; 4',6-diamidino-2-phénylindole (DAPI). The image highlights the presence of immune cells stained with CD45(+), PanCK(+), and PD-L1(+). (**C**) The image highlights the presence of unlabeled cells (CD45(-), PanCK(-), and PD-L1(-). Scale bar = 10 µm.

Figure 5: Overview of analysis of the fluorescent images. (**A**) The main steps are described: microscopy scanning, the channel split according to the fluorescence, and the import of files into image analysis software. (**B**) Description of the three different steps for the workflow of the image analysis software.

Table 1: Manual enumeration of patient cell enrichment. Enumeration of cells based on DAPI staining. Enumeration of other objects based on FITC, PE and CY5 staining.

Table 2: Image analysis software enumeration of patient cell enrichment. Enumeration of cells based on DAPI staining. Enumeration of other objects based on FITC, PE and CY5 staining. Comparison of manual count and image analysis software enumeration.

DISCUSSION:

Two major points were raised in the present study, the first with regards to performance of the workflow for its transfer to clinical applications, and the second concerning the decrease in subjectivity for the analysis of fluorescence images obtained.

A performant and optimized workflow for CTC enumeration was initially determined using customizable IF assay after cell enrichment via a CTC label-free microfluidic system (spiral microfluidic device). Using this workflow, a pilot study confirmed that all samples from metastatic NSCLC patients contained atypical cells, which were all CD45(-). They may alternatively be labeled with PanCK and/or PD-L1 biomarkers; however, they can also be completely negative for all tested biomarkers [CD45(-), PanCK(-), and PD-L1(-) as observed in the S19 sample (**Table 2**)]. This strongly highlights the need for additional biomarkers for phenotyping CTC sub-populations. Consequently, it has been proposed to add epithelial-mesenchymal biomarkers such as EpCAM, Vimentin, and N-Cadherin; markers of cancer stem cells including CD44 and CD133; and specific tumor markers including TTF1 for lung adenocarcinoma.

In the pilot study, the range of atypical cell was [40; >400] from 3.5 mL of whole blood. For 80% of the patient samples, the atypical cell count was over 50. Indeed, in cytological[19,20,21] samples from Endo Bronchial Ultra Sonic Guide Trans Bronchial Needle Aspiration or CT-guided trans-thoracic punctures, PD-L1 analysis is suitable in most of samples, but a threshold of ≥100 tumor cells is commonly admitted to produce a statistical and clinical interpretation of the value. However, in the particular case of blood CTCs, it should be noted that the issue of spatial tumor heterogeneity is bypassed by contrast to small on-site tumor samples.

The second point was to avoid the impact of the handler on analysis of immunofluorescence images. Image analysis software of fluorescent images was thus set up to standardize cell enumeration and provide statistical data for these samples. This automated process highlighted the need for powerful calculation clusters for the analysis of all cells contained in the same sample. In addition, quality of the IF staining has to be in the same plane (to avoid use of confocal systems), and the density of cells on the cytospin must be calibrated to enable the image analysis software to recognize all cells separately on the slide. Finally, the results were not validated

regarding clinical outcomes in a cohort of patients, but this point should be addressed in another dedicated study.

ACKNOWLEDGMENTS:

This work was supported by research grants from AstraZeneca (London, United-Kingdom), Biolidics (Singapore) and the Ligue Contre le Cancer (Saone et Loire, France). The authors thank AstraZeneca and Biolidics companies for their financial support.

DISCLOSURES:

Jean-Philippe Aurel and Kathryn Weiqi Li are employees of Biolidics company that produces instruments used in this article. The other authors have nothing to disclose.

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Figure 1

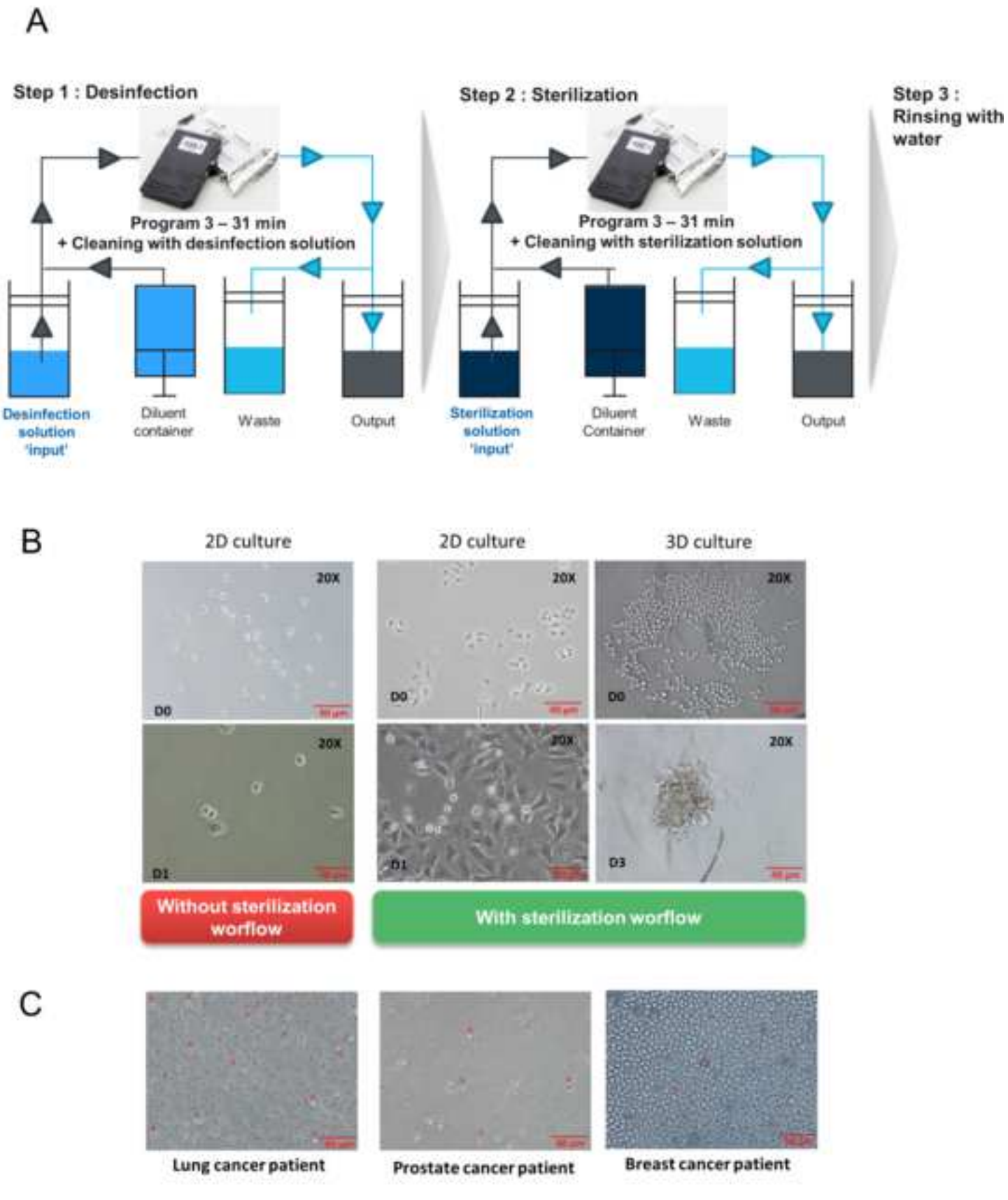


Figure 2 Click here to access/download;Figure;Diapositive2.TIF

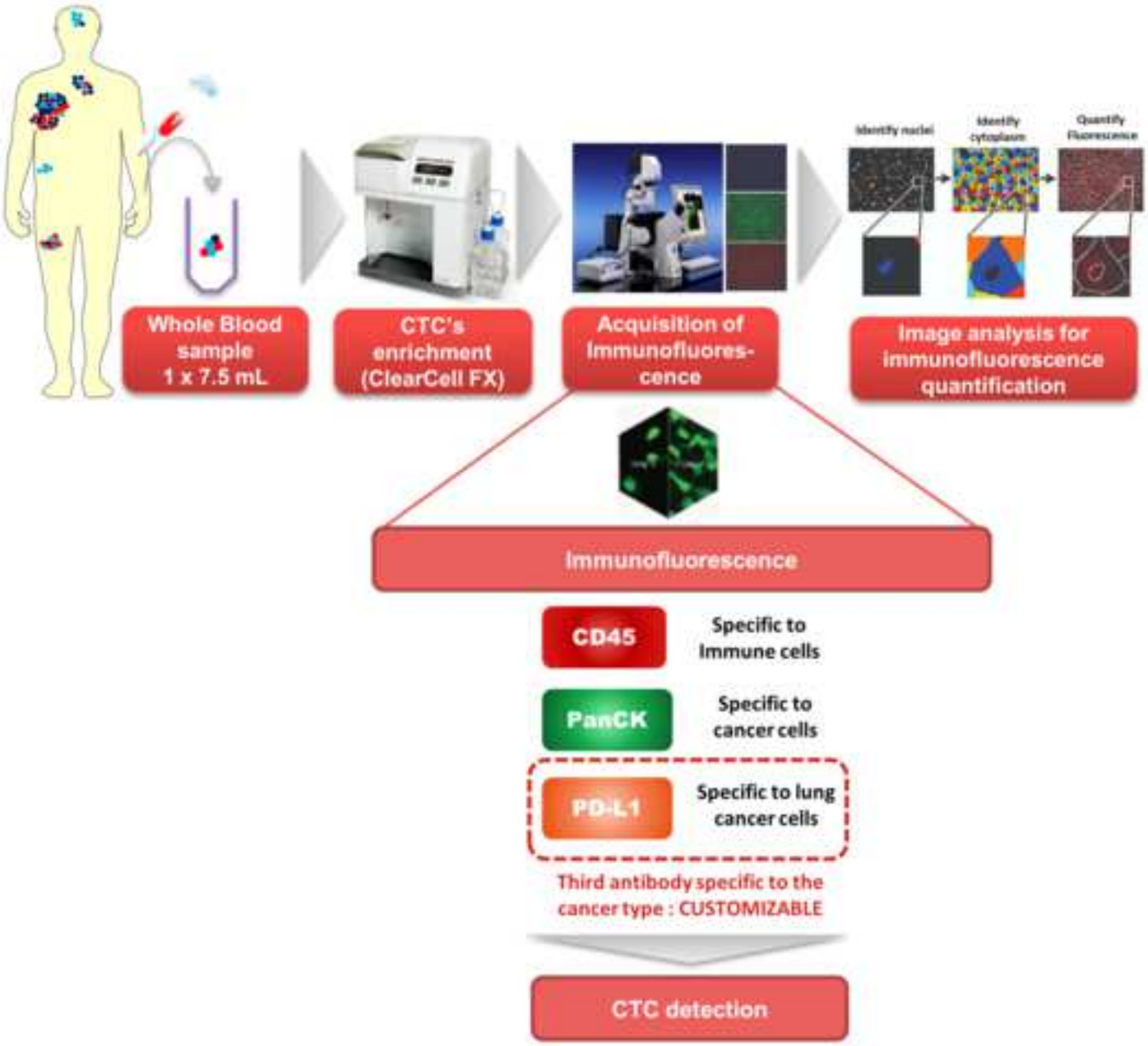
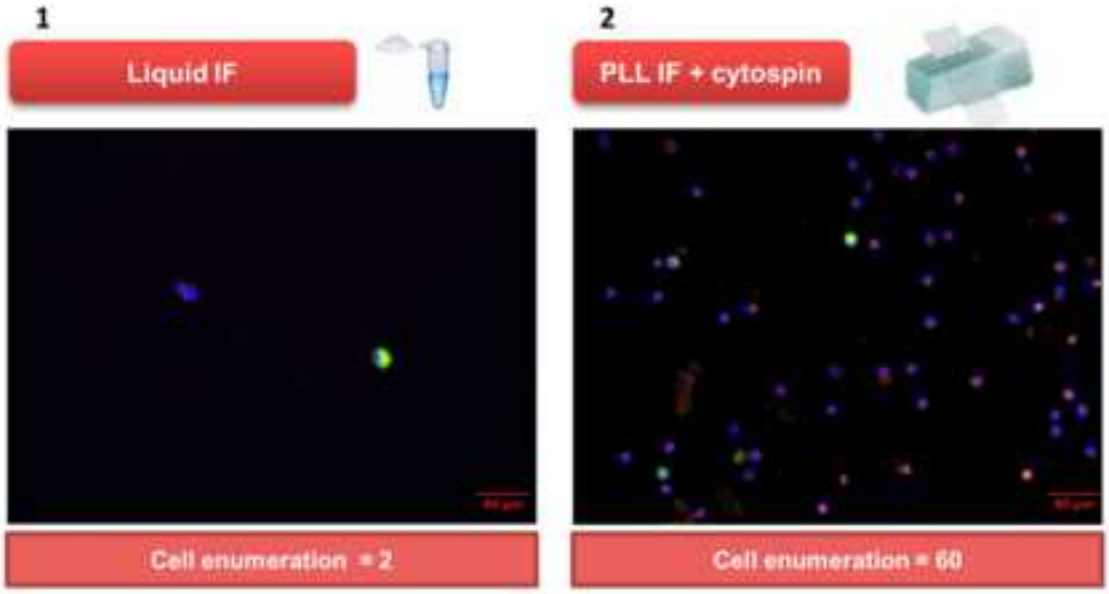


Figure 3

A

#Condition	Number of spiked cells	Liquid IF	Polylysine IF	PLL IF + cytospin
		Absolute Count (%) (n=3)		
100 cells	100	31 (31%)	62 (62%)	x
50 cells	50	36 (72%)	36 (72%)	36 (76%)
10 cells	10	1 (10%)	8 (80%)	5 (64%)

B



C

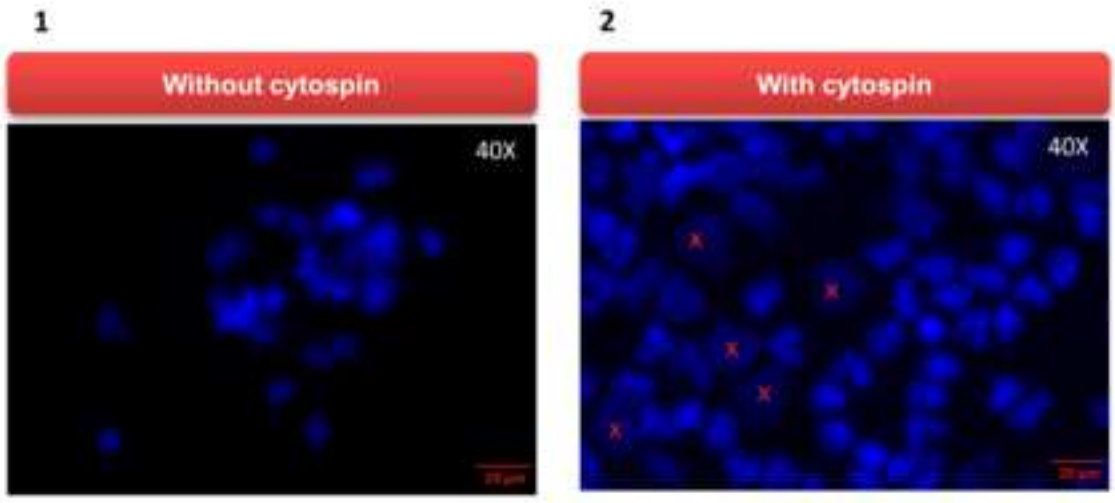


Figure 4

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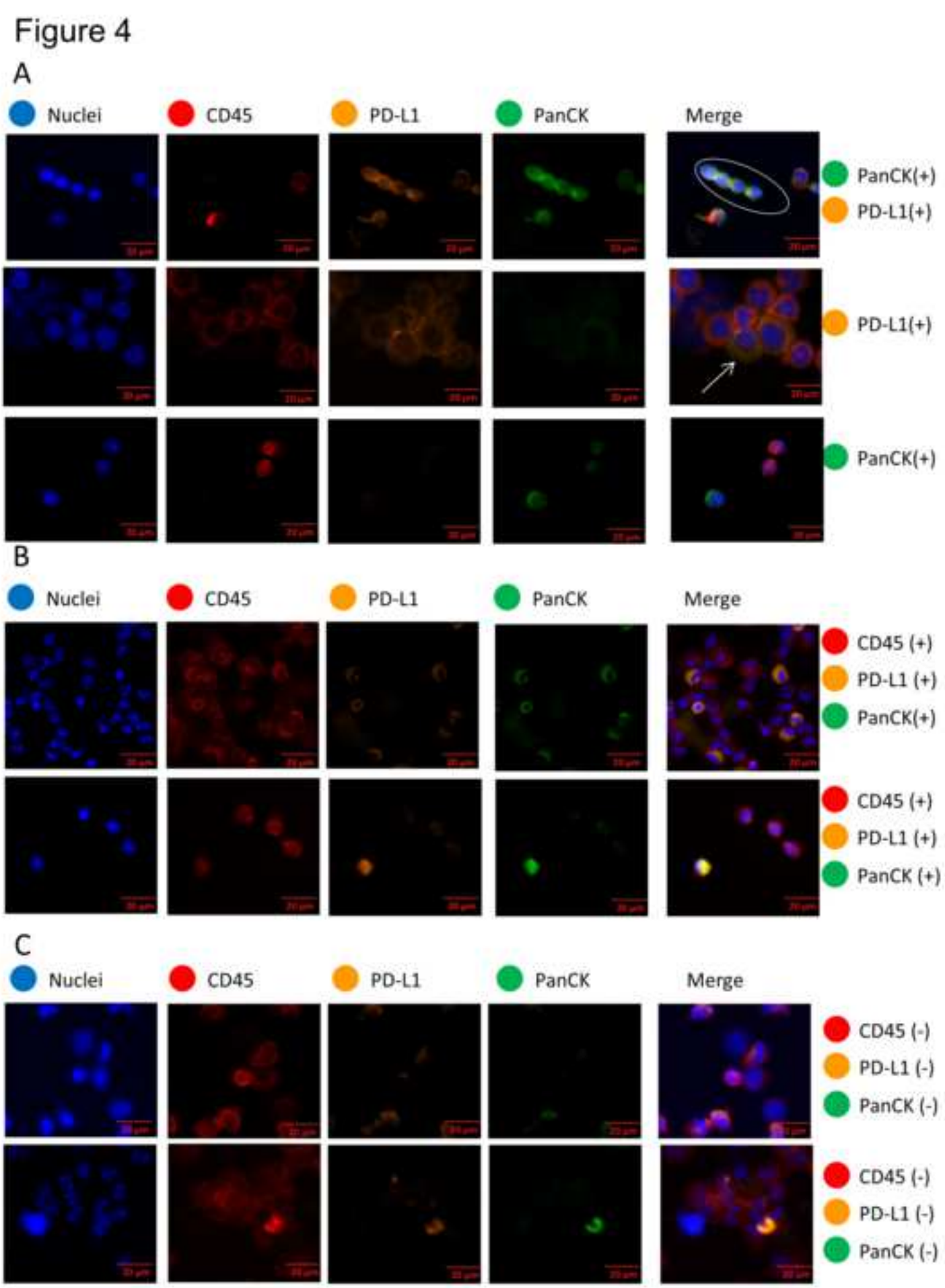

Figure 5

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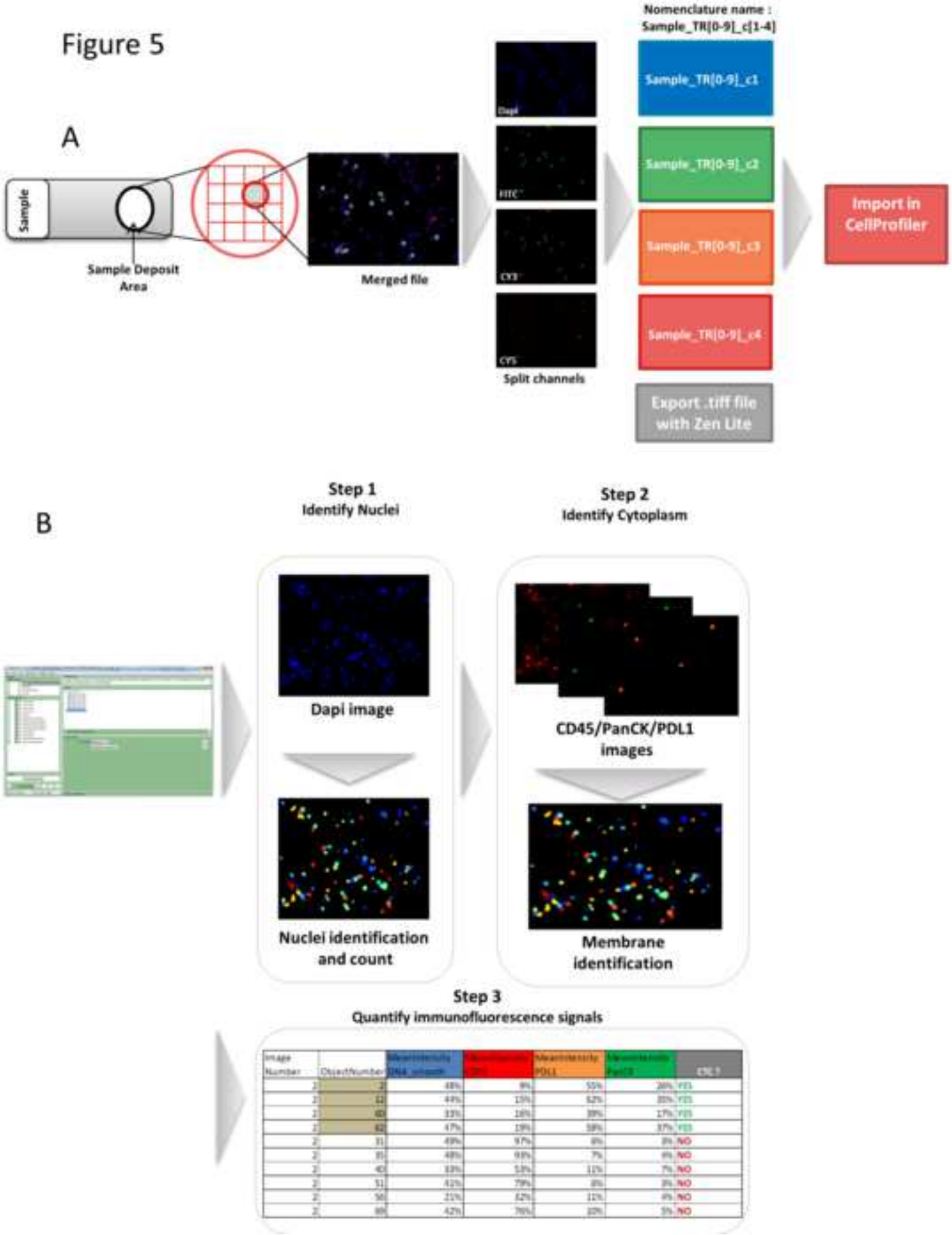

Table 1

Click here to access/download;Table;59873_R3_RE_Table_1.xlsx

No.	Volume of whole blood (mL)	Total count of CD45(-) and PD-L1(+) or PanCK(+)	CD45(-) / PanCK(-) / PD-L1(-)	PanCK(+)/PD-L1(-) (% of cells)
S01	3.5	36	0	0
S02	3.5	8	38	0
S03	3.5	150	125	29 (19.3)
S04	3.5	22	141	0
S05	3.5	293	81	0
S06	3.5	4	58	0
S07	3.5	13	94	1 (7.69)
S08	3.5	33	170	0
S09	3.5	6	126	0
S10	3.5	42	24	0
S11	3.5	56	66	23 (41.2)
S12	3.5	9	9	1 (11.1)
S13	3.5	48	81	19 (39.6)
S14	3.5	32	41	0
S15	3.5	25	85	0
S16	3.5	36	72	0

Cells subtypes (CD45-)		Count of CD45(-) and PD-L1(+) or PanCK(+) /mL whole blood	WBC(CD45+)	**WBCs/mL**
PanCK(-)/PDL1(+) (% of cells)	PanCK(+)/PD-L1(+) (% of cells)			
0	15 (41.7)	10.29	648	**86.4**
1 (12.5)	7 (87.5)	2.29	3779	**99.4**
0	121 (80.6)	42.86	4581	**99.4**
4 (18.2)	18 (81.8)	6.29	7469	**611**
0	293 (100)	83.71	8126	**1083**
0	4 (100)	1.14	42280	**5637**
2 (15.4)	10 (76.9)	3.71	32894	**4386**
0	33 (100)	9.43	112785	**15038**
0	6 (100)	1.71	30101	**4013**
26 (61.9)	16 (38.1)	12.00	25674	**3423**
5 (8.93)	28 (0.50)	16.00	2598	**346**
3 (33.3)	5 (55.6)	2.57	1681	**224**
23 (47.9)	6 (12.5)	13.71	5648	**753**
0	32 (100)	9.14	8681	**1157**
1 (4.00)	24 (96.0)	7.14	1256	**167**
0	36 (100)	10.29	11827	**1577**

Table 2

Click here to access/download;Table;59873_R3_RE_Table_2.xlsx

		Total cell count	CD45(+)	PanCK(+)/PD-L1(+) (% of cells)	PanCK(-)/PDL1(+) (% of cells)	PanCK(+)/PD-L1(-) (% of cells)
S17	Cell_Profiler	976	769	35	10	8
	Manual count	ND	ND	24	0	0
S18	Cell_Profiler	5397	5393	0	0	0
	Manual count	ND	ND	2	0	0
S19	Cell_Profiler	6570	5336	19	14	0
	Manual count	ND	ND	16	26	0
S20	Cell_Profiler	3662	3640	1	0	1
	Manual count	ND	ND	1	0	0

ND: Not determined

CD45(-)/PanCK(-)/PD-L1(-)
154
>25
4
9
1172
>25
20
26

Table of Materials

Click here to access/download;Table of Materials;59873_R3_RE_Table_of_Materials_JoVE-GARCIA.xlsx

Reagent & Materials	Manufacturer	Cat. Number	Storage Conditions
4',6-diamidino-2-phénylindole (DAPI)	Ozyme	BLE 422801	+4°C
BD Facs Clean – 5L	BD Biosciences	340345	Room temperature
Bleach 1% Cleaning Solution 100 mL	Biolidics	CBB-F016012	Room temperature
Bovine Serum Albumin (BSA) 7.5%	Sigma	A8412	+4°C
CD45 monoclonal antibody (clone HI30) Alexa Fluor 647	BioLegend	BLE304020	+4°C
CellProfiler Software	Broad Institute		
Centrifuge device	Hettich	4706	Room temperature
Centrifuge tube 50 mL	Corning	430-829	Room temperature
Centrifuge Tube 15 mL	Biolidics	CBB-F001004-25	Room temperature
ClearCell FX-1 System	Biolidics	CBB-F011002	Room temperature
Coulter Clenz Cleaning Agent – 5L	Beckman Coulter	8448222	Room temperature
CTChip FR1S	Biolidics	CBB-FR001002	Room temperature
Cytospin 4	ThermoFisher	A78300003	Room temperature
Diluent Additive Reagent – 20 mL	Biolidics	CBB-F016009	+4°C
EZ Cytofunnels	ThermoFisher	A78710003	Room temperature
FcR blocking Agent	Miltenyi Biotec	130-059-901	+4°C
Fetal Calf Serum (FCS)	Gibco	10270-106	+4°C
Fluoromount	Sigma	F4680	Room temperature
Fungizone - 50 mg	Bristol-Myers-Squibb	90129TB29	+4°C
FX1 Input Straw with lock cap	Biolidics	CBB-F013005	Room temperature
KovaSlide	Dutscher	50126	Room temperature
PanCK monoclonal antibody (clone AE1/AE3) Alexa Fluor 488	ThermoFisher	53-9003-80	+4°C
Paraformaldehyde 16%	ThermoFisher	11490570	+4°C
PD-L1 monoclonal antibody (clone 29E2A3) - Phycoerythrin	BioLegend	BLE329706	+4°C
Petri Dish	Dutscher	632180	Room temperature
Phosphate Buffered Saline (PBS)	Ozyme	BE17-512F	+4°C
Phosphate Buffered Saline Ultra Pure Grade 1X – 1L	1st Base Laboratory	BUF-2040-1X1L	Room temperature
Pluronic F-68 10%	Gibco	24040-032	Room temperature
Polylysine slides	ThermoFisher	J2800AMNZ	Room temperature
Polypropylene Conical Tube 50 mL	Falcon	352098	Room temperature
RBC Lysis Buffer – 100 mL	G Biosciences	786-649	+4°C
RBC Lysis Buffer – 250 mL	G Biosciences	786-650	+4°C

Resuspension Buffer (RSB)	Biolidics	CBB-F016003	+4°C
Shandon Cytopsin4 centrifuge	ThermoFisher	A78300003	Room temperature
Silicon Isolator	Grace bio-Labs	664270	Room temperature
Sterile Deionized Water – 100 mL	1st Base Laboratory	CUS-4100-100ml	Room temperature
Straight Fluorescent microscope Axio Imager D1	Zeiss		Room temperature
Surgical Sterile Bag	SPS Laboratoires	98ULT01240	Room temperature
Syringe BD Discardit II 20 mL sterile	BD Biosciences	300296	Room temperature
Syringe Filter 0.22 µm 33 mm sterile	ClearLine	51732	Room temperature
Zen lite 2.3 Lite Software	Zeiss		

Comments

Bleach-based cleaning agent
Bleach

Image Analysis Software

Spiral microfluidic device
All-purpose cleaning reagent
Microfluidic chip

Sample chamber with cotton

Mounting solution
Anti-fungal reagent
Straw
Chambered slide

Fixation solution

Anti-binding solution

Dedicated centrifuge

Microscope associated software

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Author(s): Jessica Garcia1,2,3, David Barthelemy1,3, Florence Geiguer1,3, Julie Ballandier1,3, Kathryn Weiqi Li4, Jean-Philippe Aurel4, Frédérique Le Breton5, Claire Rodriguez-Lafrasse1, Brigitte Manship2, Sébastien Couraud3,6,7, Léa Payen1,2,3

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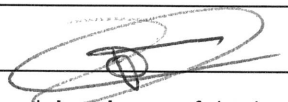
CORRESPONDING AUTHOR

Name:	PAYEN-GAY Léa
Department:	3Hospices Civils de Lyon Cancer institute, CIRculating CANcer (CIRCAN) program, Pierre Bénite, France
Institution:	Hospices Civils of LYON
Title:	Professor of Toxicology and molecular biology
Signature:	
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Pr. Léa PAYEN
Tel: 0033(0)687018829
Fax: 0033(0)478782720
E-mail: lea.payen-gay@univ-lyon1.fr

Manuscript ID #: JoVE59873_R3

JoVE
Xiaoyan Cao, Ph.D.
Review Editor

Dear Colleague,
We would like to thank you for your interest in our study titled ”Semi-automatic PD-L1 characterization and enumeration on Circulating Tumor Cells from Non-Small Cell Lung Cancer patients by immunofluorescence”, as well as the referees for their insightful remarks. As suggested, we have successfully addressed all of the points raised by the referees. We are convinced that this revised version has gained in quality and that this work is of interest to the scientific community.

We are, therefore, confident that our study, in its present form, will meet your expectations and be considered as suitable for publication in JoVE.

Sincerely Yours,

Léa Payen PharmD, PhD
Corresponding author

Answers to referees

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1. Please address the specific comments marked in the attached manuscript.
2. Figures: Please revise the scale bars so that they are easier to read.
3. Please provide high-resolution figures. If submitting as a .tiff, please ensure that the image is 1,440 pixels x 480 pixels or 300 dpi.
4. Table of Materials: Please review the attached Table of Materials to ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol.

Answers:

1. **We addressed the specific comments in the attached manuscript.**
2. **We revised the scale bars.**
3. **We provide high quality TIFF files.**
4. **We revised the Table of Materials addin relevant informations and some missing equipment and software.**