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Minimizing false positives for CTC identification

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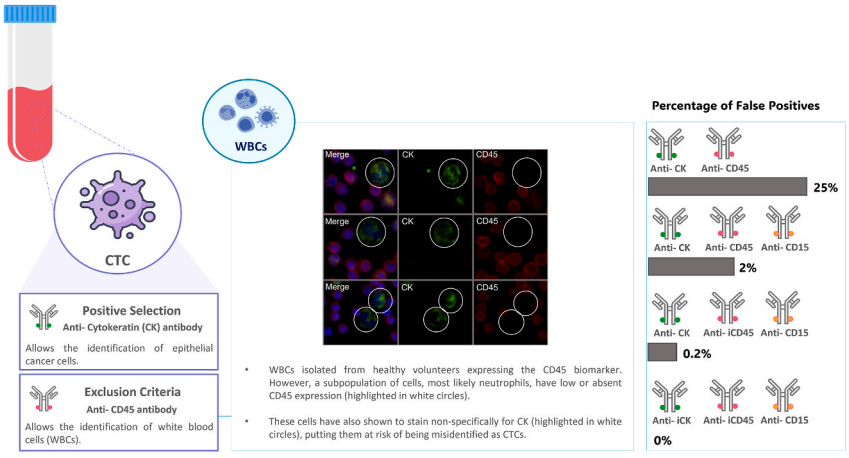
HIGHLIGHTS

- Circulating Tumour Cells (CTCs) can provide important real-time insights into tumour heterogeneity and clonal evolution.
- CTCs are identified using biomarkers for positive selection and exclusion, typically cytokeratin (CK) and CD45.
- Granulocytes often express low levels of CD45 and stain non-specifically for CK, risking to be misclassified as CTCs.
- Sensitivity and specificity of CTC assays are improved by using high-performing antibodies and double exclusion (CD15).
- False positives can be totally eliminated by combining these immunolabelling strategies.

GRAPHICAL ABSTRACT



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ABSTRACT

Background: Cancer is a leading cause of death worldwide, with metastasis playing a significant role. Circulating Tumour Cells (CTCs) can provide important real-time insights into tumour heterogeneity and clonal evolution, making them an important tool for early diagnosis and patient monitoring. Isolated CTCs are typically identified

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by immunocytochemistry using positive biomarkers (cytokeratin) and exclusion biomarkers (CD45). However, some white blood cell (WBC) populations can express low levels of CD45 and stain non-specifically for cytokeratin, increasing their risk of misclassification as CTCs. There is a clear need to improve CTC detection and enumeration criteria to unequivocally eliminate interfering WBC populations.

Results: This study showed that, indeed, some granulocyte subpopulations expressed low levels of CD45 and stained non-specifically for cytokeratin, misidentifying them as CTCs. These same cells, however, strongly expressed CD15, allowing them to be identified as WBCs and excluded from CTC classification. Flow cytometry confirmed the specificity of the CD15 antibody for the granulocyte subpopulation. False positives were considerably reduced from 25 % to 0.2 % by double exclusion, combining a CD15 antibody with a highly specific CD45 antibody. Furthermore, complete elimination of potential false positives was achieved using double exclusion in combination with improved selection of cytokeratin antibody. The study emphasises the importance of a robust exclusion criteria and high antibody specificity in CTC immuno-assays for accurate identification of CTC candidates and thorough exclusion of interfering WBC subpopulations.

Significance: This study demonstrated how misidentifying a granulocyte subpopulation can lead to inaccurate CTC evaluation. However, sensitivity and specificity of CTC identification may be improved by using high-performing antibodies and by including a second exclusion biomarker, in turn, allowing for a more comprehensive clinical application of CTCs.

List of abbreviations

BSA	(Bovine Serum Albumin)
CTC	(Circulating Tumour Cell)
CK	(Cytokeratin)
CRC	(Colorectal Cancer)
EGFR	(Epidermal Growth Factor Receptor)
EpCAM	(Epithelial Cell Adhesion Molecule)
EMT	(Epithelial to Mesenchymal Transition)
HER2	(Human Epidermal Growth Factor Receptor)
ICC	(Immunocytochemistry)
PBS	(Phosphate Buffer Saline)
PFA	(Paraformaldehyde)
PSA	(Prostate-specific Antigen)
WBC	(White Blood Cell)

1. Introduction

Circulating tumour cells are defined as cancer cells shed from a solid tumour into the bloodstream, hence becoming detectable in the blood. These cells have gained much attention since they may act as metastatic precursors responsible for disease progression and, ultimately cancer-related death [1]. Also, being living cells from the active tumour, CTCs hold the advantage of reflecting intra-tumour and inter-tumour heterogeneity and providing cancer-related information on RNA, DNA and protein expression, which may be useful to stratify patients and guide therapeutic decisions [2]. Several technologies to capture CTCs have been developed in the last years. However, it is extremely challenging to capture such a rare population of cells hidden by billions of blood cells [3]. Furthermore, the lack of universal cancer-specific markers adds further complexity to CTCs capture [4].

Most enrichment technologies that depend on EpCAM for the capture of CTCs miss to identify tumour cells undergoing epithelial-to-mesenchymal transition (EMT), which favours downregulation of epithelial markers. As such, whenever CTCs have low or absent expression of EpCAM their capture is missed. Hence attempting to overcome this limitation, CTC technologies steered towards EpCAM-independent approaches. Consequently, many rely on the combination of epithelial and mesenchymal biomarkers to increase sensitivity in the identification of the EMT CTCs [5–7]. The presence of EMT CTCs has been demonstrated as an independent predictor of prognosis, treatment resistance and aggressiveness [5,8], highlighting the importance of including such biomarkers.

Regardless of the chosen mechanism for the capture of CTCs, after enrichment these cells still need to be identified and distinguished from blood cells using an immune assay. Typically, CTC identification is done by immunostaining and relies on a combination of positive and negative selection markers to identify CTCs and exclude WBCs, respectively. Positive identification often requires epithelial markers, such as EpCAM or cytokeratin (CK), while CD45 is the most widely used negative biomarker to detect WBCs. Despite the fact that size-based isolation methods may promote the capture of a more heterogeneous pool of CTCs, the standard protocol for CTC identification primarily consists of immunostaining with antibodies against CK (often a pan-CK including CK1-8, CK10, CK14-16, CK18 and CK19) and CD45 [9]. Gradually reports started to combine the use of conventional epithelial markers with several other markers for positive CTC detection enabling the identification and classification of CTC subtypes, hence disease-specific biomarkers are often selected in particular tumour types such as PSA, HER2 and EGFR on prostate [9], breast [10,11] and lung cancer [12–14], respectively. The introduction of a second positive biomarker has proven to be useful for the identification of patient subtypes or to assess the presence of resistance mechanisms or druggable targets.

Due to the versatility of CTC isolation technologies, the CTC classification criteria is adjusted according to the immune assay used. Each user applies an independent protocol and, consequently, the selection of antibodies, clones, concentrations and fluorophore conjugations vary greatly among studies. Nonetheless, the use of the hematopoietic marker CD45 for the identification and exclusion of WBCs appears to be standardised across all studies. The CD45 is a receptor protein tyrosine phosphatase that it is reported to be expressed on the surface of all nucleated hematopoietic cells and their precursors [15]. Other studies, however, have demonstrated that, due to the diversity of WBCs in circulation, some of them, such as neutrophils [16], myeloid-derived suppressor cells [17], or other immature blasting myeloid populations [18], have low or absent CD45 expression ($CD45^{-/lo}$). Moreover, there is evidence that the granulocyte subpopulation of WBCs can stain non-specifically for CK, in particular neutrophils (specific type of granulocytes) [19]. Further complicating the issue, neutrophil concentration increases in patients with progressive cancer, increasing the WBC contamination after CTC enrichment and, the risk of CTC misclassification [20–22].

These arguments raise questions about the specificity of conventional CTC identification protocols, which are particularly challenging for isolation methods that process whole blood directly (potential more WBC contamination than those using a pre-processed samples consisting of mononucleated cells only), and for those based on cell size (since neutrophils present multi-lobed nuclei and can reach 15 μm in diameter) [18]. Thus, to accurately identify CTCs, these potentially interfering WBC populations must be properly excluded during CTC identification.

This study aims to demonstrate technical strategies to mitigate the

risks underlying current CTC identification protocols when using whole blood samples without any type of pre-treatment and, present a novel and robust assay that broadens existing exclusion criteria by introducing a second biomarker, CD15, highly expressed in $CD45^{-/lo}$ neutrophils [23,24]. Improved CTC identification protocols will reduce potential false-positive, and ultimately increase the accuracy of CTC technologies for patient diagnosis and monitoring, regardless of tumour type.

2. Materials and methods

2.1. RUBYchip™ microfluidic device fabrication and functionalisation

The RUBYchip™ (PCT/EP2016/078406) is a microfluidic device for the rapid and efficient isolation of CTCs from 7.5 mL of unprocessed whole blood. As previously described [10,25], the filtering area is comprised of a single row of anisotropic micropillars, carefully designed to allow blood cells to deform and flow through, while retaining larger and more rigid cells. Chips are fabricated by soft-lithography using silicon wafers, polydimethylsiloxane (PDMS) replicas are irreversibly bonded against 25 × 75 mm^2 glass slides using a plasma activation process as described [26]. A syringe pump was used to drive flow from the inlet to the outlet of the device (NE-1200, New Era Syringe Pumps, Farmingdale, NY, USA). The devices were then primed with 350 μL of Ethanol (Sigma Aldrich/Merck, KGaA, Darmstadt, Germany), 350 μL of 10 mM Phosphate Buffer Saline (PBS, Sigma Aldrich), and 350 μL of 1 % Pluronic F-127 (Sigma Aldrich).

2.2. Cell culture

Human colorectal SW480 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, ThermoFisher Scientific, Darmstard, Germany) supplemented with 1 % Penicillin/Streptomycin (Pen/Strep, Corning, Inc., Corning, NY, USA) and 10 % Fetal Bovine Serum (FBS, Gibco). Cells were cultured at 37 °C in a 5 % CO_2 atmosphere. Adherent cells were harvested using 0.25 % Trypsin-EDTA, resuspended in complete growth medium.

2.3. Whole blood sample processing

Samples were obtained from healthy volunteers and a colorectal cancer (CRC) patient after informed consent. Whole blood samples (7.5 mL) were collected in EDTA tubes and, processed in the RUBYchip™ at a pre-selected flow rate [25]. After sample processing, trapped cells were stained using immunocytochemistry. CTCs were identified and characterized by morphological (cell-like morphology, membrane integrity and nucleus) and phenotypical (DAPI+/CK+/CD45−/CD15−) criteria. This study was approved by the Ethics Committee at Instituto Português de Oncologia do Porto (IPO-Porto) following international guidelines.

2.4. Immunocytochemistry

Immunocytochemistry studies were performed to carry out cell identification and phenotyping in all samples studied. Whole blood samples of healthy donors were used to isolate WBCs, these were obtained by on-chip isolation or by blood fractionation protocol. Briefly, erythrocytes were removed using a 10-min incubation with lysis buffer (Biosearch™ Technologies, Teddington, United Kingdom) followed by centrifugation to obtain an enriched WBCs pellet. Cell labelling was performed using either a monoclonal pan-CK antibody (clone C-11) or a recombinant antibody able to recognise selected keratins: 7, 8, 18 and 19 (clone REA831), both labelled with FITC. WBCs identification was achieved using a monoclonal anti-CD15 and two different monoclonal anti-CD45 (clone 35-Z6 and clone MEM-28), all conjugated with Alexa Fluor 647. Cell nucleus were stained with DAPI (NucBlue™, Invitrogen, MA, USA). Cells were fixed with 4 % PFA, permeabilized with 0.25 % Triton X-100 solution, washed with 10 mM PBS and blocked with 2 % BSA (all reagent from Sigma Aldrich) followed by antibody incubation. CD15 antibody was always incubated separately, for an hour at room temperature, following a first incubation with the antibody cocktail. Afterwards, cells were washed with 0.5 % BSA and PBS. All reagents were pumped onto chip at 100 μL/min. Cell imaging was achieved using an inverted fluorescence microscope (Nikon Eclipse Ti-E microscope, Nikon, Amsterdam, Netherlands). The presence of DAPI, CK, and CD15/CD45 was analysed using the blue, green, and red fluorescence channels, respectively.

2.5. Flow cytometry

Whole blood samples from healthy volunteers were incubated with a FACS lysis buffer to remove erythrocytes. Isolated WBCs were subsequently incubated with membrane antibodies (CD45 and CD15) for 30-min, then fixed, washed and permeabilized using a FIX & PERM™ Cell Permeabilization kit (Invitrogen™). Afterwards, the cytoplasmatic antibody (CK) was incubated for 15-min. The same immune-labelling protocol was performed using SW480 cells and non-labelled cells were included as negative control. Experiments were performed in a Navios EX 10-color flow cytometer (Beckman Coulter Life Sciences, Indianapolis, IN, USA).

3. Results

3.1. Relevance of CD15 as a suitable exclusion biomarker for CTC detection

Given previous reports that CK may bind non-specifically to neutrophils [19], we considered the possibility that these cells could be misidentified as CTCs. Thus, the risk for misidentification was evaluated by standard ICC assays using healthy WBCs. Cells were stained with CD45 (clone 35-Z6) and a pan-CK antibody (CK). It was possible to distinguish WBC subpopulations with varying expression of CD45, $CD45^{lo}$ and $CD45^{hi}$, even $CK^{+}/CD45^{-}$ cells (Fig. 1). Moreover, these $CK^{+}/CD45^{-}$ cells had a clear distinct multi-lobated nucleus, which is typically associated with granulocytes. $CK^{+}/CD45^{lo}$ cells, pose a risk for CTC misidentification based on fluorescence imaging, whereas $CK^{+}/CD45^{hi}$, despite of unspecific signal or undesirable CK signal, are straightforwardly excluded as CTCs thus do not constitute a risk for CTC identification.

Since this WBC subpopulation is at risk of being misidentified as CTCs, CTC scoring with a single WBCs exclusion marker is insufficient, evidencing the need to include a second exclusion marker. Granulocytes, particularly neutrophils, are found to be $CD15^{+}$. As such, we hypothesized that the CD15 biomarker would be a suitable candidate to label and exclude these unspecific CK^{+} events with low or absent levels of CD45 expression and mitigate the risk of being misidentified as CTCs. Thus, CD15 expression was evaluated in WBCs from healthy samples and tested for optimal antibody dilution. Different concentrations of CD15 were tested to evaluate antibody performance (Fig. 2A). CD15 signal is detectable in isolated WBCs, and the brightest and clearer CD15 signal was observed at 1:50 dilution, which was the chosen concentration to proceed.

Secondly, CD15 specificity was assessed, isolated WBCs and SW480 cells were stained against both CD15 and cytokeratin antibodies. As expected, results showed that only a subpopulation of WBCs, typically with a granulocyte-like morphology, appeared to be positive for CD15 (Fig. 2B). Co-localization of CK and CD15 was also observed in these cells, demonstrating the presence of unspecific CK staining in granulocytes. These findings evidenced feasibility to use a granulocyte marker, CD15, to adequately identify $CK^{+}/CD15^{+}$ WBCs and assist in excluding them. Meanwhile, CD15 signal is not present in SW480 cells, as expected for a negative control, demonstrating specificity of CD15 antibody (Fig. 2C).

To further confirm CD15 expression and antibody specificity, flow

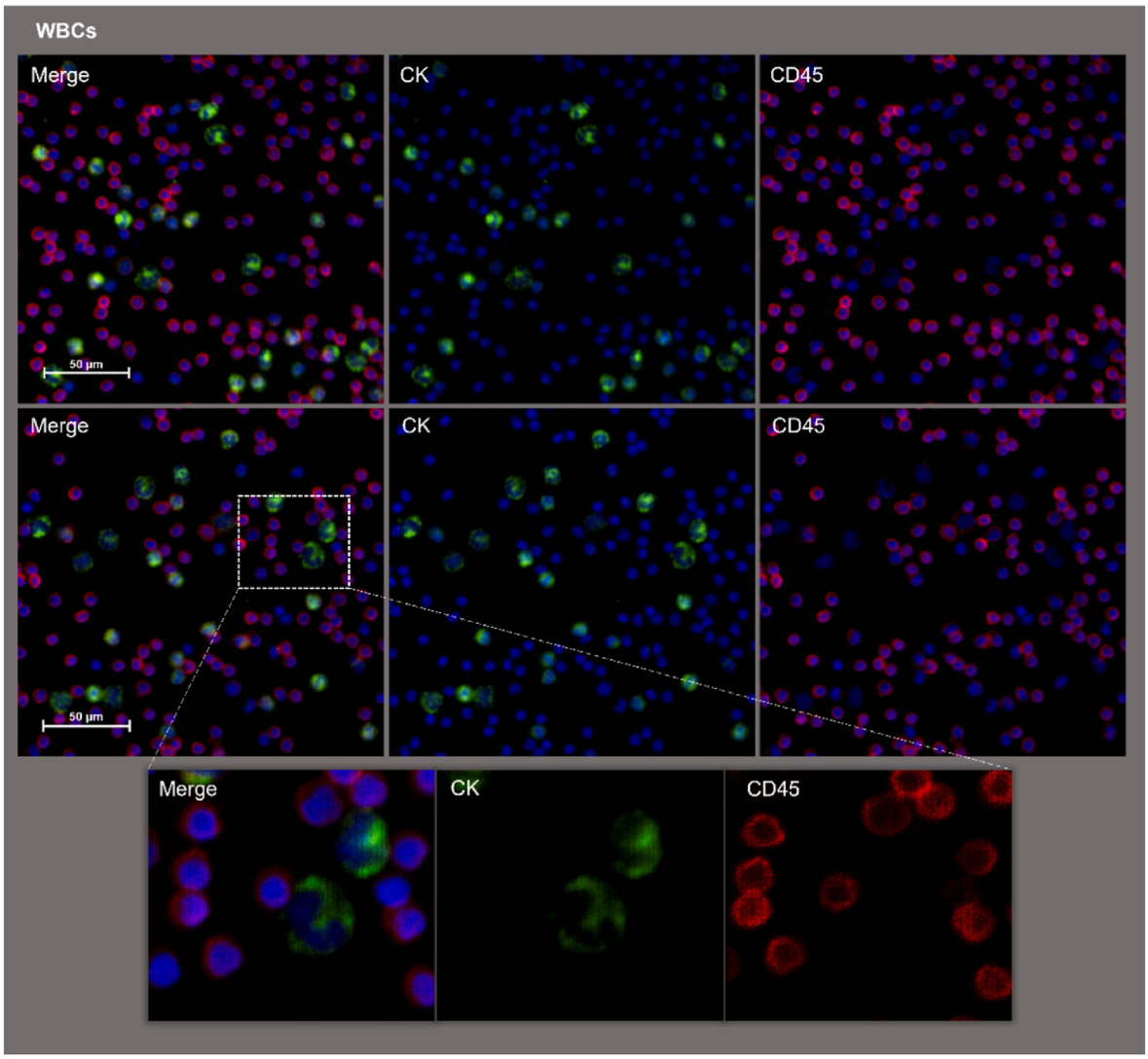

Fig. 1. WBCs from healthy whole blood samples directly labelled by immunocytochemistry against pan-Cytokeratin (green), CD45 (red) antibodies and, DAPI (blue). CD45 stain, displays variable expression in WBCs. All images are at the same magnification. Bottom insert (zoom in) to highlight staining characteristics. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

cytometry assay was carried out on WBCs isolated from healthy whole blood samples and, SW480, as negative control. WBCs and SW480 cells labelled against both CK and CD15 are shown in red and green respectively and, non-labelled WBCs are shown in grey. As expected, the neutrophil subpopulation stained positively for CD15 (dark red subpopulation), while monocytes, lymphocytes (lighter red subpopulations) and SW480 cells were confirmed negative, confirming CD15 specificity. CK positivity in SW480 was confirmed, and WBCs showed residual non-specific CK signal (Fig. 3), similarly to ICC findings.

3.2. Experimental and clinical assessment of CD15 staining on-chip

Once CD15 proved to be specific to the WBC sub-population of interest (neutrophils), we replicated the ICC protocol using the microfluidic device, to confirm whether these $CK^+/CD15^+$ WBCs are captured by the microfilter after whole blood processing. Healthy whole blood samples were processed on-chip, and the captured cells were labelled directly with DAPI, pan-CK and CD15. As expected, some WBCs trapped in the device showed CD15 positive signal co-localizing with a non-specific CK signal (Fig. 4), these contaminating $CK^+/CD15^+$ WBCs risk being misidentified as CTCs in a conventional CTC assay, suggesting CD15 has potential for the exclusion of CK^+ WBCs contaminating populations.

Knowing that some of these cells can be isolated in the device together with the CTCs, it is critical to ensure that these neutrophils are not misidentified as CTCs, based on pan-CK and CD45 staining alone. Hence, we tested the WBCs exclusion adding CD15 to the antibody cocktail and compared it against the standard CK/CD45 assay, paying particular attention to those $CK^+/CD45^{-/lo}$ cells. Hence, whole blood samples from healthy donors were processed in the RUBYchip™. Next, isolated cells were labelled inside the device with the standard DAPI/CK/CD45 (single exclusion) antibody cocktail and, in parallel, a second sample from the same donor was processed simultaneously in another device, to be stained with the novel antibody combination DAPI/CK/(CD45/CD15) (double exclusion). After imaging, all trapped cells were counted to achieve quantitative assessment. In the first device, cellular events showing non-specific signal for CK were observed and, some of those were not excluded by CD45 ($CK^+/CD45^-$ events) (Fig. 5A). Specifically, 25 % of all events were CK positive without exclusion from CD45 (Fig. 5B, left). Whereas using both exclusion markers, only 2 % of all events were CK positive without being excluded by either CD45 and/or CD15 (Fig. 5B, right).

Despite double exclusion proved to be successful in considerably reducing the percentage of misclassified events (CK^+ WBCs), this

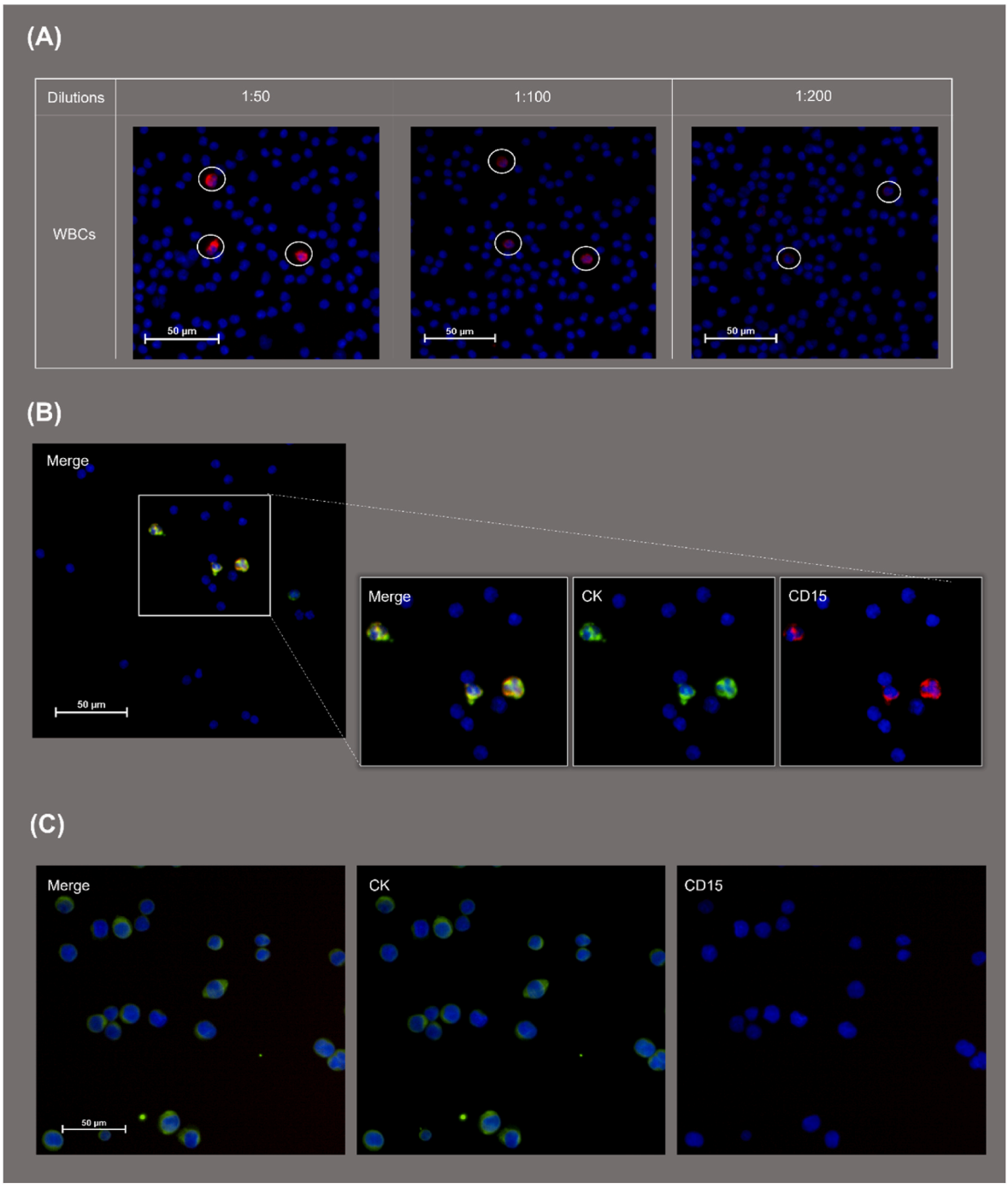

Fig. 2. (A) WBCs from healthy whole blood samples, labelled by immunocytochemistry against CD15 (red), using different antibody concentrations. (B) WBCs from healthy whole blood samples, labelled by immunocytochemistry against CK (green) and CD15. Image inserts highlights co-localization. (C) SW480 cells labelled against CK and CD15 antibodies and DAPI (blue). All images are at the same magnification. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

strategy still leaves a considerable number of cells as potential false positives. Hence, in order to achieve a more robust CTC immunolabelling protocol, the experiments were repeated using an improved CD45 antibody (iCD45, clone MEM-28). WBCs trapped on chip were stained against pan-CK and iCD45 alone (single exclusion), while, simultaneously, the second device was labelled against CK and iCD45, combined with CD15 (double exclusion). This experiment was conducted in duplicate, two different healthy volunteers were selected (N = 2, 4 devices analysed). All trapped cells imaged were counted and analysed. Of note, CD15 and CD45 biomarkers were observed in the same filter channel (Red). Results show that using this improved iCD45 antibody increased again the exclusion rate, only 1 % of unspecific CK^+ WBCs events were not excluded (Fig. 5C, left), compared to the 25 % with the former CD45 antibody used. Moreover, by using both the iCD45 and the CD15 antibodies in combination, almost all CK positive events were excluded by both these biomarkers, only 0.2 % of all non-specific CK^+ WBC events were not excluded (Fig. 5C, right).

Since CTCs are such a rare population of cells, all experimental efforts focused on minimizing potential false positives. An improved and highly specific CK antibody (iCK) was also tested, recognising

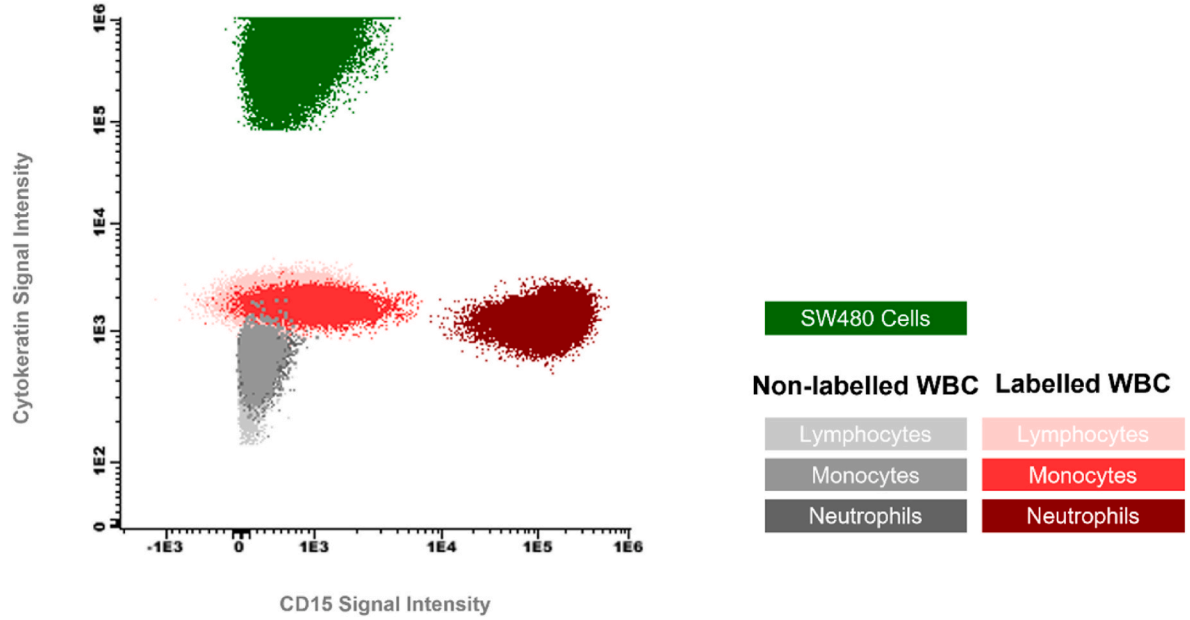

Fig. 3. Flow cytometry analysis of CK (green) and CD15 (red) in WBCs from healthy whole blood samples (red gradient color population) and SW480 cultured cells (green population). Cell population distribution by CK and CD15 biomarker, neutrophils exhibit higher signal intensity (dark red population). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

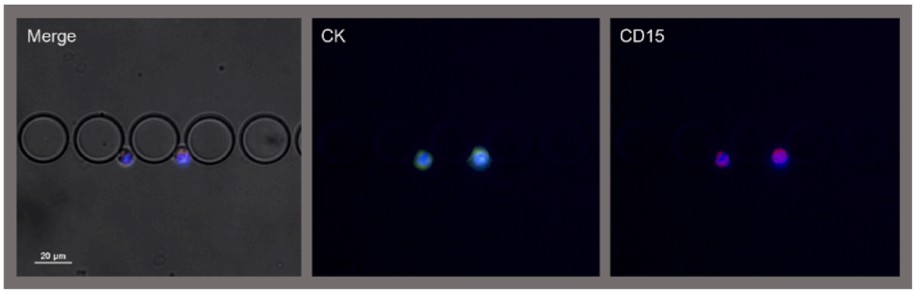

Fig. 4. Healthy whole blood sample processed on the RUBYchip™ and directly labelled by immunocytochemistry against a pan-CK (green), CD15 (red) antibodies and DAPI (blue). All images are at the same magnification. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

cytokeratins 7, 8, 18 and 19, reported to be the most frequent in many tumours [27]. The same quantitative assessment of exclusion rate was performed with iCD45 and CD15 antibodies (double exclusion), in duplicates (N = 2, 4 devices analysed). All trapped cells were counted and analysed. Non-specific $CK^+/(CD45^+/CD15^+)$ WBC events were observed in substantially lower number than previously, although these did not pose risk of misclassification, just 2 % of the total events were detected, indicating the high iCK antibody specificity. Most importantly, all of the non-specific CK^+ WBC events were excluded, 0 % of $CK^+/(CD45^-/CD15^-)$ WBCs using the combination of iCK, iCD45 and CD15 (Fig. 5D), evidencing that this antibody combination is the most effective and reliable to classify CTCs in clinical samples.

In summary, improved CD45 staining decreased the false positives from 25 % to 1 %, and represents the most significant improvement in assay reliability for WBC exclusion. Nevertheless, it is observed that introducing an additional exclusion marker, CD15 antibody, considerably decreased the percentage of potential false positives (WBCs $CK^+/CD45^-/CD15^-$). Whenever comparing double exclusion and single exclusion, we observe the lowest potential false positive percentages when CD15 is added to the assay *versus* when CD45 is used alone, regardless of the selection of CD45 antibody, which reflects a benefit in specificity to the assay beyond CD45 optimization and selection. Moreover, the adequate selection of the iCD45 antibody further lowered the percentage of potential false positives (WBCs $CK^+/CD45^-/CD15^-$), remarkably reaching 0.2 %. Along the same line, an improved selection of cytokeratin antibody, a highly specific antibody (Fig. 6) combined with a double exclusion strategy critically contributed to achieve zero percent of potential false positives (WBCs $iCK^+/iCD45^-/CD15^-$). As such, combining highly performing antibodies with robust double exclusion allowed to achieve effective and reliable classification of CTCs to be used in clinical samples.

Finally, this staining strategy was tested in a clinical sample from a colorectal cancer patient. It was possible to observe WBC events displaying unequivocal lobulated nucleus, and still exhibiting CK^+ staining, thus at risk to be misclassified as CTCs. However, the combination of CD45/CD15 antibodies clearly enables their exclusion (Fig. 7, top), highlighting the importance of not only selecting good performing antibodies, as well as adopting robust double exclusion. Moreover, in this clinical sample, CK^+ cells which were not excluded by CD45 and CD15 were observed and accurately classified as CTCs (Fig. 7, bottom).

4. Discussion

Considering their role in metastatic spreading, the study of CTCs is extremely important to understand disease progression. CTC capture and characterization is of high relevance. Microfluidic devices for CTC

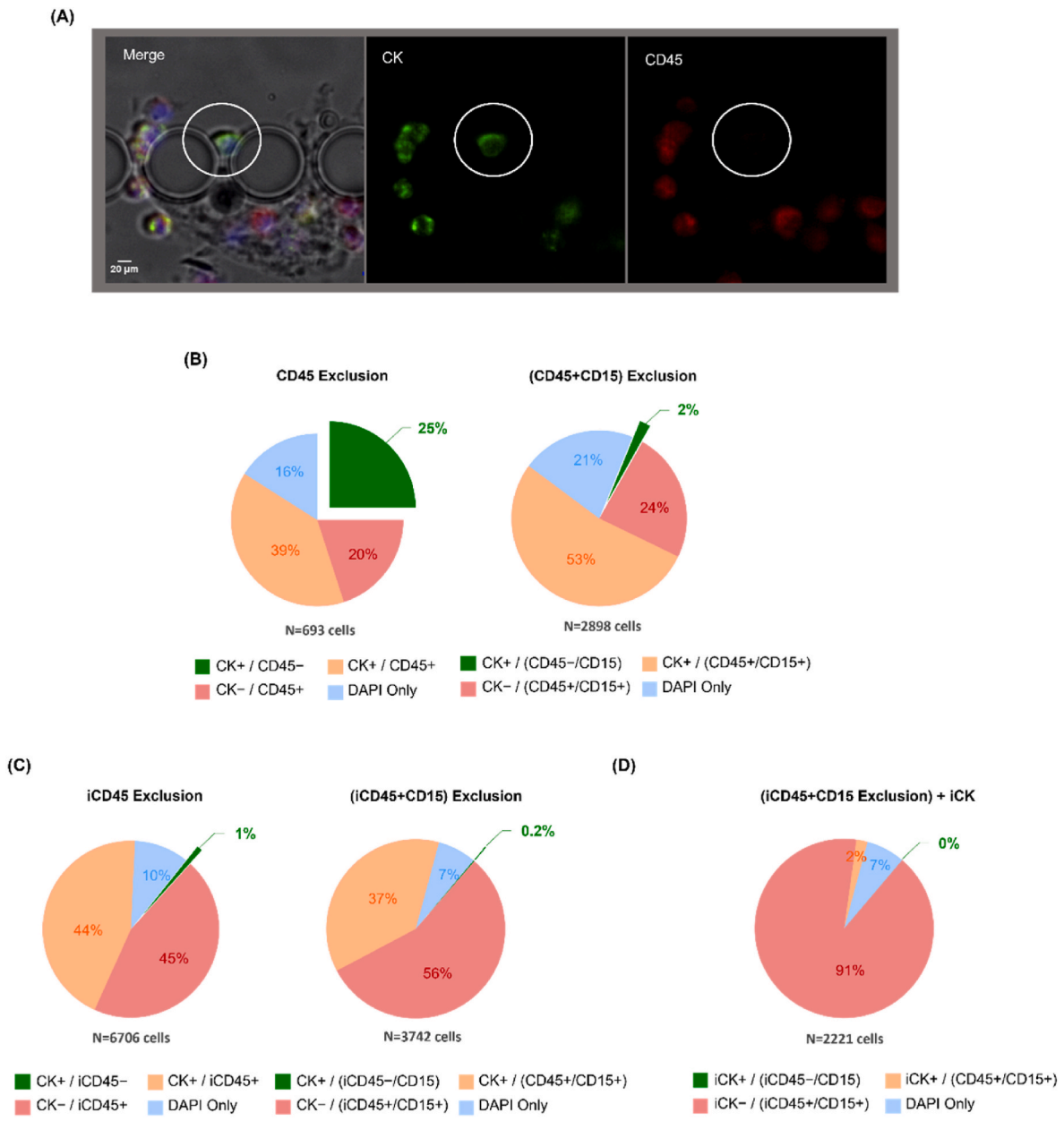

Fig. 5. (A) False positive: CK+ WBC captured on-chip labelled against pan-CK (green), CD45 (red) and DAPI (blue). Pie charts show quantitative analysis of potential false positives using (B) single exclusion and double exclusion (C) using an improved CD45 antibody (highly specific) for single exclusion and double exclusion tests (D) using an improved CK antibody (highly specific) for double exclusion tests. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

enrichment based on size and deformability are able to successfully isolate such cells with a variety of phenotypes directly from whole blood samples of cancer patients. Still, a successful CTC enumeration and characterization, requires a robust and specific cell identification criteria to avoid inaccurate CTC evaluation.

In most microfluidic technologies, CTC identification after enrichment relies on immunocytochemistry, using a selection of biomarkers for the identification of CTCs, and for the exclusion of WBCs. There is evidence that standard enumeration of CTCs, based solely on staining for CD45 and CK may be compromised by interfering WBCs, such as neutrophils, which express low levels of CD45 ($CD45^{-/lo}$) and these also increase in frequency during cancer progression [28]. Besides, it was previously described that non-specific CK staining in WBCs is associated with an intracellular staining artefact of the granulocyte subpopulation, which may contribute to misidentification of $CD45^{-/lo}$ cells as CTCs [19].

In this study, a $CK^{+}/CD45^{-/lo}$ cell population was identified among white blood cells from healthy donors, which risk being misclassified as CTCs. Their large and granular morphology raised the hypothesis of these cells likely being of myeloid origin. Besides, there is a considerable cell size overlap between CTCs and leucocytes, in fact these $CK^{+}/CD45^{-}$ cells can be found as part of the contaminating product of CTC isolation in several technologies [29–31] potentiating the risk of misclassification. This highlights the need for such technologies to include robust WBC exclusion strategies in order to achieve an accurate CTC identification and subsequent enumeration and downstream analysis.

Thus, different strategies are used to exclude this type of confounding events. For instance, other reports using microfluidic devices for CTC isolation, in the presence of undesirable CK^{+} staining on WBCs, have successfully explored double staining of activated granulocytes using CD66b (granulocyte marker) in combination with CD45 to

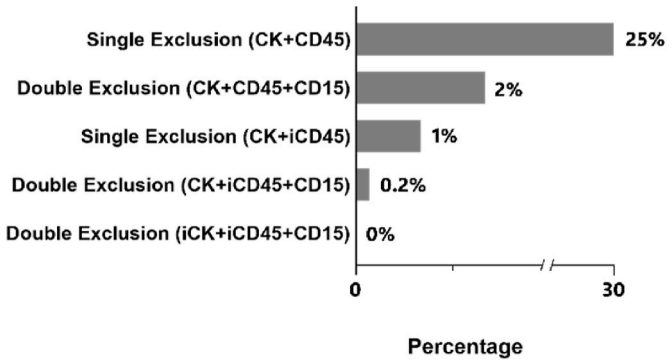

Fig. 6. Percentage of potential false positive events at each immunoassay iteration.

unequivocally identify these WBCs [29]. A similar labelling approach in lung cancer, also combined CD45 with CD11b (myeloid marker) to double exclude interfering myeloid cell populations. According to their conclusions, only by adding a third marker to the assay, CD11b, it was possible to discriminate different subpopulations of WBCs, particularly those posing a risk of being misclassified as CTCs ($CK^+/CD45^-/CD11b^+$), allowing to properly exclude these events [30].

Another study, also using a microfluidic technology, evaluated the isolation of CTCs through a negative selection mode in which the blood sample was depleted of leukocytes by immunomagnetically targeting both the common leukocyte antigen CD45 and the granulocyte marker CD15 [31]. They summarize that using this negative selection mode which includes CD45 and CD15 combined for the capture of CTCs, is of particular relevance for the isolation of CTCs from nonepithelial cancer and from cancer that has undergone EMT and lost detectable EpCAM expression, thus applicable to a broad spectrum of cancer types [31].

Similarly, this work adopted double exclusion criteria combining CD45 and CD15, which resulted in a considerably reduction of $CK^+/CD45^{-/lo}$ events misclassified as CTCs. On top of this, results showed that an appropriate selection of a highly specific CD45 antibody combined with the double exclusion strategy further reduced misclassified events to 0.2 %. Noteworthily, these findings highlight that the antibody selection should be made accordingly to the intended application; as such it is crucial to evaluate and optimise each antibody prior to its final use.

Despite being a residual occurrence, unspecific CK positive events can still negatively impact CTC enumeration. Still, adequate CK antibody selection also enabled additional reduction of misclassified cells. Adding a CK antibody, targeting 7, 8, 18 and 19 keratins, further improved assay specificity, resulting in complete elimination of false positives. Taken together, these findings emphasise the importance of a robust exclusion criteria and high antibody specificity in CTC immune assays for accurate identification of CTC candidates.

Overall, we found that granulocyte-specific markers like CD15 critically contribute to separate overlapping populations, minimizing false-positives in CTC enumeration. In sum, including CD15 as a second exclusion marker and combining it with highly specific antibodies, enables unequivocal discrimination of the $CK^+/CD45^-$ cell population of interest into true CTCs and false-positive myeloid cell derivatives allowing CTC biomarker analysis to become more accurate and, ultimately contributing to improve sensitivity and specificity of CTC identification.

5. Conclusion

To conclude, our findings show how granulocytes can be misidentified as CTCs, which can lead to inaccurate biomarker evaluation and CTC identification. However, the specificity of CTC identification may be improved with the selection of specific and good performance antibodies and with the introduction of a second exclusion marker able to efficiently exclude interfering WBCs. Establishing an accurate and robust CTC classification will enable us to harness the true diagnostic potential of liquid biopsies to predict response to treatment and, ultimately guide clinical decision making.

Patent

The RUBYchip™ design is based on the patent PCT/EP2016/078406, filed by INL in front of the EPO on November 22, 2016, covering the geometry of the microfluidic system for CTC isolation, and currently licensed exclusively to RUBYnanomed.

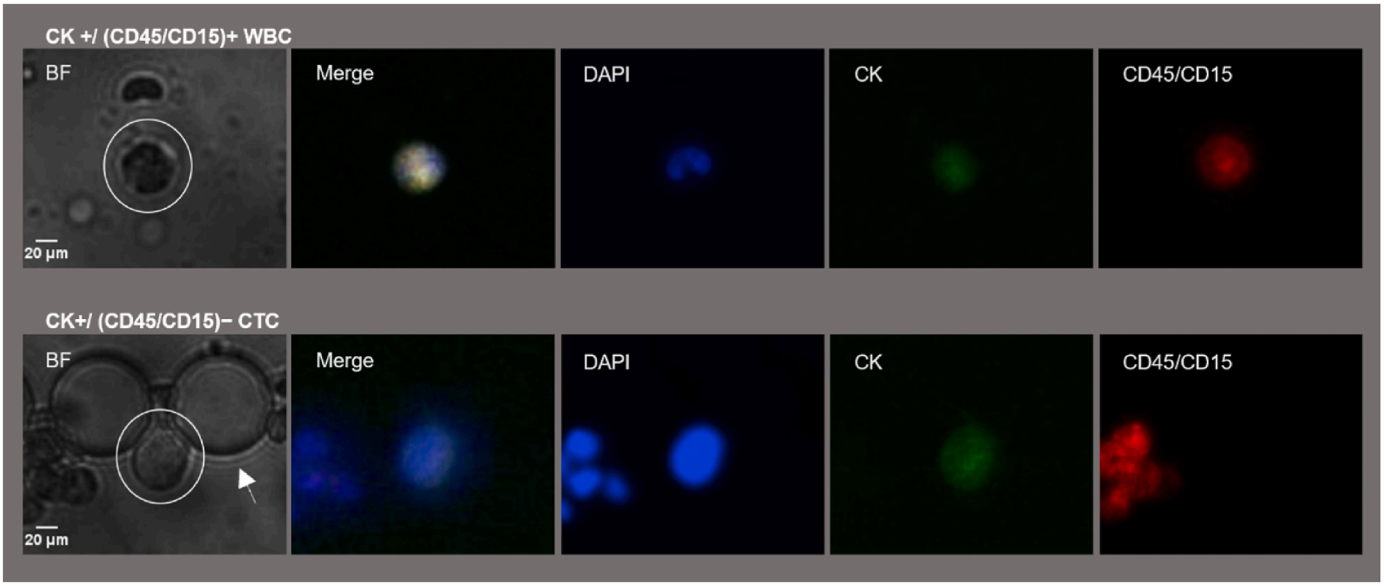

Fig. 7. Colorectal cancer whole blood sample immunostained against the complete cocktail of antibodies composed of iCK (green), iCD45, CD15 antibodies (red) and DAPI (blue). All images are at the same magnification. WBC on top (CK+/(CD45/CD15)+). No filtering pillars displayed at the bright field image. CTC at the bottom (CK+/(CD45/CD15)−). Cell (white circle) at filtering area surrounded by pillars (white arrow). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

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Institutional review board statement

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Ethics Committee of IPO-Porto (protocol code 043/021 approved on February 11, 2021).

CRediT authorship contribution statement

Adriana Carneiro: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Paulina Piairo:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Beatriz Matos:** Investigation, Methodology. **Daniela A.R. Santos:** Investigation, Methodology. **Carlos Palmeira:** Investigation, Methodology. **Lúcio Lara Santos:** Conceptualization, Supervision. **Luís Lima:** Conceptualization, Data curation, Formal analysis, Supervision, Validation, Writing – review & editing. **Lorena Diéguez:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Project administration, Supervision, Validation, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

The RUBYchip™ is based on the patent (PCT/EP2016/078406), for which Lorena Diéguez is an author. Moreover, the exploitation rights of the RUBYchip™ technology have been licensed to the spin-off company RUBYnanomed Lda., incorporated by Lorena Diéguez, Paulina Piairo and co-workers.

Data availability

Data will be made available on request.

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