Heliyon 9 (2023) e13044

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

CTC-5: A novel digital pathology approach to characterise circulating tumour cell biodiversity

B. Ffrench [a,b,*], E. Kashdan [c], Y. Huang [a,d], C.D. Spillane [a], S. Cocchiglia [b], S. Charmsaz [b], D. Varešlija [b], C. O'Brien [e], D. Scholz [f], C. Martin [a], M. Gallagher [a], D.A. Brooks [a,g], R.D. Brooks [g], S. Selemidis [h], N. Gleeson [i,j], F. AbuSaadeh [i,j], C. O'Riain [k], W. Kamran [j], R. Flavin [k], L. Young [b], S.A. O'Toole [a,i,1], J.J. O'Leary [a,k,1]

[a] *Department of Histopathology, Trinity College Dublin, St. James's Hospital, Dublin, Ireland, Trinity St. James's Cancer Institute and Emer Casey Molecular Pathology Research Laboratory, Coombe Women's & Infants University Hospital, Dublin, Ireland*
[b] *Endocrine Oncology Research Group, Department of Surgery, Royal College of Surgeons in Ireland, Dublin, Ireland*
[c] *Systems Biology Ireland, University College Dublin, Ireland*
[d] *School of Forensic Medicine, Xinxiang Medical University, Xinxiang, Henan, China*
[e] *Cancer Molecular Diagnostics, St. James's Hospital, Dublin 8, Ireland*
[f] *Conway Institute, University College Dublin, Ireland*
[g] *Cancer Research Institute, University of South Australia, Adelaide, 5001, Australia*
[h] *School of Health and Biomedical Sciences, RMIT University, Bundoora, Victoria, 3083, Australia*
[i] *Department of Obstetrics & Gynaecology, Trinity College Dublin, Trinity Centre for Health Sciences, St James's Hospital, Dublin, Ireland*
[j] *Department of Gynaecological Oncology, St James's Hospital, Dublin 8, Ireland*
[k] *Department of Histopathology, Central Pathology Laboratory, St James's Hospital, Dublin 8, Ireland*

ARTICLE INFO

Keywords:
CTC Screening
CTC Characterization
CTC Biodiversity
Merging microscopy datasets
Diagnostic imaging
Computer-assisted diagnosis
Heterotypic CTCs

ABSTRACT

Metastatic progression and tumor evolution complicates the clinical management of cancer patients. Circulating tumor cell (CTC) characterization is a growing discipline that aims to elucidate tumor metastasis and evolution processes. CTCs offer the clinical potential to monitor cancer patients for therapy response, disease relapse, and screen 'at risk' groups for the onset of malignancy. However, such clinical utility is currently limited to breast, prostate, and colorectal cancer patients. Further understanding of the basic CTC biology of other malignancies is required to progress them towards clinical utility. Unfortunately, such basic clinical research is often limited by restrictive characterization methods and high-cost barrier to entry for CTC isolation and imaging infrastructure. As experimental clinical results on applications of CTC are accumulating, it is becoming clear that a two-tier system of CTC isolation and characterization is required. The first tier is to facilitate basic research into CTC characterization. This basic research then informs a second tier specialised in clinical prognostic and diagnostic testing.

This study presented in this manuscript describes the development and application of a low-cost, CTC isolation and characterization pipeline; CTC-5. This approach uses an established 'isolation by size' approach (ScreenCell Cyto) and combines histochemical morphology stains and multiparametric immunofluorescence on the same isolated CTCs. This enables capture and

Abbreviations: CTC, circulating tumor cell; ISET, isolation by size of epithelial tumor cells.

* Corresponding author. Department of Histopathology, Trinity College Dublin, St. James's Hospital, Dublin, Ireland, Trinity St. James's Cancer Institute and Emer Casey Molecular Pathology Research Laboratory, Coombe Women's & Infants University Hospital, Dublin, Ireland.
E-mail address: ffrenchg@tcd.ie (B. Ffrench).

[1] Joint senior authors.

https://doi.org/10.1016/j.heliyon.2023.e13044
Received 26 June 2022; Received in revised form 17 December 2022; Accepted 12 January 2023
Available online 18 January 2023

characterization of CTCs independent of biomarker-based pre-selection and accommodates both single CTCs and clusters of CTCs. Additionally, the developed open-source software is provided to facilitate the synchronization of microscopy data from multiple sources (https://github.com/CTC5/). This enables high parameter histochemical and immunofluorescent analysis of CTCs with existing microscopy infrastructure without investment in CTC specific imaging hardware.

Our approach confirmed by the number of successful tests represents a potential major advance towards highly accessible low-cost technology aiming at the basic research tier of CTC isolation and characterization. The biomarker independent approach facilitates closing the gap between malignancies with poorly, and well-defined CTC phenotypes. As is currently the case for some of the most commonly occurring breast, prostate and colorectal cancers, such advances will ultimately benefit the patient, as early detection of relapse or onset of malignancy strongly correlates with their prognosis.

1. Introduction

Increasingly, the clinical impact of cellular heterogeneity within tumours is being recognised as pivotal to the emergence of chemoresistance and metastasis [1–3]. Multiple genetic 'hits' result in the generation of a malignant lineage of cells that can rapidly diversify both in terms of genetic mutation as well as cell type, metabolism, and differentiation status. This means that at the point of initial diagnosis, the tumor is often a highly heterogeneous population of cells [4,5]. Pathological assessment of the tumor at diagnosis is the primary determinant of systemic treatment strategies. However, these assessments are based on one snapshot in time of the still rapidly evolving malignancy. Thus, the most effective therapy at diagnosis may not remain optimal over the entire disease course. This caveat generates a defined need for more sophisticated, real-time, personalized-medicine solutions.

Liquid biopsies such as CTCs, offer the potential for longitudinally tracking tumour evolution over the course of a patient's treatment. To date, CTCs have proven to be valuable tools for patient prognosis [6–8] evaluating biomarkers in clinical trials [9,10] and have the potential to identify the emergence of therapeutic resistance/clinical recurrence [11,12]. However, outside of breast, prostate, and colorectal malignancies (via CELLSEARCH [13–15]), CTCs have yet to realise their full potential to provide real-time reporting and to guide therapeutic strategies in response to the emergence of resistant phenotypes and clinical relapse [6,16]. It is now accepted that supplementary CTC approaches are needed to further unlock the clinical potential of CTCs as liquid biopsies. Current thinking indicates a need for morphologic and phenotypic characterisation, rather than pure enumeration [17–20]. To achieve this, the field requires accessible tools to enable basic research for malignancies that currently cannot clinically utilise CTC enumeration and characterisation in treatment management. Herein, we designed, tested, and present a powerful approach for CTC characterization with a multiparametric readout method.

The FDA approved CELLSEARCH® CTC Test has pioneered CTC testing in the clinic. However, it is not without limitations, CELLSEARCH relies on a single protein marker (Epithelial Cell Adhesion Molecule [EpCAM]) to enrich CTCs [13,21,22]. It is becoming clear that many malignancies have more diverse CTC phenotypes, that will require a more heterogeneous set of CTC enrichment biomarkers. CELLSEARCH is arguably not best suited to support the basic research required to identify such heterogeneous biomarkers. Its initial biomarker enrichment step limits its utility as a basic research tool. Malignancies that do not yet have a defined set of biomarkers to identify CTCs require a biomarker independent method to facilitate the characterisation of their CTCs.

The CTC-5 approach described herein combines; wet-lab protocols, *in silico* approaches, morphology slide scanning and immunofluorescence microscopy to generate a digital slide, designed for cancer patient CTC enumeration and characterisation from liquid biopsies. As a foundation, it uses ScreenCell Cyto as the protein biomarker independent CTC isolation device. ScreenCell is a well described isolation device and is frequently found to identify phenotypic heterogeneity within cancer patients [23–26]. It is based on the principle of isolation by size of epithelial tumour cells [27,28] and has a proven track record of isolating CTCs from many different malignant indications including melanoma, colorectal, prostate, breast, lung, adrenocortical and pancreatic cancers [29–34]. ScreenCell has been shown to have 91.7% recovery of cancer cells spiked as low as 5 cells in 1 mL of whole blood [29]. Additionally, when compared against other CTC enrichment technologies that do not require specialised infrastructure, it was found to have best recovery of CTCs from spiked and patient samples [35]. Herein we use a proof-of-concept immunofluorescent panel: DAPI, EpCAM/PanCK, Her3, CD45. However, the CTC-5 approach is readily adaptable to characterise the expression of any set of protein biomarkers using appropriate immunohistochemistry and cytochemistry. As ScreenCell has a proven track record across multiple malignancies, and isolates cells to a slide-like format, we anticipate that with the appropriate immunofluorescent panel, this approach is applicable to most, if not all, solid tumour malignancies. Moreover, it has a low technical and monetary barrier to entry, as it uses hardware and infrastructure that are already established in most research institutes and many hospital laboratories. This current study describes the development and application of the CTC-5 approach using cell line/animal models and cancer patient samples.

The manuscript is organised as follows: In addition to the Methods section below, Supplementary File 1 provides detailed stepwise methods and describes the isolation and characterisation of CTCs as the basis of our methodology. In the data post-processing subsection, we introduce a novel approach to matching microscopic images using advanced optimisation techniques adapted for the type and the features of the images produced as part of the CTC-5 method and describe how to align them using the open-source software package developed for this project. The detailed description of the algorithm is provided in the Supplementary File 3 and source code is provided in the Supplementary File 4 (as well as via GitHub: https://github.com/CTC5/). The Results section shows the proof-of-concept of CTC-5 using multiple model systems and cancer patients. Finally, we wrap up our manuscript with the Discussion,

where we summarise our solution, detail its limitations and provide the reader with potential applications beyond the cancers presented in this work.

2. Methods

Detailed stepwise methods are described in Supplementary File 1 and summarised below.

Cell lines: The MCF7 cell line was obtained from ECACC. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 2 mM L-glutamine and 2% penicillin/streptomycin. The LY2 cell line was gifted by R. Clarke, Georgetown University, Washington DC and transduced with a luciferase and GFP tag (LY2-LUC-GFP) and cultured in phenol red-free MEM supplemented with 1× L-glutamine and 10% FBS. All cells were maintained in a cell culture incubator at 37 °C containing 5% CO_2.

Animal Models: All animal experiments were approved by the Research Ethics Committee, RCSI and performed under license from The Health Products Regulatory Authority in accordance with the European Communities Council Directive (2010/63/EU) and European Union (Protection of Animals Used for Scientific Purposes) Regulations 2012 (SI No543 2012). 5×10^4 LY2-LUC-GFP cells, in 100 μL PBS, were injected into the left ventricle of 5-6-week-old female NOD/SCID mice (Charles Rivers, UK) under isoflurane anaesthesia using a 30 G needle. Bioluminescence was measured by IVIS imaging and blood was drawn from submandibular vein, prior to recovering the mouse from anaesthesia. Blood samples were subsequently lysed and analyzed on a BD FACS Aria II Flow cytometer.

Patients: Blood samples were obtained from patients undergoing treatment for melanoma, breast and ovarian cancer at St. James's Hospital, Dublin 8, Ireland between 2015 and 2016. All patients gave informed consent, and the study was approved by the St James's and AMNCH (Adelaide and Meath incorporating the National Children's Hospital) research ethics committee. Patients with a pre-operative indication at multidisciplinary team discussion for neoadjuvant chemotherapy followed by surgery were recruited. A 6 mL K2EDTA blood sample was taken from each patient prior to the initiation of chemotherapy. Blood samples were stored at ambient temperature prior to CTC isolation. CTCs were isolated, and staining was started within eight hours of blood collection.

2.1. Circulating tumour cell isolation and characterisation using CTC-5

Patients: CTCs were captured using the ScreenCell®Cyto filtration device (ScreenCell) and stained using a modified Giemsa stain followed by immunofluorescence labelling. A 6 mL K_2-EDTA blood sample was taken from each patient. CTCs were isolated as per manufacturer's instructions. Filters were slide mounted, modified Giemsa stained and digitised. Using modified Giemsa staining, CTCs were defined as intact cells with a high nuclear:cytoplasmic ratio, and hyperchromatic nucleus with coarse chromatin. Such morphologicial features have been used across multiple malignancies and CTC capture/enumeration platforms to facilitate identification of CTCs [17,36–40].

Post modified Giemsa staining, slides were then washed, to remove Giemsa staining, and labelled for epithelial [EpCAM 1:20 (BD BioSciences) and panCK 1:200 (Dako)], lymphocyte [CD45 1:20 (2BScientific)] and cancer characterising [Her3 1:200 (Santa Cruz)] markers, as well as DAPI (nuclear stain), and then digitally imaged. This biomarker is a proof-of-concept panel, there is no intrinsic limitation for the process to incorporate different biomarker panels. Full protocol available as Supplementary File 1. Giemsa and 4-colour immunofluorescent digital images were processed *in silico* as detailed below in the CTC-5 post-processing software, generating a single multi-paged digital image for pathological review.

Cell lines: 6 mL of healthy patient blood was collected and spiked with MCF7 cells or 59 M cells. Cells were isolated using the ScreenCell®Cyto filtration device and stained as above. As per manufacturer's instructions, 3 mL of this cell spiked blood was processed via ScreenCell®Cyto device.

CTC-5 post processing software: The CTC-5 pipeline combines wet lab protocols and a custom-made open-source software package to allow for the generation of a five-channel digital slide (Giemsa [full colour], DAPI [blue], PanCK/EpCAM [green], HER3 [yellow], CD45 [far red]). Briefly, the digital slides are extracted from '.ndpi' files using the FIJI plugin: NDPITools [41–43], the various focal planes are focused into one single in-focus plane using FIJI plugin: Extend Depth of field [44,45]. The fluorescent images are stitched using the FIJI plugin: Grid/collect stitching [43,46,47]. The custom CTC-5 software automatically identifies randomly dispersed pores in the ScreenCell device, and uses these pores as common reference points in all images to facilitate scaling, rotation and alignment via our custom alignment algorithm (published herein) in combination with the FIJI plugin TurboReg [48]. The utilisation of cell independent features such as ScreenCell pores, makes the CTC-5 process robust to changes in biomarker expression, staining panels used and number of CTCs and leucocytes captured on the filtration device. Full protocol available as Supplementary File 1.

3. Results

Outside of the CTC field, flow cytometry is the current gold standard for characterising cellular heterogeneity. While there has been some reported success in characterising CTCs via flow cytometry [49,50], it is often prohibitively challenging due to low cell numbers and variable morphology. Intra cardiac injection of 5×10^4 LY2-LUC-GFP cells in 5 to 6-week-old NOD.SCID mice reliably produced clinically relevant, metastatic tumours in our hands [51]. Assuming 1.8 mL total blood volume [52], this model would be predicted to yield ~ 5500 CTCs per 200 μL mouse whole blood. In our hands, only 0 to 26.1 CTCs per 200 μL could be identified in blood drawn from the submandibular vein using a flow cytometric approach (Fig. 1A–C). While the murine model showed widespread CTC dissemination when measured by bioluminescence (Fig. 1D), it is possible that vasculature mechanics or rapid CTC

seeding/extravasation resulted in substantially reduced CTC burden in the submandibular vein. As such, this small pilot study does not preclude the use of flow cytometry for examining CTC models. However, it does indicate that physical capture of rare CTCs for characterisation via microscopic imaging may produce a more robust dataset compared to individual events on a flow cytometry density dot plot.

To this end, two specialised technologies for CTC capture were assessed: 1) antibody targeted magnetic bead enrichment (Fluxion IsoFlux™ using the IsoFlux Circulating Tumor Cell Enrichment Kit [EpCAM]) and 2) isolation by size (ScreenCell® Cyto). As the IsoFlux™ uses bead enrichment, it is not considered a biomarker independent approach to CTC capture. However, it does allow captured cells to be returned to a culture plate or cytospin slide for standard light microscopy and immunofluorescent staining. Both devices could isolate MCF7 cells spiked into whole blood (Fig. 1E – G). The IsoFlux™ had numerous antibody-coated magnetic beads in the isolate that obstructed downstream imaging (Fig. 1E and F). The Cyto device presented the cancer cells cleanly for imaging (Fig. 1G–K). In addition to the Cyto device being a protein biomarker independent CTC capture device, its compatibility with downstream imaging made it a suitable starting point for the CTC-5 staining protocol. In clinical samples, the Cyto device detected multiple different morphologies of CTCs: microemboli, doublets and single cells across multiple malignancies: including breast,

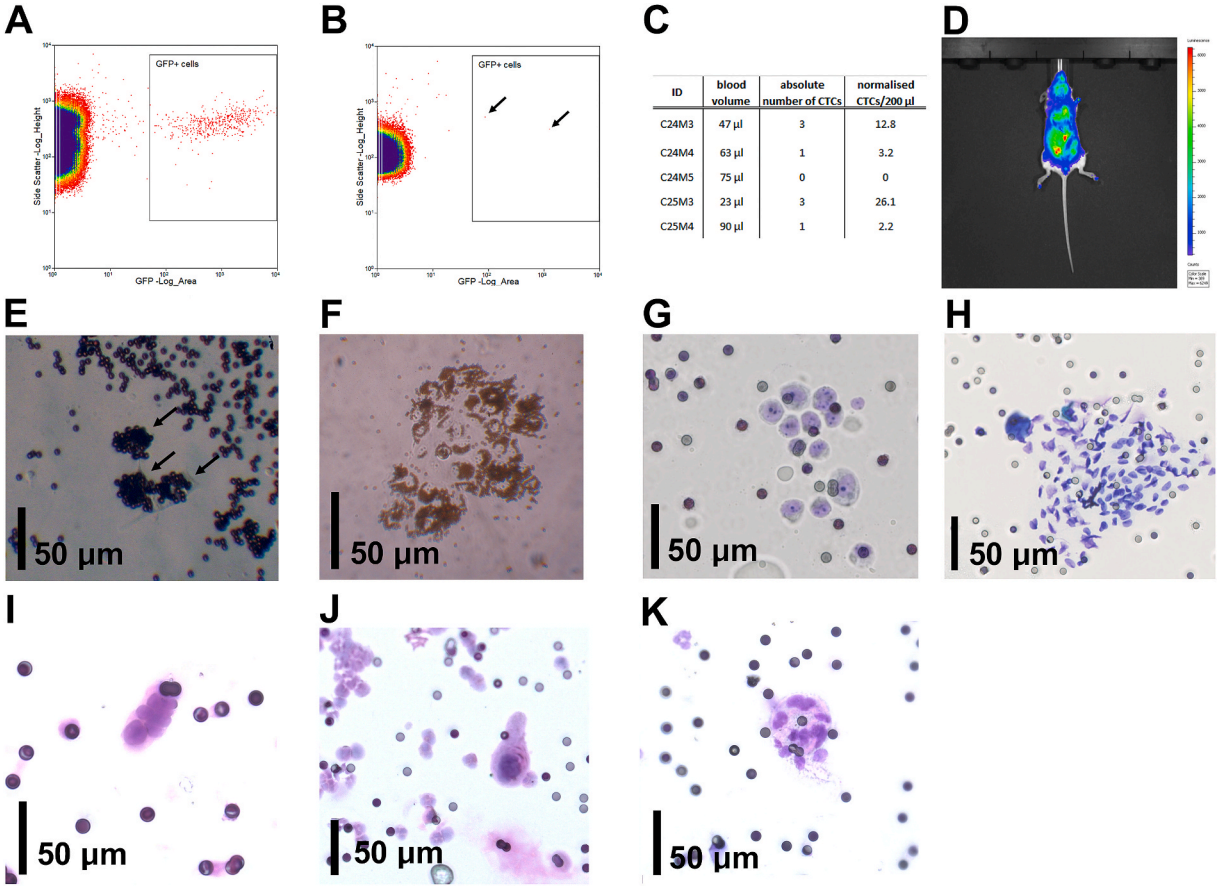

Fig. 1. Assessment of CTC isolation methods for downstream phenotypic characterization. A) LY2-LUC-GFP cells can be distinguished from whole blood via flow cytometry. 1000 cells spiked into 3 mL whole blood showed a 59.6±16.8% recovery (mean±1 sd; n = 3). B) Flow cytometric analysis of the intra-cardiac mouse model identified few if any CTC events. C) Summary of CTC detection rates using the intra-cardiac mouse model. D) Luminescence shows widespread dissemination immediately following intracardiac injection of LY2-LUC-GFP cells into immunodeficient NOD SCID nude mice. E) unstained MCF7 cells cultured for 24 h post isolation from a spiked human whole blood sample using the Fluxion Isoflux isolation method and returned to tissue culture. Black arrows indicate three cells coated in isolation beads. F) unstained MCF7 cells cultured for eight days post isolation using the Fluxion Isoflux isolation method. G) modified Giemsa stained MCF7 cells, following isolation from spiked human whole blood using the ScreenCell Cyto Isolation method. H) modified Giemsa stained microemboli of CTCs isolated using ScreenCell Cyto approach from an ER + PR + HER2-breast cancer patient. The sample was taken post-neoadjuvant chemotherapy. The patient had poor response to neo-adjuvant therapy and is still surviving with malignancy at three-year follow-up. I) modified Giemsa stained cluster of seven CTCs isolated using ScreenCell Cyto approach from a patient with BRAF + metastatic melanoma. The patient was still surviving two months post sample draw. J) modified Giemsa stained single CTC isolated using ScreenCell Cyto approach from a stage IV, high grade serous ovarian cancer patient. The sample was collected pre-neoadjuvant chemotherapy. The patient was alive with no evidence of disease 20 months post sample draw. K) modified giemsa stained cluster of eleven CTCs isolated using ScreenCell Cyto approach from a patient with stage 4, non-small cell lung cancer. The patient passed away 2 months post sampling.

melanoma, ovarian, and lung cancers (Fig. 1H–K).

The criteria for positively identifying CTCs have been continuously refined by the field. Broadly, there are a set of morphological criteria based on size, nuclear cytoplasmic ratio and chromatin condensation [17,36–40], with single cells and homotypic/heterotypic clusters, and a separate set of protein biomarker criteria based mainly on CD45 negativity and EpCAM and/or pan-cytokeratin positivity [6,21]. However, additional malignancy specific biomarkers have shown improved prognostic power over and above these 'classic' criteria. The CTC-5 staining approach combines the strengths of each set of criteria and in doing so overcomes their main weaknesses. The classic CTC markers of EpCAM and pan-cytokeratin were included in the CTC-5 panel to positively identify CTCs and CD45 was included as a negative selection criterion for WBCs [21]. Additionally, HER3: a potential biomarker for increased metastatic potential across numerous malignancies [53] including breast cancer [54] and ovarian cancer [55], was included in the antibody staining panel [55–57] (Supplementary File 1).

Modified Giemsa staining was used to assess morphological criteria, followed by immunofluorescence labelling for three protein biomarkers and DAPI nuclear stain. The modified Giemsa staining must be washed out prior to immunofluorescence imaging as the Giemsa reacts with the mountant and exhibits strong autofluorescence. It was found that modified Giemsa dye could be washed out using Tris Buffered Saline with 0.1% Tween20 (TBST; Supplementary File 1; Figure S1). Post-washing, the cells demonstrated staining that matched our initial positive and negative staining controls (Supplementary Figure S2; Fig. 2). Giemsa staining was digitised via a Hamatzu NanoZoomer slide scanner and fluorescence was digitised on an epifluorescent microscope with automated stage. Downstream *in silico* processing was required to merge all the imaging data into one coherent multichannel digital slide. As a proof of principle, MCF7 cells were spiked into normal whole blood and processed via the CTC-5 pipeline to generate a composite digital slide (Fig. 2; with full composite digital slide available as Supplementary File 2).

Manually processing the microscope images into a digital slide has a steep learning curve and is prohibitively time consuming. Moreover, we could not find an alignment algorithm that could automatically align images with such sparsely dispersed features (cells). Most currently published algorithms detect larger features in histology sections and are not suited to aligning the more cytology-like slides such as those produced by CTC isolations. To overcome these limitations, an ImageJ plugin was developed. This software acts as an interface to multiple existing plugins and builds from there to generate the composite multi-paged digital slide. A batch of samples can be set up for *in silico* processing in approximately 30 min and allowed to run for several hours/overnight on a standard configuration personal computer/laptop.

Our custom image registration algorithm primes the source images with coarse scaling, followed by iterative rotation to identify the optimal starting point for matching of images also known as "image registration". It then applies non-linear optimisation [58,59] to identify the correct affine transform, analysing both original and reflected images – to account for all possible microscope configurations. Interestingly, of the three parameters of affine transform: scaling, transition and rotation, rotation is the most important for correct alignment. The development of this alignment algorithm and software plugin was a critical step in ensuring that this composite digital slide technique could be widely applied and not limited to image processing specialists (See Supplemental File 5 for a screen capture showing the digital slide). Fig. 3 shows a schematic of the image processing software. A walkthrough tutorial and sample data set are provided (https://www.tcd.ie/medicine/histopathology/research/ctc-5/index.php). Further workings and explanation of the algorithm, including its default settings and potential limitations, is available as Supplementary File 3.

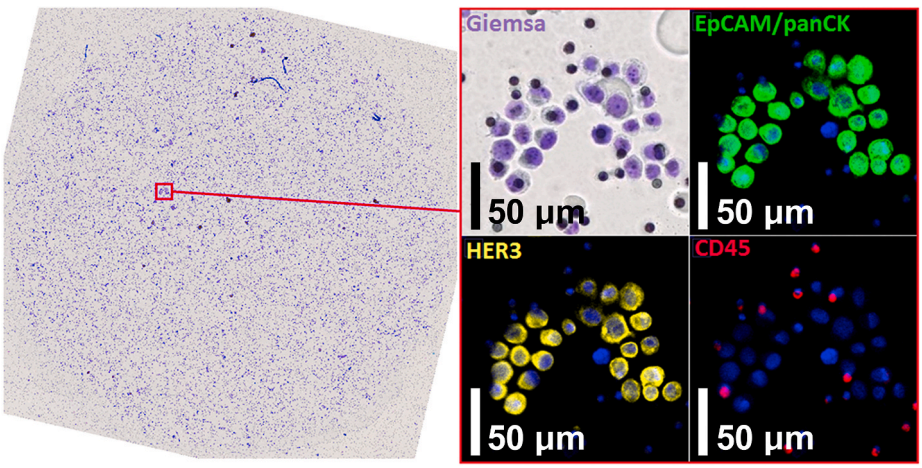

Fig. 2. CTC-5 combines Giemsa staining, with four color immunofluorescence into one easy to assess digital slide. Snapshots of a fully digitised slide containing MCF7 cells spiked into whole blood and stained for Giemsa, EpCAM/panCK, HER3, CD45 and DAPI. The full multi-channel digital slide is available as a multi-paged.tiff (Supplementary File 2). This digital slide can be viewed in all image viewing applications that support multi-paged.tiff images, including FIJI and is compatible with FIJI analytical packages. Giemsa staining was digitised on a Hamatzu NanoZoomer slide scanner. DAPI, EpCAM/PanCK and HER3 immunofluoresence was imaged on a three-channel epi-fluoresent microscope. After which, the 568 nm LED was swapped for a 647 nm LED to image the CD45 channel. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

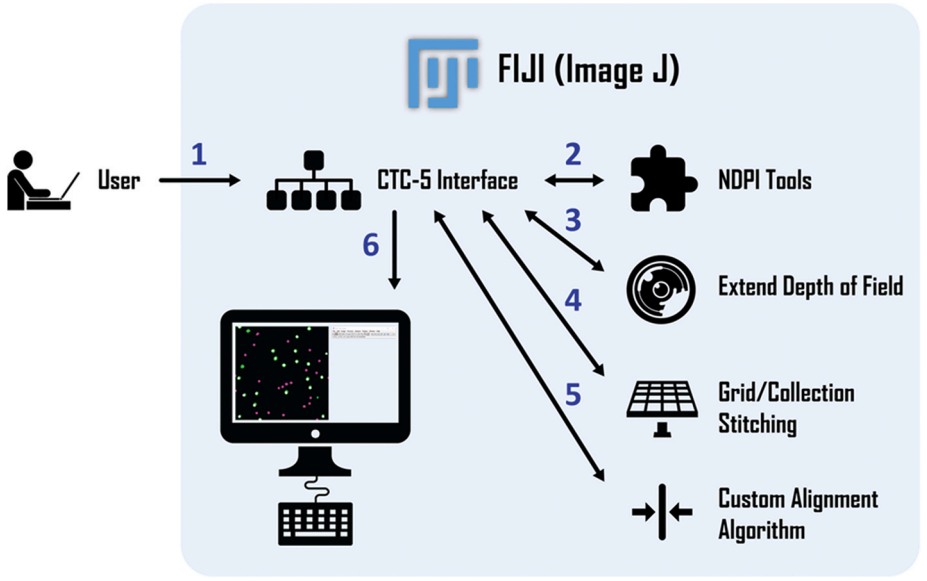

Fig. 3. Structure of CTC-5 software package. The user selects the files (images) to be processed and inputs data describing focal planes of interest and scanning direction of the microscope. The software then passes this data through several FIJI plugins, following which, our custom alignment algorithm automatically carries out feature recognition, scale adjustments and alignment. Slides scanned from the Hamatzu slide scanner are saved as proprietary files (.ndpi) that are not immediately available for processing alongside the '.tiff' files of the epi-fluorescent microscope. The digital slides are extracted from '.ndpi' files using the FIJI plugin: NDPITools [41–43], the various focal planes are focused into one single 'in focus' plane using FIJI plugin: Extend Depth of field [44,45]. The fluorescent images are stitched using the FIJI plugin: Grid/collect stitching [43,46,47]. Finally, all images are scaled rotated and aligned using our custom alignment algorithm (published herein). To our knowledge this is the first time morphology and immunofluorescence CTC data has been captured from the same slide (Fig. 2; Supplementary file 2).

To further demonstrate the ability of the CTC-5 approach to characterise heterogeneous CTCs, cells from the 59 M cell line (high grade serous ovarian adenocarcinoma derived from ascites [60]) were spiked into 6 mL healthy donor blood. 3 mL of this whole blood spike was processed via the CTC-5 pipeline. These cancer cells were readily identifiable by morphologically malignant phenotypes supported by CD45 negativity and had heterogeneous EpCAM/panCK and Her3 expression (see supplemental File 6, Figure S14). Healthy donor samples were found to have no false positive CTCs (Supplemental File 6, Figure S15). Finally, these observations of heterogeneous CTC populations were further illustrated via CTCs isolated from a breast cancer patient. This ER/PR + Her2-patient had what appeared to be clonally derived clusters of CTCs. One cluster was completely Her3-while the other cluster of cells was wholly Her3+ (Fig. 4).

The CTC-5 pipeline combines wet lab protocols and a custom made open source software package to allow for the generation of a five-channel digital slide (Giemsa [full colour], DAPI [blue] [55], PanCK/EpCAM [green], HER3 [yellow], CD45 [far red]). Its utility is demonstrated through the imaging of rare cancer cells captured from whole blood. However, it also has potential for widespread application in other sample types where access to cells of interest is limited or where co-staining of the same cells is required as opposed to staining of sequential sections from a tissue block.

4. Discussion

Understanding cancer heterogeneity is central to the development and prescription of targeted therapies. The prime examples of such precision therapies are Herceptin (Her2+ breast cancer [61]) and Gleevec (BCR-ABL and c-KIT mutated cancers [62,63]), which alongside their companion diagnostics have revolutionized treatment strategies for the respective patients. On the back of these successes, there has been a concerted drive to more holistically understand and target tumor heterogeneity. To this purpose, multiplexed biomarker technology and digital composites offer substantive solutions.

All CTC detection and capture devices have intrinsic selection biases. For example, flow cytometry and IsoFlux™ are biased by the proteins used to classify/capture cells as CTCs. As an 'Isolation by Size of Epithelial Tumour Cells' (ISET) based device, ScreenCell is biased by cell size and rigidity for CTC capture. ISET based capture requires histochemical and/or biomarker staining to further identify CTCs. This decoupling of the CTC capture criteria from the biomarker characterisation offers ISET technologies a key advantage: the antigens/epitopes utilised in biomarker-based capture of the CTCs are often blocked for use in downstream characterisation. For example, if EpCAM is used to capture CTCs, this epitope is blocked for characterisation of percentage EpCAM positive CTC in the sample. ISET based devices do not have any such limitation on downstream protein-based biomarker characterisation. The CTC-5 approach offers a further advantage along this vein: if a protein biomarker is used to capture CTCs, there is no indication of how many CTCs were missed due to that biomarker not being expressed. As CTC-5 combines modified giemsa with protein biomarker

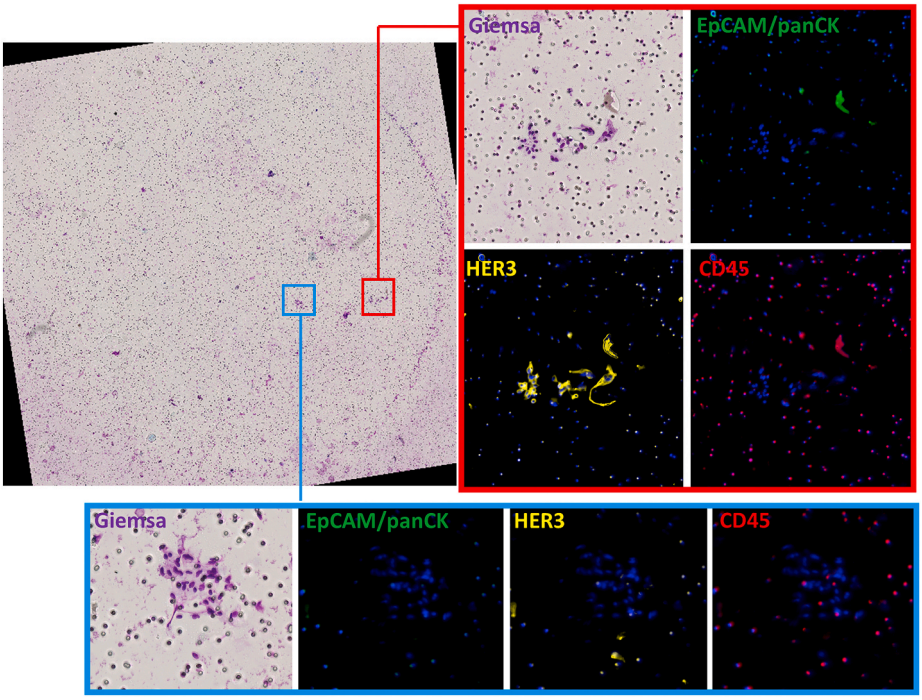

Fig. 4. CTC-5 staining identifies heterogeneous expression of HER3 in a breast cancer patient. Snapshots of a fully digitised slide from an ER/PR + Her2-breast cancer patient in the post neo-adjuvant treatment setting. This patient is alive with no evidence of disease 5 years post diagnosis. The panels show two sets of clusters of CTCs isolated from this patient: One cluster (red panel) has high HER3 expression while the other cluster has no HER3 expression (blue panel). This example shows the utility of the CTC-5 approach in the identification and characterisation of heterogeneous CTC populations. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

characterisation, even if no CTCs express any of the biomarkers in a panel, malignant CTC phenotypes identified via the modified giemsa staining and verified as CD45 negative, will still identify CTCs that were completely biomarker negative (as exemplified in Figure S14).

CTC-5 is a new and highly accessible tool for characterising CTC heterogeneity, providing proof-of-principle for combining morphology and multiple biomarker technologies into a single system.

While it is a substantive advance, the CTC-5 approach is not without limitations:

1. The underlying ScreenCell Cyto technology isolates CTCs from 3 mL whole blood, as opposed to the current gold standard of 7.5 mL whole blood via the CellSearch platform. This lower blood volume could potentially result in a lower qualitative detection rate of CTCs in a given sample.
2. The immunofluorescent panel described herein (DAPI/EpCAM/PanCK/HER3/CD45) is for proof-of-principle purposes only and it is highly likely that different panels will need to be developed to investigate malignancies and CTCs that do not follow the classic EpCAM/panCK positive CD45 negative biomarker profile.
3. While microscopy infrastructure may be widely available in research settings there is substantial microscopy expertise required to become proficient at immunofluorescent panel design and implementing processes for whole slide scanning. Therefore, while highly suitable for novel biomarker discovery, in a research setting, we envisage such newly discovered malignancy/phenotype specific biomarkers would need to be transitioned to a more clinically appropriate system such as CellSearch, that is more robust to operator experience.
4. Additional technical limitations to the alignment algorithm are detailed in Supplemental File 3, step 9.

Despite these limitations, the true power of the CTC-5 approach is its scalability. It is founded on ubiquitously used techniques: histochemistry and immunofluorescence, and a CTC capture device that has a proven track record across multiple malignancies [29–34]. As such, it can be applied to the study of any solid malignancy; and the digitisation of CTCs provides real-time verifiable data on the cancer cells being characterised.

Recent studies have found heterotypic CTC clusters to be especially potent vectors of solid tumour metastasis [64–66]. As such, they have become an area of special interest within the CTC field. To date, the best approaches to identify and characterise heterotypic CTC clusters require specialised infrastructure that reduces accessibility [67]. With an appropriately designed biomarker panel the CTC-5 approach should be well suited to the characterisation of heterotypic clusters. The pairing of histochemical and immunofluorescence staining with a filtration-based CTC capture device should contribute to a high sensitivity, high specificity, and accessible approach to

CTC cluster characterisation. This would potentially overcome some of the current limitations in CTC isolation technologies recently described by Schuster et al. [67]. Larger clinical trials could help establish heterotypic specific biomarkers suitable for the identification of heterotypic CTC clusters. For example, the literature discusses CD44 and ICAM1 junction proteins as mediators of homotypic CTC clusters [68,69] and VCAM-1, TTF-1 and CD56 as biomarkers of heterotypic clusters [67,70]. Platelet biomarkers such as CD42a or CD42b could also be used to identify platelet cloaking in heterotypic clusters [71].

In our opinion, co-location of CTC clusters with WBCs on a filtration-based CTC capture device (as seen in Fig. 2 and S14) is not sufficient to identify heterotypic CTC clusters. This co-location may be an artefact of the filtration process as opposed to a representation of the *in vivo* phenotype. We believe high magnification visualisation of cell-cell junctions is required to definitively identify heterotypic clusters. Scanning entire slides at resolutions high enough to visualise cell-cell junctions would be very inefficient. Instead, an approach of screening, followed by secondary imaging of any detected clusters at higher resolution would likely need to be adopted. Finally, as heterotypic clusters appear to be a rare event within CTC populations, which themselves are rare events, the blood sampling strategy may need to be adjusted to ensure a sufficient volume of blood is screened per patient to optimise the detection rate of heterotypic clusters.

In addition to offering a potentially high specificity, high sensitivity and accessible method to characterise CTC clusters, we believe a further strength of the CTC-5 approach is that the process results in a high-resolution digital slide. Such digital data can also be used as a data source for development of new explainable artificial intelligence (xAI) techniques for cancer diagnostics, evolution, and forecasting. Moreover, as this method is powered by open-source software and has the capacity to scale to support multiple image formats, it can reduce the capital expenditure required to develop and implement CTC screening and characterisation programs in clinical settings.

Commonly available immunofluorescent microscopes typically support four to six wavelength detection channels, each amenable to report on a different biomarker. Currently, clinically validated antibodies are frequently the same species and immunoglobulin, thus preventing multiplexed usage. Cyclic Immunofluorescence methods [72] combined with the alignment methods described herein, could be developed to support highly multiplexed staining with such primary antibodies already validated for clinical use. Multiplexed staining approaches for decisions on cancer treatment and management are already well established in blood borne malignancies with flow cytometry and it is expected that similar approaches will improve treatment management in solid tumours. There is already an emerging clinical need for such multiplexed panels in lung cancer [73] and other solid tumour malignancies are poised to follow suit in the coming years [74]. Furthermore, the CTC-5 approach can achieve additional functionality out of existing hardware. For example, a four-channel microscope can scan a set of slides once, the LEDs and/or filter cubes can be swapped out and slides can be re-scanned and an eight-channel slide can be aligned and processed *in silico*. Indeed, as proof-of-principle, the four-colour immunofluorescence images shown in Fig. 2 are a three-colour scan followed by an LED swap and a one colour scan.

High-end slide scanners are capable of digitising slides in both brightfield and multichannel fluorescence. However, this information cannot currently be captured from the same slide. More commonly, brightfield slides tend to be imaged in colour on slide scanners using Horse Radish Peroxidase labelling to identify protein expression, while multichannel immunofluorescence tends to be imaged on dedicated epi-fluorescent microscopes. These fluorescent microscopes tend to be equipped with sensitive black and white cameras and as such, are not optimal for imaging morphological staining. The pipeline published herein overcomes these limitations by allowing scans of the same slide from different microscopes to be merged. To the best of our knowledge, this is the most comprehensive combination of histochemical and immunofluorescence labelling that has been achieved to date in the study of CTCs.

In its current iteration, the CTC-5 processing software is custom-tailored to alignment of ScreenCell Cyto devices imaged on Hamatzu slide scanners and '.tiff' format immunofluorescence images. However, our open-source code can be adapted to accommodate virtually any ImageJ/FIJI supported format. Indeed, the same approach has applications beyond CTC characterisation and can be used to maximise characterisation capabilities for any rare samples such as: cytology samples or tissue microarrays in a cost-effective manner.

Author contribution statement

Brendan Ffrench: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Eugene Kashdan; Sharon O'Toole: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Yanmei Huang: Performed the experiments; Analyzed and interpreted the data.

Cathy Spillane; Sinead Cocchiglia; Sara Charmsaz: Performed the experiments.

Damir Varešlija; Cara Martin; Michael Gallagher; Doug Brooks; Robert Brooks; Stravos Selemidis; Leonie Young: Conceived and designed the experiments.

Cathal O'Brien; Dimitri Scholz; Noreen Gleeson; Feras AbuSaadeh; Ciaran O'Riain; Waseem Kamran; Richard Flavin: Contributed reagents, materials, analysis tools or data.

John O'Leary: Conceived and designed the experiments; Wrote the paper.

Funding statement

This study was funded by a Health Research Board Clinician Scientist Award to Prof John O'Leary (CSA/2012/9). This research was also supported by a research grant from Science Foundation Ireland, Centres for Science Engineering and Technology (CSETs) award to the Biomedical Diagnostic Institute (10/CE/B1821). The authors are grateful for the support of the Royal City of Dublin Hospital Trust.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of interest's statement

The authors declare no competing interests.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.heliyon.2023.e13044.

References

[1] I.J. Fidler, G. Poste, The cellular heterogeneity of malignant neoplasms: implications for adjuvant chemotherapy, Semin. Oncol. 12 (1985) 207–221.
[2] S.B. Lim, et al., Addressing cellular heterogeneity in tumor and circulation for refined prognostication, Proc. Natl. Acad. Sci. U. S. A. 116 (2019) 17957–17962.
[3] B. Rybinski, K. Yun, Addressing intra-tumoral heterogeneity and therapy resistance, Oncotarget 7 (2016) 72322–72342.
[4] A. Marusyk, K. Polyak, Tumor heterogeneity: causes and consequences, Biochim. Biophys. Acta (2010) 105–117.
[5] P.L. Bedard, A.R. Hansen, M.J. Ratain, L.L. Siu, Tumour heterogeneity in the clinic, Nature 501 (2013) 355–364.
[6] M.G. Krebs, J.M. Hou, T.H. Ward, F.H. Blackhall, C. Dive, Circulating tumour cells: their utility in cancer management and predicting outcomes, Ther. Adv. Med. Oncol. 2 (2010) 351–365.
[7] H. Wang, N.H. Stoecklein, P.P. Lin, O. Gires, Circulating and disseminated tumor cells: diagnostic tools and therapeutic targets in motion, Oncotarget 8 (2017) 1884–1912.
[8] L. Keller, K. Pantel, Unravelling tumour heterogeneity by single-cell profiling of circulating tumour cells, Nat. Rev. Cancer 19 (2019) 553–567.
[9] C. Paoletti, et al., Circulating biomarkers and resistance to endocrine therapy in metastatic breast cancers: correlative results from AZD9496 oral SERD phase I trial, Clin. Cancer Res. 24 (2018) 5860–5872.
[10] E.S. Lianidou, A. Markou, A. Strati, The role of CTCs as tumor biomarkers, Adv. Exp. Med. Biol. 867 (2015) 341–367.
[11] J. Sparano, et al., Association of circulating tumor cells with late recurrence of estrogen receptor-positive breast cancer: a secondary analysis of a randomized clinical trial, JAMA Oncol. 4 (2018) 1700–1706.
[12] S. Abalde-Cela, P. Piairo, L. Dieguez, The significance of circulating tumour cells in the clinic, Acta Cytol. 63 (2019) 466–478.
[13] M. Cristofanilli, et al., Circulating tumor cells, disease progression, and survival in metastatic breast cancer, N. Engl. J. Med. 351 (2004) 781–791.
[14] J.S. de Bono, et al., Circulating tumor cells predict survival benefit from treatment in metastatic castration-resistant prostate cancer, Clin. Cancer Res. 14 (2008) 6302–6309.
[15] S.J. Cohen, et al., Relationship of circulating tumor cells to tumor response, progression-free survival, and overall survival in patients with metastatic colorectal cancer, J. Clin. Oncol. 26 (2008) 3213–3221.
[16] M. Tellez-Gabriel, M.F. Heymann, D. Heymann, Circulating tumor cells as a tool for assessing tumor heterogeneity, Theranostics 9 (2019) 4580–4594.
[17] S.A. O'Toole, et al., Circulating tumour cell enumeration does not correlate with Miller-Payne grade in a cohort of breast cancer patients undergoing neoadjuvant chemotherapy, Breast Cancer Res. Treat. 181 (2020) 571–580.
[18] A. Toss, Z. Mu, S. Fernandez, M. Cristofanilli, CTC enumeration and characterization: moving toward personalized medicine, Ann. Transl. Med. 2 (2014) 108.
[19] J. Li, S.G. Gregory, M.A. Garcia-Blanco, A.J. Armstrong, Using circulating tumor cells to inform on prostate cancer biology and clinical utility, Crit. Rev. Clin. Lab Sci. 52 (2015) 191–210.
[20] S.B. Lim, W. Di Lee, J. Vasudevan, W.-T. Lim, C.T. Lim, Liquid biopsy: one cell at a time, NPJ Prec. Oncol. 3 (2019) 23.
[21] L.M. Millner, M.W. Linder, R. Valdes Jr., Circulating tumor cells: a review of present methods and the need to identify heterogeneous phenotypes, Ann. Clin. Lab. Sci. 43 (2013) 295–304.
[22] L. Keller, S. Werner, K. Pantel, Biology and clinical relevance of EpCAM, Cell Stress 3 (2019) 165–180.
[23] T.M. Gorges, et al., Improved detection of circulating tumor cells in metastatic colorectal cancer by the combination of the CellSearch(R) system and the AdnaTest(R), PLoS One 11 (2016), e0155126.
[24] A. Lopresti, et al., Identification of atypical circulating tumor cells with prognostic value in metastatic breast cancer patients, Cancers 14 (2022).
[25] M. Mascalchi, et al., Prevalence and number of circulating tumour cells and microemboli at diagnosis of advanced NSCLC, J. Cancer Res. Clin. Oncol. 142 (2016) 195–200.
[26] C. Nicolazzo, et al., Circulating tumor cells in right- and left-sided colorectal cancer, Cancers 11 (2019).
[27] G. Vona, et al., Isolation by size of epithelial tumor cells : a new method for the immunomorphological and molecular characterization of circulatingtumor cells, Am. J. Pathol. 156 (2000) 57–63.
[28] P. Pinzani, et al., Isolation by size of epithelial tumor cells in peripheral blood of patients with breast cancer: correlation with real-time reverse transcriptase-polymerase chain reaction results and feasibility of molecular analysis by laser microdissection, Hum. Pathol. 37 (2006) 711–718.
[29] I. Desitter, et al., A new device for rapid isolation by size and characterization of rare circulating tumor cells, Anticancer Res. 31 (2011) 427–441.
[30] C.L. Chen, et al., Single-cell analysis of circulating tumor cells identifies cumulative expression patterns of EMT-related genes in metastatic prostate cancer, Prostate 73 (2013) 813–826.
[31] J. Adebayo Awe, et al., Three-Dimensional telomeric analysis of isolated circulating tumor cells (CTCs) defines CTC subpopulations, Transl. Oncol. 6 (2013) 51–65.
[32] P. Pinzani, et al., Detection of circulating tumor cells in patients with adrenocortical carcinoma: a monocentric preliminary study, J. Clin. Endocrinol. Metab. 98 (2013) 3731–3738.
[33] B. Kulemann, et al., Circulating tumor cells found in patients with localized and advanced pancreatic cancer, Pancreas 44 (2015) 547–550.
[34] Z. Mu, et al., Detection and characterization of circulating tumor associated cells in metastatic breast cancer, Int. J. Mol. Sci. 17 (2016).
[35] A. Drucker, et al., Comparative performance of different methods for circulating tumor cell enrichment in metastatic breast cancer patients, PLoS One 15 (2020), e0237308.
[36] T. Fehm, et al., Methods for isolating circulating epithelial cells and criteria for their classification as carcinoma cells, Cytotherapy 7 (2005) 171–185.
[37] S.J. Tan, et al., Versatile label free biochip for the detection of circulating tumor cells from peripheral blood in cancer patients, Biosens. Bioelectron. 26 (2010) 1701–1705.
[38] S.T. Ligthart, et al., Circulating tumor cells count and morphological features in breast, colorectal and prostate cancer, PLoS One 8 (2013), e67148.

[39] C. Reduzzi, et al., Circulating tumor cell clusters are frequently detected in women with early-stage breast cancer, Cancers 13 (2021).
[40] M. Mascalchi, et al., Circulating tumor cells and microemboli can differentiate malignant and benign pulmonary lesions, J. Cancer 8 (2017) 2223–2230.
[41] C. Deroulers, et al., Analyzing huge pathology images with open source software, Diagn. Pathol. 8 (2013) 92.
[42] V. Della Mea, G.L. Baroni, D. Pilutti, Di Loreto, C. SlideJ, An ImageJ plugin for automated processing of whole slide images, PLoS One 12 (2017), e0180540.
[43] J. Schindelin, et al., Fiji: an open-source platform for biological-image analysis, Nat. Methods 9 (2012) 676–682.
[44] A. Intarapanich, S. Kaewkamnerd, M. Pannarut, P.J. Shaw, S. Tongsima, Fast processing of microscopic images using object-based extended depth of field, BMC Bioinf. 17 (2016) 516.
[45] Y. Wu, et al., Three-dimensional virtual refocusing of fluorescence microscopy images using deep learning, Nat. Methods 16 (2019) 1323–1331.
[46] S. Preibisch, S. Saalfeld, P. Tomancak, Globally optimal stitching of tiled 3D microscopic image acquisitions, Bioinformatics 25 (2009) 1463–1465.
[47] T. Peng, et al., A BaSiC tool for background and shading correction of optical microscopy images, Nat. Commun. 8 (2017), 14836.
[48] P. Thevenaz, U.E. Ruttimann, M. Unser, A pyramid approach to subpixel registration based on intensity, IEEE Trans. Image Process. 7 (1998) 27–41.
[49] Y. Lu, et al., Isolation and characterization of living circulating tumor cells in patients by immunomagnetic negative enrichment coupled with flow cytometry, Cancer 121 (2015) 3036–3045.
[50] N. Bhagwat, et al., An integrated flow cytometry-based platform for isolation and molecular characterization of circulating tumor single cells and clusters, Sci. Rep. 8 (2018) 5035.
[51] D. Vareslija, et al., Transcriptome characterization of matched primary breast and brain metastatic tumors to detect novel actionable targets, J. Natl. Cancer Inst. 111 (2019) 388–398.
[52] K.H. Diehl, et al., A good practice guide to the administration of substances and removal of blood, including routes and volumes, J. Appl. Toxicol. 21 (2001) 15–23.
[53] F. Ledel, K. Stenstedt, M. Hallstrom, P. Ragnhammar, D. Edler, HER3 expression in primary colorectal cancer including corresponding metastases in lymph node and liver, Acta Oncol. 54 (2015) 480–486.
[54] C. Xue, et al., ErbB3-dependent motility and intravasation in breast cancer metastasis, Cancer Res. 66 (2006) 1418–1426.
[55] S. Pradeep, et al., Hematogenous metastasis of ovarian cancer: rethinking mode of spread, Cancer Cell 26 (2014) 77–91.
[56] C.H. Lee, et al., Assessment of Her-1, Her-2, and Her-3 expression and Her-2 amplification in advanced stage ovarian carcinoma, Int. J. Gynecol. Pathol. 24 (2005) 147–152.
[57] Y.W. Chung, et al., Overexpression of HER2/HER3 and clinical feature of ovarian cancer, J. Gynecol. Oncol. 30 (2019) e75.
[58] M. Styner, C. Brechbuhler, G. Szekely, G. Gerig, Parametric estimate of intensity inhomogeneities applied to MRI, IEEE Trans. Med. Imag. 19 (2000) 153–165.
[59] J.P. Pluim, J.B. Maintz, M.A. Viergever, Image registration by maximization of combined mutual information and gradient information, IEEE Trans. Med. Imag. 19 (2000) 809–814.
[60] J. Mei, et al., Cellular models of development of ovarian high-grade serous carcinoma: a review of cell of origin and mechanisms of carcinogenesis, Cell Prolif 54 (2021), e13029.
[61] K. Reynolds, S. Sarangi, A. Bardia, D.S. Dizon, Precision medicine and personalized breast cancer: combination pertuzumab therapy, Pharmgen. Pers. Med. 7 (2014) 95–105.
[62] C.L. Sawyers, Opportunities and challenges in the development of kinase inhibitor therapy for cancer, Genes Dev. 17 (2003) 2998–3010.
[63] K.S. Bhullar, et al., Kinase-targeted cancer therapies: progress, challenges and future directions, Mol. Cancer 17 (2018) 48.
[64] N.R. Campbell, et al., Cooperation between melanoma cell states promotes metastasis through heterotypic cluster formation, Dev. Cell 56 (2021) 2808–2825 e2810.
[65] A. Dovas, A. Patsialou, A.S. Harney, J. Condeelis, D. Cox, Imaging interactions between macrophages and tumour cells that are involved in metastasis in vivo and in vitro, J. Microsc. 251 (2013) 261–269.
[66] M. Roh-Johnson, et al., Macrophage contact induces RhoA GTPase signaling to trigger tumor cell intravasation, Oncogene 33 (2014) 4203–4212.
[67] E. Schuster, et al., Better together: circulating tumor cell clustering in metastatic cancer, Trends Cancer 7 (2021) 1020–1032.
[68] X. Liu, et al., Homophilic CD44 interactions mediate tumor cell aggregation and polyclonal metastasis in patient-derived breast cancer models, Cancer Discov. 9 (2019) 96–113.
[69] R. Taftaf, et al., ICAM1 initiates CTC cluster formation and trans-endothelial migration in lung metastasis of breast cancer, Nat. Commun. 12 (2021) 4867.
[70] I. Messaritakis, et al., TTF-1- and/or CD56-positive Circulating Tumor Cells in patients with small cell lung cancer (SCLC), Sci. Rep. 7 (2017), 45351.
[71] K. Egan, et al., Platelet adhesion and degranulation induce pro-survival and pro-angiogenic signalling in ovarian cancer cells, PLoS One 6 (2011), e26125.
[72] J.R. Lin, M. Fallahi-Sichani, P.K. Sorger, Highly multiplexed imaging of single cells using a high-throughput cyclic immunofluorescence method, Nat. Commun. 6 (2015) 8390.
[73] P. Hofman, et al., Multiplexed immunohistochemistry for molecular and immune profiling in lung cancer-just about ready for prime-time? Cancers 11 (2019).
[74] N. Eissing, et al., Easy performance of 6-color confocal immunofluorescence with 4-laser line microscopes, Immunol. Lett. 161 (2014) 1–5.