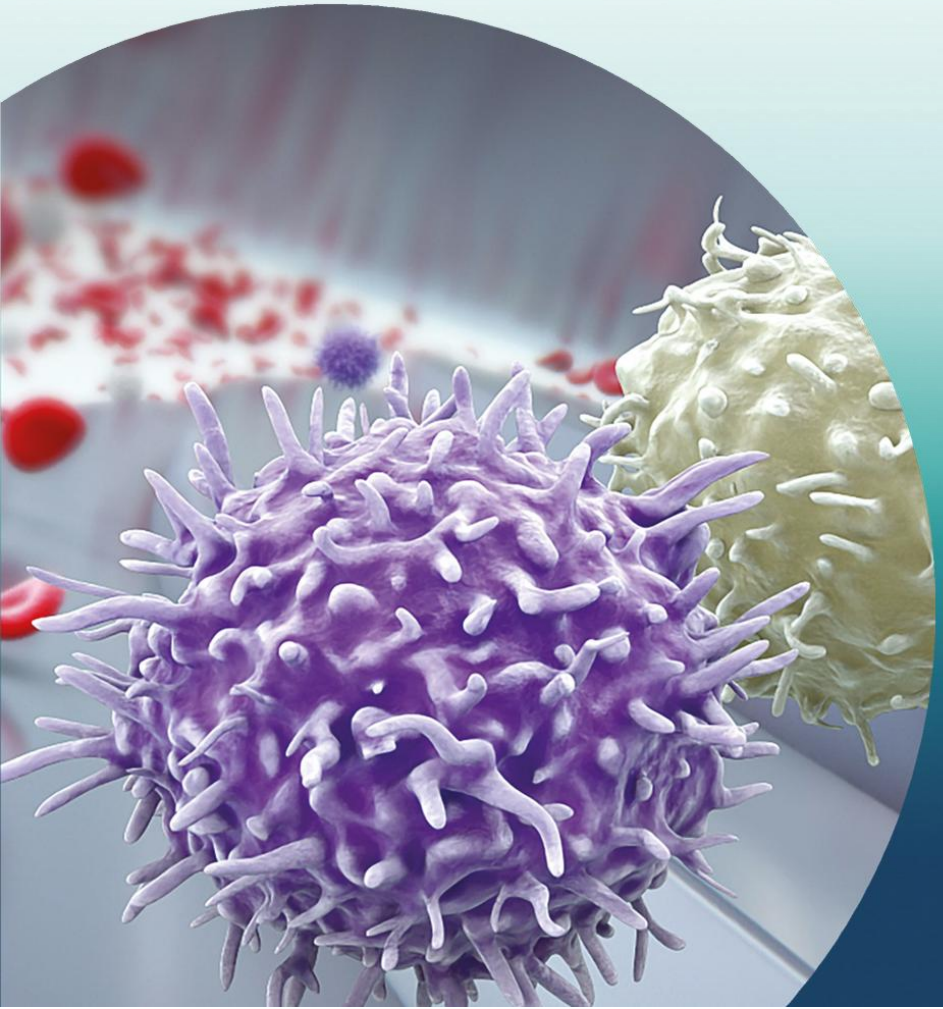
CellLBx Health

WHITE PAPER

Circulating Tumor Cells & Xenografts

The Parsortix® Platform as a tool to support innovative xenograft-based discovery projects



1.0 Executive Summary

The total number of new global cancer cases is continually rising with 18.7 million new cases in 2022 forecast to rise to 30.9 million cases by 2050[1]. As such there is a need for efficient drug discovery and development to enable precision medicine and the optimisation of cancer care.

Liquid biopsy is a minimally invasive technique that can involve the analysis of various fluids, such as blood or urine, to detect and monitor diseases, particularly cancer. Common liquid biopsy analytes include circulating tumor cells (CTCs), circulating tumor DNA (ctDNA) and exosomes.

Liquid biopsy assessment of blood-based biomarkers is a rapidly emerging field in cancer research which holds vast potential for drug discovery and development. Furthermore, liquid biopsies have the potential to be utilised across the cancer care pathway, for diagnosis, prognosis, treatment selection, the monitoring of treatment response and resistance, as well as indicating disease progression and relapse[2,3].

Preclinical models are a key tool in translational cancer research used to enhance our understanding of the biology of cancer and therapeutic discovery. Preclinical testing of anticancer agents *in vitro* and *in vivo* has been utilised for over 40 years and is a regulatory requirement prior to clinical development in many jurisdictions. 3D modelling of cancer, including the transplantation of cancer cells *in vivo* (xenotransplantation) has rapidly advanced in recent years to recapitulate the development of disease[4], mechanisms of metastasis, anticancer profiling and assessment for novel drug targets in zebrafish, chicken embryos and more commonly mouse xenografts. More recently, the isolation of circulating tumor cells (CTCs) from blood samples via liquid biopsy, as potential precursors of metastasis, is emerging in the field of preclinical models. Intact, living CTCs recovered from liquid biopsies are shed from both primary and metastatic tumors and enter the blood stream. These CTCs can be utilised in transplantation to mimic human disease, model the metastatic process and facilitate drug discovery projects.

CelLBxHealth's Parsortix Platform, is a novel epitope independent liquid biopsy technology which enables the enrichment and harvest of CTCs and CTC clusters, based on cell size and deformability for downstream analysis. CTCs enriched and harvested by the Parsortix Platform have been utilised to investigate prognosis, targeted treatment selection, monitor resistance and clonal evolution, and identify recurrence. Moreover, the Parsortix Platform can harvest live and intact CTCs facilitating cell culture and transplantation into preclinical models such as mouse or zebrafish embryo xenografts to:

- **Model the metastatic cascade and study the biology of cancer,** by enriching patient derived CTCs for xenograft transplantation, or enriching CTCs from cell line derived xenograft mouse models
- **Study CTC clusters and CTC-WBC clusters,** by facilitating epitope independent, deformability and sized based enrichment
- **Facilitate discovery projects** by creating a model to mimic human disease

2.0 Introduction

Preclinical testing is a vital stage in the development of medicines to assess efficacy and safety before clinical trials can begin. The first human cell line to grow and divide *in vitro*, was isolated in 1952. This cell line, isolated from a 31 year old woman diagnosed with an aggressive form of cervical cancer, was the beginning of 2D cell culture that enabled numerous breakthroughs in biomedical research, including the polio vaccine, the human papilloma virus (HPV) vaccine, helping understanding Ebola and HIV, and numerous other significant anti-cancer research milestones[5].

Simple pre-clinical models such as 2D cell culture are a fast and cost-effective approach for drug discovery and testing. However, there are limitations to this technique. 2D cell culture can fail to recapitulate treatment-limiting hallmarks that are present in the tumor microenvironment[4]. Moreover, the unique and complex characteristics of cancer challenge pre-clinical-to-clinical translation[4]. Instead, *in vitro* 3D modelling of cancer has rapidly advanced to provide closer biomimicry and more effective preclinical testing. This includes the development of organoids, spheroids, polymeric scaffolds and xenografts to map the tumor microenvironment, and assess cell-cell and cell-matrix interactions in treatment profiling[4].

Historically xenograft models have been critical in successfully identifying cytotoxic drugs and continue to model disease towards robust preclinical assessments to improve the likelihood of clinical success[6]. The ability to enrich and harvest viable CTCs that have shed from a tumor, into the bloodstream via a liquid biopsy can facilitate CTC culture which holds positive implications for xenograft modelling. Moreover, translational research involving the xenotransplantation of CTCs are emerging in literature to display a unique ability to spontaneously mirror human disease in mouse models to study the biology of cancer and novel drug targets[7]. Furthermore, the analysis of CTCs from liquid biopsy holds significant potential as a tool to harness living cells from a patient's tumor, which carry large amounts of up-to-date information as the tumor changes and mutates. These living whole cells enable a more complete picture of the cancer to be understood as they allow multiomic analysis of DNA, RNA and proteins and hold the potential to answer a wide range of clinical questions to enable personalised treatment for cancer patients.

The advancements in incorporating CTCs from liquid biopsy into preclinical models has only recently become possible with technologies that enrich and harvest intact, live CTCs, as well as large CTC clusters that have high metastatic potential, such as the Parsortix Platform. There is a significant opportunity for discovery projects using cell culture and/or xenograft transplantation of CTCs to support the development of agents targeting the metastatic cascade as the leading cause of cancer-related deaths.

Note on Preclinical Applications:

The use of the Parsortix Platform in xenograft modelling, CRISPR screening, and other research contexts described in this whitepaper is **intended for preclinical investigational use only**. These applications are exploratory in nature, and **clinical utility has not been established in humans**.

3.0 Evidence supporting the use of the Parsortix Platform for the development and assessment of xenograft models

The Parsortix System is a next generation liquid biopsy technology. Starting from a blood draw, the Platform isolates and harvests intact and viable CTCs, providing a real-time sample for subsequent analyses using widely adopted laboratory techniques.

Whole blood samples are processed using CelLBxHealth's Parsortix Platform for marker independent enrichment of epithelial and mesenchymal CTCs, along with those CTCs undergoing epithelial-to-mesenchymal transition (EMT), and CTC clusters. The Parsortix Platform uses a patented microfluidic technology in the form of a single use cassette to enrich and harvest CTCs based on their size and lack of deformability, compared to other blood cells. Intact and viable CTCs are harvested from the Platform and can be utilised for a range of downstream assays including CTC culturing.

Currently, there are 12 peer reviewed publications in which the Parsortix Platform has been utilised to (1) develop Parsortix Platform enriched CTC derived cancer cell lines for cell culture and mouse xenotransplantation (Figures 1&2), (2) analyse CTCs and CTC clusters from CTC or cell line derived mouse xenografts (Figure 3), and (3) develop CTC Zebrafish embryo xenografts (Figures 4&5). These publications use various techniques such as single cell picking, RNA sequencing and CRISPR, generating significant findings on the biology of CTC driven metastasis including neutrophils escorting CTCs, hypoxic influence on CTC invasion, and the influence of the circadian rhythm on CTC production. More specifically, research groups have developed and optimised their own protocols involving Parsortix enriched CTC derived xenograft models. These papers and associated methodologies are summarised in Table 1, and a representative selection are discussed in sections 3.1 - 3.3 below.

3.1 Parsortix enriched CTCs for the development of mouse xenografts

Professor Nicola Aceto's laboratory at the Institute of Molecular Health Sciences, Zurich Switzerland, have established a protocol utilising the Parsortix Platform to enrich, harvest and culture CTCs from a patient sample, for transplantation into mouse models to mimic human disease. Scheidmann *et al.* (2022) describe this protocol (Figure 2). Briefly, blood from a breast cancer patient was drawn into EDTA vacutainers and processed via the Parsortix Platform using a 6.5 µm cassette. CTCs were identified using EPCAM, HER2, EGFR, CD45. Human CTC-derived cells, (BR16), were successfully cultured under hypoxic conditions (5% O_2) in ultra-low attachment 6-well plates, T75 flasks or CellSTACK and specific growth medium were added every third day (RPMI-1640 medium containing 20 ng/mL recombinant HER2, 20 ng/mL recombinant human FGF, 1xB27 supplement and 1x antibiotic-antimycotic)[7].

After CTC culture, this research group describes transplantation of cells into anesthetized eight-to-ten-week-old female NSG mice via injection into the right mammary fat pad. Before injection cells were resuspended into PBS and growth factor basement membrane extract. CTCs, CTC clusters and organs with metastasis were then sampled 22-28 weeks post injection. For CTC and CTC cluster evaluation, approximately 1 mL of blood was drawn via cardiac puncture, thereafter, the blood was processed via the Parsortix Platform[7] (Figure 2), using a 50 mL falcon tube at a constant pressure of 50 mbar.

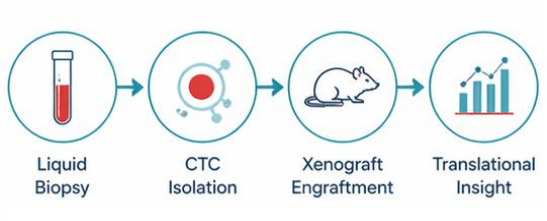


Figure 1. Parsortix enriched CTCs for the development of mouse xenografts

This xenograft model was utilised to mimic human disease progression and perform loss-of-function CRISPR studies to identify critical steps in the metastatic cascade[7]. The research reported the identification of specific genetic dependencies for three main steps of cancer metastasis (growth of primary tumor lesion, intravasation of CTCs and adaptation in various distant metastatic sites). PLK1 was shown to be important for intravasation of CTCs, both single and clusters, and treatment with readily available inhibitors prevented this process[7]. The authors stated that this workflow allowed for the identification of actionable targets in the spread of cancer and may hold clinical potential for discovering and testing new targets[7].

3.2 The Parsortix Platform to enable assessment of CTCs from xenograft models

There are numerous publications that use mouse xenografts developed using patient derived CTCs or cancer cell lines to model disease and use the Parsortix Platform to subsequently assess CTCs. For example, Reimer, F. *et al.* (2023) created a triple negative breast cancer cell line derived xenograft using MDA-MB-231 and its brain-seeking subline MDA-MB-231-BR to investigate the expression of the structural protein, desmocollin 2, to reported that reduced desmocollin 2 expression decreases metastatic potential[8]. Similarly, Stamatakis, K. *et al.* (2022) utilised cell line derived xenografts and the Parsortix Platform enriched CTCs to compare cyclooxygenase 2 effector (COX2) genes as potential inflammation-related biomarkers. The author's identified two biomarkers, Egr1 and Klf4, in which a reduction in gene expression level was a potential marker for the presence of CTCs[9].

The Aceto laboratory utilised the Parsortix Platform to investigate the impact of the circadian rhythm on the release of CTCs in breast cancer patients to further understand the metastatic cascade. Diamantopoulou *et al.*, (2022) reported on four different mouse models, including xenografts derived from human breast CTCs, human breast cancer cells, mouse breast cancer cells, and an immunocompetent syngeneic breast cancer model[10]. At length, this study reported that in patient samples, most CTCs (78%) were obtained at night (4am), including single CTCs, CTC clusters and CTC-white blood cell clusters. Consistent with patient data, most CTC events (87% - 99.2%) were present in samples obtained

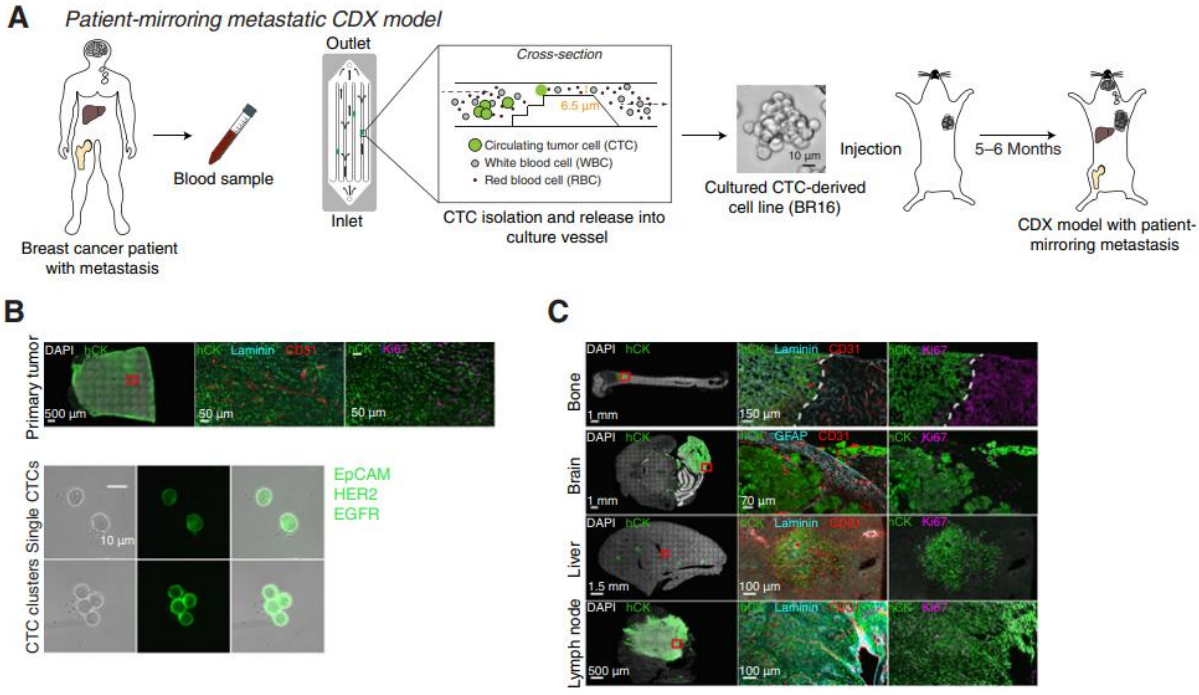


Figure 2. Generation and characterization of a CTC-derived xenograft model. A, Schematic of the establishment of human CTC-derived xenografts (CDX) from a patient with breast cancer (BR16). CTCs from BR16 patient blood were isolated, expanded ex vivo, and injected in the mammary fat pad of NSG mice. Upon tumor development, spontaneously formed CTCs seed metastases to defined organs, mirroring the metastatic pattern of the patient-of-origin. B, Representative pictures of primary tumor, single CTCs, and CTC clusters from the BR16-CDX model. The primary tumor was stained for human pan-cytokeratin (hCK; green), laminin (turquoise), CD31 (red), Ki67 (purple), and DAPI (nuclei; white), whereas single CTCs and CTC clusters were live-stained for epithelial cell adhesion molecule (EpCAM), HER2, and EGFR (green) within the microfluidic cassette, directly after capture. C, Representative pictures of spontaneous bone, brain, liver and lymph node metastasis of BR16-CDX mice, stained for hCK (green), laminin (turquoise), glial fibrillary acidic protein (GFAP; turquoise), CD31 (red), Ki67 (purple), and DAPI (white)[7].

during mouse rest phase[10]. This effect was mirrored in jetlag induced mice, revealing the impact of melatonin expression. The authors suggest that the gene expression changes they observed provide novel insight into the role of the circadian rhythm in the generation of CTCs with high metastatic potential and highlights the potential need for time-controlled delivery of treatments[10].

Moreover, Donato *et al.,* (2020) have also enriched CTCs from patient CTC derived xenografts using the Parsortix Platform to investigate hypoxic CTC clusters and their metastatic potential, reporting that therapy promoting vascularisation may supress intra-tumor hypoxia and clustering of CTCs leading to reduced metastasis[11]. In addition, research by Szczerba *et al.,* (2019) into CTC neutrophil escorts as enablers of cell cycle progression has provided a rationale for targeting this interaction in treatment of breast cancer[12].

More recently, research into DNA methylation of CTC clusters impacting on metastasis and the dissociation of CTC clusters into single cells when treated with Na+/K+ ATPase inhibitors has highlighted this as a potential strategy to suppress the spread of cancer[13]. More specifically, xenograft models treated with Na+/K+ ATPase inhibitors saw an 80.7-fold suppression in metastatic burden[13] as compared to untreated animals. More recently, the latter work expanded into a phase I clinical trial, and first-in-human confirmation that treatment with the Na+/K+ ATPase inhibitor, digoxin, leads to a partial CTC cluster dissolution in women with metastatic breast cancer[14]. Although clinical endpoints were not included, this is the first evidence to suggest novel approaches for the prevention of metastasis. As no drug class currently exists to target the metastatic cascade this holds potential to introduce a paradigm shift in cancer treatment with new drugs being developed. The Parsortix Platform was instrumental in this breakthrough finding enabling the enrichment and the study of CTC clusters used in preclinical research that led to drug development and a first-in-human clinical trial[14].

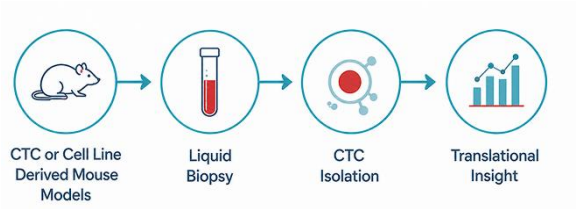


Figure 3. CTC or cell line derived mouse models and CTC enrichment using the Parsortix Platform for translational research.

Professor Dario Marchetti's laboratory at the University of New Mexico, reported breakthrough findings into CTC clusters and the metastatic cascade using CTC derived humanised and immunodeficient mouse xenografts and the Parsortix Platform. The group reported, for the first time, the directionality of cancer[15]. They found that the RPL/RPS gene expression signature in CTC: B cell clusters acts as a "post code" creating a tumor promoting niche in a specific target

organ, in this case, a melanoma brain-liver metastasis axis. They reported a higher number of humanised CTC derived mouse models developed metastatic brain melanoma (MBM) as compared to immunodeficient mice[15]. It is hypothesised that immune cells not only cluster with CTCs but educate them on secondary metastasis. This is a novel finding providing insight into the metastatic cascade that may have implications for other tumor types and have potential applications for the development of new drug classes to target CTC clusters. The authors state that the Parsortix Platform is the most suitable technology to capture CTC clusters from preclinical models and patient blood.

Overall, the assessment of the Parsortix Platform derived CTCs and CTC clusters in xenograft models has been reported utilising a range of techniques (such as via fluorescence microscopy, flow cytometry, DNA and RNA interrogation via NGS[7] and single cell picking via the CellCelector for further DNA and RNA interrogation[10]) towards understanding the metastatic cascade, CTC clusters and the development of potential drug discovery projects.

3.3 Parsortix enriched CTCs for the development of a Zebrafish xenograft

Reinhardt, F. *et al.* (2023) reported on a workflow in which Parsortix Platform enriched CTCs from metastatic breast cancer patients were transplanted into zebrafish embryos to track the development of metastatic disease. Following enrichment of CTCs using the Parsortix Platform, CTCs were stained (EpCAM, MUC1, HER2, CD45) and cells positive for EpCAM/MUC1/HER2 and negative for CD45 were additionally labelled with CellTracker Red. CellCelector was used to pick 50 single CTCs, and these were injected into Zebrafish embryos. Zebrafish were imaged at one and three-days post injection using a fluorescence microscope to assess the spread of tumor cells. This method (Figure 5) is described as the first of its kind, and may be used in the future to further study metastasis and potentially inform the development of novel targets[16].

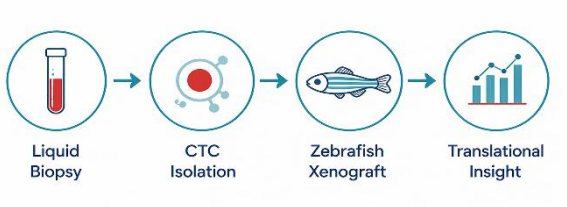
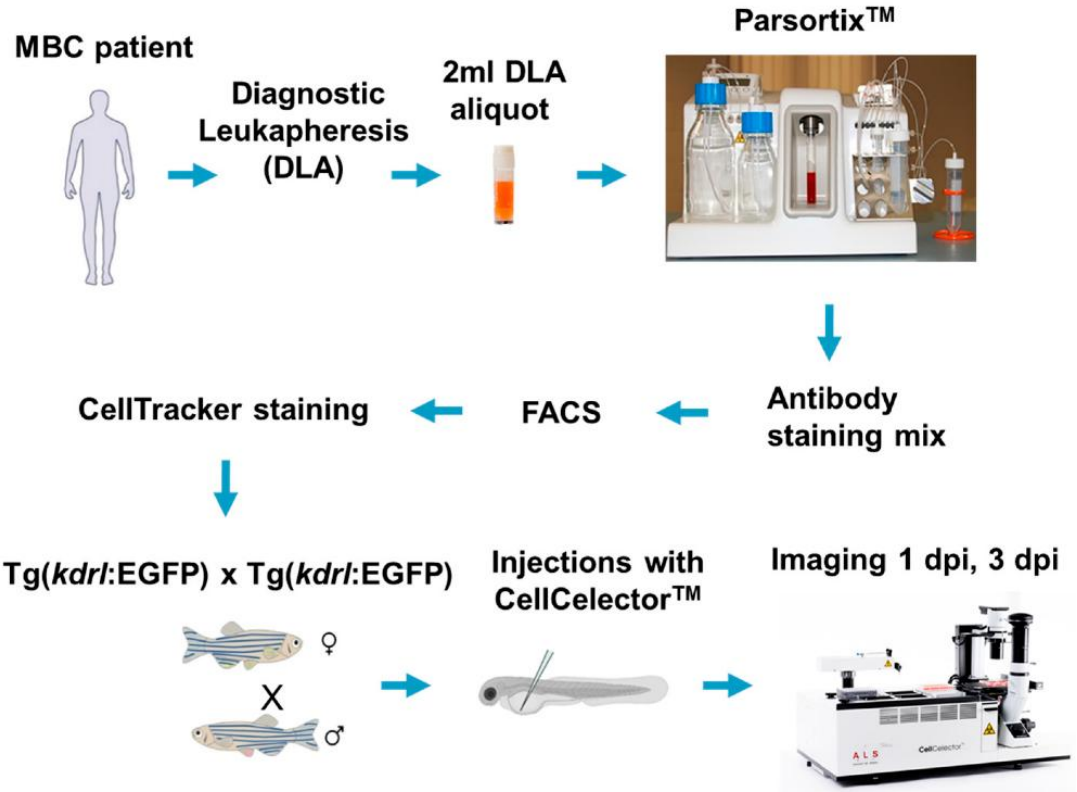


Figure 4. Parsortix enriched CTCs for the development of zebrafish xenografts.

Figure 5. Schematic representation of the zebrafish xenograft assay workflow[16]



3.4 Summary of publications leveraging the Parsortix Platform for Xenograft models

Table 1. Independent peer reviewed literature utilising Parsortix enriched CTCs and xenograft models

Author & Title	Date	Xenograft model	Key methods of analysis	Key findings
Bowley, T. Y. et al.[13] A melanoma brain metastasis CTC signature and CTC:B cell clusters associate with secondary liver metastasis: a melanoma brain-liver metastasis axis	2025	CTC-derived melanoma brain metastasis xenograft	Bioluminescence imaging, RNA sequencing	Higher number of humanised (human immune cells) CTC derived mouse models developed MBM as compared to immunodeficient mice. RNA-seq confirmed RPL/RPS gene signature was upregulated in CTC clusters, showing a tumor promoting niche (or a "post code"). This is the first report of the directionality of metastasis (spread of metastatic brain cancer to the liver). It is hypothesised that immune cells not only cluster with CTCs but educate them. The number of CTC:B cell clusters was 15-20 fold higher in primary patients then metastatic patients.
Kurzeder, C. et al.[12] Digoxin for reduction of circulating tumor cell cluster size in metastatic breast cancer: a proof-of-concept study	2025	Cell line derived breast cancer xenograft	Bioluminescence imaging	First-in-human, phase I study of digoxin treatment leading to partial CTC cluster dissolution towards stopping the metastatic spread of cancer. Xenograft metastatic potential assay showed that CTC clusters of at least 4 cells, exhibited higher metastatic potential compared to smaller clusters via bioluminescence imaging.
Reinhardt, F. et al.[15] DanioCTC: Analysis of Circulating Tumor Cells from Metastatic Breast Cancer Patients in Zebrafish Xenografts	2023	**Parsortix enriched CTC Zebrafish embryo breast cancer xenograft**	Fluorescence microscopy	Novel workflow for injecting a few (~50) Parsortix Platform enriched CTCs from MBC patients into zebrafish embryos, which provides an important model to investigate the metastatic capabilities of CTCs. The authors claim this novel method could help to enhance understanding of MBC biology and facilitate the development of targeted interventions for the disease.
Bowley, T. Y. et al.[17] Targeting Translation and the Cell Cycle Inversely Affects CTC Metabolism but Not Metastasis	2023	CTC-derived metastatic brain melanoma xenograft	Immunofluorescence imaging, RNA sequencing	Investigation of four treatment groups of CTC-derived metastatic brain melanoma xenografts. Parsortix Platform enriched CTCs from xenograft models were utilised to interrogate 21 previously identified gene RPL/RPS signatures that drive onset and progression. The authors state that the examination of gene expression in CTCs could be pivotal in prescribing more targeted treatments.
Reimer, F. et al.[8] The role of the desmosomal protein desmocollin 2 in tumor progression in triple negative breast cancer patients	2023	Cell line derived triple negative breast cancer xenograft	Ex vivo bioluminescence imaging, Western blot analyses	Triple negative breast cancer cell line MDA-MB-231 and its brain-seeking subline MDA-MB-231-BR were injected into mouse models to investigate metastatic potential. The model reported that reduced desmocollin 2 expression decreases metastatic potential.

Author & Title	Date	Xenograft model	Key methods of analysis	Key findings
Bowley, T. Y. et al.[18] The RPL/RPS Gene Signature of Melanoma CTCs Associates with Brain Metastasis	2022	CTC-derived metastatic brain melanoma xenograft	Immunofluorescence imaging, RNA sequencing	Developed CTC-derived metastatic brain melanoma xenografts to mimic human disease development. Parsortix Platform enriched CTCs from xenograft models were interrogated via immunofluorescence imaging and RNA sequencing to identify a new CTC gene expression signature which may have potential to provide new prognostic and therapeutic tools.
Diamantopoulou, Z. et al.[10] The metastatic spread of breast cancer accelerates during sleep	2022	Patient CTC derived human breast cancer xenograft	Immunofluorescence imaging, RNA sequencing	Parsortix Platform enriched CTCs from a patient CTC derived breast xenograft model were analysed, authors reported that the metastatic ability of CTCs may be associated with sleep (or rest phase) towards time controlled treatment strategies.
Scheidmann, M. C. et al.[7] An in vivo CRISPR screen identifies stepwise genetic dependencies of metastatic progression	2022	**Parsortix enriched CTC derived human breast cancer xenograft (BR16)**	RT-qPCR	Developed a Parsortix Platform enriched CTC derived human breast xenograft model to perform loss-of-function CRISPR screen to identify specific genetic dependencies for three main steps of cancer metastasis (growth of primary tumor lesion, intravasation of CTCs and adaptation in various distant metastatic sites).
Stamatakis, K. et al.[9] Cyclooxygenase 2 Effector Genes as Potential Inflammation-Related Biomarkers for Colorectal Cancer Circulating Tumor Cells Detection by Liquid Biopsy.	2022	Cell line derived colorectal cancer xenograft	RT-qPCR	Colorectal cancer cell line expressing GFP and luciferase xenografts were developed. Parsortix Platform enriched CTCs from these xenografts were used to compare COX2 gene profiles. The author's identified two biomarkers, Egr1 and Klf4, in which reduction in gene expression level presented as potential markers for the presence of CTCs.
Donato, C. et al.[11] Hypoxia Triggers the Intravasation of Clustered Circulating Tumor Cells	2020	Patient CTC derived human breast cancer xenograft And cell line derived triple negative breast cancer xenograft	Immunofluorescence imaging	Utilised xenograft models to show that the majority of CTC clusters were undergoing hypoxia, while single CTCs were normoxic. The authors propose therapy promoting vascularisation may supress intra-tumor hypoxia and clustering of CTCs leading to reduced metastasis.
Szczerba, B. M. et al.[12] Neutrophils escort circulating tumor cells to enable cell cycle progression	2019	Patient CTC derived human breast cancer xenograft	RNA sequencing	Xenografts and patient samples were interrogated for single CTC-associated WBCs, as well as corresponding cancer cells within each CTC–WBC clusters. The authors show that CTC and neutrophil association drives cell cycle progression within the bloodstream and expands the metastatic potential of CTCs, providing a rationale for targeting this interaction in treatment of breast cancer.
Gkountela, S. et al.[13] Circulating Tumor Cell Clustering Shapes DNA Methylation to Enable Metastasis Seeding	2019	Patient CTC derived human breast cancer xenograft	Whole-genome bisulfite sequencing Methylation analysis	The research demonstrated the ability to harvest intact metastatic CTC clusters, using the Parsortix Platform and highlights the potential of targeting CTC clusters to intercept the metastatic spread. Treatment to dissociate CTC clusters with Na+/K+ ATPase inhibitors saw an 80.7-fold suppression in metastatic burden in mouse models as compared to untreated animals.

4.0 Conclusion

The independent literature reviewed here demonstrates that CelLBxHealth's Parsortix Platform can be used to successfully (1) develop Parsortix Platform enriched CTC derived cancer cell lines for cell culture and mouse xenotransplantation, (2) analyse CTCs and CTC clusters from patient CTC or cell line derived mouse xenografts, and (3) develop CTC Zebrafish embryo xenografts. These models have been utilised in various peer reviewed publications investigating CTC cluster driven metastasis, hypoxic influence on CTC invasion, neutrophil escort of CTCs, the influence of the circadian rhythm on the release of tumor cells into the bloodstream and the assessment of CTC cluster dissolution. The research discussed here utilised various techniques such as single cell picking, RNA sequencing, and CRISPR for repeatable, longitudinal assessment of xenograft models.

New discovery projects are urgently needed to better understand the spread of cancer and to develop treatment strategies targeting metastasis. CelLBxHealth's Parsortix Platform can allow for dynamic assessment of xenograft models to study the metastatic cascade, CTC and CTC clusters, and facilitate drug discovery projects.

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