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NanoVelcro Rare-Cell Assays for Detection and Characterization of Circulating Tumor Cells

Yu Jen Jan^{1,2,†}, Jie-Fu Chen^{1,†}, Yazhen Zhu^{2,†}, Yi-Tsung Lu^{3,†}, Szu Hao Chen², Howard Chung², Matthew Smalley^{2,4}, Yen-Wen Huang^{2,4}, Jiantong Dong², Hsiao-Hua Yu⁵, James S. Tomlinson^{6,8,9}, Shuang Hou⁶, Vatche G. Agopian^{6,7}, Edwin M. Posadas¹, and Hsian-Rong Tseng²

¹Urologic Oncology Program and Uro-Oncology Research Laboratories, Samuel Oschin Comprehensive Cancer Institute, Cedars-Sinai Medical Center, Los Angeles, California, USA

²Department of Molecular and Medical Pharmacology, California NanoSystems Institute, Crump Institute for Molecular Imaging, University of California, Los Angeles, Los Angeles, California, USA

³Norris Comprehensive Cancer Center, Keck School of Medicine, University of Southern California, Los Angeles, California, USA

⁴CytoLumina Technologies Corp., Los Angeles, California, USA

⁵Institute of Chemistry, Academia Sinica, Taipei, Taiwan

⁶Department of Surgery, University of California, Los Angeles, Los Angeles, California, USA

⁷Liver Transplantation and Hepatobiliary Surgery, University of California, Los Angeles, Los Angeles, California, USA

⁸Center for Pancreatic Disease, University of California, Los Angeles, Los Angeles, California, USA

⁹Department of Surgery, Greater Los Angeles Veteran's Affairs Administration, Los Angeles, California, USA

Abstract

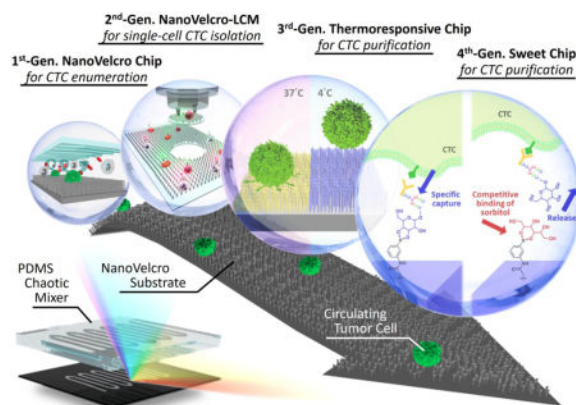
Circulating tumor cells (CTCs) are cancer cells shredded from either a primary tumor or a metastatic site and circulate in the blood as the potential cellular origin of metastasis. By detecting and analyzing CTCs, we will be able to noninvasively monitor disease progression in individual cancer patients and obtain insightful information for assessing disease status, thus realizing the concept of “tumor liquid biopsy”. However, it is technically challenging to identify CTCs in patient blood samples because of the extremely low abundance of CTCs among a large number of hematologic cells. In order to address this challenge, our research team at UCLA pioneered a

[†]These authors contribute equally to this work.

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unique concept of “NanoVelcro” cell-affinity substrates, in which CTC capture agent-coated nanostructured substrates were utilized to immobilize CTCs with remarkable efficiency. Four generations of NanoVelcro CTC assays have been developed over the past decade for a variety of clinical utilities. The 1st-gen NanoVelcro chips, composed of a silicon nanowire substrate (SiNS) and an overlaid microfluidic chaotic mixer, were created for CTC enumeration. The 2nd-gen NanoVelcro chips (i.e., NanoVelcro-LMD), based on polymer nanosubstrates, were developed for single-CTC isolation in conjunction with the use of the laser microdissection (LMD) technique. By grafting thermoresponsive polymer brushes onto SiNS, the 3rd-gen Thermoresponsive NanoVelcro chips have demonstrated the capture and release of CTCs at 37 and 4 °C respectively, thereby allowing for rapid CTC purification while maintaining cell viability and molecular integrity. Fabricated with boronic acid-grafted conducting polymer-based nanomaterial on chip surface, the 4th-gen NanoVelcro Chips (Sweet chip) were able to purify CTCs with well-preserved RNA transcripts, which could be used for downstream analysis of several cancer specific RNA biomarkers. In this review article, we will summarize the development of the four generations of NanoVelcro CTC Assays, and the clinical applications of each generation of devices.

Graphical abstract



1. Introduction

1.1. Circulating tumor cell (CTC)

The gold standard for cancer diagnosis is based on pathological analysis of tumor tissues, which relies upon tissue specimens acquired by invasive procedures, e.g., surgical excision or needle biopsy. Crucial information including histopathology and molecular profiling can be generated to achieve accurate diagnosis and classification of the disease. However, these invasive procedures impose risks to cancer patients. First, the invasive procedures can be quite costly. The risk of injury to the patient may limit the implementation of the invasive procedures (e.g., pneumothoraxes that can be caused by lung biopsies). Further, certain malignancies pose technical challenges due to the anatomical locations of metastasis. For instance, advanced prostate cancer metastases are commonly found in the bone and are sclerotic in nature. In such cases, typical small needle biopsies are avoided and larger, drill-based sampling is required. Moreover, the well-recognized tumor temporospatial heterogeneity[1–7] raises severe concerns over how accurately a given biopsied sample

represents a disease whose biological and molecular nature varies from site to site and changes over time in the course of treatment interventions. Despite its difficulty, a re-biopsy procedure is often recommended to detect a possible new biology profile of cancer cells during the clinical treatment course in some solid tumors (e.g. lung cancer).

As a non-invasive alternative to tumor biopsy, researchers have been exploring the use of circulating tumor cells (CTCs) as “liquid biopsies” of solid tumors. CTCs are blood borne tumor cells shed from either primary or metastatic sites. Through a simple blood draw, CTCs can be detected and recovered throughout the course of disease development without needing invasive and painful biopsy procedures. In addition to conventional diagnostic imaging and serum marker detection, detecting and characterizing CTCs in patient blood provides an opportunity for early diagnosis of cancer metastasis. Further, serial CTC tests can be performed over the disease progression with relatively high frequency, creating an opportunity to perform real-time, dynamic monitoring of an evolving and adapting malignant process[8, 9]. To address this unmet need, there have been significant research endeavors[10], especially in the fields of chemistry, materials science, and bioengineering, devoted to developing CTC detection, isolation, and characterization technologies[11]. However, identifying CTCs in blood samples has been technically challenging due to the extremely low abundance (a few to hundreds per milliliter) of CTCs among a large number (10^9 mL^{-1}) of hematologic cells in the blood. Initial CTC studies focused on enumeration and protein expression analysis [12–14]. More recent research efforts have demonstrated that CTCs and their matching tumor tissues share significant similarities at the genomic[15–17] and transcriptomic[18, 19] levels. Mounting evidence has consistently shown CTCs to be a powerful tool with which we can study the underlying biology of cancer, guide therapeutic interventions, and monitor the progression of disease.

1.2. Existing CTC assays

In order to effectively conduct detection and analysis of CTCs, a variety of methodologies have been developed. (i) Immunomagnetic separation: Positive selection/enrichment of CTCs[13, 20, 21] can be achieved using capture agent-labeled magnetic beads. For epithelial-origin solid tumors, anti-EpCAM [22] is the most widely used capture agent. Alternatively, negative depletion [23, 24] of white blood cells (WBCs) using magnetic beads tagged by anti-CD45 can result in purified CTCs. CellSearch™ [12–14] is the only FDA-approved CTC assay with prognostic utility in metastatic breast, colorectal, and prostate cancers. CellSearch™ Assay enriches CTCs with magnetic beads tagged with anti-EpCAM, followed by immunocytochemistry (ICC) treatment to distinguish CTCs (DAPI+/cytokeratin –CK+/CD45–) from WBCs (DAPI+/CK–/CD45+) and cell debris in the background. New immunomagnetic technologies including MagSweeper[25], magnetic sifters[26], AdnaGen/Qiagen[27], IsoFlux[28], VerIFAST[29] and Cynvenio[30], were developed to improve the speed, efficiency, and cost of CTC detection and characterization. (ii) Flow cytometry: Although one of the most commonly used cell-sorting technologies for the analysis of sub-populations of fluorescently labeled cells [31, 32], flow cytometry lacked the ability to reveal sufficient morphological information to satisfy the standards set by pathologists for CTCs. The development of ensemble decision aliquot ranking (eDAR) [33, 34] addressed this drawback. (iii) Microfluidics-enabled CTC assays: Massachusetts General Hospital

(MGH) developed the microfluidic devices [35], propelling recent research efforts focusing on the development of microfluidic CTC assays. Their 1st-generation (gen) microchips [35] (i.e., CTC-Chip) covalently attached anti-EpCAM to silicon microposts, thus maximizing the contact between the flow-through cells and device surfaces. Biocept's CTC assay [36] and GEDI approach [37] were created using similar device configurations. Moreover, utilizing microposts (self-assembled from magnetic beads) in a microchip, the "Ephesia" approach [38] demonstrated the advantages of both immunomagnetic and microchip-based methods. The MGH team's 2nd-gen microchips [39] (i.e., herringbone-chip) featured herringbone structures micro-engineered into a polydimethylsiloxane (PDMS) block to enhance contact between CTCs and the antibody-tagged device surfaces. Most recently, the MGH team developed the 3rd-gen iChip [40], which integrates inertial focusing of blood cells with immunomagnetic WBC depletion in a microfluidic chip. Notably, "Unmanipulated" CTCs can be recovered from whole blood samples with well-preserved viability and molecular integrity, enabling CTC-based transcriptomic profiling [18], drug susceptibility testing, [41] and *ex vivo* culturing. However, it was shown that the sorting mechanism of iChips might hamper recovery of CTC clusters [42]. "Cluster-Chip", was developed to overcome this problem [43]. Several other types of microfluidics-based CTC assays, including cell rolling [44], micro-nuclear magnetic resonance (μ NMR) [45], Vortex technology [46, 47], and lipid bilayer-deposited microchips [48] have also been developed. Moreover, Velocity Valley Chip was developed to separate CTCs into subpopulations [49] according to their EpCAM expression levels. (iv) Microscopy imaging [50–52]: Using high-speed fluorescent microscopy, CTCs can be identified in smeared peripheral blood mononuclear cell (PBMC) samples, with high-quality morphological features. Epic Sciences' CLIA-certified (Clinical Laboratory Improvement Amendments) services establish them as one of the current leaders in this technology. Similarly, RareCyte's fluorescence microscopy-based platform has demonstrated CTC identification in clinical samples [53], as well as the feasibility of performing single-CTC genotyping.

While all the aforementioned technologies utilize capture agents to isolate CTCs (e.g., EpCAM), CTC assays are not inherently required to use such markers. Instead, these "label-free" technologies exploit the physical properties of CTCs. (v) CTC filters: Filter-based techniques [54–58] are established to sort CTCs based on their size difference. A variety of systems/kits including Clearbridge [56, 57], Rarecells [59], Creatv MicroTech, ScreenCell [60], Celsee [61] are now commercially available to support utilities in research laboratories. Since these filter devices only capture CTCs, which exhibit larger sizes than WBCs, small-sized CTCs might be overlooked in this kind of approach. (vi) Dielectrophoresis: A dielectrophoretic field can be used to sort CTCs apart from the background WBCs as a result of CTCs' differential dielectric properties (which depend on their diameter, membrane area, density, conductivity and volume). ApoCell's technology [62] introduces a dielectrophoretic field in a microchip to achieve CTC sorting. DEPArray™ (Silicon BioSystems) combines dielectrophoretic sorting and microscopy imaging [63] to isolate pre-sorted CTC mixtures, allowing for downstream CTC-based molecular characterizations with single-cell resolution. DEPArray™ is frequently used as a downstream single-CTC isolation technique using the recovered CTC samples from CellSearch™ Assays. [64] (vii) Several outstanding review

articles[65–67] that highlight CTC detection and characterization technologies provide different coverage and scope from this review article.

2. NanoVelcro CTC Assays

2.1. Development and evolution

Over the last few decades, medical research in the field of nanotechnology has made significant progress in reducing the costs of CTC characterization [68–70]. The goal of personalized care is one-step closer with the deployment of emerging advances in oncology. Knowing that the nanoscale tissue microenvironment (particularly the cellular membrane and extracellular matrix) can help mediate cellular behavior, the Tseng Lab at UCLA launched the nanosubstrate microfluidic platform (i.e., silicon nanowire substrate, SiNS[71]) for capturing CTCs, colloquially deemed the “NanoVelcro” assay [71, 72].

This novel approach utilized capture agent-coated nanosubstrates to immobilize CTCs with high efficiency, resembling the working mechanism of Velcro™. In much the same way that two fabric strips of a Velcro fastener attach tightly together, the NanoVelcro cell-affinity substrates interact with CTCs to form strong binding. The team developed a three-color ICC protocol [73] with DAPI, anti-CK, and anti-CD45 to identify CTCs immobilized on the nanosubstrate. Nonspecifically captured WBCs (DAPI+/CK-/CD45+, sizes<12 μm) are differentiated from CTCs (DAPI+/CK+/CD45-, sizes>6 μm) by cell imaging criteria with CK/CD45 expression, object size, and DAPI staining. In the time since the initial proof-of-concept of the NanoVelcro substrates and optimization of the ICC protocol, four generations of NanoVelcro CTC Assays have been developed since the launch of the first model for a variety of clinical utilities[74, 75].(Figure 1)

2.2. Proof-of-Concept demonstration of NanoVelcro Cell-Affinity substrates - stationary NanoVelcro CTC Assay

The working mechanism (Figure 2a) of NanoVelcro assay [71, 76–78] lies in the use of SiNS, which allows for Velcro-like interactions [79] between SiNS and nanoscale cell-surface components. Anti-EpCAM was selected as the capture agent for its specificity for capturing CTCs and drastically improved capture efficiency compared to flat SiNS. (Figure 2b). The anti-EpCAM capture agent is grafted onto SiNS via biotin-streptavidin conjugation (Figure 2c). CTC capture in whole blood samples was achieved in a stationary device setting via comprehensive optimization. SEM characterization of CTCs on SiNS (Figure 2d) and imprinted PLGA NanoVelcro substrates (Figure 2e) supported the effectiveness of the proposed NanoVelcro working mechanism. Through testing, NanoVelcro cell-affinity substrates demonstrate CTC capture efficiencies ranging from 40 to 70% in a stationary device setting. [69]

2.3. General applicability of the concept of NanoVelcro Cell-Affinity Assay

In addition to SiNS[71], extensive research endeavors have been devoted to exploring the use of different nanomaterials on the NanoVelcro cell-affinity assay. Such materials include TiO₂ nanowires[77], polymer dots[76], nanopillars[78], nanoparticles[80], Fe₃O₄ nanoparticles[81], gold clusters on silicon nanowires[82], layer-by-layer-assembled

nanostructures[83], graphene oxide nanosheets[84], and DNA networks[85]. These efforts have demonstrated the ability to efficiently capture CTCs and other types of rare cells. Other groups of researchers have utilized nanomaterials for rare cell assays. Dr. Wang's group [86] at the Chinese Academy of Science focused on structural adjustments and introduced a hierarchically-assembled ITO nanowire array with vertical and horizontal branches fabricated as a three-dimensional fractal nano-biointerface for CTC capture with enhanced efficiency. Dr Cui's group at Stanford employed a dual-function lipid coating on traditional nanostructured surfaces. Based on their investigation of interactions between cells and the nanopillar array, both prevention of nonspecific cell adhesion and CTC capture by a functionalized layer of antibodies were demonstrated. [87, 88] A capture-agent free approach was carried out by Dr. Fu's group[89] at the University of Michigan. A nanoroughened glass substrate on slides exhibited a differential adhesion affinity for CTCs rather than normal blood cells. This approach demonstrated CTC capture ability independent of the surface biomarker[22].

2.4. 1st-Gen NanoVelcro Chip

On the basis of the stationary NanoVelcro cell-affinity substrates [71, 76–78], we sought for further improvement of the capture performance. This effort led to the development of the 1st-gen NanoVelcro Chip[8, 90], which consists of a lithographically patterned SiNS and an overlaid microfluidic chaotic mixer (Figure 3a and b). The PDMS chaotic mixer has herringbone microstructures on the roof and induces[91] vertical flows in the microchannel, which increases the contact frequency between cells and nanosubstrates. CTCs captured by this device are stained by a 3-color ICC protocol with DAPI, anti-CK and anti-CD45. Single-cell image cytometry [72] (Figure 3c and d) analyzing CK/CD45 expressions and CTC footprint sizes is employed to identify CTCs from nonspecifically captured WBCs and cellular debris. Using artificial CTC samples, we achieved >85% capture efficiency with the 1st-gen NanoVelcro Chips. After performing side-by-side validation studies using patient blood samples, we found that the sensitivity of the 1st-gen NanoVelcro Chip [8] is superior to the FDA-cleared CellSearch™ Assay. The 1st-gen NanoVelcro Chips along with the ICC protocol have since been used for CTC enumeration in multiple cancers.

2.5. Alternative capture agents: Aptamers

Despite being the most widely utilized CTC capture agent, anti-EpCAM possesses several shortcomings, including its high cost and poor stability. These constrain the use of CTC-based diagnostics, especially in low-resource environments. Among other choices, aptamers stood out as a strong candidate for CTC-based diagnostics because of their many desirable properties. First, the ease of selection and synthesis make aptamers more affordable than antibodies like anti-EpCAM. Second, they possess comparable binding affinity and specificity to antibody-based capture agents. Third, low immunogenicity of aptamers erases concerns for compatibility issues[92]. Recently, we have generated two singled-stranded DNA aptamers via an in vitro cell-SELEX[93] (systematic evolution of ligands by exponential enrichment) that has shown success in capturing non-small cell lung cancer (NSCLC) CTCs. With the aptamer-grafted NanoVelcro Chip (Figure 4), we demonstrated high efficiency of capturing NSCLC CTCs from blood. We were also able to recover the nanosubstrate-bounded CTCs using enzymatic treatment. Our recent study suggests that

rationally-designed aptamer cocktails may elicit a synergistic effect for capturing CTCs in a microfluidic setting, achieving enhanced and differential capture of CTCs for non-small cell lung cancer (NSCLC) patients[94].

2.6. 2nd-Gen NanoVelcro Chip

The 2nd-gen NanoVelcro Chip (Figure 5) was developed to address the need to isolate CTCs for subsequent molecular analysis[16, 95, 96]. Known as the NanoVelcro-LCM (laser capture microdissection) approach, we engineered a polymer-based CTC capture substrate, which can be dissected by an LCM microscope for molecular analysis, especially at the genomic level.

The commercial LCM slides are polymer coated microscope slides with their thermoplastic membrane dissectible by laser dissection microscopes[97]. LCM technology was used in dissecting visually selected areas of tissues from the pathological samples, and the quality of the genomic materials was well preserved. By electrospinning nanofibers made of PLGA (poly(lactic-co-glycolic acid)), onto the LCM slides (Figure 5a-c), the PLGA NanoVelcro substrates were able to interact with nanoscale cellular structures in much the same way as the SiNS on the 1st-gen NanoVelcro Chips, while remaining transparent and laser dissectible. The negatively charged PLGA reduced non-specific adsorption of cell-free DNA by the substrate, thus decreasing the background contamination in subsequent analysis[98]. The PLGA nanofibers were then covalently attached to streptavidin, followed by conjugation of biotinylated antibodies to confer specific capture of CTCs from whole blood samples. (Figure 5b) As with the 1st-gen NanoVelcro Chip, CTC enrichment was achieved by introducing an overlaid PDMS microfluidic chaotic mixer.

Once CTCs were immobilized on the substrate, the CTC-bearing substrate was fixed with methanol and stained with anti-cytokeratin (CK) and anti-CD45. DAPI nuclear staining was excluded from the process to avoid interference in the genome amplification process. Under an LCM microscope, single-CTCs were visually identified by their CK+/CD45-characteristics and were specifically isolated. (Figure 5d and e) To analyze the genomes of single cells, whole genome amplification (WGA) was employed prior to sequencing. Initially, we used PCR-based WGA, and were successful in performing Sanger sequencing targeting a short fragment[96]. However, similar technology only yielded 25–80% coverage in whole exome sequencing (WES), and significantly limited analysis[32]. To improve our sequencing quality, we incorporated a multiple displacement amplification (MDA) reaction to amplify CTC DNA in long fragments. MDA was better than PCR-based WGA in reducing amplification errors[99]. An additional rigorous quality check was employed to select high-quality CTC samples using a multiplexed PCR based on eight housekeeping genes. The successful amplification of the housekeeping genes directly correlated with the final coverage of sequencing. By choosing only the best quality CTC samples, we avoided the unnecessary waste of the sequencing power, and demonstrated the successful whole genome sequencing (WGS) of 4 CTCs with more than 95% coverage[16].

In addition to CTC analysis, our team utilized the 2nd-gen NanoVelcro Assay for enrichment, isolation and characterization of circulating fetal nucleated cells (CFNCs) in maternal blood, showing the feasibility of non-invasive prenatal diagnostics (NIPD)[100].

Using the newly developed imprinted PLGA nanosubstrate with the optimized capture condition and immunocytochemistry (ICC) protocol, this 2nd-gen NanoVelcro Assay was able to effectively enrich a subcategory of CFNCs, i.e., circulating trophoblasts (cTBs) from maternal blood. After we captured and identified single cTBs (*Hoechst*+/*CK7*+/*HLA-G*+/*CD45*−, 20 μm >sizes> 12 μm), we utilized similar LCM techniques for cTB isolation, followed by downstream genetic testing by array Comparative Genomic Hybridization (aCGH) and Short Tandem Repeats (STR) analysis. Using maternal blood samples collected from expectant mothers with a single fetus, the cTB-derived aCGH data was able to detect fetal genders as well as chromosomal aberrations, which had been confirmed by standard clinical practice. This non-invasive prenatal testing (NIPT) method could potentially replace the current gold standard for diagnosing fetal genetic abnormalities, which involves invasive and higher-risk procedures such as amniocentesis and chorionic villus sampling.

2.7. 3rd-Gen NanoVelcro Chips

Since functional[41] and molecular[15] analyses of captured CTCs have gained more recognition recently, the need for isolating high quality CTCs with higher efficiency and simpler techniques has become essential. Although our 2nd-gen NanoVelcro-LCM approach[71, 101] demonstrated great precision in single-CTC isolation, problems stemming from the time and labor-intensive process and poor viabilities of isolated cells still need to be overcome. As we continued to advance our CTC capture technology with improved efficiency and higher viability of captured CTCs, the 3rd-generation Thermoresponsive NanoVelcro approach was developed to address such needs[102, 103]. Thermoresponsive polymer brushes (i.e., poly(N-isopropylacrylamide), PIPAAm) that were grafted onto SiNS gave the 3rd-gen NanoVelcro Chips (Figure 7) the ability to capture and release CTCs at 37 and 4°C, respectively. Our innovative technique of temperature-dependent conformational changes to polymer brushes could effectively transform the accessibility of the captured CTCs on the NanoVelcro chip, providing a more efficient CTC purification method with enhanced cell viability and molecular integrity. By strategically introducing biotin groups onto the polymer brushes, we facilitated their conjugation with anti-EpCAM, a CTC-capture agent. At 37°C, CTCs are captured on the NanoVelcro substrates due to their interactions with anti-EpCAM grafted on hydrophobic domains of the polymer brushes. On the other hand, at 4°C, CTC release occurs due to conformational changes of the polymer brushes that induce internalization of anti-EpCAM. In addition to superior CTC-capture efficiency, thermoresponsive NanoVelcro demonstrated high recovery rates (>70%) and high recovered cell viability (>90%) at 4°C[103].

We continue to strive for rapid purification of CTCs from clinical blood samples, serving as a foundation for downstream CTC analysis for both molecular and functional aspects. Furthermore, obtaining viable CTCs for ex vivo expansion could be useful for a variety of applications, such as generating CTC-derived cancer lines and in vitro real-time monitoring of disease status. In the era of personalized and precision medicine, our device could assist the development of potential therapeutics for individual cancer patients.

There are other groups developing techniques of CTC capture and release using different thermoresponsive nanomaterials. Dr. Agarwal's team at University of Miami[104] also

utilized the thermoresponsive property of PIPAAm on a CTC separation slot filter. The filter is made of a parylene C membrane which binds CTCs using non-specific electrostatic interactions. When the temperature is lower than 32°C, PIPAAm demonstrates a hydrophilic property, allowing the non-specific electrostatic interactions between CTC membranes and the parylene C membrane of the filter. When the temperature is heated up over 37°C, PIPAAm becomes hydrophobic, thus releasing the electrostatically bound cells. Dr. Nagrath's team at the University of Michigan[105] also used a similar approach by coating thermal-sensitive polymers combined with Graphene oxide (GO) to a chip. The chip demonstrated the ability to capture CTCs with anti-EpCAM at room temperature and release them at the lower critical solution temperature (LCST) of 13°C. Another thermoresponsive technique was carried out by Dr. Liu's team at Beijing National Laboratory for Molecular Sciences, which combined a thermoresponsive nanostructured surface with hydrophobic interaction for its synergistic effect to capture and release CTCs[106]. In addition to thermoresponsive methods, other approaches, including photoresponsive[107] or electroresponsive[108] methods, have also been reported for capturing and releasing CTCs.

2.8. 4th-Gen NanoVelcro Chips

Although the 3rd-gen NanoVelcro Chip provided a stable and robust way to recover CTCs, we have also been exploring other methods to improve throughput and cell viability. Hence, we developed another method for CTC capture and release by utilizing surface chemistry with competitive binding. The 4th-gen NanoVelcro Chip (Sweet chip) [75] is fabricated with poly(3,4-ethylene-dioxythiophene)s (PEDOT)-based nanomaterial on the chip surface. In this platform, phenylboronic acid (PBA)-grafted PEDOT NanoVelcro combines the three-dimensional PEDOT nanosubstrate, and provides high specificity for CTC capture upon antibody conjugation. Furthermore, sorbitol competitive binding gently releases the captured cells. CTCs purified by this PEDOT NanoVelcro chip provide well-preserved RNA transcripts for the analysis of expression levels of several prostate cancer specific RNA biomarkers (e.g. AR-FL, AR-V7, KLK3, FOLH1 and SchLAP1), which may provide clinical insights into the disease. (Figure 8)

There are other endeavors in the capture and release of viable CTCs using surface chemical reactions. Dr. Stott's team at MGH utilized enzymatic reactions to release chip-bound CTCs by inducing cleavage of the bio-conjugation ligands of capture agents [109]. They also used small molecule Glutathione (GSH) to trigger ligand exchange to release CTCs [110]. Moreover, Dr. Liu's team at Beijing National Laboratory for Molecular Sciences demonstrated the capture of CTCs by utilizing a dual responsive surface composed of poly(acrylamidophenylboronic acid) brush grafted from aligned silicon nanowire array. This surface can capture and release CTCs by controlling pH and glucose concentration[111].

3. CTC enumeration with the 1st-gen NanoVelcro CTC Assay

3.1. Prostate cancer

With the proof-of-concept studies demonstrating the CTC capture performance of the 1st-gen NanoVelcro CTC Assay[90], a joint research team was formed by UCLA and Cedars-Sinai Medical Center to further test the use of this assay for prostate cancer CTC

enumeration. We first focused on patients with advanced metastatic disease, in order to address the problem of CellSearch™ assay failing to detect CTCs in most metastatic castration-resistant prostate cancer patients [14]. The initial study [8] tested forty patients (8 with localized disease and 32 with metastatic disease). NanoVelcro CTC Assay was able to detect CTCs in all 40 patients, suggesting its consistent performance for CTC enumeration, particularly in the setting of advanced diseases. Serial enumerations were also performed to study the change of CTC count after initiation of therapies. Consistent with the observations made using CellSearch™ assay, the clinical responders had a statistically significant drop in CTC counts. Furthermore, in a patient who was able to provide serial blood specimens for as long as 460 days, we observed changes of CTC count corresponding to the patient's initial response and subsequent failure during sipuleucel-t and nilutamide treatment. This initial observational study not only supported the use of 1st-gen NanoVelcro CTC Assay for CTC enumeration, but also suggested the potential clinical value of serial CTC measurements to monitor disease progression and response to treatment.

As the clinical studies continued to expand, the 1st-gen NanoVelcro CTC Assays also underwent improvements, including the introduction of a user-friendly system and protocols. The current version of the NanoVelcro CTC Enumeration Assay (Figure 9) has undergone extensive testing from calibration/interference tests with cancer cell lines. More than 100 prostate cancer patient samples have been tested for side-by-side comparison with CellSearch™ Assay. The research team noted the differences of the results of these two assays. The experience of the UCLA/CSMC team in combining NanoVelcro CTC Assay with higher resolution fluorescence microscopy suggested that certain immune cells, particularly neutrophils, might be falsely characterized as CTCs given the high CK false-positivity, resulting in the unusual high CTC readouts in the CellSearch™ Assay. To further improve the accuracy of CTC identification, the nature of CTCs as well as the mechanism of the assay will need to be further investigated. Clinical studies of larger scale are also needed to validate the performance of the assay and the clinical value of CTC enumeration in various disease settings. Meanwhile, this has led to the use of cytopathology standards and morphologic analysis in the NanoVelcro Assay, which not only helps minimize the false-positive events, but also results in the discovery of morphologic subsets of CTCs and their association with lethal visceral progression in prostate cancer.

3.2. Subclassification of prostate cancer CTCs

NanoVelcro CTC Assay combined with high-resolution fluorescence microscopy[73, 112] has allowed for better visualization and morphology analysis of the sub-cellular structures of CTCs. An observational study was conducted by UCLA/CSMC with serial blood samples collected from patients with different stages of prostate cancer. Mathematical modeling and unsupervised clustering of CTC nuclear size distribution was applied to identify three distinct subsets of CTCs. The CTC subsets were termed large-nuclear CTCs (lnCTCs, nuclear size > 15.0 μm), small-nuclear CTCs (snCTCs, nuclear size between 8.5 μm and 15.0 μm), and very-small-nuclear CTCs (vsnCTCs, nuclear size < 8.5 μm). vsnCTCs and snCTCs were more commonly seen in patients with metastatic disease. More importantly, we found that vsnCTCs predominantly occur in patients with visceral metastasis (i.e. metastasis to visceral organs such as the lung or liver). Moreover, patients with visceral

metastasis had higher numbers of vsnCTCs compared to those without visceral lesions. Serial CTC analysis in this study also found several patients whose vsnCTCs emerged before clinical radiographic detection of visceral metastasis[113]. This observation has now been investigated by analyzing CTCs from serially collected blood specimens from larger patient cohorts focused on patients with metastatic prostate cancer who had progression under androgen receptor signaling inhibitors (ARSIs), such as abiraterone, enzalutamide, or other equivalent drugs. Initial analysis of another retrospective cohort consisting of 28 patients (13 had bone-only metastasis and 15 with visceral metastasis at the first CTC enumeration [113]) found vsnCTCs in all the patients with visceral metastasis. Six out of thirteen initially non-visceral metastatic patients developed visceral metastasis during continuous follow-up, and vsnCTCs were detected mostly within 150 days prior to clinical detection of visceral lesions by radiographic imaging; the only exception was a patient whose vsnCTCs were seen more than 400 days before radiographic detection of his visceral metastasis. In addition to the patients who developed visceral lesions, three other patients were found to have vsnCTCs but had not developed visceral metastasis by the time of this analysis, and will continue to be monitored for visceral metastasis in the future. It is worth noting that in this study, none of the vsnCTC-negative patients developed visceral metastasis. By incorporating with morphologic analysis (i.e., nuclear size) to CTC enumeration, it allows for further investigation of the association between emergence of vsnCTCs and visceral progression in patients with prostate cancer. (Figure 10)

3.3. Pancreatic cancer

In addition to prostate cancer, several research teams have also explored the use of NanoVelcro CTC Assay in other types of solid tumors. As one of the most difficult cancers to detect CTCs [114, 115], pancreatic ductal adenocarcinoma (PDAC) has attracted the attention of the research team at UCLA to investigate[116] the feasibility of applying the NanoVelcro Assay. The team hypothesized that NanoVelcro CTC Assay could detect CTCs in PDAC patients, especially when conventional image studies (e.g. CT scan, MRI) cannot reveal the tumors due to imaging thresholds, and that the CTC count could serve as an adjunctive biomarker for staging. With the initial success in cell line models, a prospective study was conducted to analyze CTCs using NanoVelcro assay from 100 consecutive, pre-treatment PDAC patients. Among the 36 patients without prior chemotherapy, a median of eight CTCs (IQR 3–12) were found in the 10 patients with occult metastatic disease, versus one CTC (IQR 0–2) in the 26 patients with localized disease ($p < 0.0001$). With CTC enumeration from the patient's venous blood, we can predict successfully resected tumors and occult metastatic disease among untreated patient with PDACs (Figure 11a.). Evaluation of CTC counts revealed the presence of CTCs in 54/72 patients with confirmed PDAC (sensitivity = 75.0%, specificity = 96.4%). Furthermore, a cut-off of >3 CTCs in 4-mL blood was able to distinguish patients with metastatic disease from those with local/regional PDAC. In comparison with CA19-9 as one of the conventional PDAC metastasis detection tools, CTC enumeration can clearly provide superior sensitivity, specificity, PPV, NPV and AUROC in distinguishing patients with occult metastatic disease from those with potentially curable and localized tumors (Figure 11b.). This study not only demonstrated that NanoVelcro assay can be readily adopted in different cancers, but also suggested a promising role of CTCs in early detection and occult cancer metastasis of PDAC. We

envision that CTC enumeration can be utilized as an effective biomarker for clinical decision-making.

3.4. Kidney cancer

Another example of the clinical applications of NanoVelcro CTC enumeration assay is the detection of CTCs in patients with renal cell carcinoma (RCC). With the clear cell subtype as the vast majority, RCC also encounters challenges in early detection. To capture CTCs from RCC patients, a new combination of capture antibodies (i.e., anti-CA9 and anti-CD147) were tested. A research team from the Fourth Military Medical University in China then led the effort to test the revised protocol in clinical samples [117]. The results showed CTCs in 72/76 clear cell RCC patients, with the CTC counts significantly higher than those in benign kidney tumors (12.8 ± 6.9 vs. 1.7 ± 1.7). No CTCs were detected in healthy volunteers. Moreover, CTC counts in patients with advanced disease (stages III and IV) were higher than counts in patients with early disease (stages I and II). Further analysis on CTC subsets found that the number of CTCs with vimentin expression correlated with the clinical stage of RCC, indicating a prognostic role of CTCs in RCC. Together these results demonstrated the versatility of NanoVelcro CTC Assay to be adopted in different cancers, and the role of CTCs in RCC as a tool for early detection and prognostication.

4. Molecular Analysis of CTCs using 2nd-gen and 3rd-gen NanoVelcro Chip

4.1. Mutational analysis of circulating melanoma cells (CMCs) and pancreatic CTCs

Utilizing the 2nd-gen NanoVelcro Chips, our first attempt to perform single-CTC mutational analysis was to detect a signature oncogenic mutation (i.e., BRAF^{V600E}) in individual melanoma CTCs[96]. BRAF^{V600E} is present in 50% of melanoma patients[118], and its presence predicts the treatment response to the specific inhibitors, including vemurafenib[119].

By utilizing melanoma-specific anti-CD146 as the capture agent, we isolated single CTCs from several stage IV melanoma patients with known BRAF^{V600E} mutations by standard sequencing of tissue biopsy. After PCR-based WGA, the genomic materials from the isolated single CTCs were subjected to Sanger sequencing specifically looking for the BRAF^{V600E} mutation. (Figure 12a) We not only demonstrated the detection of the oncogenic mutation in the CTCs, but also displayed a strong signal-to-noise ratio. This finding confirmed the purity of our isolated single CTC, which was different from the sequencing results of biopsied melanoma tissues, where melanoma cells inevitably mixed with normal cells surrounding the cancer. Our finding also suggested the feasibility of detecting BRAF^{V600E} in melanoma CTC as a companion diagnostic tool for vemurafenib to avoid invasive tissue sampling.

Knowing that mutation can be detected in single-CTC sequencing, our team designed a study to assess the sensitivity of such detection utilizing pancreatic CTCs as a model system. In pancreatic cancer, KRAS oncogene mutations are present in more than 95% of the patients, and 98% of those mutations are located in codon 12[120]. Utilizing epithelial cancer specific anti-EpCAM as the capture agent, we identified pancreatic CTCs by their

CEA+/CD45– characteristics under fluorescence microscopy. The dissected single CTCs were subjected to PCR-based WGA and Sanger sequencing.

Utilizing the 2nd-gen NanoVelcro Chips, we demonstrated that both KRAS^{G12V} (Figure 12b) and KRAS^{G12D} mutations [121] were detected in the pancreatic CTCs. 92% of the patients with known KRAS mutations were demonstrated to have the same mutation in their CTCs. We also revealed a low mutation detection rate in each CTC, indicating that PCR-based WGA was the most error-prone step in the CTC sequencing process. However, the sequencing of 10 CTCs from each patient was sufficient to compensate the false negative detection caused by random loss of allele in the WGA process.

4.2. Whole exome sequencing of CTCs

We further explored the feasibility of performing whole genome analysis on single-CTC DNA. In our first report, we performed whole exome sequencing on isolated single CTCs [95], and demonstrated the streamlined process of our platform. After PCR-based WGA, single-CTC DNA obtained from 2nd-gen NanoVelcro chips was sequenced by WES. WES was performed using a standard exome capture kit and Illumina HiSeq. 25 to 80% whole exome coverage was achieved with a mean depth of 29 to 48X. We observed no chromosomal loss in any CTCs, indicating no chromosomal loss during the extraction of single-CTC DNA. Limited by the coverage, we only compared the shared mutation between individual CTCs, CTCs and WBCs, and demonstrated the difference between CTCs and WBCs. This was the first report to demonstrate the feasibility of performing single-CTC WES to verify the difference between CTCs and WBCs at the whole genome level.

4.3. Whole genome sequencing (WGS) of CTCs

With the improvement of our processing technique and WGA method, we then sought to compare the single-CTC DNA with the paired tumor tissues from the same patient. In an index prostate cancer patient, we performed high-quality whole genome sequencing on isolated single CTCs as well as his primary prostate cancer and liver metastasis[16]. In the four sequenced single CTCs, we achieved high-quality sequencing with above 95% sequencing coverage with a standard 30X depth (Figure 13a). The data quality was comparable to the sequencing of the primary prostate tissue and the biopsied tissue from liver metastasis. 29% of the somatic single nucleotide variants (SSNVs) shared between the primary tumor and liver metastasis were found in the CTCs, and 86% of the mutations shared in CTCs were traced back to either the primary or metastatic tumor (Figure 13c). As the first team to perform WGS on CTCs, we also demonstrated the same rearrangements (in chr 3) in the tumor tissues and CTCs (Figure 13d) that were not present in the comparison WBCs or adjacent normal tissue (Figure 13b). Utilizing the high-quality WGS of single CTCs, we demonstrated the similarity between CTCs and tumor tissue at the whole genome level. Our report set the foundation for future studies to utilize isolated single CTCs as a surrogate source of tumor tissue to study cancer biology *in vivo*.

4.4. Molecular analysis of CTCs using 3rd-gen NanoVelcro Chip

In order to utilize the 3-gen Thermo-responsive NanoVelcro Chip for CTC capture and release, we first optimized performance by using artificial blood samples containing anti-

EpCAM-positive H1975 non-small cell lung cancer (NSCLC) cell line. We performed 2 rounds of CTC capture and release to ensure higher purity, enabling point mutation analysis of EGFR (Figure 14a). Strong correlations with tumor tissues and molecular signatures of purified CTCs were found by Sanger sequencing of the 7 NSCLC patients' CTCs. Additionally, we analyzed molecular signatures of serial CTCs for a NSCLC patient who was treated with EGFR inhibitor. We initially observed L858R mutation in the CTCs, which correlated to good drug response with EGFR inhibitor (i.e. Gefitinib). However, the emergence of T790M mutation was noticed after the treatment and the patient's disease progressed, indicating possible drug resistance (Figure 14b-d). These results showed promising utility of CTCs for detecting resistance of targeted therapies.

5. Technical summary for four generations of NanoVelcro CTC Assays

In summary, the four generations of NanoVelcro CTC Assays presented above introduced unique properties comparing to other existing systems. These four generations of NanoVelcro CTC Assays have similar operating mechanisms allowing the assays to achieve optimized CTC capture efficiency. First of all, the combination of embedded nanostructures and capture agents results in enhanced affinity between NanoVelcro substrates and CTCs. Second, increased contact frequency between NanoVelcro substrates and CTCs results from the chaotic flow created by the overlaid PDMS chaotic mixer. In addition, the reproducibility, scalability, and cost-effectiveness of producing these NanoVelcro CTC Assays can be guaranteed by employing micro-fabrication techniques for the two essential functional components (i.e., PDMS chaotic mixer and chip nanosubstrates). Other advantages include (i) Speed: Images of all CTCs are obtained via semi-automated microscopy in a short period of time allowing for rapid pathological review. A total of 4 hours is required to complete enumeration and isolation of individual CTCs with the 1st-gen NanoVelcro Enumeration Assay or 2nd gen NanoVelcro-LCM assay, which is significantly less than the time required for the FDA-approved CellSearch™ assay (4 hours for enumeration only). (ii) Flexibility: the four generations of assays demonstrated utilities for detecting and isolating CTCs with molecular intactness and cell viability; (iii) Sample utilization capacity: CTCs can be recovered from 2 mL blood samples (compared to 7.5 mL samples used by CellSearch™ Assay) given the high sensitivity of NanoVelcro Assays. Up to 5-mL of blood can be processed in one assay if needed, and high capacity can be achieved through multiple rounds performed in parallel. (iv) Cost efficiency: A CellSearch™ assay costs approximately \$1,200. In comparison, a NanoVelcro CTC enumeration or isolation assay, including the materials, device fabrication, surface coating, and antibodies, can be performed at a much cheaper price; (v) Simple user interface: the user-friendly chip holder, the fluid handler (Figure 8) and computerized interface allow for easy setup and reduce variation among different users. Unique capacities to each generation of NanoVelcro Assay has been developed over the past decade including high-resolution fluorescent imaging for CTC morphological analysis, LCM technique and temperature-dependent purification for CTC isolation and enrichment. The NanoVelcro Assays have demonstrated the ability to address unmet needs in the field of oncology with their unique capacities, such as the stratification of heterogeneous CTC populations (1st-gen), molecular characterization of CTCs (2nd to 4th gens), and rapid CTC purification/enrichment (3rd and 4th gens).

5.1. Future scientific and clinical developments

With the rapid advancements in therapeutics and a strong drive towards personalization of cancer care, there is a clear, urgent, and unmet need for a liquid biopsy in the oncology clinic. This need is being addressed by many advancements in the field of nanotechnology-based diagnostics. Future research directions will focus on particular needs of nanotechnology-based CTC assays. These needs include (i) Exploring other possible capture/release mechanisms for CTC purification with physiologically compatible conditions for instant CTC isolation or purification that could provide high cell viability and molecular integrity for molecular profiling of the disease. (ii) Further understanding of the interactions between nanointerfaces and CTCs that could affect CTC-capture efficiency, molecular integrity and cell viability. (iii) Exploring the techniques of *ex vivo* expansion and rare-cell culture of purified CTCs for various research utilities such as drug susceptibility testing and xenograft models. (iv) Exploring the utilities of multi-omic analytical technologies to characterize CTC heterogeneity with single-cell resolution. (v) Characterization of other circulating rare cells such as circulating stromal cells and tumor-associated immune cells that harbor information about disease characteristics and tumor microenvironment.

5.2. Conclusion and outlook

The application of nanotechnology in clinical oncology has shown a promising future to address a myriad of unmet needs. As the current knowledge and understanding of cancer continues to evolve, cancer biologists and physician scientists look to characterize the dynamic biology of the disease. In such an evolving biological environment, oncologists are already familiar with handling temporal variation of data. Analysis of CTCs and other circulating biomarkers has shown promise for investigating the dynamic biology in the individual patient. From the molecular biological concepts of DNA, RNA and proteins, it is evident that there is a great deal of variability in the identities of patients and cancers. *Utilizing a noninvasive means to explore these differences can help connect the laboratory and the clinic.* Early successes in this field have served as motivation for continued work and inspired others to pursue interdisciplinary research in translational medicine.

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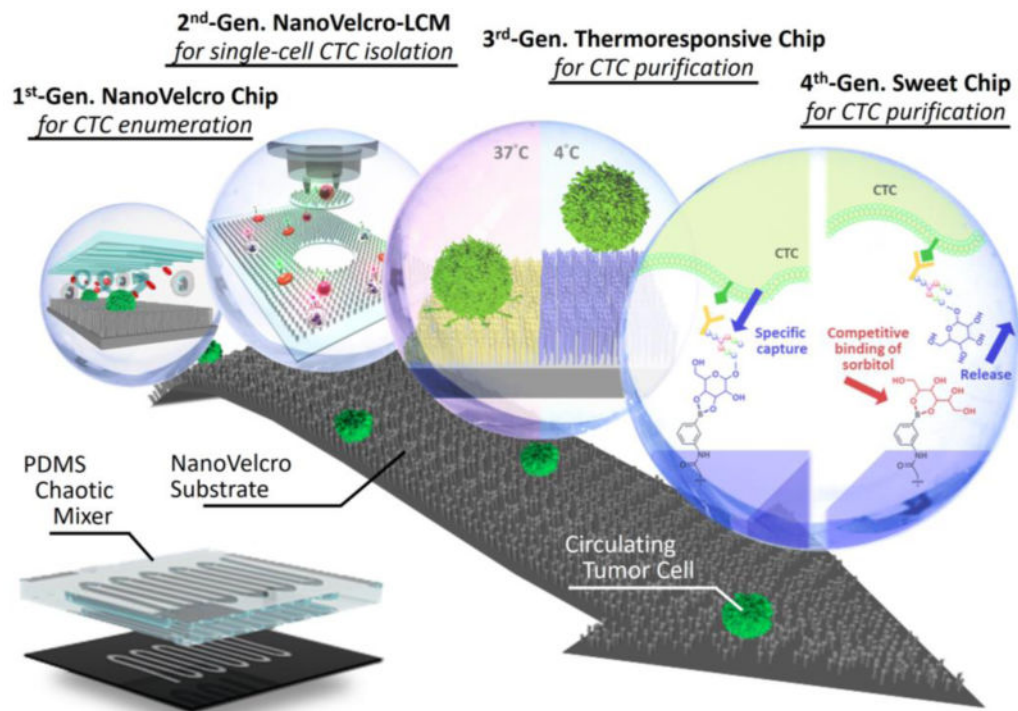
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	1 st -Gen.	2 nd -Gen.	3 rd -Gen.	4 th -Gen.
Mechanism	Combine use of capture agents and embedded nanosubstrates increases the affinity between CTCs and surface			
	Overlaid chaotic mixer introduces helical flows in microchannels, enhancing contact frequency			
		LCM technique enables single-CTC identification and isolation	Temperature-dependent conformational changes of polymer brushes , allowing for capture and release of CTCs	Competitive binding of sorbitol to phenylboronic acid (PBA) , enabling release of antibody captured CTCs
Capacity & Utility	CTC enrichment and morphologic analysis		Ex vivo expansion of purified CTCs	
	CTC enumeration for cancer prognosis, staging, and dynamic monitoring	Single-CTC isolation , followed by molecular analysis	Temperature-dependent purification to recover CTCs with high viability and molecular intactness	Competitive binding technique to recover intact CTCs for molecular analysis

Figure 1. Graphic illustration and table comparison of the NanoVelcro CTC Assays among four different generations.

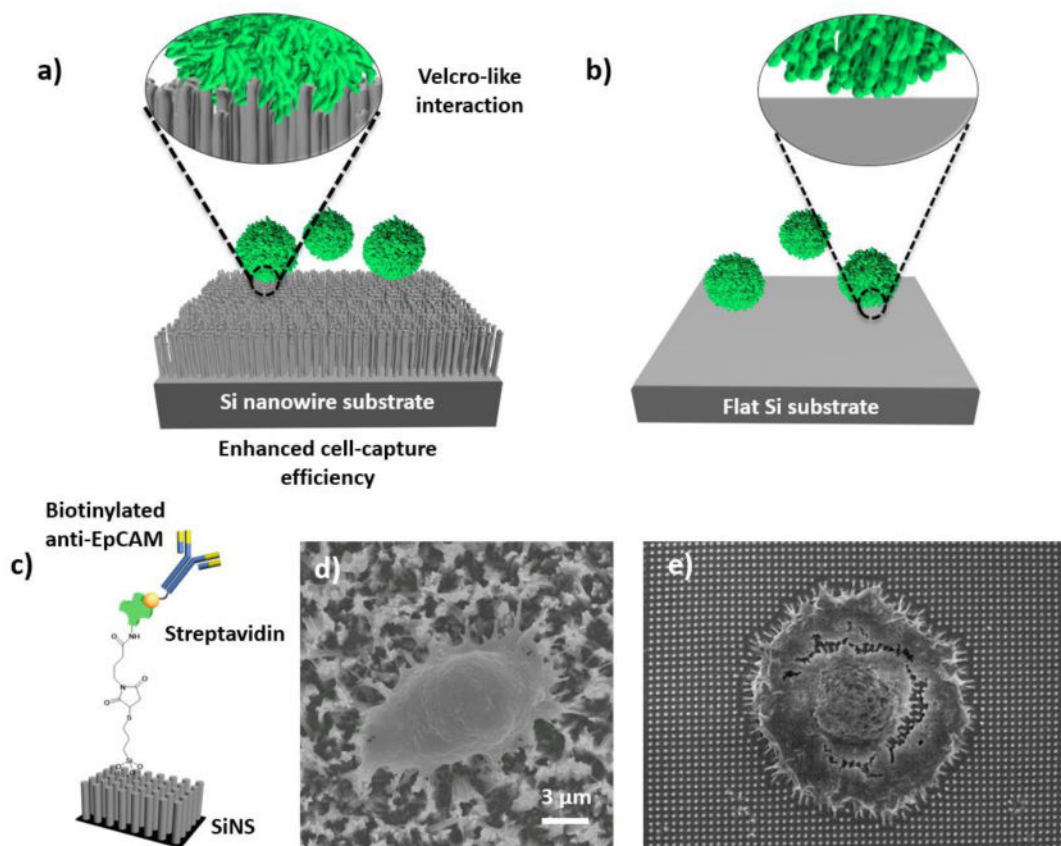


Figure 2. Velcro-like working mechanism of NanoVelcro cell-affinity substrates

a) An anti-EpCAM-coated SiNS was employed to achieve significantly enhanced capture of CTCs in contrast to **b)** an anti-EpCAM-coated flat silicon substrate. **c)** Anti-EpCAM is grafted onto SiNS to confer specificity for recognizing CTCs. **d)** and **e)** SEM images of a SiNS and PLGA NanoVelcro substrate, on which MCF7 cells and prostate cancer CTCs were captured, respectively.

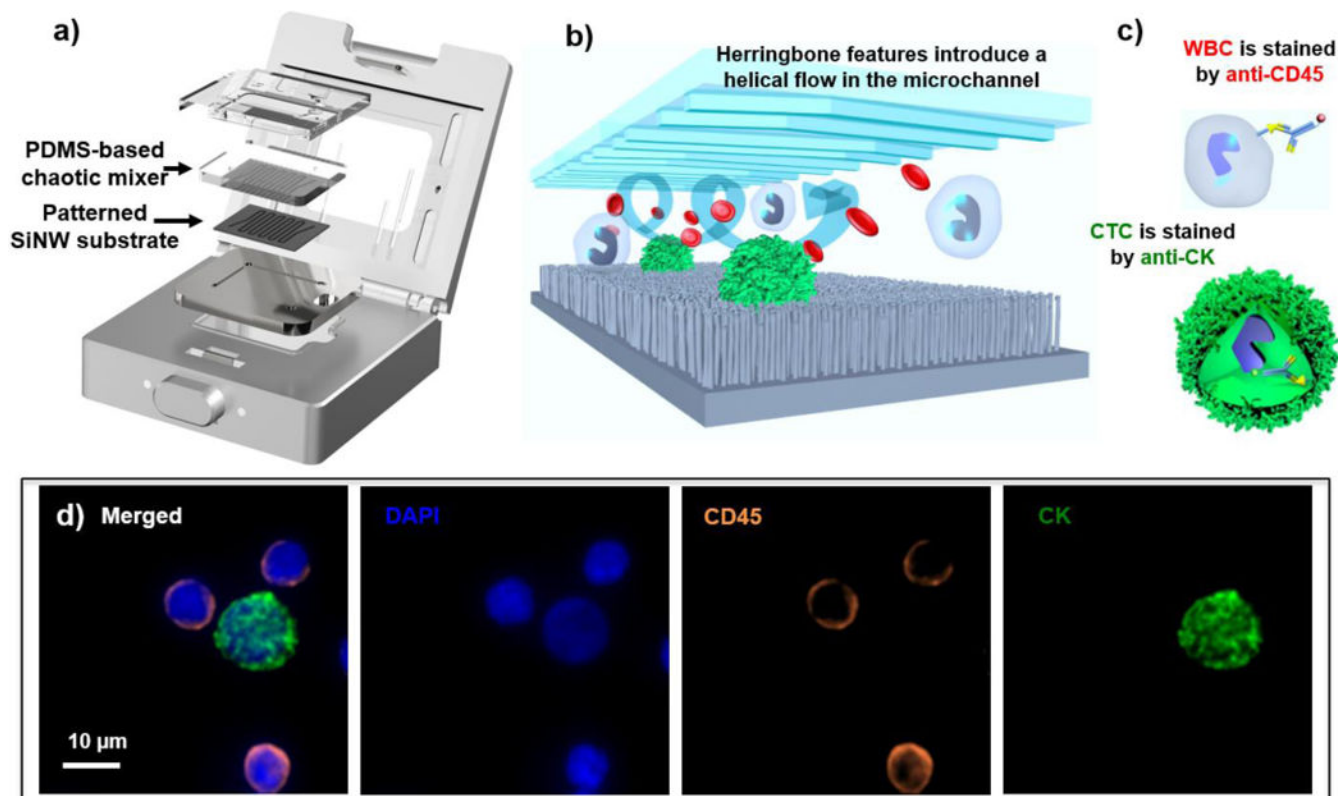


Figure 3. Workflow of NanoVelcro and Imaging CTC System

a) and **b)** Configuration of 1st-gen NanoVelcro CTC chip. The device is composed of a patterned NanoVelcro substrate and an overlaid PDMS chaotic mixer. **c)** Using a 3-color ICC protocol, CTCs (DAPI+/CK+/CD45-, sizes > 6 µm) can be clearly distinguished from nonspecifically captured WBCs (DAPI+/CK-/CD45+, sizes < 12 µm [72]) by a 3-color ICC protocol, **d)** Fluorescent images of 2 prostate cancer CTCs captured on the substrate along with non-specifically captured WBCs.

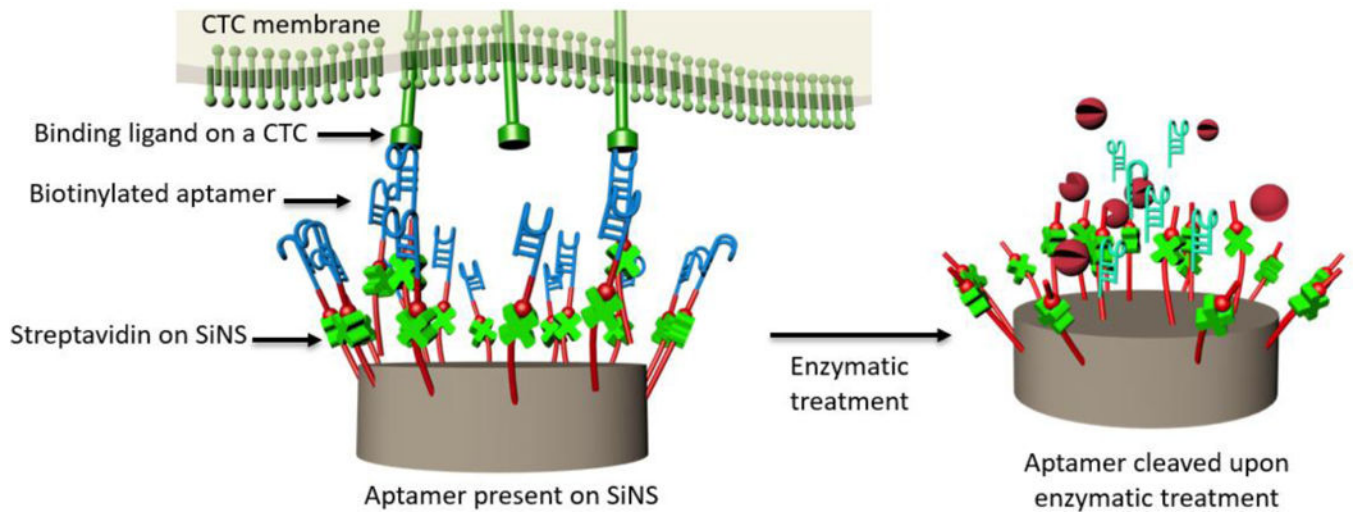


Figure 4. Molecular mechanism governing the capture and enzymatic release of NSCLC CTCs from the aptamer-coated SiNS.

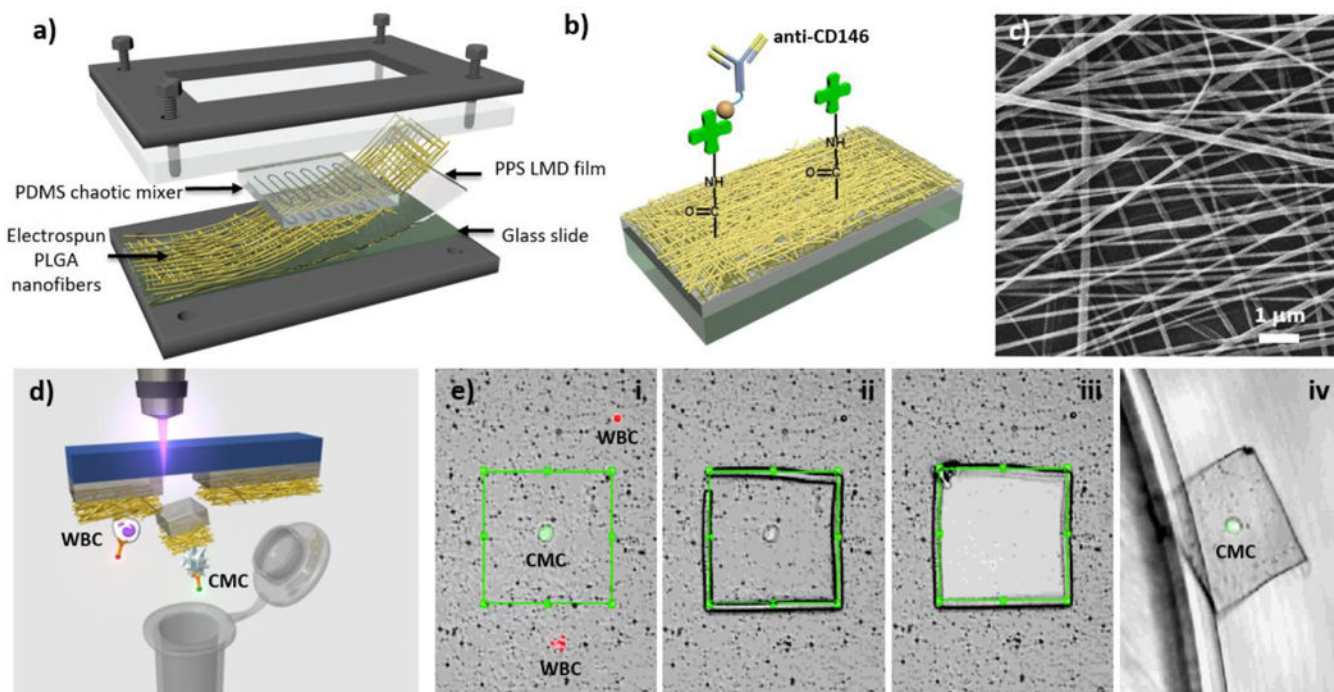


Figure 5. Illustrations of 2nd-Gen NanoVelcro-LCM technology and its application of single-CTC isolation

(a) The PDMS chaotic mixer is laid on top of PLGA nanofibers [96] to increase cell capture efficiency. (b) Streptavidin is covalently bound to PLGA nanofibers to interact and bind with biotinylated capture agents. (e.g., anti-CD146 for Circulating Melanoma Cells (CMCs) and anti-EpCAM for pancreatic cancer CTCs). (c) Image of PLGA nanofibers under scanning electron microscope. (d) The graphic illustration of single CMC/CTC isolation from NanoVelcro chips with the use of LMD. (e) Steps of single cell isolation: (i) CMC/CTC identification (ii) CMC/CTC isolation with laser cutting the PLGA around the cell and followed by (iii) moving the PLGA piece containing CMC/CTC from the chip (iv) into a 200 μ L PCR tube.

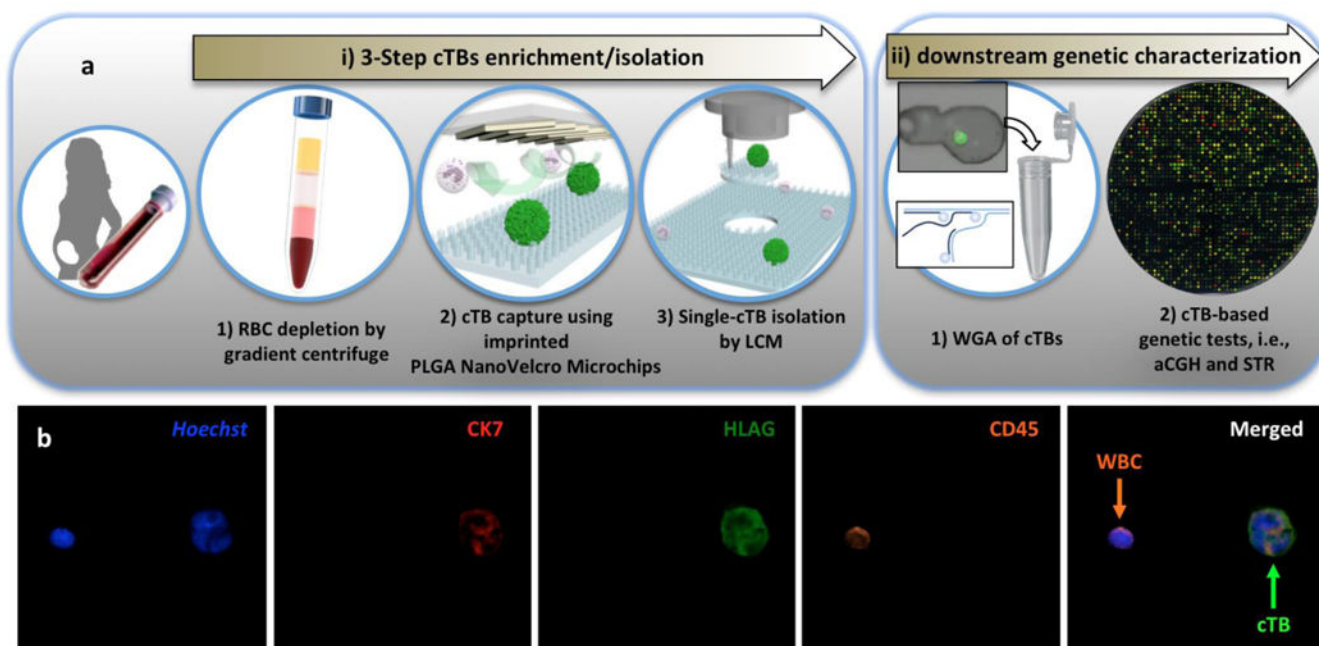


Figure 6. Examination of circulating trophoblasts (cTBs) from maternal blood by using single-cell isolatable system, the 2nd-gen NanoVelcro Assay, to be characterized by its phenotype and genotype

a) The workflow of rare cell enrichment and isolation. After centrifugation of the maternal blood, buffet coat (PBMC layer) was extracted to capture the target cells by NanoVelcro chips. Individual cell isolation is positioned by LCM to extract the targeted cell. The whole genome amplification (WGA) of target cells was conducted for further genetic analysis. **b).** Immunostaining of cTB by various specific markers. cTB is identified by specific markers, CK7 (red) and HLAG (green), to distinguish from other lymphocytes by CD45 (orange). Hoechst is utilized to stain cell nucleus. Adapted with permission from (Hou, et al., 2017) [100]. Copyright (2018) American Chemical Society.

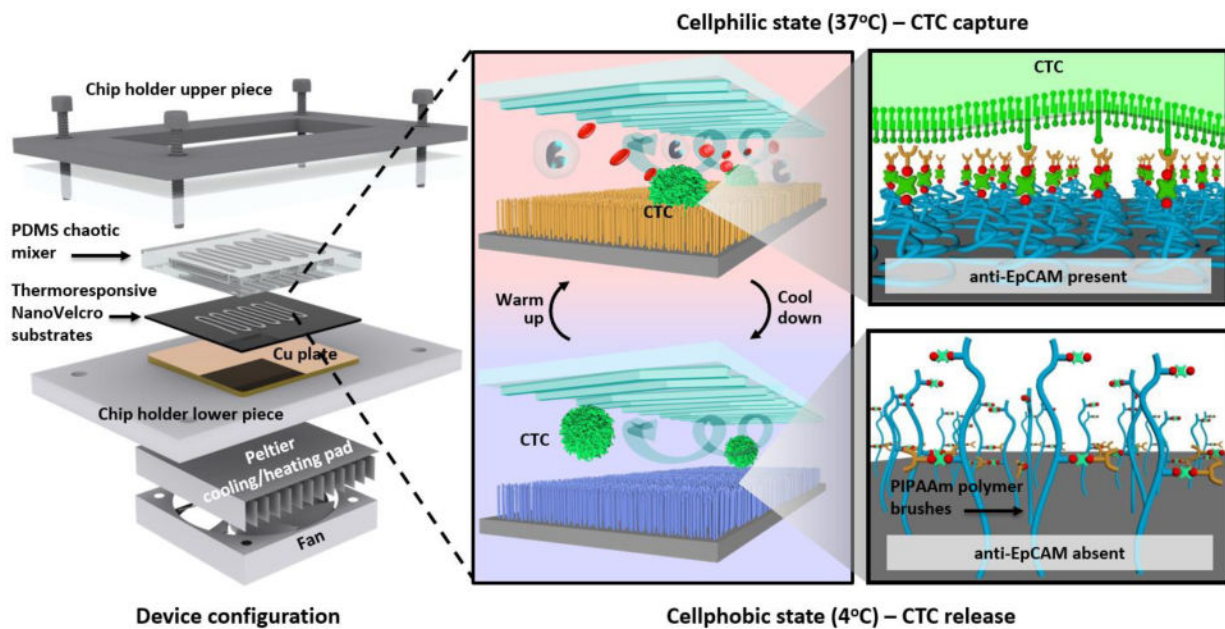


Figure 7. Operating mechanism of the thermoresponsive NanoVelcro CTC substrate
 For capturing and releasing CTCs at 37 and 4°C, respectively. The temperature-dependent conformational changes of polymer brushes effectively alter the accessibility of anti-EpCAM on NanoVelcro substrates.

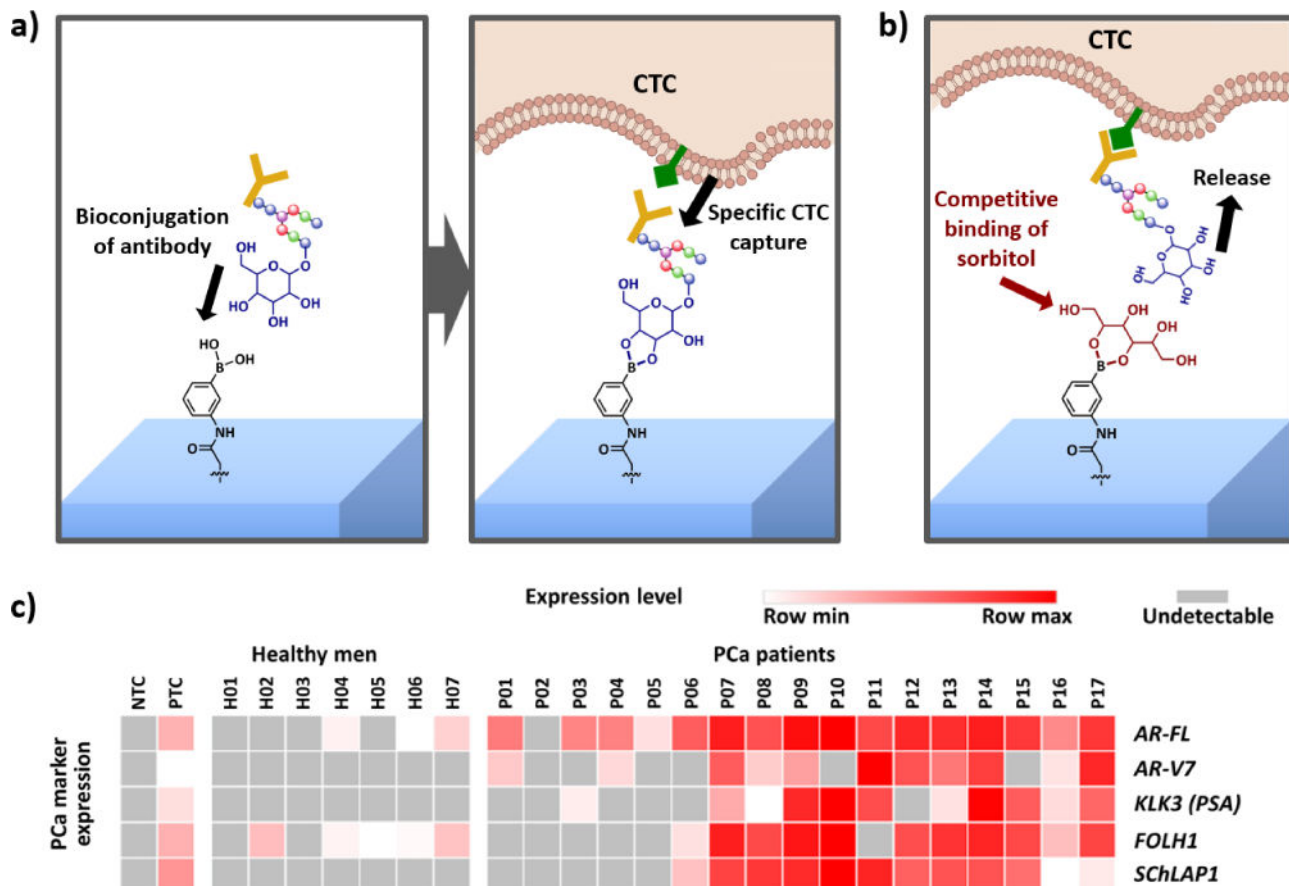


Figure 8. Illustration of 4th-gen NanoVelcro Chip (Sweet chip)

a) The mechanism for circulating tumor cell (CTC) capture is that the surface-grafted phenylboronic acid (PBA) conjugates with antibody, subsequently enabling specific CTC capture. **b)** The mechanism for CTC release is that the introduction of glycan with stronger affinity to PBA (i.e., sorbitol) results in competitive binding, allowing CTC release. **c)** RNA signature detection in 7 healthy men and 17 prostate cancer patients using 4th-gen NanoVelcro Chip. Expression of AR-FL, AR-V7, KLK3, FOLH1 and SchLAP1 is summarized in heatmap. PTC = positive control (RNA from 100 LNCaP cells + 100 22Rv1 cells in 1 million WBCs from a healthy man purified by PEDOT-NanoVelcro Chips); NTC = negative control (nuclease free water)[75].

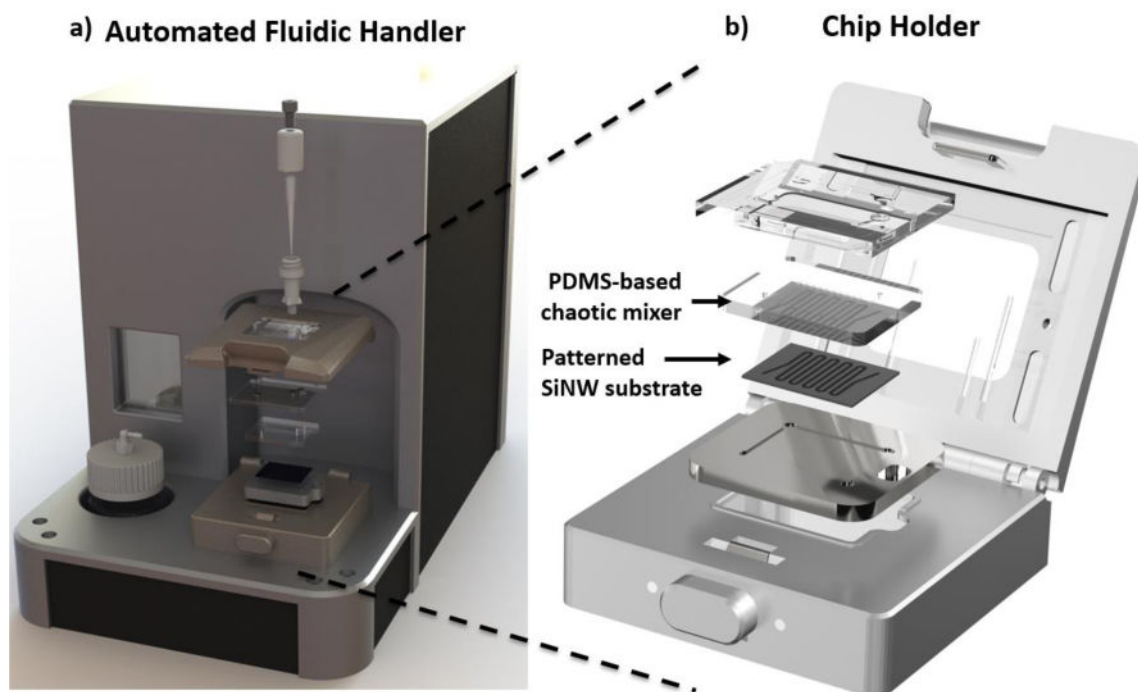


Figure 9. The current version of NanoVelcro CTC Enumeration Assay
a) A fluidic handler designed to process blood samples into NanoVelcro Chips. **b)** The mechanical click-on approach enables the NanoVelcro assay to assemble instantly.

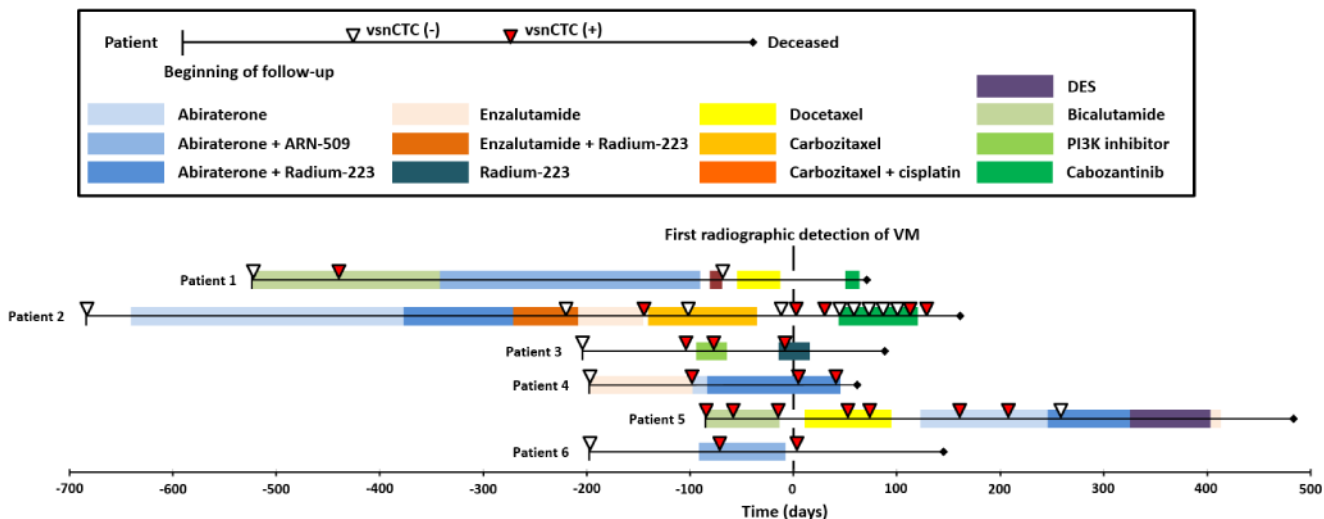


Figure 10. 1stGen NanoVelcro CTC Device characterized very-small-nuclear CTCs (vsncTCs) in visceral metastasis of prostate cancer

The correlation between presence of vsnCTCs and the presence of visceral metastasis is demonstrated. The presence of vsnCTCs in serial CTC enumerations before the detection of visceral metastasis suggests its predictive potential for visceral progression in prostate cancer patients.

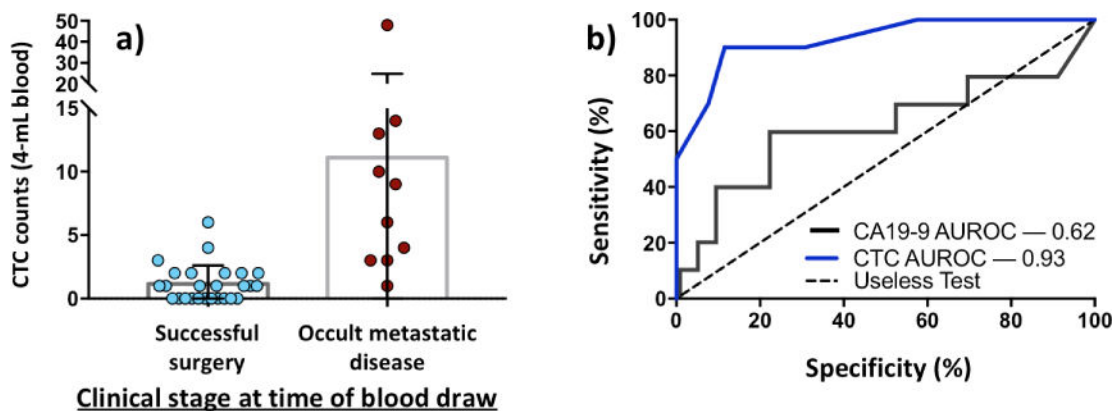


Figure 11. CTC circulating tumor cell, CA19-9 cancer antigen 19-9, ROC receiver operating characteristic curve, AUROC area under the receiver operating characteristic curve
a) CTC count between successfully resected tumors and occult metastatic disease patients. The distinct difference in CTC number indicates that CTC enumeration can be utilized as a preoperative predictor of occult metastatic disease among untreated patients. **b)** The ROC curve of CTCs and CA19-9 in preoperative detection of patients with occult metastatic disease. AUROC difference between two methods (0.62 vs 0.93) shows effectiveness of CTC enumeration in occult metastatic disease detection.

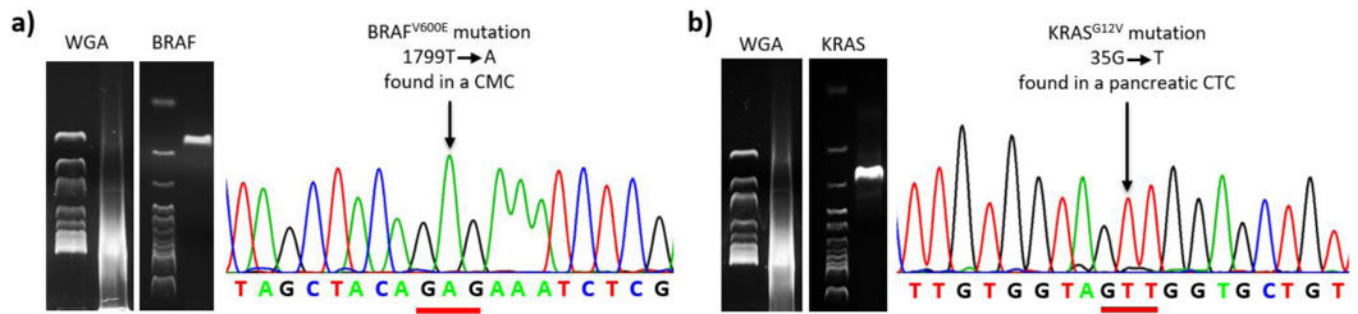


Figure 12. a) Single-CMC WGA result and gel electrophoresis

The CMCs exhibit the unique BRAF^{V600E} mutation confirmed by Sanger sequencing [96].

b) KRAS^{G12V} mutation present in pancreatic CTCs [121].

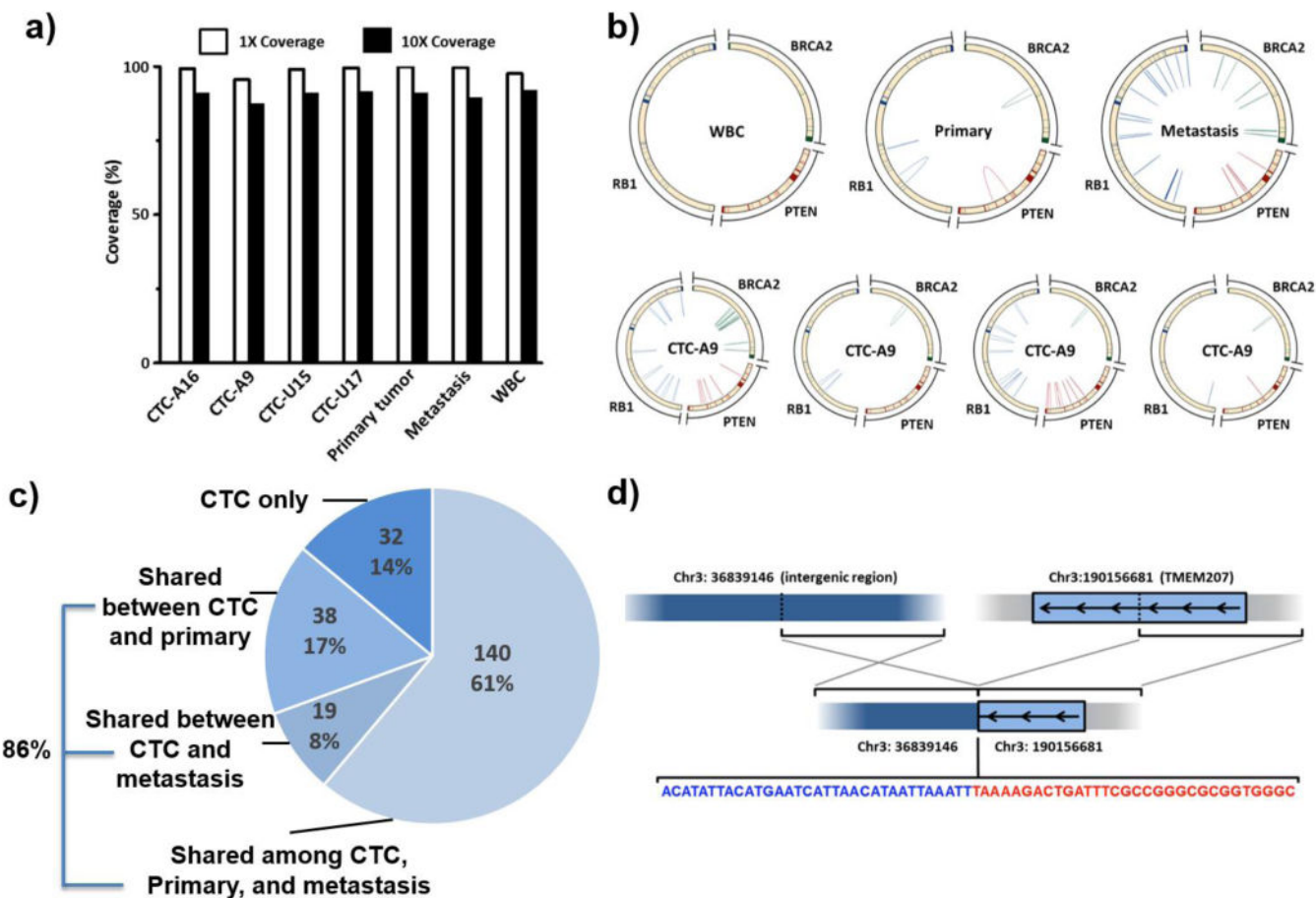


Figure 13. 2nd-gen NanoVelcro CTC Chips enabled high-quality WGS on single-CTCs
a) Above 95% coverage was demonstrated for single-CTC WGS[16]. **b)** Tumor suppressor gene rearrangements including RB1, BRCA2 and PTEN were detected in CTCs and tumors. No mutation was found in WBCs. **c)** We identified that more than 3 CTCs shared clonal somatic single nucleotide variants (SSNVs), which can be considered high confident mutations. 86.0% of CTC-clonal SSNVs are shared among either the primary tumor or metastatic tissue. **d)** TMEM27 inter-chromosomal rearrangement was detected in both tumor tissues and CTCs.

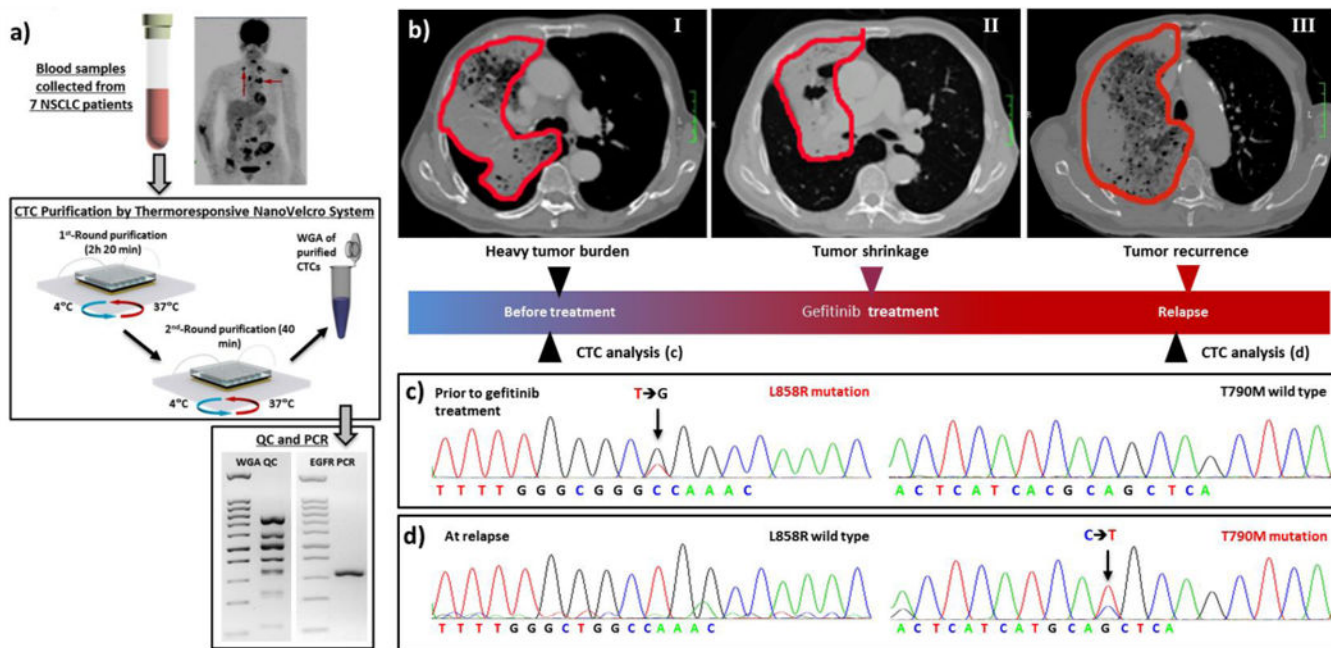


Figure 14. CTC purification of non-small cell lung cancer (NSCLC) by using 3rd-Gen NanoVelcro CTC Chips and molecular analysis
 (a) CTC purification and EGFR mutation detection are demonstrated by 3rd-gen NanoVelcro Chips and electrophoresis. (b-d) Disease course and longitudinal mutational analysis of CTCs from a NSCLC patient. (b) Tumor regression and progression during gefitinib (1st-generation EGFR inhibitor) treatment course. (c) L8585R mutation was detected both in CTCs and tumor tissue before the patient was treated with gefitinib. (d) The secondary T790M mutation was detected in the CTCs after disease progression.