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TITLE: Molecular Characterization of Very Small Nuclear Circulating Tumor Cells: A Putative Biomarker for Visceral Metastasis in Prostate Cancer

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Cedars Sinai Medical Center
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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Metastatic, castration-resistant prostate cancer (mCRPC) may exhibit varied clinical courses. Some mCRPC patients will develop metastasis beyond the bone and lymph nodes, including the liver, lungs, and adrenal glands. These conditions are termed visceral metastases (VM). Patients with VM have significantly poorer overall survival than patients with non-VM, as their clinical course involves rapid deterioration from organ failure. Certain CRPC treatments have been shown to push the progression of the cancer to its more aggressive VM form. This highlights the importance of developing a means of detecting and predicting cancer progression to the viscera. Using the NanoVelcro Chip designed to capture circulating tumor cells (CTCs), we identified a morphologically unique subgroup of these cells, which we termed very-small-nuclear CTCs (vsnCTCs). We found that these vsnCTCs appear in patients with VM and begin emerging before the disease progresses to VM. Thus we hypothesize that vsnCTCs are associated with the development of VM and are biologically distinct from non-vsnCTCs, which lead to a different clinical course. We aim to analyze the association between vsnCTCs and VM, as these cells could play a key role in detection VM. Additionally, we aim to compare the gene expression of vsnCTCs and non-vsnCTCs to gain greater insight into the biology of VM.

15. SUBJECT TERMS
Prostate Cancer, biomarkers, circulating tumor cells.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
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INTRODUCTION: **Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.**

In this proposed study, we hypothesized that very-small-nuclear circulating tumor cells (vsnCTCs) are associated with and favor the development of visceral metastasis (VM) in metastatic castration-resistant prostate cancer (mCRPC) and harbor expression signatures distinct from other non-vsnCTCs on several oncogenic pathways. The hypothesis will be tested by a retrospective analysis for the association between vsnCTCs and VM, and comparison of gene expression signatures of vsnCTCs to non-vsnCTCs.

1. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

Very-small-nuclear circulating tumor cells (vsnCTCs), prostate cancer, visceral metastasis (VM), NanoVelcro assay

2. **ACCOMPLISHMENTS:** The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Training-Specific Tasks:
Major Task 1: Training and educational development in prostate cancer research.
Milestone(s) Achieved: Presentation of project data at a national meeting or preparation for publication.
- Presentation on AACR 2017 Annual Meeting on April 4, 2017; Publication on Advanced HealthCare Materials in September, 2017

Research-Specific Tasks:
Specific Aim 1: Retrospective analysis for the association between vsnCTCs and VM.
Major Task 1: CTC enumeration studies using NanoVelcro Chip on the specimens from the blood specimen/CTC bank.
Milestone(s) Achieved: identify at least 15 patients and their specimens for each of the following metastatic categories: no metastasis, osseous/lymph node metastasis only, visceral metastasis present.
- Accomplished on February 11, 2017

Milestone(s) Achieved: Complete the CTC enumeration studies and match the clinical annotation for all the identified specimens.
- 80% completion

Major Task 2: Mathematical modeling of CTC nuclear size
Milestone(s) Achieved: Complete the association analysis
- 60% completion

(see following page)

(continued)
Specific Aim 2: Comparing gene expression signatures of vsnCTCs and non-vsnCTCs.
Major Task 3: Optimize NanoVelcro-LCM platform for CTC isolation and downstream expressional analysis.
Milestone(s) Achieved: Identify vsnCTC-specific and/or VM-specific expression signatures.
- 60% completion

What was accomplished under these goals?
For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

See **Appendix A**.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Training
- One-on-one work with mentor, Dr. Edwin Posadas, for clinical study design, execution, data collection, and interpretation
- One-on-one work with co-mentor, Dr. Hsian-Rong Tseng, for optimization of NanoVecro CTC assay and development of subsequent approaches for CTC-based RNA measurement
- Monthly meeting with consultant, Dr. Leland Chung, for experimental design, data analysis and interpretation
- Quarterly meeting with consultant, Dr. Michael Freeman, for experimental design, data analysis and interpretation
- Attendance of Biostatistics and Bioinformatics Research Center Presentation at Cedars-Sinai Medical Center

Professional development

- Attendance of courses at 2016 United States and Canadian Academy of Pathology (USCAP) Annual Meeting, including:
  - Clinical Application of Next Generation Sequencing
  - Genitourinary Pathology
  - Diagnostic Challenges in Prostate Pathology
  - Pathology in the Era of Genomic Medicine: New Frontiers and New Challenges
- Attendance of 2017 USCAP Annual Meeting.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Conference presentations:

- Hsian-Rong Tseng and Edwin M. Posadas. Clinical Applications of NanoVelcro Rare-Cell Assays for Detection and Characterization of Circulating Tumor Cells. 2017 NCI Alliance of Nanotechnology in Cancer Principal Investigator Meeting.
- Jie-Fu Chen and Edwin M. Posadas. Clinical Applications of NanoVelcro Rare-Cell Assays for Detection and Characterization of Circulating Tumor Cells. 5th Annual National Cancer Institute (NCI) Center for Strategic Scientific Initiatives (CSSI) Science Day (invited oral presentation).
- Jie-Fu Chen, et al. Bio-Competition-based Smart NanoVelcro Chip for isolation and Expressional Analysis of Circulating Tumor Cells from Prostate Cancer Patients. [abstract] In: Proceedings of the 108th Annual Meeting of the American Association for Cancer Research; 2017 Apr 1-5; Washington DC: AACR; Cancer Research 77 (13 Supplement), 3780-3780.
- Hsian-Rong Tseng and Edwin M. Posadas. Clinical Applications of NanoVelcro Rare-Cell Assays for Detection and Characterization of Circulating Tumor Cells. 2016 NCI Alliance of Nanotechnology in Cancer Principal Investigator Meeting.
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- Jie-Fu Chen, et al. Very-small-nuclear circulating tumor cell (vsnCTC) as a putative biomarker for visceral metastasis in metastatic castration-resistant prostate cancer (mCRPC). [abstract] In: Proceedings of the 107th Annual Meeting of the American Association for Cancer Research; 2016 Apr 16-20; New Orleans, LA. Philadelphia (PA): AACR; Cancer Res 2016;76(14 Suppl):Abstract nr 4962.
- Jie-Fu Chen, et al. Very small nuclear circulating tumor cell (vsnCTC) as a putative biomarker for visceral metastasis (VM) in metastatic castration-resistant prostate cancer (mCRPC). Laboratory Investigation 96, 221A.
- Jie-Fu Chen, et al. Very small nuclear circulating tumor cell (vsnCTC) as a putative biomarker for visceral metastasis in metastatic castration-resistant prostate cancer (mCRPC). J Clin Oncol 34, 2016 (suppl 2S; abstr 64).

Publications:

- Chen J-F, Zhu Y, Lu Y-T, et al. Clinical Applications of NanoVelcro Rare-Cell Assays for Detection and Characterization of Circulating Tumor Cells. Theranostics. 2016;6(9):1425-1439.
- Mo□Yuan Shen, **Jie□Fu Chen**, Chun□Hao Luo, Sangjun Lee, Cheng□Hsuan Li, Yung□Ling Yang, Yu□Han Tsai, Bo□Cheng Ho, Li□Rong Bao, Tien□Jung Lee, Yu Jen Jan, Ya□Zhen Zhu, Shirley Cheng, Felix Y Feng, Peilin Chen, Shuang Hou, Vatche Agopian, Yu□Sheng Hsiao, Hsian□Rong Tseng, Edwin M Posadas, Hsiao□hua Yu. Glycan Stimulation Enables Purification of Prostate Cancer Circulating Tumor Cells on PEDOT NanoVelcro Chips for RNA Biomarker Detection. Adv Healthc Mater. 2017 Sep 11.
- The Gift of Grab - An experimental device snags tumor cells like Velcro to speed cancer diagnosis and predict disease spread. Discoveries (Cedars-Sinai) Spring 2017.

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

Specific Aim 1: Retrospective analysis for the association between vsnCTCs and VM.

- With the completion of the first Milestone in Major Task 1, the specimens and clinical information available for have been identified. The CTC enumeration effort will continue be carried out at current rate with an expected completion of Major Task 1 in early 2018
- The association analysis (Major Task 2) is expected to complete within 1 to 2 months after the completion of Major Task 1.
- Presentation of the study results to relevant society is expected in early 2018 (AACR Annual Meeting); manuscript will be finalized before the end of funding period, and is expected in late 2018 or early 2019.

Specific Aim 2: Comparing gene expression signatures of vsnCTCs and non-vsnCTCs.

- CTC-based RNA analysis with NanoVelcro-LCM protocol will continue to be carried out at current rate, and is expected to complete in early 2018.
- A VM-associated expression signature (PCS, see **Appendix A.**) has been identified in early 2017. CTC-based RNA analysis will also be carried out for PCS classifiers. The data collection is expected to complete in early 2018, and analysis will conclude in mid 2018.

Combined analysis between retrospective clinical study (Specific Aim 1) and CTC-based RNA analysis (Specific Aim 2) is expected to complete in mid 2018. Results will be reported in conferences of relevant society in late 2018 or early 2019. Publications are expected in early 2019.

4. IMPACT: Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge,

theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Through our research, we aim to confirm the association between vsnCTCs and VM. Moreover, we intend to characterize these cells and identify the mechanism underlying VM disease progression. The overarching goal of our research inquiry is to develop of a new assay for predicting VM. This is an important an unmet need for prostate cancer clinical care as hormonal treatments drive more patients' conditions towards VM. By identifying men early in their transition to this more aggressive, VM-disposed disease, oncologists can implement therapy that will alter the natural history of VM in prostate cancer. Our studies will elucidate the biological differences between VM and non-VM prostate cancer, as revealed through CTC and tissue analysis, and lead to refined therapeutic strategies. Our work will lead to significant progress toward a putative biomarker for aggressive prostate cancer.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to report.

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to report.

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

The ultimate goal of this research is to pave the way for developing the use of CTC as a putative biomarker for aggressive prostate cancer, which will allow oncologists to implement therapy that will alter the natural history of VM in prostate cancer.

5. **CHANGES/PROBLEMS:** The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Nothing to report.

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to report.

Significant changes in use or care of vertebrate animals

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS: List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

1. Chen J-F, Zhu Y, Lu Y-T, et al. Clinical Applications of NanoVelcro Rare-Cell Assays for Detection and Characterization of Circulating Tumor Cells. Theranostics. 2016;6(9):1425-1439.
2. Mo□Yuan Shen, **Jie□Fu Chen**, Chun□Hao Luo, Sangjun Lee, Cheng□Hsuan Li, Yung□Ling Yang, Yu□Han Tsai, Bo□Cheng Ho, Li□Rong Bao, Tien□Jung Lee, Yu Jen Jan, Ya□Zhen Zhu, Shirley Cheng, Felix Y Feng, Peilin Chen, Shuang Hou, Vatche Agopian, Yu□Sheng Hsiao, Hsian□Rong Tseng, Edwin M Posadas, Hsiao□hua Yu. Glycan Stimulation Enables Purification of Prostate Cancer Circulating Tumor Cells on PEDOT NanoVelcro Chips for RNA Biomarker Detection. Adv Healthc Mater. 2017 Sep 11

  Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

1. Jie-Fu Chen and Edwin M. Posadas. Clinical Applications of NanoVelcro Rare-Cell Assays for Detection and Characterization of Circulating Tumor Cells. 5th Annual National Cancer Institute (NCI) Center for Strategic Scientific Initiatives (CSSI) Science Day (invited oral presentation).
2. Jie-Fu Chen, et al. Bio-Competition-based Smart NanoVelcro Chip for isolation and Expressional Analysis of Circulating Tumor Cells from Prostate Cancer Patients. [abstract] In: Proceedings of the 108th Annual Meeting of the American Association for Cancer Research; 2017 Apr 1-5; Washington DC: AACR; Cancer Research 77 (13 Supplement), 3780-3780.
3. Jie-Fu Chen, et al. Very small nuclear circulating tumor cell (vsnCTC) as a putative biomarker for visceral metastasis in metastatic castration-resistant prostate cancer (mCRPC). The Journal of Urology 195 (4), e78.
4. Jie-Fu Chen, et al. Very-small-nuclear circulating tumor cell (vsnCTC) as a putative biomarker for visceral metastasis in metastatic castration-resistant prostate cancer (mCRPC). [abstract] In: Proceedings of the 107th Annual Meeting of the American Association for Cancer Research; 2016 Apr 16-20; New Orleans, LA. Philadelphia (PA): AACR; Cancer Res 2016;76(14 Suppl):Abstract nr 4962.
5. Jie-Fu Chen, et al. Very small nuclear circulating tumor cell (vsnCTC) as a putative biomarker for visceral metastasis (VM) in metastatic castration-resistant prostate cancer (mCRPC). Laboratory Investigation 96, 221A.
6. Jie-Fu Chen, et al. Very small nuclear circulating tumor cell (vsnCTC) as a putative biomarker for visceral metastasis in metastatic castration-resistant prostate cancer (mCRPC). J Clin Oncol 34, 2016 (suppl 2S; abstr 64).

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

The Gift of Grab - An experimental device snags tumor cells like Velcro to speed cancer diagnosis and predict disease spread. Discoveries (Cedars-Sinai) Spring 2017.

- **Website(s) or other Internet site(s)**

List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

No websites to report.

- **Technologies or techniques**

Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

In this research, we developed a new CTC purification platform based on a phenylboronic acid (PBA)-grafted poly(3,4-ethylenedioxythiophene)s (PEDOT) NanoVelcro chip. In this platform, the covalently-grafted PBA groups allow direct conjugation of antibody (i.e., anti-EpCAM) onto a PEDOT nanosubstrate via PBA-oligosaccharide bonding, enabling specific capture of CTCs. Upon exposure to a glycan molecule (i.e., sorbitol) which has a stronger affinity to PBA, competitive binding leads to release of the captured CTCs. Through the affinity capture, followed by the "on-demand" release with minimal changes in the microenvironments, CTCs can be purified with their RNA better preserved.

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to report.

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Example:

Name:	*Mary Smith*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*1234567*
Nearest person month worked:	*5*
Contribution to Project:	*Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support:	*The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

Name:	*Jie-Fu Chen, MD*
Project Role:	*PI*
Unchanged	
Name:	*Edwin M. Posadas, MD*
Project Role:	*Co-mentor*
Unchanged	
Name:	*Hsian-Rong Tseng, PhD*
Project Role:	*Co-mentor*
Unchanged	
Name:	*Leland W.K. Chung, PhD*
Project Role:	*Consultant*
Unchanged	
Name:	*Michael Freeman, PhD*
Project Role:	*Consultant*
Unchanged	
Name:	*Ker-Chau Li, PhD*
Project Role:	*Consultant*
Unchanged	

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Change of PI
Jie-Fu Chen, MD, the submitting PI of this research, has left the position of postdoctoral fellow at Cedars-Sinai Medical Center in June, 2017, due to personal career plan. The proposed work will be continued by his successor, Yu-Jen Jan, MD, starting from June, 2017. The proposed research has not changed and will be carried out according to the abovementioned plan.

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Organization Name: University of California, Los Angeles (UCLA)
Location of Organization: 500 Westwood Plz, California NanoSystems Institute (CNSI)
Partner's contribution to the project

- Facilities

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to **https://ers.amedd.army.mil** for each unique award.

QUAD CHARTS: If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.

9. APPENDICES:

Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

Appendix A.

Specific Aim 1: Retrospective analysis for the association between very-small-nuclear circulating tumor cells (vsnCTCs) and visceral metastasis (VM) in prostate cancer (PCa) patients.

- **Introduction**

Our working hypothesis is that *vsnCTCs can be detected in PCa patients with existing or developing VM*. This work will be carried out by performing NanoVelcro CTC enumeration studies on existing cryopreserved blood samples collected from > 100 patients over the course of their disease progression. The resulting vsnCTC counts will be compared to the natural history of disease progression with a focus on VM development and response to therapeutic interventions.

- **Major activities**

Using the cryopreserved blood samples in our Cedars-Sinai Medical Center (CSMC) Urologic Oncology Program (UOP) blood specimen/CTC bank, we have been conducting a retrospective CTC enumeration study using imprinted NanoVelcro PLGA (i.e., poly(lactic-co-glycolic acid) Chips PCa patients across a spectrum of metastatic states similar to our published study. We analyzed the population of CTCs using our statistical modeling approach focusing on nuclear size distribution, and compared the temporal variations in vsnCTC counts with the annotated clinical history in our existing database and refine the association between vsnCTC counts and VM.

Study Population: UOPBBB. This is an ongoing, annotated, blood, tissue, and data collection that grows on a weekly basis. **Table 1** shows the demographic characteristics of the patients at the time of this progress report.

Characteristics	Patient #	Percentage
Metastatic site		
Visceral metastasis (VM)	18	31%
non-VM	27	47%
Evolving from non-VM to VM	13	22%
Total	**58**	**84%**
Race		
White	47	81%
Black	6	10%
American Indian	0	0%
Asain	2	3%
Other	1	2%
N/A	2	3%
Total	**58**	**100%**
Ethnicity		
Hispanic	4	7%
Non-Hispanic	52	90%
N/A	2	3%
Total	**58**	**100%**
Age		
41-50	3	5%
51-60	3	5%
61-70	19	33%
71-80	24	41%
81-90	7	12%
N/A	2	3%
Total	**58**	**100%**

Table 1. Characteristics of patients in our current repository immediately available retrospective CTC enumeration study.

CTC enumeration – capturing, imaging, and morphologic assessment.

(i) CTC capture. The general procedure for CTC enrichment by NanoVelcro PLGA Chips is summarized in a workflow (**Figure 1**). From our annotated blood specimen/CTC bank, the cryopreserved PBMC samples (containing CTCs) are thawed and incubated with a capture agent cocktail (containing biotinylated anti-human EpCAM and CD147 antibodies).[1] After washing free capture agents away, the cell suspension is loaded (flow rate = 0.5 mL/h) into the NanoVelcro Chip (freshly conjugated with streptavidin via NHS chemistry), using the fluid handler. After fixation using 95% methanol, the cells immobilized on the NanoVelcro substrate are subjected to 3-color ICC staining with DAPI, anti-CK, and anti-CD45.

(ii) Imaging captured CTCs. NanoVelcro Chips with immobilized cells are imaged using an upright fluorescence microscope (Eclipse 90i, Nikon) with NIS-Element imaging software (Nikon). An automatic scan is carried out by the imaging system under 40X magnification with DAPI, FITC and TRITC channels corresponding to nuclear, CK, and CD45 staining respectively. The DAPI+/CK+/CD45- events are selected as candidate CTCs and are further subjected to another scan at 100X and/or 400X magnifications.

(iii) Morphologic assessment and nuclear size measurement of the CTCs. Nuclear size of all identified CTCs is measured along the longest axis and the perpendicular width.[2,3] The nuclear size is defined as previously described.[4] Additional morphologic features (e.g. cell size, circularity, and nuclear-cytoplasmic ratio) are also obtained using the NIS-Element morphometric software package and other open-source cellular image analysis tools (e.g., CellProfiler and ImageJ).

Retrospective analyses for the association of CTC nuclear size and vsnCTCs with VM. We aimed to confirm the association between CTC nuclear size and metastatic status from our initial study. We hypothesize that small CTC nuclear size is associated with an increased risk of VM progression. A second analysis on this same sample will be performed using the definition of vsnCTC from our initial study (DAPI+/CK-/CD45+, nuclear size< 8.5 μm),[4] and the vsnCTC count of each patient will be determined. We hypothesize that the proportion of VM patients harboring vsnCTCs is greater than that in patients with non-visceral metastases. Here we assume that those CTCs with nuclei < 8.5 μm represent a distinct pathologic entity and we treat them as such (rather than as continuous variable). An association will be made between vsnCTC counts and metastatic state determined by contemporary imaging. We will confirm the relationship between presence of vsnCTCs and VM by categorizing patients as either non-VM or VM. As each subject's nuclear size and the presence or absence of VM will be noted over time, statistical models taking into account correlated data will be employed for both analyses.

Exploratory analysis of the vsnCTC-VM relationship. In addition to the vsnCTC-VM association, several potential relationships will also be explored, including (i) the dynamic change in vsnCTC counts after key clinical events such as

initial castration, progression to CRPC, and initiation of next-generation AR therapies; (ii) Change in vsnCTC counts at/near time of disease progression through the above-mentioned treatments; (iii) Temporal relationship between the appearance of vsnCTC and radiographic detection of VM. Since metastatic status is subject to change over the disease course, multiple enumeration studies from individual patients are included for constructing the dynamic change of vsnCTC counts along the disease evolution.

Statistical analysis. The main hypothesis to be tested is whether progression to VM (dichotomous response variable; Yes/No) is associated with CTC nuclear size (μm). This analyses are done in two ways in parallel: we associate the presence or absence of vsnCTCs using the existing definition from our published study (nuclear size <8.5 μm), looking at vsnCTCs as a dichotomous variable. To avoid statistical biases attributable to our sample set in the published series, we also conduct an analysis of nuclear size coded as a continuous variable and the presence of VM. This approach removes any dependence on pre-specified definitions of CTC subsets. The statistical analyses are performed in three steps: 1) exploratory data analysis, 2) univariate analysis, and 3) multivariable analysis. As each subject's nuclear size and the presence or absence of VM are collected over time, a generalized method of moments logistic regression model[5], which takes into account the correlation due to repeated measures and time dependent covariates, is used in both uni- and multivariable analyses to model the relationship of progression to VM with CTC nuclear size and the presence or absence of vsnCTCs. Logistic regression diagnostics is employed to ensure that the logistic model is appropriate. In the multivariable analysis the possibility of collinearity is reduced through the careful initial assessment of correlations among all study covariates. Cross-validation techniques are used to assess and compare the performance of the prediction models constructed. All tests of hypotheses are two-sided with a significance level of 0.05. Statistical analyses are conducted using SAS and available macros provided by Lalonde et al.[5]

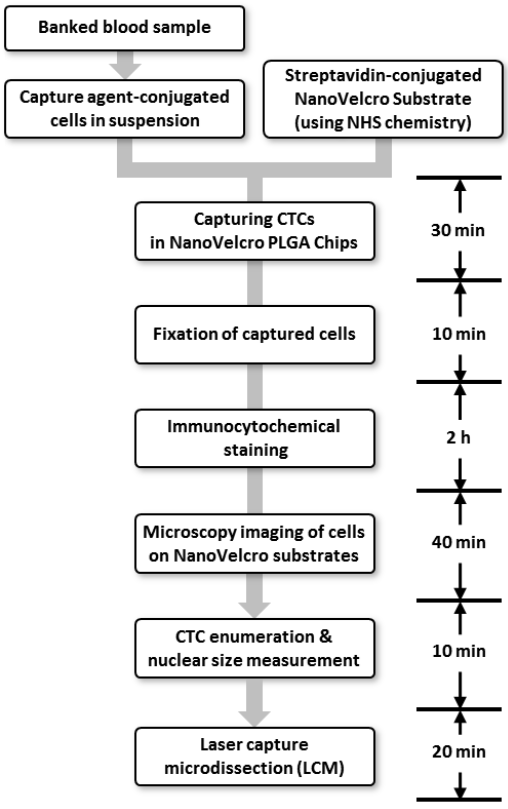


Figure 1. Workflow for NanoVelcro CTC enumeration assay

- **Significant results**

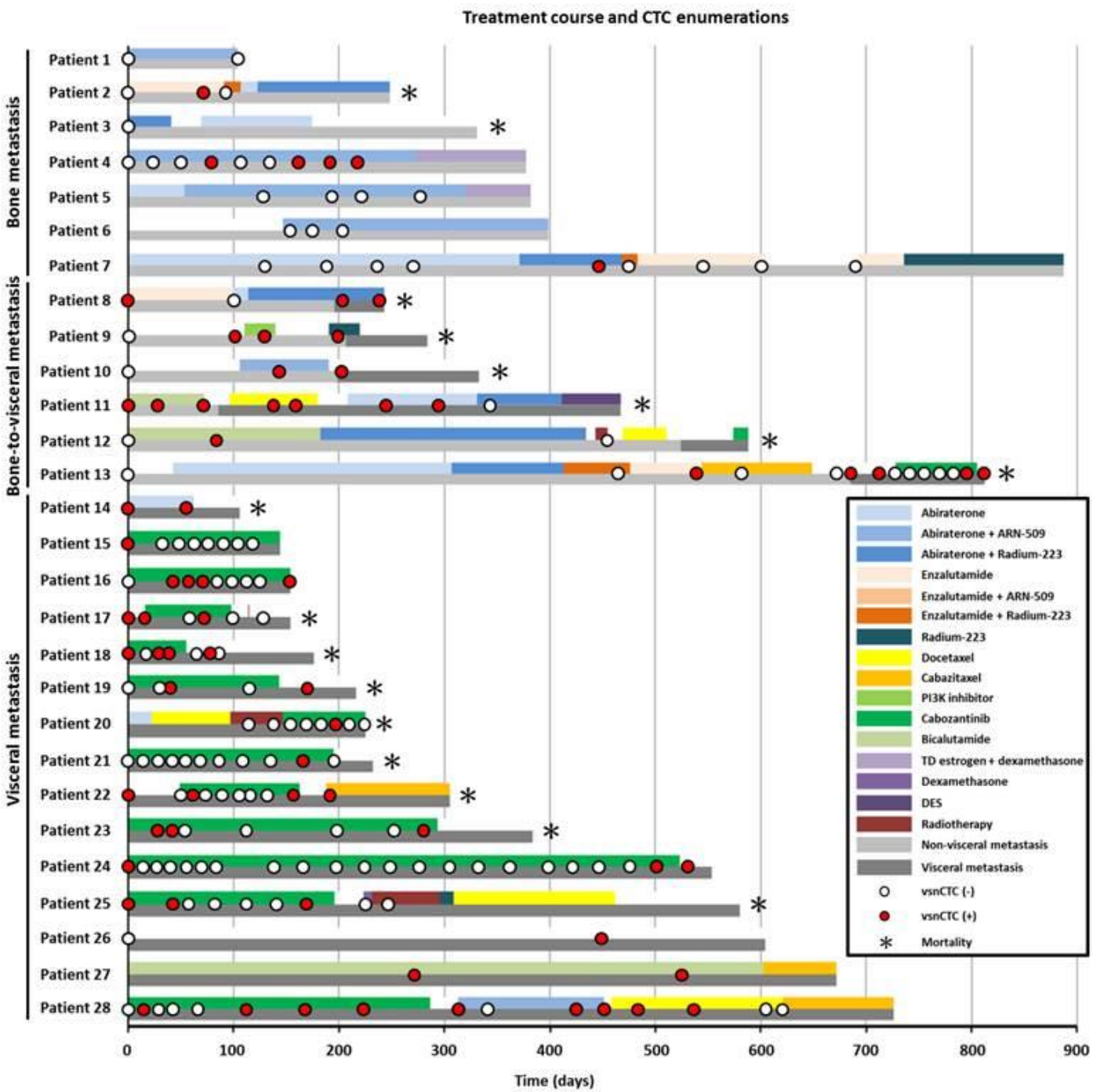
Retrospective analysis of the association between vsnCTCs and VM. As our team further expand the investigation for this phenomenon in a bigger patient database, we have identified 28 metastatic PCa patients who had progressed through next generation hormonal maneuvers such as abiraterone, enzalutamide, or an equivalent drug. Serial blood specimens were used retrospectively for CTC enumeration and subgroup analysis (**Figure 2**). Fifteen out of 28 patients presented with visceral lesions and 13 had bone-only disease at their first CTC enumeration. Six out of 13 non-visceral metastatic patients developed visceral lesions during follow-up, and vsnCTCs were detected 86-196 days prior to radiographic detection of the visceral lesions. Four patients had vsnCTCs detected without the presence of visceral lesions by the time of this analysis, but we are still following some of them for visceral progression in the future. Overall, vsnCTCs were detected in all the patients with VM, and none of the patients without vsnCTCs developed VM in this study. The proposed study aimed to identify serial blood specimens from at least 15 patient for each metastatic category (i.e.,VM, non-VM, evolving from non-VM to VM), which has been accomplished in February, 2017. We are currently completing the retrospective CTC enumeration and subsequent association analysis.

Aside from the potential predictive utility of vsnCTCs, we also analyzed the relationship between vsnCTCs and patients' response to therapeutic interventions. The team observed reduction of vsnCTC count occurred at initiation of anti-cancer treatment. Conversion from vsnCTC(-) to vsnCTC(+) was seen prior to progression of visceral lesions under the treatment (**Figure 3**).

- **Reporting**

The findings in above-mentioned 28 patients have been reported in national meetings including 2016 American Association for Cancer Research (AACR) Annual Meeting, 2016 American Society of Clinical Oncology (ASCO) Genitourinary Cancer Symposium, 2016 American Urology Association (AUA) Annual Meeting, and 2016 United States and Canadian Academy of Pathology (USCAP) Annual Meeting, as well as our review article published in *Theranostics*.[6]

Figure 2. Retrospective analysis of the association between vsnCTCs and VM in an expanded patient cohort (next page). NanoVelcro CTC Assay identified very-small-nuclear CTCs (vsnCTCs) in prostate cancer patients with visceral metastasis. The presence of vsnCTCs has been associated with the presence of visceral metastasis. Serial CTC enumerations also suggested that vsnCTCs emerge before the development/detection of visceral lesions and thus may be a predictive biomarker for visceral metastasis in prostate cancer patients.



Treatments received	Site of visceral metastasis
Abiraterone + ARN-509	N/A
Enzalutamide, enzalutamide + Radium-223, abiraterone, abiraterone + Radium-223	N/A
Abiraterone + Radium-223, abiraterone	N/A
BIND-014, enzalutamide, enzalutamide + Radium-223, abiraterone + ARN-509, TD estrogen + dexamethasone	N/A
Bicalutamide, abiraterone, abiraterone + ARN-509, TD estrogen + dexamethasone	N/A
Degarelix, abiraterone, abiraterone + ARN-509	N/A
Leuprolide, bicalutamide, docetaxel, abiraterone, abiraterone + Radium-223, enzalutamide + Radium-223, enzalutamide, Radium-223	N/A
Enzalutamide, abiraterone + Radium-223	Liver
Radiotherapy, PI3K inhibitor, Radium-223	Lung
BIND-014, abiraterone + ARN-509	Dura and subcutaneous soft tissue
Bicalutamide, leuprolide, docetaxel, abiraterone, abiraterone + Radium-223, DES	Lung
Leuprolide, abiraterone + ARN509, radiotherapy, docetaxel, cabozantinib	Liver
Radiotherapy, bicalutamide, DES, docetaxel, abiraterone, Radium-223, enzalutamide, cabazitaxel, cabozantinib	Liver
Docetaxel, abiraterone	Adrenal gland
Bicalutamide, enzalutamide, docetaxel, cabozantinib	Liver and adrenal gland
Bicalutamide, cabazitaxel, docetaxel + carboplatin, abiraterone + Radium-223, cabozantinib	Lung
Cabozantinib	Liver and brain
Cisplatin + etoposide, adjuvant radiotherapy, cabozantinib	Lung
Leuprolide, denosumab, abiraterone + docetaxel, docetaxel, cabozantinib	Liver and lung
Ixabepilone, leuprolide, salvage radiotherapy, bicalutamide, denosumab, prostvac, abiraterone, docetaxel, radiotherapy, cabozantinib	Liver and lung
Cabozantinib	Liver
Cabozantinib	Liver
Cabozantinib	Liver
Leuprolide, docetaxel, abiraterone, cabozantinib	Liver
Dexamethasone, cabozantinib, radiotherapy, Radium-223, docetaxel	Pleura
N/A	Lung and adrenal gland
Bicalutamide, docetaxel + carboplatin, cabazitaxel + cisplatin	Lung
Radiotherapy, bicalutamide, cabazitaxel, cabozantinib, abiraterone + ARN-509, docetaxel, cabazitaxel	Liver

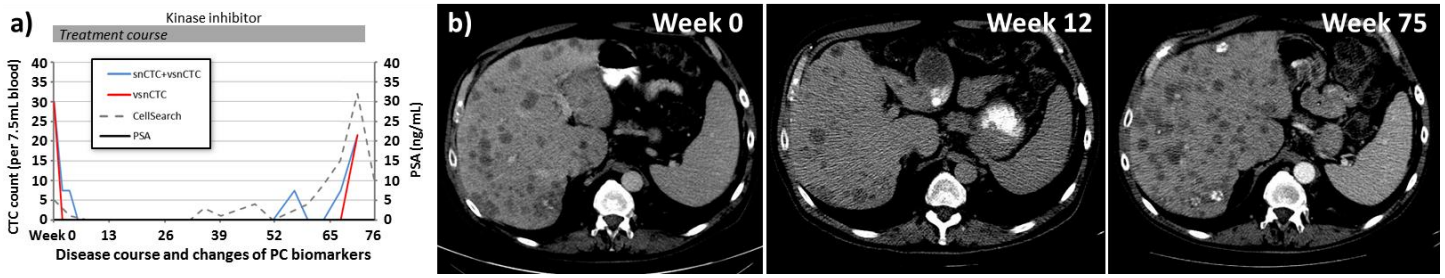


Figure 3. Dynamic change of vsnCTC count may reflect the impact from anti-cancer therapy. A VM patient experienced a clinical improvement within 2 weeks of starting a multi-targeted kinase inhibitor. Soft tissue and bone response was noted at week 12. **a)** His vsnCTC counts decreased from 30 vsnCTCs/7.5mL to 0 vsnCTCs/7.5mL within 2 weeks of starting therapy, and remained undetectable as he continued to benefit from the therapy. At week 70, vsnCTCs re-emerged with a count of 22 vsnCTCs/7.5mL, and the patient developed visceral progression at week 75. **b)** Radiographic assessment at the time of baseline (pre-treatment, week 0), partial response (week 12), and visceral progression (week 75).

Specific Aim 2: Comparing gene expression signatures of vsnCTCs and non-vsnCTCs.

- **Introduction**

Our working hypothesis is that *vsnCTCs harbor expression signatures distinct from other non-vsn CTCs* in pathways involving AR activity, NE differentiation, nuclear structure regulation, metastatic capacity, and anti-cancer immunity. Through our latest collaboration with Dr. Michael Freeman's group at CSMC, we have successfully adopted the Prostate cancer Classification System (PCS)[7] for characterization of CTCs and their association with VM. Dr. Freeman's team developed the PCS classification system using pathway activation signatures of known relevance to PCa from a large virtual cohort (n = 1,321) of human PCa transcriptome profiles computationally assembled from 38 distinct cohorts. PCS is a novel classification system for PCa consisting of three distinct subtypes (named PCS1–3). This subtyping scheme was validated in 10 independent patient cohorts and 19 laboratory models of PCa, including cell lines and genetically engineered mouse models. PCS1 tumors progress more rapidly to metastatic disease in comparison with PCS2 or PCS3. Further analysis showed enrichment of VM in PCS1 (**Figure 4**). This finding holds true in the training data set (chi-square test, P = 0.0028), and the validation dataset (SU2C mCRPC dataset, chi-square test, P = 0.00045). To apply this finding clinically, a 37-gene panel was developed that can accurately assign individual tumors to one of the three PCS subtypes (**Figure 4e**). This panel can be applied to identify CTCs that exhibit PCS1 phenotype (**Figure 4f**). Coupling NanoVelcro Chip with laser-capture microdissection (LCM) technology, we developed the NanoVelcro-LCM approach, which enables isolation of single CTCs for downstream mutational and expressional analysis. We aim to use NanoVelcro-LCM approach to isolate single CTCs for targeted gene expression analysis, focusing on PCS classification scheme, and compare the differential expression between vsnCTCs and non-vsnCTCs.

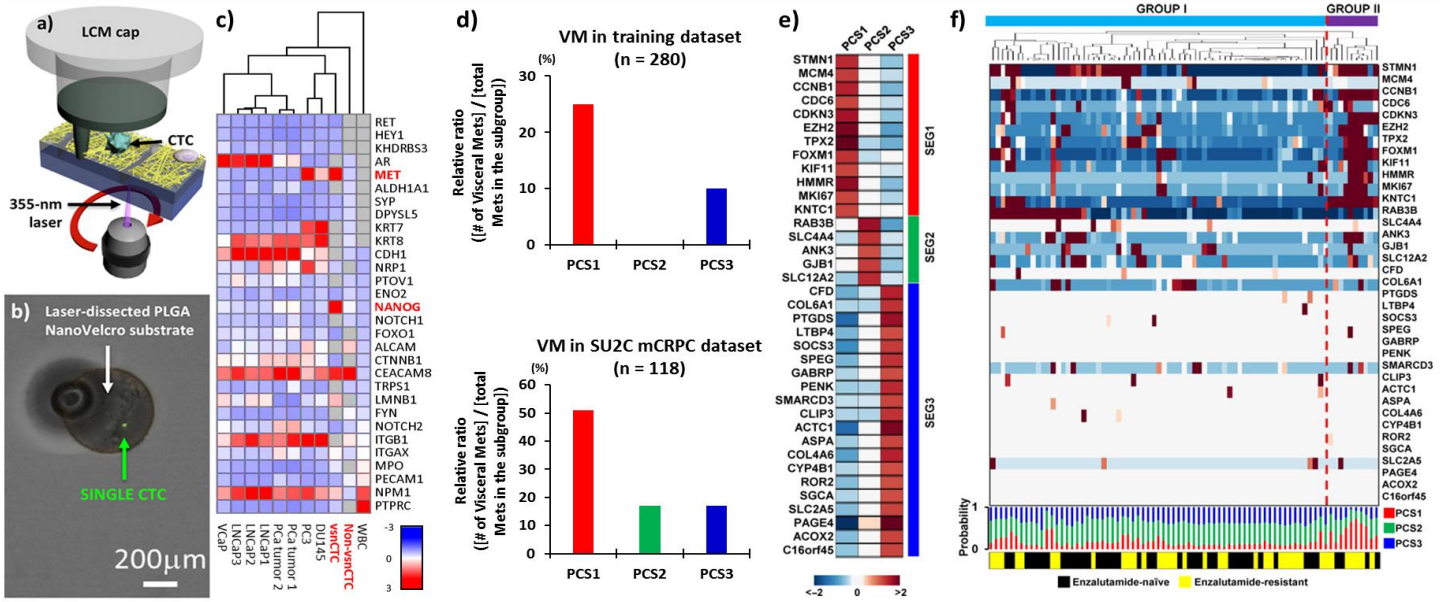


Figure 4. NanoVelcro-LCM technology for single-CTC isolation and RNA sequencing (RNA-seq). **a**) Schematic of single CTC isolation by our NanoVelcro-LCM technique. **b**) Micrograph of a PCa CTC on a laser-microdissected PLGA NanoVelcro substrate. **c**) Data from RNA-seq demonstrated the expression signature of cancer cells distinct from that of white blood cell (WBC). Several prometastatic genes (e.g. *MET, NANOG*) showed differential expression between vsnCTC and non-vsnCTC. d) Analysis of training dataset and SU2C mCRPC datasets showed enrichment of VM in PCS1 category. e) 38-gene panel for PCS classification. f) Initial test of PCS in single CTC transcriptomic datasets successfully identified CTCs with PCS1 molecular phenotype in patients with resistance to enzalutamide.

- **Major activities**

CTC isolation using NanoVelcro-LCM approach

The NanoVelcro-LCM technology is the combination of LCM technique with the transparent NanoVelcro PLGA Chip. *This approach allows precise isolation of single-CTCs for downstream molecular characterization.*[1] Similar to the approach for CTC enumeration in Specific Aim 1, the capture agents (i.e. anti-EpCAM) are linked to the NanoVelcro PLGA substrates for CTC capture. Subsequently, the nanosubstrate-immobilized CTCs can be specifically isolated using a LCM microscope (**Figure 4a/b**). The single CTCs isolated by NanoVelcro-LCM technology can be subjected to a variety of molecular analyses, including mutational analysis by PCR and targeted sequencing (e.g. $BRAF^{V600E}$ in melanoma CTCs[1] and $KRAS^{G12V}$ in pancreatic CTCs), as well as NGS at whole exome[8] and whole genome scale.[9] Moreover, we have demonstrated the feasibility of performing transcriptomic analyses for the CTCs isolated by NanoVelcro-LCM technology (**Figure 4c**). Our initial study of RNA-seq has shown upregulation of several pro-metastatic genes (i.e. *MET* and *NANOG*) in samples containing vsnCTCs compared to those containing non-vsnCTCs. Using the blood samples from the PCa patients identified in Specific Aim 1, this work continued to accumulate data focusing on a more precise analysis of the genes that are potentially associated with vsnCTC phenotype and VM progression.

Development of highly efficient NanoVelcro CTC purification approaches

Despite the enormous research efforts in CTC-based molecular testing, it remains technically challenging for existing platforms to efficiently obtain high-quality signals due to the low abundance of CTCs and the fragility of the genetic materials. To address this issue, new technologies capable of capturing and releasing CTCs with minimal contamination of white blood cells (WBCs) and maximal cellular viabilities and molecular intactness are needed. On the basis of nanostructure-embedded substrates that exhibit enhanced cell-capture affinity, we and others previously introduced stimuli-responsive materials onto the nanosubstrates [10,11] to enable "on-demand" release of the captured CTCs for downstream molecular analyses. In most cases, the captured cells were released by various physical [12-17] and biological mechanisms.[18,19] However, some external stimuli pose a harsh condition in which the genetic content of CTCs can be altered and lead to the incorrect information in molecular analysis, particularly in the cases of vulnerable genetic materials such as RNA.[10] As such, there is unmet need to develop an efficient CTC purification technology using a physiologically compatible stimulus, which provides higher quality RNA for characterization. Herein we developed a new CTC purification platform based on a phenylboronic acid (PBA)-grafted poly(3,4-ethylenedioxythiophene)s (PEDOT) NanoVelcro chip. In this platform, the covalently-grafted PBA groups allow direct conjugation of antibody (i.e., anti-EpCAM) onto a PEDOT nanosubstrate via PBA-oligosaccharide bonding, enabling specific capture of CTCs. Upon exposure to a glycan molecule (i.e., sorbitol) which has a stronger affinity to PBA, competitive binding leads to release of the captured CTCs. Through the affinity capture, followed by the "on-demand" release with minimal changes in the microenvironments, CTCs can be purified with their RNA better preserved. We then demonstrated the measurement of RNA biomarkers in purified CTCs from PCa patients focusing on *AR-FL*, *AR-V7*, *KLK3* (prostate-specific antigen, PSA), *FOLH1* (prostate-specific membrane antigen, PSMA), and long non-coding RNA *SChLAP1* (SWI/SNF Complex Antagonist Associated With Prostate Cancer 1) using the workflow illustrated in **Figure 5**.

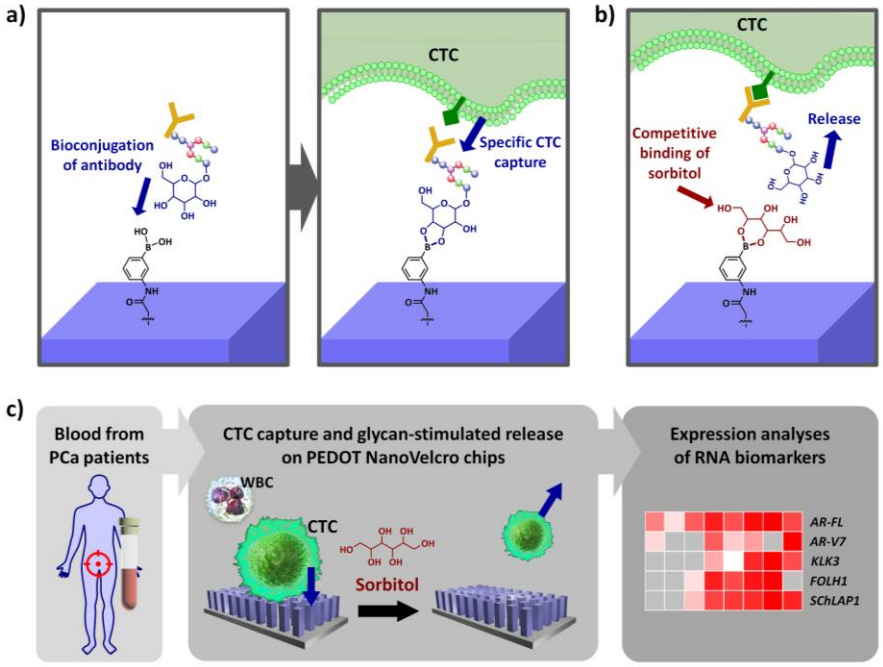


Figure 5. Illustration on the concept of PBA-PEDOT NanoVelcro CTC purification system. (a) The mechanism for CTC capture is that the surface-grafted phenylboronic acid (PBA) conjugates with antibody, subsequently enabling specific CTC capture. **(b)** The mechanism for CTC release is that the introduction of glycan with stronger affinity to PBA (i.e., sorbitol) results in competitive binding, allowing CTC release. **(c)** Workflow on using this glycan-stimulation enabled CTC purification platform on poly(3,4-ethylenedioxythiophene) (PEDOT) NanoVelcro chips for RNA biomarker analysis from purified CTCs of prostate cancer patients.

Development of CTC-based RNA assay

(i) Preparation of artificial samples. We prepared artificial samples by spiking 3 PCa cell lines obtained from American Type Culture Collection (ATCC) of different molecular characteristics (in terms of their expression levels on EpCAM), i.e.,

LNCaP, 22Rv1, and PC3, with 2-mL blood from healthy donors at densities of 400-500, 100-150, 40-50 and 1-5 cells/mL. The artificial samples will be preserved in 10-mL Acid Citrate Dextrose (ACD) tube and used within 6h after preparation. These artificial samples will be used to validate the sensitivity of our CTC-RNA assay.

(ii) Calibration studies to assess the performance of RNA quantification. We prepared standardized cDNA mixture by extracting RNA from artificial samples. The samples are prepared by spiking different PCa cell lines into 5000 WBCs at densities of 400-500, 100-150, 40-50 and 1-5 cells per sample. The cells are lysed and RNA will be converted to cDNA, and the targeted RNA expression of PCS panel is quantified using standardized methodologies including targeted digital droplet RT-PCR (ddRT-PCR), Delta Gene™ Assay on BioMark™ HD System, and NanoString nCounter® platform for each condition with five repeats.

(iii) Calibration studies to examine the complete CTC-PCS assay. To assess the performance of the CTC-PCS assay from beginning to end, we performed non-stop CTC isolation/purification and RNA quantification with artificial CTC samples with five repeats for each condition.

(iv) Development of CTC-based RNA panel focusing on VM-specific signatures

Based on the PCS classifiers, we developed a revised panel of genes for CTC-based measurement. The revised panel identifies PCS1 phenotype, which is associated with increased risk of VM. Meanwhile, genes with significant expression in WBCs were removed from the panel, allowing for a more sensitive and specific detection of CTC-based RNA signatures. The panel serves as the target for downstream molecular test following CTC isolation (by NanoVelcro-LCM approach) or purification (by NanoVelcro CTC purification systems). The revised PCS panel will undergo the aforementioned calibration studies (steps i – iii) to confirm its classification capacity.

- **Significant results**

Development of highly efficient NanoVelcro CTC purification approaches

Using the PBA-grafted PEDOT NanoVelcro chips, we observed that anti-EpCAM-conjugated PEDOT NanoVelcro chip exhibited much higher capture efficiency (72.5 ± 3.0%, $p < 0.0001$) for EpCAM-positive LNCaP cells, as compared to the non-conjugated PEDOT NanoVelcro chip, non-conjugated PEDOT (composed by poly(EDOT-PBA-co-EDOT-EG3)) film, and anti-EpCAM-conjugated PEDOT film. As expected, the anti-EpCAM-conjugated PEDOT NanoVelcro also exhibited significantly higher capture efficiency ($p < 0.001$ for each comparison) for other EpCAM-positive PCa cells (75.2 ± 3.2 % for PC3, and 67.8 ± 1.7% for 22Rv1) compared to EpCAM-negative WBCs (0.01 ± 0.004 %). The captured cells could be released most efficiently with 0.5 M sorbitol solution ($p < 0.05$ in comparison to 0.25 M). Further optimization (Figure 3f) revealed that the highest release efficiency (95%) and cell viability (96%) were achieved by incubating the cells in 0.5M sorbitol solution for 30 minutes.[20]

Under operating conditions optimized from the calibration studies, we purified CTCs from clinical blood samples and measured the expression of PCa-related RNA biomarkers. Samples were obtained from 17 PCa patients (13 metastatic and 4 localized) and 7 healthy individuals with no known malignancies. Expression data for 5 PCa-related biomarkers from all 24 subjects were summarized in **Figure 6**. Every patient (n = 17) had at least one CTC upon enumeration. We detected PCa-related biomarkers in CTCs from 94% of patients (16/17). Consistent with previous reports,[21-23] the expression of *KLK3* was seen in 77% of metastatic patients (10/13) as compared to only 25% of the non-metastatic PCa patients (1/4). The expression of *FOLH1* and *SChLAP1* was detected exclusively in metastatic PCa patients. *FOLH1* expression in CTCs was detected in 85% of metastatic PCa patients (11/13), and *SChLAP1* was detected in 92% of the same patient group (12/13). This is the first demonstration of *SChLAP1* expression in CTCs. Our results suggest that *SChLAP1* detection in CTCs may have a similar role as that in primary tumor tissues. Also consistent with prior observation, *AR-V7* was always detected in the presence of *AR-FL*. Our results showed *AR-V7* in 75% of the patients (6/8) who had

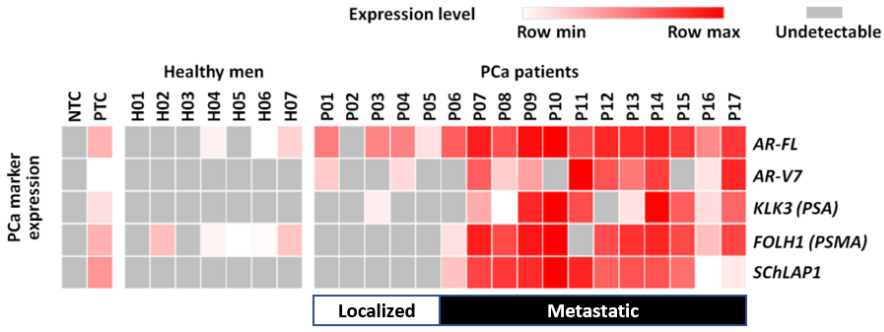


Figure 6. Detection of PCa-related RNA signatures in CTCs purified by PEDOT NanoVelcro Chips in 7 healthy men and 17 PCa patients. PTC = positive control (RNA from 100 LNCaP cells + 100 22Rv1 cells in 1 million WBCs from a healthy man purified by PEDOT-NanoVelcro Chips); NTC = negative control (nuclease free water). The expression of *KLK3* gene was more commonly seen in metastatic PCa with 77% (10/13) of the metastatic patients showing positive signal compared to only 25% (1/4) non-metastatic PCa patient having detectable expression. *FOLH1* gene expression in CTCs was only detected in metastatic PCa patients with 85% (11/13) of the metastatic PCa patients having detectable expression. *SChLAP1* expression in CTCs was only detected in metastatic PCa patients with 92% (12/13) of the metastatic PCa patients having detectable expression. Expression of *AR-V7* was can be detected in 6/8 metastatic PCa patients who had been exposed to abiraterone (A) or enzalutamide (E), and was accompanied with the expression of *AR-FL*. Some (3/5) patients who were had not received A/E treatment also showed positive *AR-V7* signals. All expression levels were normalized against *ACTB*.

been exposed to abiraterone and/or enzalutamide. Interestingly, in the abiraterone and enzalutamide naïve patients, AR-V7 was seen in 60% of subjects (3/5). Our detection rate for *AR-V7* was higher than some previously reports though others have reported even higher rates.[24,25] This reflected differences in the CTC enrichment approach (PEDOT-NanoVelcro Chips vs. no enrichment) and methodology of *AR-V7* detection (direct RNA detection via qRT-PCR vs. antibody-mediated immunofluorescence staining). Other factors such as the exposure to other anti-cancer treatments after AR-targeted therapy may have affected the expression of *AR-V7* in CTCs as well.[26] Notably, we also detected *AR-V7* in patients with localized PCa. The clinical significance of *AR-V7* in these patients has yet to be determined. The *AR-V7*(+) patients in this study continue to be monitored for the development of resistance. [20]

CTC-based RNA measurement focusing on VM-specific signatures

Through the above-mentioned calibration process, we have developed a panel of VM-specific genes based on PCS classifiers, which can be measured robustly in CTCs with minimal background noise from co-existing WBCs. Focusing on the revised PCS classifiers, we performed RNA measurement using NanoString nCounter system on PCa cell lines, artificial samples (PCa cells spiked into healthy blood), and blood samples from PCa patients. After normalization, the clustergram showed successful distinction of PCa cells (including LNCaP, PC3, and 22Rv1), artificial samples, and purified CTCs from clinical samples (**Figure 7**). The artificial samples were prepared by spiking 5 – 50 PCa cells into 5000 PBMCs from healthy blood. The clinical samples contain CTCs purified from 2 mL of whole blood from patients with histologically confirmed PCa. Among the PCa cells, 22Rv1 cells showed higher similarity to artificial samples or clinical samples, consistent with the findings in initial testing of PCS panel.[7] As shown in **Figure 7**, the artificial samples and clinical samples could be easily separated by the revised PCS classifiers. Among the clinical samples, patients with castration-sensitive and castration-resistant disease were also clustered well. Surprisingly, the revised PCS classifiers also identified patients with progressive disease at the time of blood collection from those with stable disease. An expanded patient cohort and blood samples has been identified to further investigate the classification capacity of the revised PCS panel. All the clinical samples in this study are linked to well-annotated clinical history and concomitant CTC enumeration, which is currently undergoing analysis to further clarify the association between the PCS expression profile and presence of vsnCTCs, as well as their association with VM.

- **Reporting**

The development of PBA-grafted PEDOT NanoVelcro chips has been presented in 2017 AACR Annual Meeting, and published on Advanced Healthcare Materials.[20] It is also presented along with the latest results from CTC-based PCS analysis in 2017 NCI Alliance for Nanotechnology in Cancer PI Meeting.

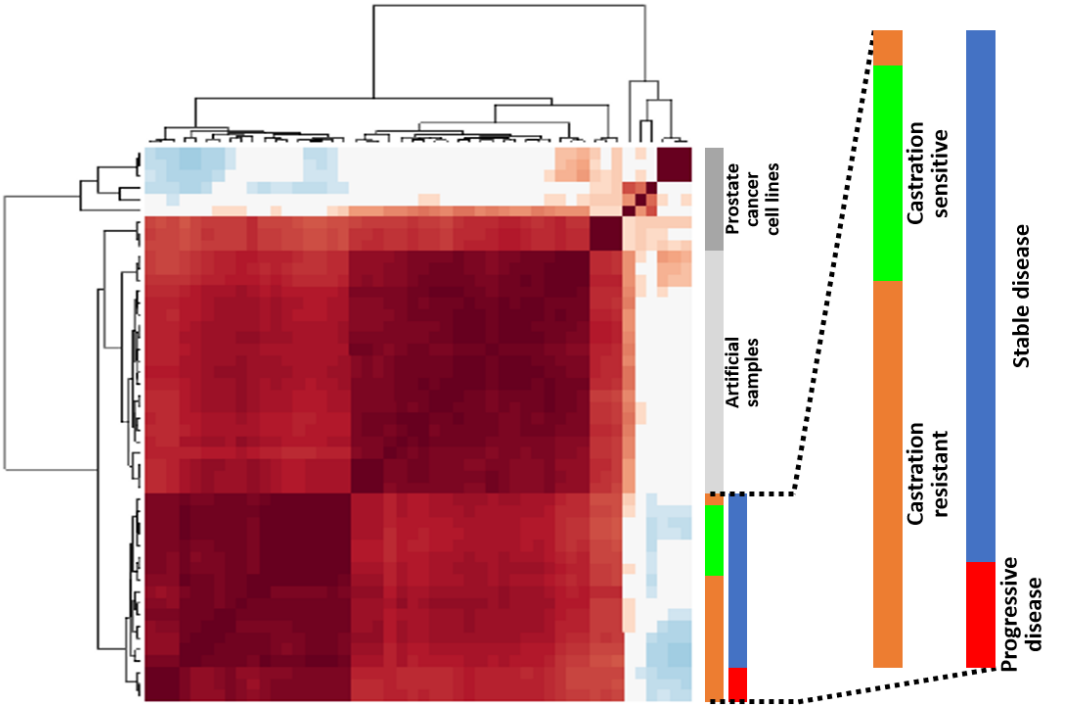


Figure 7. Clustergram showing PCS signatures in PCa cells, artificial samples, and CTCs from PCa patients.

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Glycan Stimulation Enables Purification of Prostate Cancer Circulating Tumor Cells on PEDOT NanoVelcro Chips for RNA Biomarker Detection

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A glycan-stimulated and poly(3,4-ethylene-dioxythiophene)s (PEDOT)-based nanomaterial platform is fabricated to purify circulating tumor cells (CTCs) from blood samples of prostate cancer (PCa) patients. This new platform, phenylboronic acid (PBA)-grafted PEDOT NanoVelcro, combines the 3D PEDOT nanosubstrate, which greatly enhances CTC capturing efficiency, with a poly(EDOT-PBA-*co*-EDOT-EG3) interfacial layer, which not only provides high specificity for CTC capture upon antibody conjugation but also enables competitive binding of sorbitol to gently release the captured cells. CTCs purified by this PEDOT NanoVelcro chip provide well-preserved RNA transcripts for the analysis of the expression level of several PCa-specific RNA biomarkers, which may provide clinical insights into the disease.

Circulating tumor cells (CTCs) are rare cancer cells shed or detached from solid tumors into the blood stream. Prospective clinical trials have shown the value of CTCs as clinical biomarkers where counts associate with survival and changes in number associate with response to therapy in solid tumors such as melanoma,[1] breast,[2] colorectal,[3] lung,[4] and prostate cancer (PCa).[5] Beyond enumeration, CTCs themselves are now being studied as an alternative tissue source creating a foundation for a "liquid biopsy." This holds the potential to conduct contemporary molecular characterizations that can provide insights into a cancer when tumor biopsy is difficult and/or dangerous to perform.[6] Recent research endeavors demonstrated the feasibility of CTC-based detection of clinically relevant molecular signatures, such as mutations in *EGFR*,[7] *BRAF*,[8] and *KRAS* genes.[8] In addition to the DNA mutations, CTC-based measurement of gene expression has also been explored. This is particularly important in PCa where there is a relatively low abundance of DNA mutations in tumors.[9] In PCa and other malignancies, many have focused efforts on characterization of gene expression and other RNA biomarkers.[10] In addition to more conventional genes, newer noncoding RNAs such as long noncoding RNAs (lncRNA) are showing even greater prognostic value in PCa. Expression of *SChLAP1* in PCa tumors was linked to higher risk of metastasis.[11] Profiling of clinically and biologically relevant RNA biomarkers in PCa CTCs has been reported: higher expression of *FOLH1* (prostate specific membrane

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antigen, PSMA) in CTCs is associated with more aggressive disease.[12] Studies of androgen receptor (AR) splicing variants in CTCs reveal that the expression of *AR-V7* in CTCs predicts the resistance to abiraterone and enzalutamide.[13] However, despite the enormous research efforts in CTC-based molecular testing, it remains technically challenging for existing platforms to efficiently obtain high-quality signals due to the low abundance of CTCs and the fragility of the genetic materials. To address this issue, new technologies capable of capturing and releasing CTCs with minimal contamination of white blood cells (WBCs) and maximal cellular viabilities and molecular intactness are needed.

A cell-affinity substrate using a unique concept called "NanoVelcro" was pioneered by us.[14,15] On this capture agent-coated nanostructured substrates substrate, we were able to selectively sort and purify the cells of interest (e.g., CTCs) from background cells with high efficiency. When the hairy nanostructured substrate meet the rough cancer cell surfaces, stronger binding occurred, which mimicked the working mechanism of Velcro strips. Other researchers also started to test the utilities of different nanostructure-embedded substrates[16] for capturing CTCs and other types of rare cells since our proof-of-concept publication in 2009,[17] and these works support the general applicability of nanostructure-embedded cell-affinity assays and their potential for cell-sorting applications. Among the materials which have been engineered into nanostructured substrates, poly(3,4-ethylenedioxythiophene) (PEDOT)[18] is promising given their advantages in easy introduction of functional groups through covalent bonds[19] and compatibility with various nanoengineering approaches.[20] We have demonstrated highly efficient CTC capturing on NanoVelcro chips with electropolymerized PEDOT nanodots[21] and nanoimprinted PEDOT rods array.[22]

On the basis of nanostructure-embedded substrates that exhibit enhanced cell-capture affinity, we and others previously introduced stimuli-responsive materials onto the nanosubstrates[23,24] to enable "on-demand" release of the captured CTCs for downstream molecular analyses. In most cases, the captured cells were released by various physical[25] and biological mechanisms.[26] However, some external stimuli pose a harsh condition in which the genetic content of CTCs can be altered and lead to the incorrect information in molecular analysis, particularly in the cases of vulnerable genetic materials such as RNA.[23] As such, there is unmet need to develop an efficient CTC purification technology using a physiologically compatible stimulus, which will provide higher quality RNA for characterization.

Herein, we introduce a new CTC purification platform based on a phenylboronic acid (PBA)-grafted PEDOT NanoVelcro chip. In this platform, the covalently grafted PBA groups allow direct conjugation of antibody (i.e., anti-EpCAM) onto a PEDOT nanosubstrate via PBA–oligosaccharide bonding, enabling specific capture of CTCs (**Figure 1**a). Upon exposure to a glycan molecule (i.e., sorbitol) which has a stronger affinity to PBA,[27] competitive binding leads to release of the captured CTCs (Figure 1b). Through the affinity capture, followed by the "on-demand" release with minimal changes in the microenvironments, CTCs can be purified with their RNA better preserved. We then demonstrated the measurement of RNA biomarkers in purified CTCs from PCa patients focusing on *AR-FL*, *AR-V7*, *KLK3*, *FOLH1*, and *SChLAP1* using the workflow illustrated in Figure 1c.

Given the advantages of high conductivity,[18] stability in aqueous solutions,[28] and low toxicity,[29] PEDOTs have been incorporated into the interface between biology systems and electronic devices (e.g., biosensors[30] and medical devices[31]). Choosing PEDOT as the base material provides us an easy access to meet the molecular and nanostructural requirements to build the cell purification platform. Once the PEDOT monomers are properly functionalized using organic synthetic approaches, electropolymerization utilizing PEDOTs' conductive nature gave us a quick (<1 min) and highly reproducible approach to functionalize all conductive nanosubstrates, meeting the cellular needs. As shown in **Figure 2**a, a PBA-grafted PEDOT NanoVelcro Chip was prepared by a simple two-step procedure: (1) nanoimprinting of the PEDOT rods array, followed by (2) electrochemical deposition of PBA-grafted PEDOT layer (i.e., poly(EDOT-PBA-*co*-EDOT-EG3)) in a solution containing two monomer precursors (i.e., EDOT-EG3 and EDOT-PBA). EDOT-PBA introduced the covalently grafted PBA groups to control the capture and release of CTC (Figure 1a,b). The other unit, EDOT-EG3, played a critical role in reducing the nonspecific binding of nontargeted cells and facilitating CTC release from the chips upon sorbitol exposure.[32] Images taken by scanning electron microscopy (SEM) confirmed the structures of nanoimprinted PEDOT rods array as shown in Figure 2b. The nanoimprinting and subsequent deposition of PBA-functionalized PEDOT layer were also confirmed by X-ray photoelectron spectroscopy (XPS). Once the PEDOT rods array was imprinted, we could observe the characteristic peaks from the chip, including O 1s electrons from ether groups, C 1s electrons from all $-CH_2-$ and $-CH-$ linkers and S 2s and 2p electrons from thiophenes (Figure S3, Supporting Information). Upon electropolymerizing the poly(EDOT-PBA-*co*-EDOT-EG3) layer, the detection of new peaks from B 1s (190.4 eV) and N 1s (399.2 eV) confirmed the polymer deposition as shown in Figure 2c. Energy-dispersive X-ray spectroscopy (EDX, Figure S4, Supporting Information) on the sample showed that boron signal was evenly detected, suggesting the electropolymerization occurred on all surface of the chip.

Unlike an earlier report which utilized PBA to recognize sialic acid on cancer cell surfaces,[33] we applied surface PBAs to form conjugates with the oligosaccharide residues on antibodies (i.e., anti-EpCAM) to capture CTC. We used quartz crystal microbalance experiments to confirm our central molecule scheme, bioconjugation, and sorbitol-stimulated release of antibodies on PBA-functionalized polymer. When the polymer is prepared completely from EDOT-PBA, we were able to achieve a density of 319 ± 8 ng cm^{-2} of the conjugated antibodies as shown in Figure 2d, but only 51 ± 0.01% of the antibodies were released in sorbitol solution (n = 3). This was most likely due to the nonspecific interactions between the polymer and antibodies, prohibiting the release of antibodies after the oligosaccharide–PBA binding was replaced by the stronger sorbitol–PBA interaction.[32,34] As mentioned earlier, introduction of EDOT-EG3 unit to the polymer limited the nonspecific absorptions of proteins and cells, thereby enhancing both the capture specificity and release yield. When EDOT-PBA

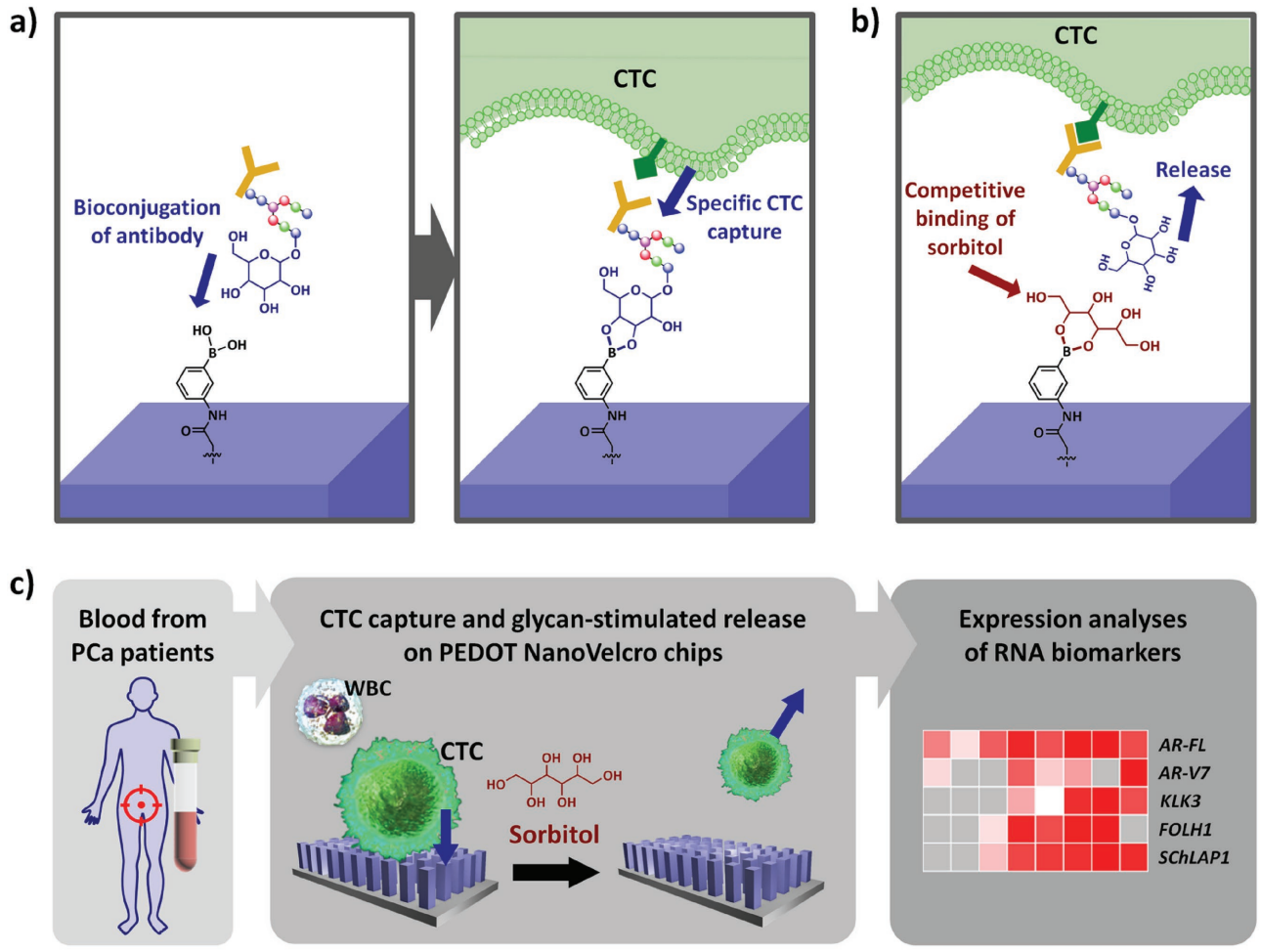

Figure 1. Illustration on the main research scheme. a) The mechanism for circulating tumor cell (CTC) capture is that the surface-grafted phenylboronic acid (PBA) conjugates with antibody, subsequently enabling specific CTC capture. b) The mechanism for CTC release is that the introduction of glycan with stronger affinity to PBA (i.e., sorbitol) results in competitive binding, allowing CTC release. c) Workflow on using this glycan stimulation enabled CTC purification platform on poly(3,4-ethylenedioxythiophene) (PEDOT) NanoVelcro chips for RNA biomarker analysis from purified CTCs of prostate cancer (PCa) patients.

and EDOT-EG3 were copolymerized, the surface density of conjugated antibodies correlated positively with the feed percentage of EDOT-PBA, and we noted a sharp increase of antibody release from below 60% to almost 100% with the feed percentage of EDOT-EG3 greater than 50% (Figure 2d).[32,34] Based on these results, the polymer composed from EDOT-EG3 and EDOT-PBA at 1:1 feed ratio showed optimal performance, combining high surface density of capture antibodies with almost complete release after sorbitol exposure (Figure S6, Supporting Information). This optimized composition was then used for the PBA-grafted NanoVelcro chip fabrication for in vitro cellular and clinical sample studies.

The synergistic effect of nanostructures and capture antibodies has been proven to enhance the CTC capture performance. We previously reported that 3D PEDOTs can successfully capture CTCs through biotinylated anti-EpCAM and streptavidin-modified interface with great efficiency.[21,22] Using the PBA-grafted PEDOT NanoVelcro chips, we also observed that anti-EpCAM-conjugated PEDOT NanoVelcro chip exhibited much higher capture efficiency (72.5 ± 3.0%, $n = 3$, $p < 0.0001$) for EpCAM-positive LNCaP cells, as compared to the nonconjugated PEDOT NanoVelcro chip, nonconjugated PEDOT (composed by poly(EDOT-PBA-*co*-EDOT-EG3)) film, and anti-EpCAM-conjugated PEDOT film (**Figure 3**a). As expected, the anti-EpCAM-conjugated PEDOT NanoVelcro also exhibited significantly higher capture efficiency ($p < 0.001$ for each comparison) for other EpCAM-positive PCa cells (75.2 ± 3.2% for PC3, and 67.8 ± 1.7% for 22Rv1, $n = 3$ for each cell line) compared to EpCAM-negative WBCs (0.01 ± 0.004%, $n = 3$, Figure 3b). When we optimized the capture efficiency by varying the incubation time, it plateaued at 60 min as shown in Figure 3c. The performance of CTC capturing remained consistent in spiking study (Figure 3d) within the tested range of 1–2000 PCa cells spiked into healthy human blood. The capture efficiency for 22Rv1 cells was constantly lower than that for LNCaP or PC3 cells, likely due to the technical difficulties in cell counting resulted from cell aggregation.

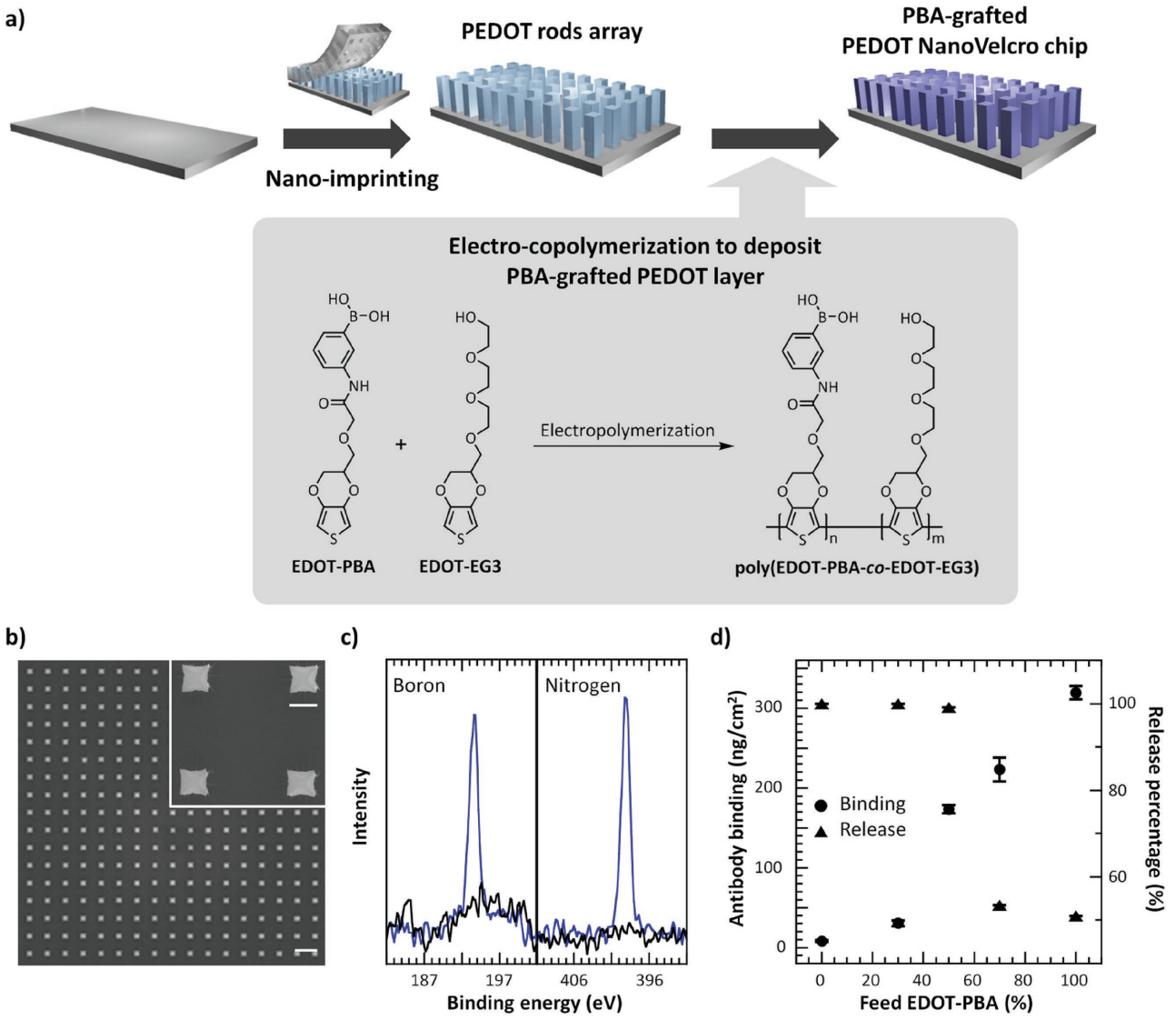

Figure 2. Materials synthesis and characterization of PBA-grafted PEDOT NanoVelcro chips. a) Schematic representation on the chip fabrication and electropolymerization to deposit the PBA-grafted poly(EDOT-PBA-*co*-EDOT-EG3). EDOT-EG3 was used to reduce nonspecific interactions for specific CTC capture and EDOT-PBA was used to introduce the grafted PBA groups. c) SEM images of the PEDOT rods array structure. The length of the scale bar in the image was 5 and 1 µm (enlarged), respectively. d) X-ray photoelectron spectra of nanoimprinted PEDOT rods array (black) and PBA-grafted PEDOT NanoVelcro chip (blue) in boron 1s and nitrogen 1s regions. e) Binding (circle) and sorbitol-stimulated release (triangle) of anti-EpCAM on top of poly(EDOT-PBA-*co*-EDOT-EG3) films examined by quartz crystal microbalance measurements ($n = 3$ for each data point).

After optimizing the capture performance, we continued to search experimental conditions for optimal cell release using sorbitol solution. As shown in Figure 3e, the cells could be released most efficiently with 0.5 M sorbitol solution ($p < 0.05$ in comparison to 0.25 M, $n = 3$). Although the release efficiency of 1 M sorbitol solution was not significantly lower than that of 0.5 M solution, cell damage was observed at concentration greater than 1 M, possibly due to the change in osmolality. Further optimization (Figure 3f) revealed that the highest release efficiency (95%) and cell viability (96%) were achieved by incubating the cells in 0.5 M sorbitol solution for 30 min. Incubation for more than 30 min did not increase the release efficiency significantly but decreased the cell viability, possibly due to the prolonged exposure to unfavorably high osmotic environment. In order to examine the PBA-grafted PEDOT NanoVelcro chips for CTC enrichment in samples from cancer patients, we incubated an artificial sample of 200 LNCaP cells spiked into 1 million WBCs on the chip. As shown in the representative experiment, only 3141 cells were captured after the chip was washed to remove unbound cells (Figure 3g). Among these cells, 141 were LNCaP cells (71% of the original LNCaP cell population) compared to 3000 WBCs (0.3% of the original WBC population). The differential binding affinity could be attributed to the introduction of cell-resisting EDOT-EG3 monomer, which decreased the binding of WBCs, and the use of capture antibodies, which specifically captured LNCaP cells.

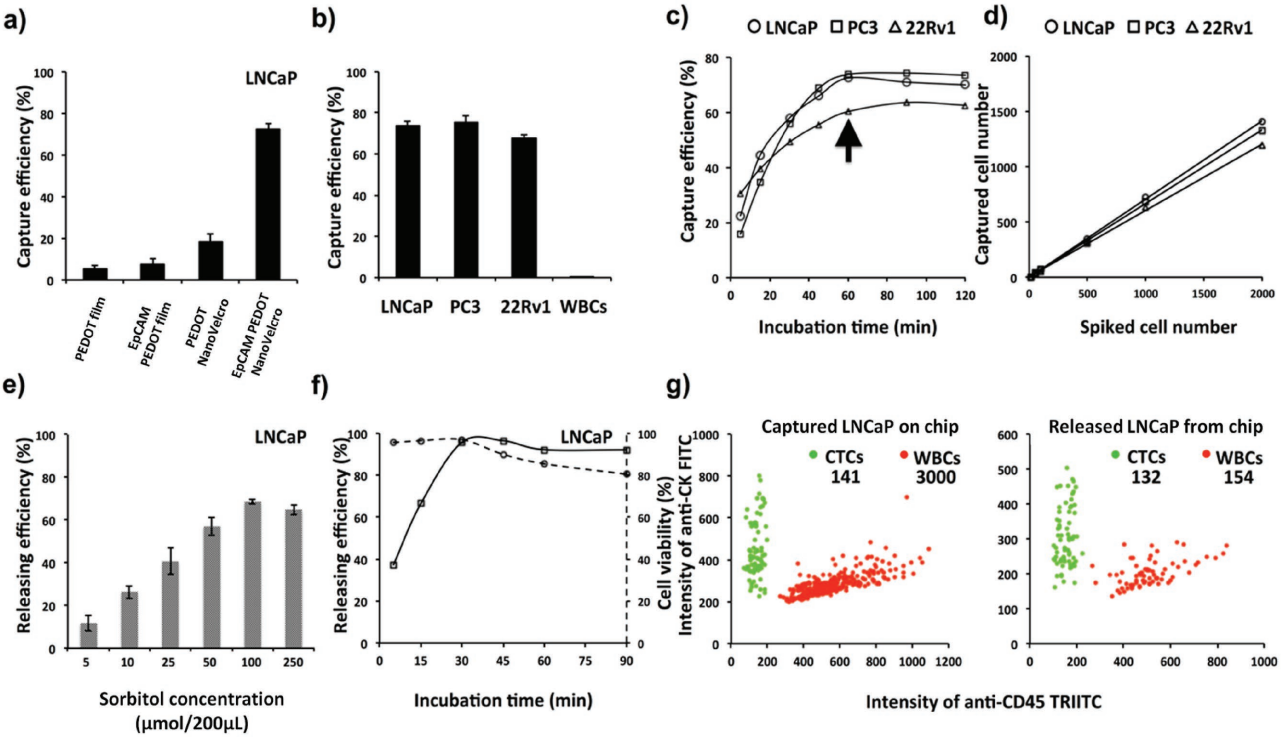

Figure 3. Optimization of PEDOT NanoVelcro chips based CTC purification assay. a) Synergistic effect of anti-EpCAM and PEDOT NanoVelcro chips largely enhanced the capture efficiency of prostate cancer (PCa) cells. The anti-EpCAM-conjugated chip (labeled as EpCAM PEDOT NanoVelcro) yielded the highest capture efficiency for EpCAM-positive LNCaP cells ($n = 3$). b) Capturing efficiency of anti-EpCAM-conjugated PEDOT NanoVelcro chips is significantly higher for EpCAM-positive PCa cells (LNCaP, PC3, and 22Rv1) than that for EpCAM-negative cells (white blood cells, WBCs) ($n = 3$ for each group). c) Capturing efficiency on the new PEDOT NanoVelcro was dependent on the incubation time. The efficiency for PCa cell lines (LNCaP, PC3, and 22Rv1) plateaued at 60 min. d) Capturing efficiency remained constant at different cell number ranging from 1 to 2000 cells. Regression analysis showed linear correlation with capturing efficiency consistent with (c). e) Releasing efficiency of PEDOT NanoVelcro chips was dependent on sorbitol concentration. The highest releasing efficiency occurred at 100 μmol/200 μL (0.5 M) ($n = 3$). f) Incubation time with sorbitol affected the releasing efficiency of PEDOT NanoVelcro chips and the cellular viability. An incubation time of 30 min provided optimal cellular viability (95%) and releasing efficiency (96%). g) Scatter plot showed the composition of cells captured on PEDOT-NanoVelcro substrates during a representative spiking study with 200 LNCaP cells spiked into 1 million WBCs. Three-color immunocytochemistry method based on FITC-labeled anti-Cytokeratin, TRITC-labeled anti-CD45, and DAPI nuclear staining was applied to identify and enumerate CTCs from nonspecifically trapped WBCs. (Left) After cell capturing process, LNCaP cells were captured by an efficiency of 71% compared to 0.3% for WBCs. (Right) After incubating with sorbitol, 94% of LNCaP could be released compared to 5.1% for WBCs. The purity of cancer cells was enhanced from 0.02 to 46% after the capturing-releasing process.

Upon the treatment with 0.5 M sorbitol, 132 captured LNCaP cells (94% of the captured LNCaP population) were released together with 154 nontargeted WBCs (5% of the captured WBC population). The most advantageous feature of this PBA-grafted PEDOT NanoVelcro chip is that within a single capturing and releasing cycle, the purity of the CTC enhanced from the original 0.02 to 46%. The cells showed 98% viability on-chip and 95% after stimulated release from the PEDOT NanoVelcro chip (Figure S7, Supporting Information). The increased purity and high cell viability would be beneficial for the subsequent molecular and biochemical analysis.

Using the PBA-grafted PEDOT NanoVelcro chips under operating conditions optimized from the aforementioned studies, we purified CTCs from clinical blood samples and measured the expression of PCa-related RNA biomarkers. Samples were obtained from 17 PCa patients (13 metastatic and 4 localized) and 7 healthy individuals with no known malignancies. The methods for reverse transcription (RT) and quantitative PCR (qPCR) were developed using serially diluted PCa cell lines to ensure the linear correlation (Figure S9, Supporting Information). The expression level of each PCa-related biomarker was normalized against that of house-keeping gene *ACTB*. Enumeration of CTCs was performed in parallel to confirm the presence of CTCs.

Expression data for five PCa-related biomarkers from all 24 subjects were summarized in **Figure 4**a. Every patient ($n = 17$) had at least one CTC upon enumeration (Figure S10, Supporting Information). We detected PCa-related biomarkers in CTCs from 94% of patients (16/17). Consistent with previous reports,[35–37] the expression of *KLK3* (prostate-specific antigen, PSA) was seen in 77% of metastatic patients (10/13) as compared to only 25% of the nonmetastatic PCa patients (1/4) (Figure 4b). The expression of *FOLH1* and *SChLAP1* was detected exclusively in metastatic PCa patients. *FOLH1* expression in CTCs was detected in 85% of metastatic PCa patients (11/13) (Figure 4c), and *SChLAP1* was detected in 92% of the same patient group (12/13) (Figure 4d). This is the first demonstration of *SChLAP1* expression in CTCs. Our

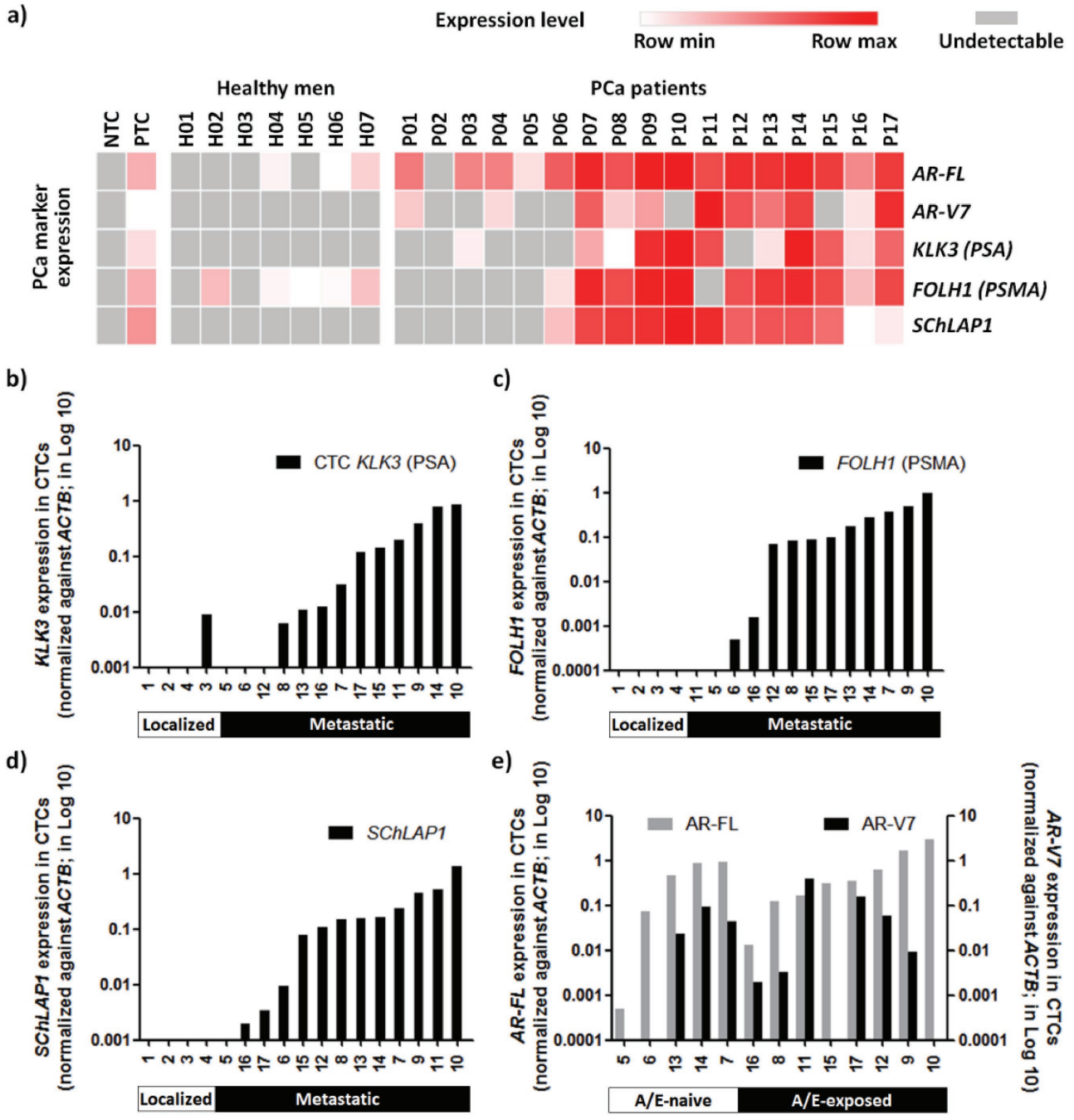

Figure 4. Detection of PCa-related RNA signatures in CTCs purified by PEDOT NanoVelcro Chips. a) Summary of RNA signature detection in 7 healthy men and 17 PCa patients. PTC = positive control (RNA from 100 LNCaP cells + 100 22Rv1 cells in 1 million WBCs from a healthy man purified by PEDOT-NanoVelcro Chips); NTC = negative control (nuclease free water). b) *KLK3* gene expression in CTCs. The expression of *KLK3* gene was more commonly seen in metastatic PCa with 77% (10/13) of the metastatic patients showing positive signal compared to only 25% (1/4) nonmetastatic PCa patient having detectable expression. c) *FOLH1* gene expression in CTCs was only detected in metastatic PCa patients with 85% (11/13) of the metastatic PCa patients having detectable expression. d) *SChLAP1* expression in CTCs was only detected in metastatic PCa patients with 92% (12/13) of the metastatic PCa patients having detectable expression. e) Expression of *AR-FL* and *AR-V7* in CTCs. *AR-V7* can be detected in 6/8 metastatic PCa patients who had been exposed to abiraterone (A) or enzalutamide (E), and was accompanied with the expression of *AR-FL*. Some (3/5) patients who had not received A/E treatment also showed positive *AR-V7* signals. All expression levels were normalized against *ACTB*.

results suggest that *SChLAP1* detection in CTCs may have a similar role as that in primary tumor tissues. Figure 4e depicted wild-type *AR* (AR-FL) expression and *AR-V7*. Consistent with prior observation, *AR-V7* was always detected in the presence of *AR-FL*. Our results showed *AR-V7* in 75% of the patients (6/8) who had been exposed to abiraterone and/or enzalutamide. Interestingly, in the abiraterone and enzalutamide naïve patients, *AR-V7* was seen in 60% of subjects

(3/5). Our detection rate for *AR-V7* was higher than some previous reports though others have reported even higher rates.[38] This reflected differences in the CTC enrichment approach (PEDOT-NanoVelcro Chips vs no enrichment) and methodology of *AR-V7* detection (direct RNA detection via qRT-PCR vs antibody-mediated immunofluorescence staining). Other factors such as the exposure to other anticancer treatments after AR-targeted therapy may have affected the expression of *AR-V7* in CTCs as well.[39] Notably, we also detected *AR-V7* in patients with localized PCa. The clinical significance of *AR-V7* in these patients has yet to be determined. The *AR-V7*(+) patients in this study continue to be monitored for the development of resistance. Further studies are required to validate the sensitivity and clinical utility of this assay.

In summary, we introduce an innovative approach for CTC purification using a new PBA-grafted PEDOT NanoVelcro chip, integrating the advantageous features of nanoimprinted 3D PEDOT rods array, which demonstrates the ability to enhance CTC capture, and a poly(EDOT-PBA-*co*-EDOT-EG3) layer, which not only provides highly selective CTC capture by conjugation with antibodies through phenylboronic-acid-oligosaccharide binding but also enables competitive glycan binding to release the captured cells. As a result, this PEDOT NanoVelcro chip is able to achieve high cell purity as well as preserve the integrity of RNA transcripts from these purified cells. We further demonstrate the feasibility of this approach to detect disease-related RNA signals by measuring the expression level of several PCa-specific biomarkers in purified CTCs, including *AR-FL*, *AR-V7*, *KLK3*, *FOLH1*, and *SChLAP1*. This capacity provides important opportunities for clinical correlation.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

circulating tumor cells, poly(3,4-ethylene-dioxythiophene)s, prostate cancer, responsive materials, RNA biomarkers

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Review

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

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Abstract

Liquid biopsy of tumor through isolation of circulating tumor cells (CTCs) allows non-invasive, repetitive, and systemic sampling of disease. Although detecting and enumerating CTCs is of prognostic significance in metastatic cancer, it is conceivable that performing molecular and functional characterization on CTCs will reveal unprecedented insight into the pathogenic mechanisms driving lethal disease. Nanomaterial-embedded cancer diagnostic platforms, i.e., NanoVelcro CTC Assays represent a unique rare-cell sorting method that enables detection isolation, and characterization of CTCs in peripheral blood, providing an opportunity to noninvasively monitor disease progression in individual cancer patients. Over the past decade, a series of NanoVelcro CTC Assays has been demonstrated for exploring the full potential of CTCs as a clinical biomarker, including CTC enumeration, phenotyping, genotyping and expression profiling. In this review article, the authors will briefly introduce the development of three generations of NanoVelcro CTC Assays, and highlight the clinical applications of each generation for various types of solid cancers, including prostate cancer, pancreatic cancer, lung cancer, and melanoma.

Key words: Circulating tumor cell

Circulating tumor cell (CTC)

Pathologic evaluation remains the gold standard for diagnosis and prognosis in the care of cancer patients. This approach typically relies on the tissue specimens obtained by surgical excision or radiographically guided biopsy. While tremendous amounts of information can be obtained from tissues, including histopathology and molecular signatures, this approach has several disadvantages. First, the procedures to obtain tissues are both invasive and costly. The risk of morbidity and psychological stress on the patients largely limit the feasibility of invasive procedures. Moreover, it has been technically challenging to biopsy lesions of certain cancer types or at certain locations, for instance, the osteoblastic metastasis in prostate cancer. Finally, recent studies

showing temporospatial heterogeneity [1-7] within a tumor raise serious concerns about how accurately a single biopsy represents a cancer that is spatially heterogeneous and evolves over time.

As an alternative to solid tumor biopsy, many propose the use of a "liquid biopsy" based on circulating tumor cell (CTC) sampling and are actively developing CTC capture techniques.[8] CTCs are rare tumor cells shed from all present disease sites that have active blood perfusion, including primary and metastatic tumors. Sampled through phlebotomy, CTCs can be obtained easily throughout the course of cancer; even during the late stages of metastatic disease without needing invasive and complex traditional biopsy procedures. The ability of serial CTC sampling performed over the course of disease offers the opportunity for real-time, dynamic monitoring of the disease evolution.[9, 10] Over the past decade, collaborative and interdisciplinary research groups including chemistry, material science, bioengineering, cancer biology, and oncology have been formed to focus their efforts upon CTC detection, isolation, and characterization.[11] These collaborative scientific endeavors have led to many important studies setting the foundation for the realization of CTCs to function as a liquid biopsy. Initial studies focused on enumeration [12-14] while recently, some groups have begun to show genomic[15-17] and transcriptomic[18, 19] similarities between CTCs and the traditional tumors biopsies. More and more evidence is supporting the use of CTCs for investigating the nature of cancer, guiding therapeutic interventions, and assessing emerging resistance.

Conventional CTC assays

The most widely used CTC detection assays include: (i) Immunomagnetic separation: these methods utilize capture agent-labeled magnetic beads to either positively select [13, 20, 21] CTCs targeting their surface markers (e.g., epithelial cell adhesion molecule [EpCAM]) or negatively deplete [22, 23] white blood cells (WBCs) using anti-CD45. The CellSearch™ Assay [12-14] is the only FDA-cleared CTC diagnostic technology for metastatic breast, prostate, and colorectal cancers. This assay harvests CTCs with anti-EpCAM-coated magnetic beads, and the subsequent immunocytochemistry (ICC) process helps to identify CTCs (DAPI+/cytokeratin, CK+/CD45-) from nonspecifically captured WBCs (DAPI+/CK-/CD45+). Recently, several new systems (e.g., MagSweeper[24], IsoFlux[25], Cynvenio,[26] magnetic sifters,[27] VerIFAST[28] and AdnaGen/Qiagen[29]) have been developed to further improve detection speed and efficiency. (ii) Flow cytometry: In conjunction with the use of fluorescent markers, flow cytometry [30, 31] is one of the most mature technologies for analyzing and sorting subpopulations of cells. However, this flow-based methodology often has limited detection power due to the low abundance of CTCs, and is unable to provide the CTCs' morphological information. An improved method, known as ensemble-decision aliquot ranking (eDAR),[32, 33] was developed to address this weakness. (iii) Microscopy imaging: Microscopy imaging [34-36] of ICC-treated blood samples allows for highly sensitive detection of CTCs, accompanied with their morphometric characteristics and protein expression. Currently, Epic Sciences is one of the leaders in the commercial sector, now providing Clinical Laboratory Improvement Amendments (CLIA)-certified laboratory tests for both CTC enumeration and characterization. In contrast to the previous three approaches, which require the use of CTC markers, the following two approaches are recognized as label-free methods. (iv) CTC filters: Filter-based approaches [37-41] have been established to trap CTCs according to their sizes. A wide collection of commercial kits/systems from Clearbridge,[39, 40] Rarecells,[42] ScreenCell,[43] and Creatv MicroTech etc. are now available to support research utility. Nevertheless, concerns regarding overlooking small-sized CTCs have been raised. (v) Dielectrophoresis: CTCs can be sorted from WBCs in the presence of a dielectrophoretic field, since the CTC's dielectric properties (depending on their diameter, membrane area, density, conductivity and volume) are different from those of WBCs. ApoCell's technology[44] leverages these differences in a microfluidic flow channel to isolate CTCs. Silicon Biosystems' DEPArray™ combines the use of microscopy imaging and dielectrophoresis sorting[45] to identify and isolate pre-sorted CTCs, paving the way for downstream single-CTC molecular characterizations. (vi) Others: Several reviews [46-48] also summarized a wide collection of CTC detection technologies which may not be included in this article.

Microfluidics-enabled CTC assays

The microfluidic affinity-capture devices [49] developed by Toner et al. sparked the recent research efforts focused on the development of nanotechnology-enabled CTC assays. This 1st-generation (gen) device [49] (i.e., CTC-Chip) featured chemically etched microposts on a silicon substrate, on which anti-EpCAM antibodies were covalently functionalized. These embedded microposts were designed to maximize the contact between the device surfaces and the flow through

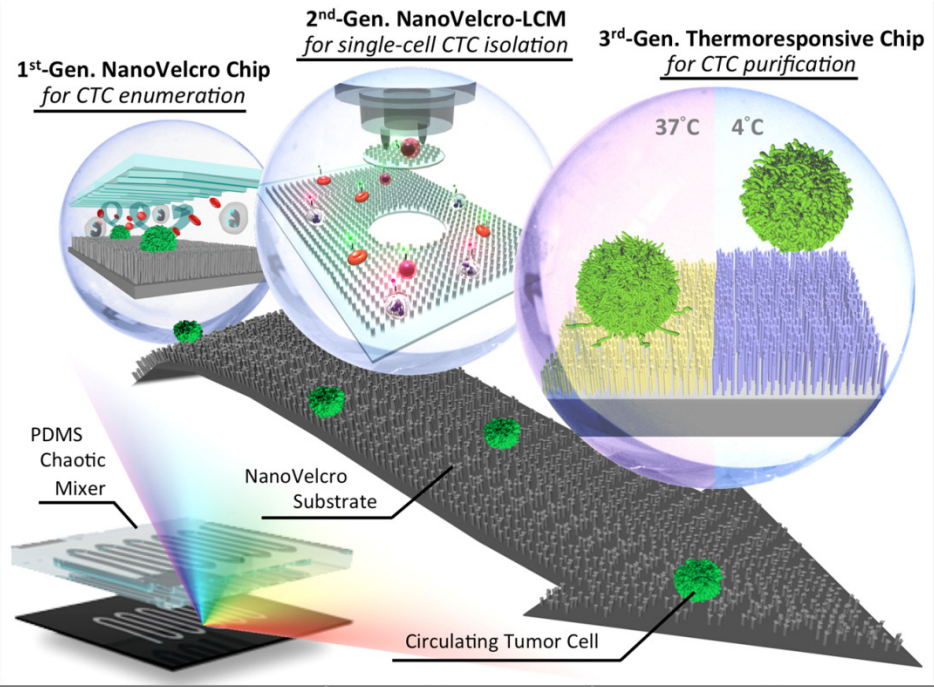
cells. Following CTC capture, ICC was conducted to identify CTCs. The CTC-Chips demonstrated significantly more success in enumeration performance than most of the conventional CTC assays. Thereafter, similar device configurations were adapted to create new microfluidic chips (e.g., geometrically enhanced differential immunocapture, GEDI [50] approach and Biocept's CTC assay [51]), and different antibody capture agents were employed. Recently, a unique "Ephesia" approach [52] based on microposts of capture agent-coated magnetic beads self-assembled in a microchip demonstrated combined advantages of both microfluidic and immunomagnetic cell sorting. The 2nd-gen device [53] (i.e., herringbone-chip, HB-Chip) from the same group was made from an imprinted polydimethylsiloxane (PDMS) component on a glass slide. Microscale herringbone patterns were engineered into the PDMS component to introduce microvortices, leading to enhanced contact between the CTCs and the antibody-coated chip surfaces. In addition to the commonly used ICC technique, the transparent nature of the HB-Chip allowed for imaging of the captured CTCs by standard clinical histopathological stains (i.e., haematoxylin and eosin stain). Although the microfluidic setting improves CTC-capture performance, the majority of the microfluidic CTC assays suffers from depth of field issues when performing microscopy imaging due to the vertical depths of 3-dimensional device configurations. Time-consuming multiple cross-sectional imaging scans that generate large image files are required in order to avoid out-of-focus or superimposed micrographs. By coupling a pair of microelectrodes at the terminal of a plastic microfluidic chip[54], enzymatic release of the captured CTCs can be electrically counted without the issue of microscopy imaging. In contrast to their 1st and 2nd-gen devices, their 3rd-gen iChip[55] represents a groundbreaking label-free approach, which combines negative immunomagnetic depletion processes with an inertial focusing setting in an integrated microchip. Most importantly, this approach allows for the recovery of unmanipulated CTCs with desired molecular integrity and viability, allowing for downstream expressional profiling[18], as well as *ex vivo* culture and drug susceptibility testing[56]. The sorting mechanism of iChip, however, was recently reported to compromise the isolation of CTC clusters, which potentially contain CTCs with high metastatic potential.[57] "Cluster-Chip", a microchip that can be used individually or in conjunction with CTC-iChips to isolate CTC clusters, was developed to address this issue.[58] Other microfluidic CTC assays based on unique principles, including micro-nuclear magnetic resonance (μNMR) platform[59], cell rolling[60], supported lipid bilayer (SLB)-coated microfluidic devices[61], and Vortex technology[62, 63] have also been developed and demonstrated. In addition to the microfluidic assays developed for the enumeration, molecular characterization, and *ex vivo* expansion of CTCs, a sectioned microfluidic device (known as the Velocity Valley Chip) that selectively captures CTCs in a manner dependent on the number of magnetic beads grafted on the surface of a given CTC [64] has been designed. This device was employed to separate CTCs into subpopulations by EpCAM expression of individual CTCs. Overall, microfluidic technology has shown its potential in enriching and isolating CTCs amenable for subsequent molecular and functional characterizations.

NanoVelcro CTC Assays: Three generations of development

Recent advances in the field of nanotechnology offer powerful solutions [65-67] resulting in a wide range of in-depth characterizations of CTCs while drastically reducing costs. Ultimately, deployment of these emerging advances will bring oncology closer to the goal of personalized care. It has long been recognized that there are nanoscale components present in the tissue microenvironment, including the extracellular matrix, and the cellular membrane. These provide structural and biochemical support that regulate cellular behavior and fate. Inspired by the nanoscale interactions observed in the tissue microenvironment, Dr. Tseng's research team at UCLA pioneered the development of "NanoVelcro" cell-affinity substrates [68, 69]. In this unique approach, capture agent-coated nanostructured substrates are utilized to immobilize CTCs with high efficiency. The working mechanism of NanoVelcro cell-affinity substrates mimics that of Velcro™ – when the two fabric strips of a Velcro fastener are pressed together, interactions between the hairy surfaces on two strips leads to strong affinity between cells and nanosubstrates. In addition to the silicon nanowire substrate (SiNS)[68], the general applicability of the NanoVelcro cell-affinity assay is supported by extensive research endeavors devoted to exploiting different nanomaterials, e.g., polymer dots[70]/nanopillars[71], TiO_2 nanowires[72]/nanoparticles[73], layer-by-layer-assembled nanostructures[74], gold clusters on silicon nanowires[75], Fe_3O_4 nanoparticles[76], DNA networks[77], and graphene oxide nanosheets[78] to achieve high affinity capture of CTCs and other types of rare cells. In parallel, the team has also established a 3-color ICC protocol[79] using DAPI, anti-CD45, and anti-CK to

identify nanosubstrate-immobilized CTCs. Single-cell image cytometry data covering DAPI staining, CK/CD45 expression and object size can be used to distinguish CTCs (DAPI+/CK+/CD45-, sizes>6 μm) from nonspecifically captured WBCs (DAPI+/CK-/CD45+, sizes<12 μm), and cellular debris. With the initial proof-of-concept demonstration of the NanoVelcro substrates and ICC protocol in place, three generations of NanoVelcro CTC Chips have been established[69] (**Figure 1**) to achieve different clinical utilities.

The 1st-gen NanoVelcro Chip [9, 80], composed of a SiNS and an overlaid microfluidic chaotic mixer, was created for CTC enumeration. The performance (>85% of CTC capture efficiency) of these NanoVelcro Chips was measured using artificial CTC samples. Side-by-side analytical validation studies using clinical blood samples show that the sensitivity of the 1st-gen NanoVelcro Chip exceeds [9] that of the FDA-approved CellSearch™ Assay. Notably, the NanoVelcro-like approach allows immobilization of CTCs onto a flat and small surface, thus facilitating the implementation of subsequent high-resolution immunofluorescence microscopy imaging of CTCs without multiple cross-sectional imaging scans required for the majority of the existing microfluidic CTC assays. Moving beyond CTC enumeration, the 2nd-gen NanoVelcro Chips[16, 81, 82], known as NanoVelcro-LCM approach were developed by replacing SiNS with a transparent substrate covered with nanofibers made of PLGA, i.e., poly(lactic-co-glycolic acid). The transparent PLGA



	1st-Gen.	2nd-Gen.	3rd-Gen.
Mechanism	The combined use of **capture agents** and **embedded nanostructures** increases the affinity between CTC		
	Overlaid chaotic mixer introduces helical flows in microchannels, enhancing contact frequency between CTCs and nanosubstrates		
		LCM technique enables single-CTC identification and isolation	**Temperature-dependent conformational changes of polymer brushes** alter the accessibility of capture, allowing for capture and release of CTCs
Capacity & Utility	**CTC enrichment** and **morphologic analysis**		**Temperature-dependent purification** to recover CTCs with high viability and molecular intactness
	CTC enumeration for cancer prognosis, staging, and dynamic monitoring	Single-CTC isolation, followed by molecular analysis	**Ex vivo expansion** of purified CTCs

Figure 1. Conceptual illustration of three generations of the NanoVelcro CTC Assays developed by the UCLA team to achieve different clinical utilities. 1st-Gen NanoVelcro Chip [9, 80], composed of a silicon nanowire substrate (SiNS) and an overlaid microfluidic chaotic mixer, was created for CTC enumeration. In conjunction with the use of the laser capture microdissection (LCM) technique, 2nd-gen NanoVelcro-LMD technology [16, 81, 82], was developed for single-CTC isolation. The individually isolated CTCs can be subjected to single-CTC genotyping. By grafting thermoresponsive polymer brushes onto SiNS, 3rd-gen Thermoresponsive NanoVelcro CTC Chips [83, 84] were developed for purification of CTCs via capture and release of CTCs at 37°C and 4°C, respectively. The surface-grafted polymer brushes were responsible for altering the accessibility of the capture agent on NanoVelcro substrates, allowing for rapid CTC purification with desired viability and molecular integrity. (Reprinted with permission from Tseng 2014, Copyright, American Chemical Society). We compare the performance and differences of the three generations of NanoVelcro CTC Assays in a table.

NanoVelcro substrate retains the desired CTC capture performance, and allows for seamless integration with a laser capture microdissection (LCM) technique to isolate immobilized CTCs with single-cell resolution. The individually isolated CTCs can be subjected to single-CTC genotyping (both Sanger sequencing [82] and next-generation sequencing [NGS][16, 81]) to serve as liquid biopsies. In order to increase throughput, lower labor, and address the need for viable/unfixed CTCs, the UCLA team developed the 3rd-gen Thermoresponsive NanoVelcro Chips [83, 84] and demonstrated the ability to capture and release viable CTCs at 37°C and 4°C, respectively. By grafting thermoresponsive polymer brushes[83] (poly(N-isopropylacrylamide, PIPAAm) onto SiNS via atom transfer radical polymerization, the temperature-dependent conformational changes of polymer brushes can effectively alter the accessibility of capture agents on SiNS, allowing for rapid CTC purification with desired viability and molecular integrity. The advent of the 3rd-gen Thermoresponsive NanoVelcro Chips is expected to open up new opportunities to connect with a wider range of molecular and functional assays. The continuous research endeavors put together by the UCLA team and its clinical collaborators have demonstrated the use of NanoVelcro CTC assays in clinical settings to facilitate the concept of CTC-based liquid biopsy. These results are briefly summarized in this review article.

Enumerating CTCs using 1st-gen NanoVelcro CTC Assay

Given the CTC detection performance observed for the 1st-gen NanoVelcro CTC Assay [80], continuous efforts were devoted to test its utility for CTC detection in different solid tumors in conjunction with the use of combined capture and ICC antibodies. The initial clinical studies focused on prostate cancer with the intention to address the issue that CellSearch™ assay is unable to detect CTCs in a large portion of late stage prostate cancer patients [14]. These clinical validation studies [9] were jointly conducted by Urologic Oncology teams at Ronald Reagan UCLA Medical Center and Cedars-Sinai Medical Center (CSMC). Forty prostate cancer patients (32 with metastatic disease and 8 with localized disease) were recruited. CTCs were identified in all 40 patients, indicating a consistent efficiency of 1st-gen NanoVelcro Assay for CTC enumeration in prostate cancer patients across different stages of disease. The team also performed serial enumeration allowing the comparison of CTC number changes after 4-10 weeks of therapy, and observed a statistically significant reduction in CTC counts in the clinical responders. Further, long-term follow ups were also performed for 460 days with serial CTC collection and enumeration. In this case, CTC numbers faithfully represented the initial response and subsequent failures during nilutamide and sipuleucel-t treatment. This study demonstrates the consistency of the 1st-gen NanoVelcro CTC Assay over time for CTC enumeration, and shows that continuous monitoring of CTC numbers can be employed to follow responses to different treatments and monitor disease progression.

In addition to the prostate cancer application described above, the pancreatic cancer research team at Ronald Reagan UCLA Medical Center investigated [85] the feasibility of applying NanoVelcro Enumeration Assay to detect CTCs in patients with pancreatic ductal adenocarcinoma (PDAC). The goal is to explore the use of CTCs as an adjunctive biomarker at the time of PDAC presentation. Venous blood was collected prospectively from 100 consecutive, pre-treatment PDAC patients. Utilizing the 1st-gen NanoVelcro CTC Chips, samples were evaluated for the presence and number of CTCs. CTC enumeration data was then evaluated as a diagnostic and staging biomarker for PDAC. Evaluation of CTCs as a diagnostic 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 discriminate between local/regional and metastatic disease. Together, our results highlight the utility of CTCs as a liquid biopsy to better inform diagnosis and staging of PDAC, importantly, at the time of disease presentation.

Incremental improvement of the 1st-gen NanoVelcro CTC Assays has led to a user-friendly system and protocol allowing convenient CTC enumeration studies at different facilities. In preparation for its FDA 510K clinical study, the latest version of NanoVelcro CTC Enumeration Assay (**Figure 2**) has been subjected to a series of studies, including calibration/interference tests using two different cancer cell lines (MCF7 and PC3), duplicated studies using clinical samples from pancreatic cancer and liver cancer patients, and side-by-side comparison with CellSearch™ Assay using clinical samples from more than 100 prostate cancer patients. These data have shown that the NanoVelcro Assay is sufficiently reproducible for 510K trials.

With continuous accumulation of CTC enumeration data, the UCLA/CSMC team has amassed a cohort of prostate cancer patients followed with serial CTC counts by NanoVelcro and CellSearch™ assays that are annotated with patient clinical data. These studies show parallels and differences in the output of these two assays. **Figure**

3a shows a comparison of the enumeration results of CellSearch™ assay and NanoVelcro assay from more than 100 samples collected from metastatic castration-resistant prostate cancer (mCRPC) patients. In this patient cohort, the NanoVelcro CTC Assay detected counts ranging from 0-98 cells per 7.5 mL of blood, in comparison to CellSearch™ that yielded 0-905 counts per 7.5 mL of blood. In most patients, changes in both NanoVelcro and CellSearch™ CTC counts reflected disease progression – counts decreased immediately after the initiation of anti-cancer therapies. Similarly, counts rose at the time of disease progression (**Figure 3b**). Both CellSearch™ and NanoVelcro assays capture cells based on EpCAM expression and identify putative CTCs based on CK and CD45 fluorescence signals. However, the differences in each system, such as the use of nanostructured substrate and different imaging modality may contribute to the variation in the dynamic range of the assay. Higher resolution fluorescence microscopy in CTC detection suggests that certain white blood cells may be falsely counted as CTCs given potential CK false-positivity. This would result in unusually high CTC counts, as many research teams have observed (**Figure 3c**).

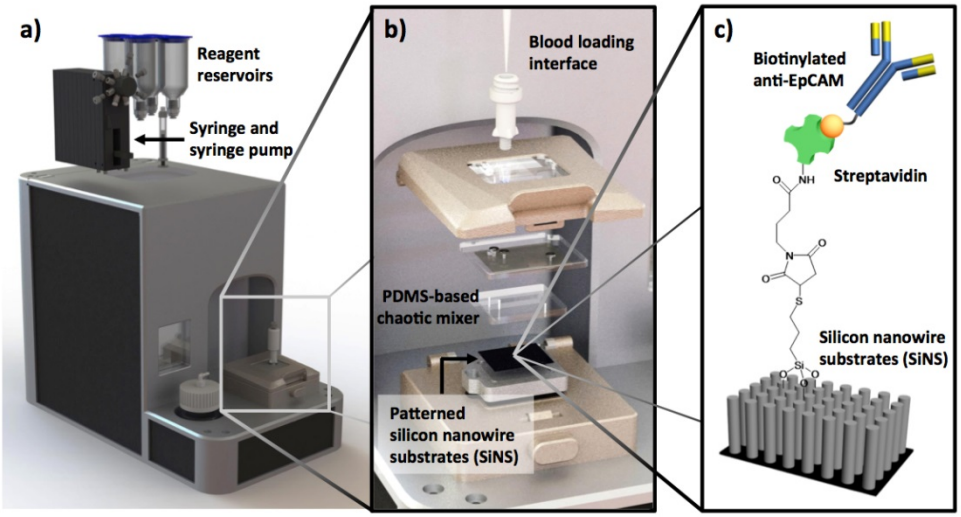


Figure 2. The latest version of NanoVelcro CTC Enumeration Assay is performed using (a) a fluidic handler that has been designed and fabricated to introduce processed blood samples into NanoVelcro Chips. (b) Engineering design of NanoVelcro Chips, in which the mechanical click-on approach allows instant assembly of the device. (c) Silanation reaction and NHS chemistry were employed to covalently link streptavidin onto the SiNW substrate, allowing conjugation of biotinylated anti-EpCAM prior to CTC detection studies.

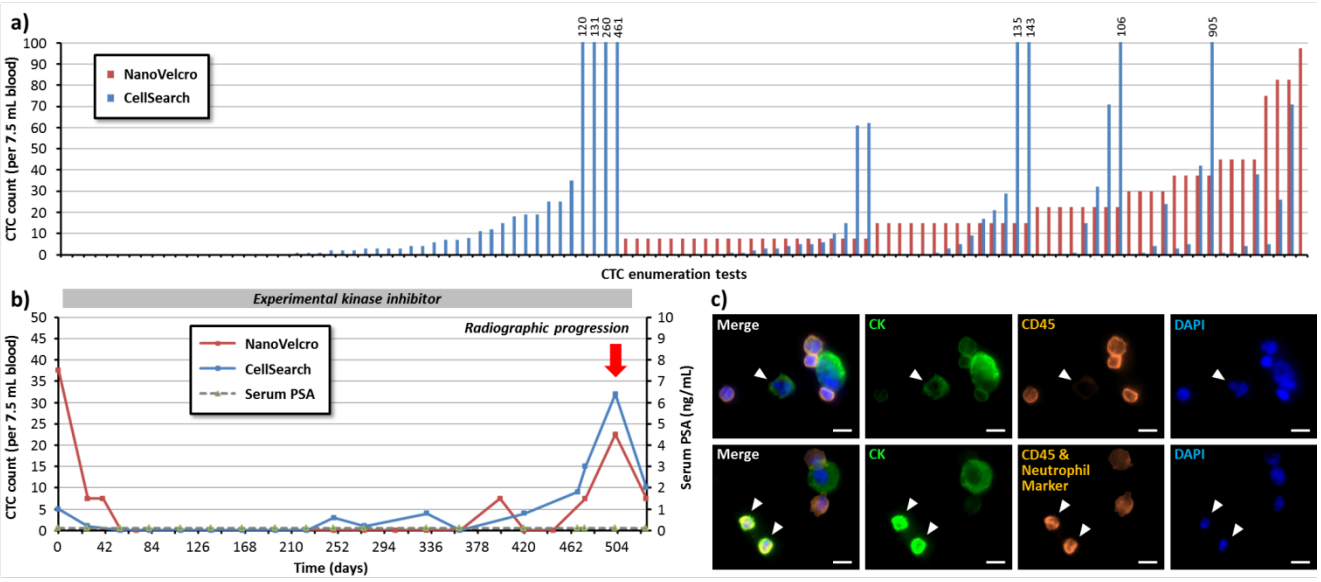


Figure 3. Comparison of NanoVelcro and CellSearch™ Assays in CTC enumeration for metastatic castration-resistant prostate cancer (mCRPC) patients. More than 100 contemporary CTC enumeration results from NanoVelcro and CellSearch™ Assays are shown in (a). The data demonstrated parallels and differences in the output of these two assays. The NanoVelcro CTC assay detected counts ranging from 0-98 cells per 7.5 mL of blood, in comparison to CellSearch™ that yielded 0-905 counts per 7.5 mL of blood. (b) A typical case showing the NanoVelcro and CellSearch™ counts in reflection of patient's disease progression. Cell counts decreased immediately after the initiation of anti-cancer therapies, and rose at the time of disease progression. (c) (Upper row) some of cells present with strong CK fluorescence signal and weak CD45 signal (arrowheads), but could be identified as WBCs with fluorescence staining neutrophil markers (lower row), suggesting the existence of false positive events that may contribute to the unusually high cell counts.

Applying conventional cytopathology standards to morphologic analysis may help eliminate the false-positive events. To further address this issue, more studies are needed to understand the assay and the nature of CTCs. Meanwhile, larger scale clinical trials with parallel tests and head-to-head comparison have to be conducted to validate the performance of the CTC assay. Careful subgroup analysis must be performed to identify the factors that may have affected the results. The UCLA/CSMC team thus explored the use of the NanoVelcro CTC Assay for studying CTC subpopulations in prostate cancer patients.

Subclassification of Prostate Cancer CTCs

Over the past 3 years, the UCLA/CSMC team has performed CTC analysis using NanoVelcro CTC Assay in conjunction with fluorescence microscopy[79, 86] that enabled visualization of cellular features of the captured CTCs and pathologic review for cellular morphology and nuclear size. In their initial observational study, the team analyzed serial blood specimens collected from men with various disease states ranging from localized prostate cancer to advanced metastatic castration-resistant disease. A mathematical modeling and unsupervised clustering of CTC nuclear size distribution identified 3 distinct subsets of CTCs, i.e., very-small-nuclear CTCs (vsnCTCs, nuclear size < 8.54 μm), small-nuclear CTCs (snCTCs, nuclear size between 8.54 μm and 14.99 μm) and large-nuclear CTCs (lnCTCs, nuclear size > 14.99 μm). snCTCs and vsnCTCs seem to appear in metastatic prostate cancer patients, while vsnCTCs occurred predominantly in patients with metastasis in visceral organs such as the liver or lungs, and vsnCTC counts were significantly higher in patients with visceral lesions compared to those without. The UCLA/CSMC team also found that vsnCTCs emerge prior to appearance of visceral metastasis on clinical imaging.[87]

As the UCLA/CSMC team further expand the investigation for this phenomenon in a bigger patient database, they identified 28 metastatic prostate cancer patients who had progressed through next generation hormonal maneuvers such as abiraterone, enzalutamide, or an equivalent drug. Serial blood specimens were used retrospectively for CTC enumeration and subgroup analysis (**Figure 4**). Fifteen out of 28 patients presented with visceral lesions and 13 had bone-only disease at their first CTC enumeration. Six out of 13 non-visceral metastatic patients developed visceral lesions during follow-up, and vsnCTCs were detected 86-196 days prior to radiographic detection of the visceral lesions. Four patients had vsnCTCs detected without the presence of visceral lesions by the time of this analysis, but the UCLA/CSMC team is still following some of them for visceral progression in the future. Overall, vsnCTCs were detected in all the patients with visceral metastasis, and none of the patients without vsnCTCs developed visceral metastasis in this study.

Aside from the potential predictive utility of vsnCTCs, the UCLA/CSMC team also analyzes the relationship between vsnCTCs and patients' response to therapeutic interventions. The team observed reduction of vsnCTC count occurred at initiation of anti-cancer treatment. Conversion from vsnCTC(-) to vsnCTC(+) was seen prior to progression of visceral lesions under the treatment.

The data summarized above point toward a potential benefit in adding simple morphologic categorization (i.e., nuclear size) to CTC enumeration, and prompt the group to further enhance the understanding of the association between vsnCTCs and visceral metastasis in prostate cancer patients.

Detecting NSCLC CTCs using aptamer-grafted NanoVelcro Chips

Antibody capture agents (e.g., anti-EpCAM) are commonly used with marker-based CTC enrichment platforms. Considering the high production cost and poor storage stability known for antibodies, researchers have been exploiting alternative CTC capture agents, e.g., aptamers. Aptamers are single-stranded oligonucleotides with function similar to antibodies because they are both able to differentiate between other molecules and cells. These molecules can be produced by the process called in vitro cell-SELEX (systematic evolution of ligands by exponential enrichment)[88-90], allowing for custom productions of aptamer-based CTC capture agents. In collaboration with the Chinese Academy of Science, a group of aptamers specific to non-small cell lung cancer (NSCLC) cells was generated through in vitro cell-SELEX process. By coating these aptamers onto the NanoVelcro Chips, the study team was not only able to capture NSCLC CTCs with high efficiency, but also recover the nanosubstrate-immobilized CTCs upon treatment with a nuclease solution. On the basis of the dual aptamer capture agents, the joint team recently showed a rational design of several aptamer cocktails based on a panel of existing aptamers. By grafting these aptamer cocktails onto NanoVelcro Chips, the devices exhibited [91] enhanced and differential capture performances using blood samples collected from a cohort of NSCLC patients. This study also shows the feasibility of exploiting the aptamer-grafted NanoVelcro Chips to dissect CTC heterogeneity and to monitor treatment responses.

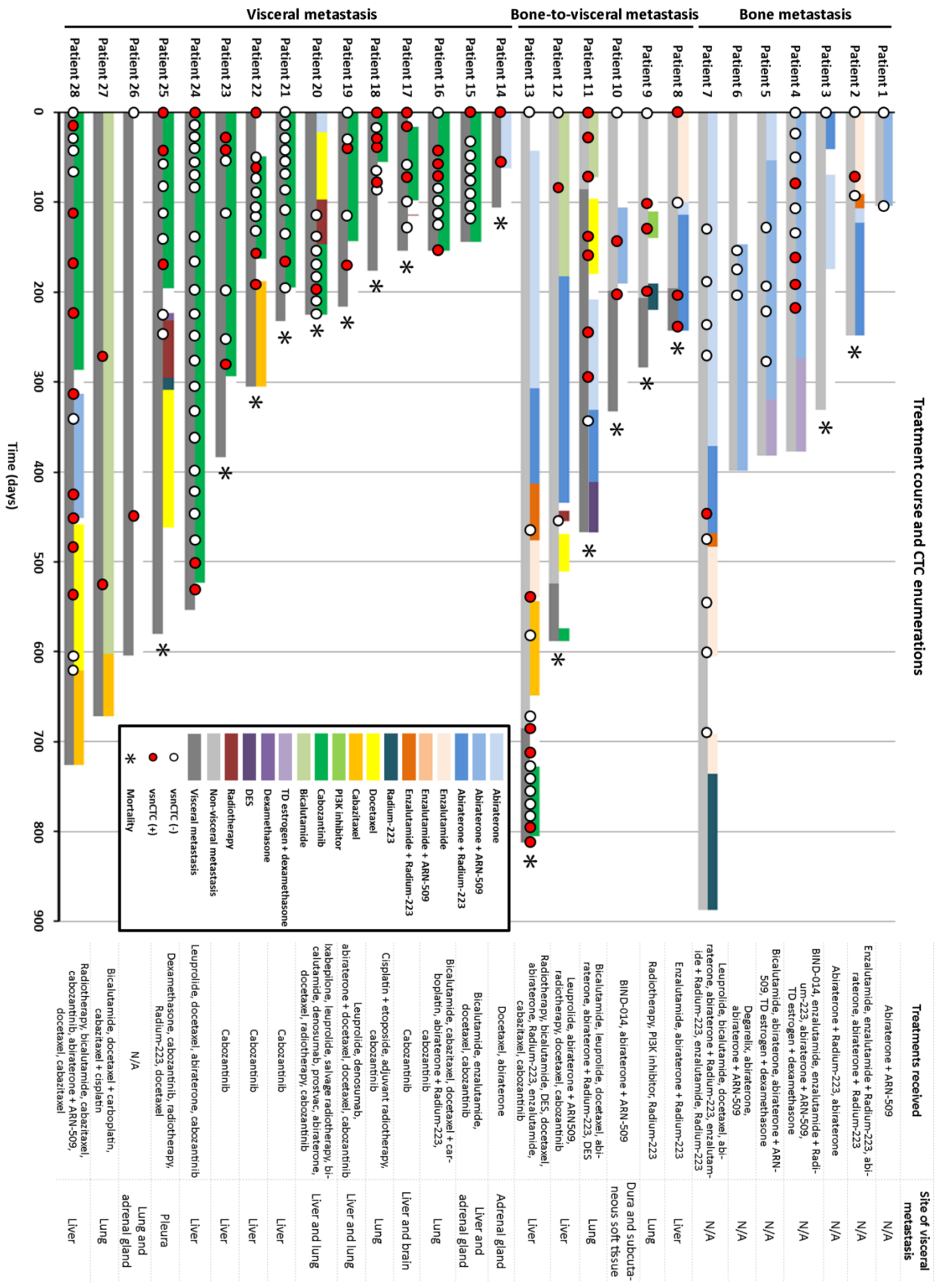


Figure 4. 1st-Gen. NanoVelcro CTC Assay identified very-small-nuclear CTCs (vsnCTCs) in prostate cancer patients with visceral metastasis. The presence of vsnCTCs has been associated with the presence of visceral metastasis. Serial CTC enumerations also suggested that vsnCTCs emerge before the development/detection of visceral lesions and thus may be a predictive biomarker for visceral metastasis in prostate cancer patients.

Molecular Analysis of CTCs using 2nd-gen NanoVelcro Chip

Mutational Analysis of Circulating Melanoma and Pancreatic Cancer Cells

Although 1st-gen NanoVelcro Chips allow efficient and reproducible detection and subclassification of CTCs in patient blood, challenges remain in 1) broadening its general applicability for detecting other types of solid-tumor CTCs that exhibit surface markers other than EpCAM, and 2) enabling the isolation of single CTCs for subsequent molecular analyses. To test the general applicability of the NanoVelcro-based cell-affinity assay, the UCLA team explored a melanoma-specific capture agent[92] (i.e., anti-CD146) to capture circulating melanoma cells (CMCs; a subcategory of solid-tumor CTCs). On the foundation of the 1st-gen NanoVelcro Chips, 2nd-gen NanoVelcro-LCM technology (**Figure 5**) was demonstrated to not only capture CMCs with high efficiency, but also enable highly specific isolation of single CMCs immobilized on the nanosubstrates without contamination by background WBCs. The non-transparent SiNS in 1st-gen NanoVelcro Chips was replaced with a transparent PLGA-nanofiber-embedded substrate, prepared by depositing electrospun PLGA nanofibers onto a commercial laser microdissection (LMD) slide (**Figure 5a**), followed by streptavidin-mediated conjugation of anti-CD146 (**Figure 5b** and **c**). Both artificial and patient blood samples were obtained to optimize and validate the performance of the PLGA-NanoVelcro Chips. Here, a new 4-color ICC protocol for parallel staining of FITC-labeled anti-Mart1, TRITC-labeled anti-HMW-MAA, DAPI, and Cy5-labeled anti-CD45 was established to identify CMCs (DAPI+/Mart1+/HMW-MAA+/CD45-, and 40 mm>diameter>10 mm) among nonspecifically captured WBCs (DAPI+/Mart1-/HMWMAA-/CD45+ and diameter<10 mm) and cellular debris, immobilized on the PLGA NanoVelcro substrates. These PLGA NanoVelcro Chips exhibit similar performance to capture CMCs compared to those observed for 1st-gen NanoVelcro Chips. Most importantly, the transparent PLGA NanoVelcro substrate allows for seamless integration with a LCM technique to isolate immobilized CMCs with single-cell resolution (**Figure 5d** and **e**). After CMC capture and ICC melanoma lineage validation, a LMD microscope (Leica) or a LCM microscope (Arcturus™ Life Technology) was used to cut out and harvest single CMCs. After conducting whole genome amplification (WGA) and targeted PCR amplification on the isolated CMCs, the amplified DNA materials were subjected to Sanger sequence analysis (**Figure 5f**). To examine the clinical utility of the optimized 2nd-gen NanoVelcro-LCM technology, the UCLA team then performed single-CMC isolation and genotyping using peripheral blood samples collected from multiple stage-IV melanoma patients, whose melanomas have been previously characterized by conventional PCR-based techniques (cobas 4800, Roche) to contain a signature oncogenic mutation, i.e., $BRAF^{V600E}$. Over the past five years the oncology field has experienced a paradigm shift in treatment of metastatic melanoma. BRAF inhibitors (e.g., vemurafenib), designed for treating melanomas harboring the $BRAF^{V600E}$ oncogenic mutation, have had unprecedented response rates in excess of 60-80%.[93, 94] The $BRAF^{V600E}$ mutation (present in 60% of melanomas) is an indispensable requisite for response to this agent. Thus, detecting the $BRAF^{V600E}$ mutation can be employed as a companion diagnostic solution that can guide the implementation of vemurafenib treatment, as well as facilitate the clinical development of the new BRAF inhibitors. The ability to detect $BRAF^{V600E}$ mutation in CMCs could evolve into a companion diagnostic that would avoid invasive tissue sampling.

As a second model, the 2nd-gen NanoVelcro-LCM technology was utilized to pursue characterization of KRAS mutation in pancreatic cancer CTCs as more than 95% of patients have activating mutations.[95],[96] Using a similar approach to that described above, both $KRAS^{G12V}$ (**Figure 5g**) and $KRAS^{G12D}$ mutations[97] were detected in the pancreatic CTCs immobilized on the PLGA NanoVelcro substrates.

Whole Exome Sequencing of CTCs

Knowing that identification of genetic alteration was possible, it was hypothesized that the NanoVelcro-LCM approach could be used to monitor the dynamic tumor biology of a cancer by profiling CTCs. As a result, a streamlined process [81], for whole exome sequencing (WES) of CTCs based on the 2nd-gen platform, was established. Single CTCs were isolated from a prostate cancer patient, and WBCs were utilized here as control representing germline DNA. After whole genome amplification (WGA), DNA was sequenced by standard exon-capture targeted sequencing. The results indicated that 25 to 80% of the targeted exome regions were sequenced with a mean coverage of 29 to 48X, and that no chromosomal loss occurred during the isolation and sequencing processes. In looking across the genomic information from the samples, the study team concluded that there were more shared mutations among individual CTCs than there were between

CTCs and WBCs. The similarity of CTCs and differences between CTCs and the WBC control verified the feasibility of using the NanoVelcro-LCM platform to capture pure CTCs for WES.

Whole Genome Sequencing (WGS) of CTCs

Given the success with WES on single-CTCs isolated by the 2nd-gen NanoVelcro Assay, the team further refined the protocol for high-quality WGS on single-CTCs. The major technological breakthroughs included (i) the utilization of ethanol instead of paraformaldehyde fixation to better preserve single-CTC DNA integrity [98]; (ii) the utilization of multiple displacement amplification (MDA) to decrease the loss of unamplified segments [81] as well as bias introduced by polymer chain reaction (PCR)-based amplification [99]; and (iii) the rigorous quality check to select high-quality CTC samples by a multiplexed PCR based on eight housekeeping genes. Utilizing a streamlined protocol, the mutational landscapes of serially-collected single CTCs were compared with the primary and metastatic tumor tissues in a mCRPC patient at the whole genome level.

In 4 sequenced individual CTCs, 30X sequencing depth (data not shown) and above 95% sequencing coverage in WGS (**Figure 6a**) were achieved. These single-CTC WGS data allow the subsequent comparisons. Twenty nine percent of founder single nucleotide variants (SSNVs) in tumor tissues were found in CTCs. In addition, 86% of clonal mutations in CTCs were traced back to either the primary or metastatic tumor (**Figure 6b**). The research team found and validated an intrachromosomal rearrangement in chr3 (**Figure 6c**) and an interchromosomal rearrangement between chr13 and chr15. These tumor specific rearrangements were shared between both tumor tissues and most of the CTCs (**Figure 6d**), but not identified in WBCs and normal adjacent tissue. At the same time, highly heterogeneous short rearrangements were discovered in important tumor suppressor genes, including *PTEN*, *RB1* and *BRCA2*, in all tumor and CTC samples [16].

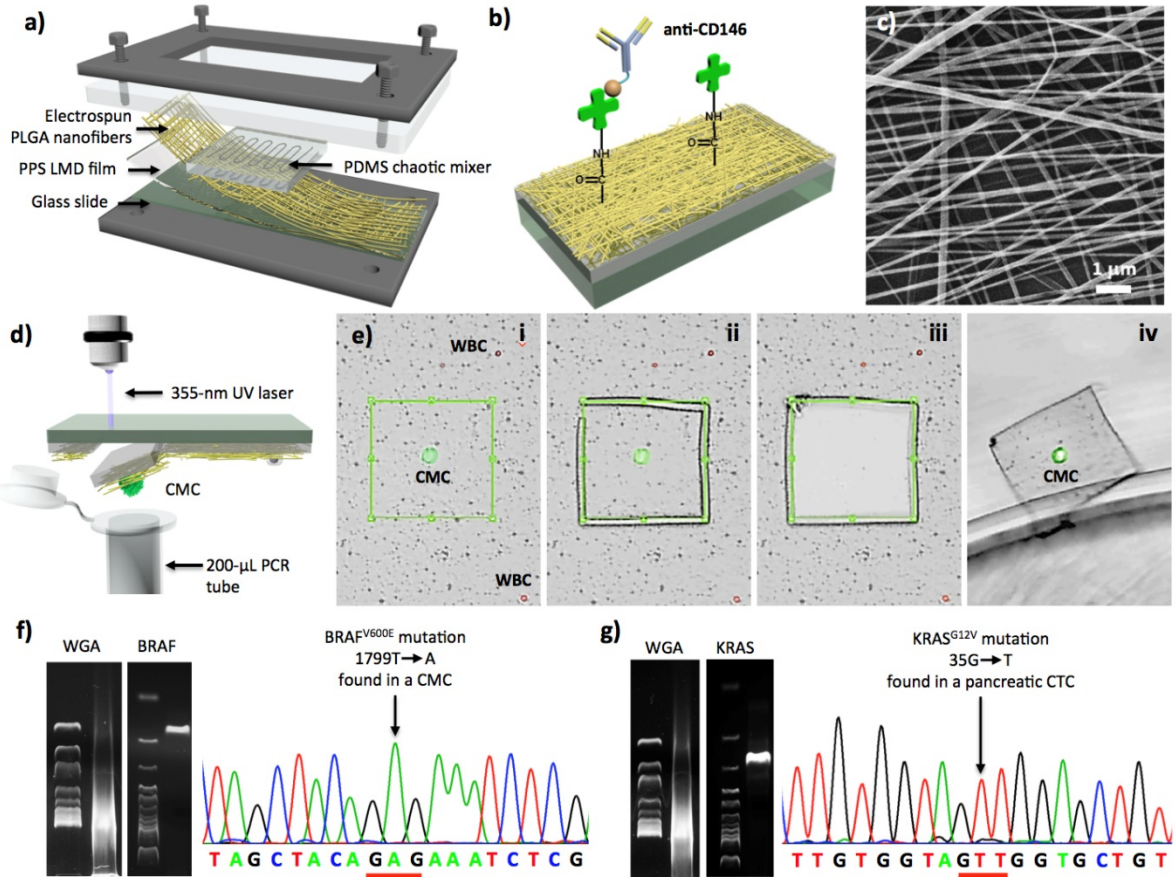


Figure 5. 2nd-Gen NanoVelcro-LCM technology for single-CTC isolation, followed by mutational analyses. (a) The PDMS chaotic mixer is layered on top of a NanoVelcro chip that contains PLGA nanofibers [82]. (b) For the binding of biotinylated capture agents (i.e., anti-CD146 for CMC and anti-EpCAM for pancreatic cancer), streptavidin is conjugated to PLGA nanofibers. (c) An image of the electrospun PLGA nanofibers by using SEM. (d) The graphic illustration of LMD-based single-CMC isolation. (e) The process to isolate single CMCs consists of (i) identification of CMC, (ii) isolation of the selected CMC using laser dissection, followed by (iii), and (iv) discharge of CMC from the silicon substrate into a 200 μl PCR tube. (f) Results of single-CMC WGA and gel electrophoresis after amplification in PCR with BRAF-specific primer. Through Sanger sequencing, further affirmation is gained because of the display of CMCs exhibiting the unique $BRAF^{V600E}$ mutation. (g) Pancreatic CTCs and the $KRAS^{G12V}$ mutation present [97].

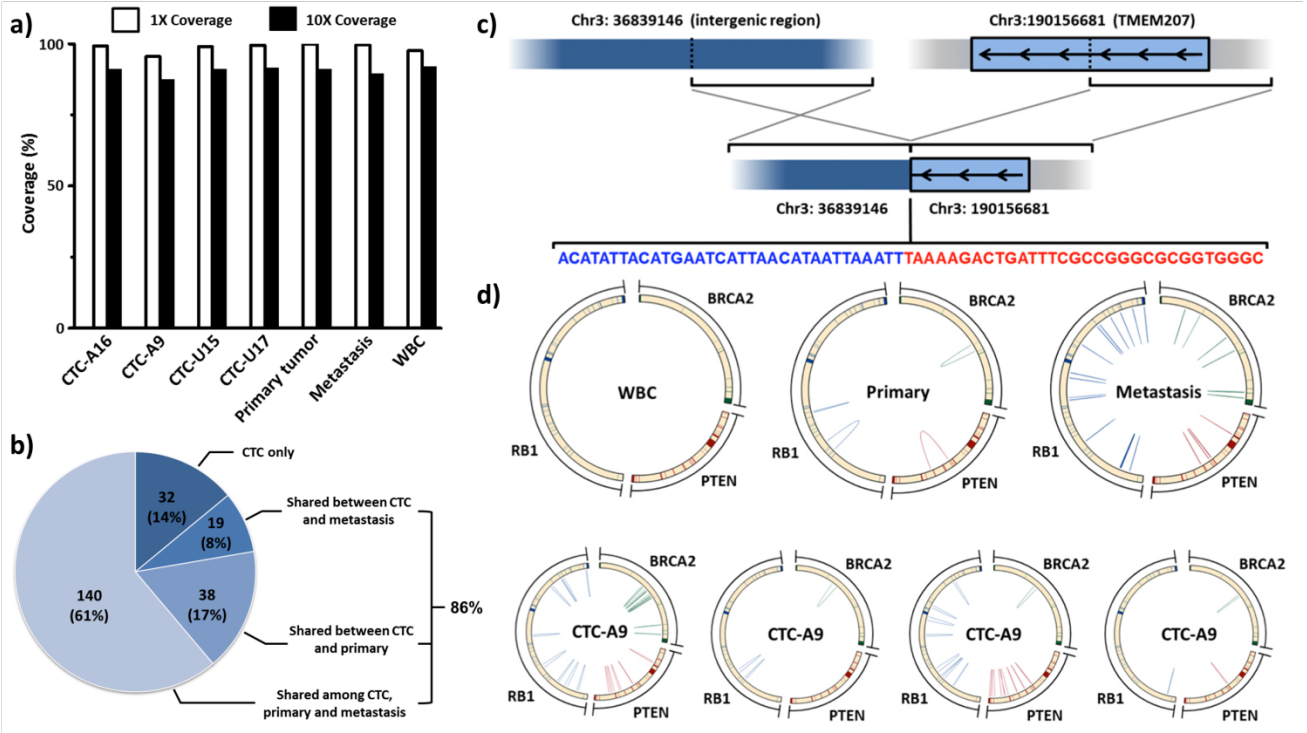


Figure 6. 2nd-Gen NanoVelcro CTC Chips for single-CTC isolation, followed by whole genome sequencing (WGS). (a) Single-CTC whole genome sequencing successfully demonstrated [16] above 90% coverage. (b) Clonal single nucleotide variants (SSNVs) shared by more than three CTCs were identified. These SSNVs were repeatedly detected in more than three single-cell sequencing runs and can be considered high confident mutations. It is interesting to note that 86.0% of these clonal SSNVs in CTCs can be traced back to either the primary or metastatic tissues. (c) Interchromosomal rearrangement involving *TMEM207* was found in CTCs and tumor tissues. (d) Complex rearrangements involving tumor suppressor genes, including *PTEN*, *RB1* and *BRCA2*, were found in CTCs and tumor tissues but not in WBCs.

Molecular Analysis of CTCs using 3rd-gen Thermoresponsive NanoVelcro Chip

Though the 2nd-gen NanoVelcro-LCM technology [16, 81, 82] exhibited exceptional precision in single-CTC isolation, this approach suffered from its limited throughput due to the labor-intensive procedure of LCM. Since fixation is needed for ICC, this approach does not yield viable CTCs. There have been significant research endeavors devoted to developing new CTC purification methods that overcome this technical challenge. For example, the MGH CTC-iChips[55] are capable of sequential depletions of normal blood cells, to recover unmanipulated CTCs which can be cultured *ex vivo* for drug susceptibility testing. Several issues, e.g., lack of control over CTC purity after their recovery, and high operation costs associated with complicated device design, need to be resolved in order to translate these new methods into research and clinical settings. The 3rd-gen Thermoresponsive NanoVelcro Chips [83, 84] will have the potential to overcome all of the challenges encountered in the field.

The move toward molecular characterization and functional analysis of CTCs creates an urgent need for (i) efficient isolation, (ii) improved cell quality, and (iii) lower technical demand on the end user. The 3rd-gen thermoresponsive NanoVelcro Chip[83, 84] was engineered with these issues in mind. This chip can successfully capture and release CTCs at 37 °C and 4 °C, respectively, via grafting thermoresponsive polymer brushes onto the SiNS. This unique idea takes advantage of the fact that the polymer brushes undergo temperature-dependent conformational changes. This alters the accessibility of the CTC surface to capture agents on the NanoVelcro Chips. Ultimately, this allows for rapid, viable CTC purification with intact nucleic acid content. To optimize the CTC purification parameters for the 3rd-gen Thermoresponsive NanoVelcro Chips, we used H1975 anti-EpCAM-positive non-small cell lung cancer (NSCLC) cells as a model system. We found that by performing two rounds of capture/release CTCs can be obtained with higher purity, allowing for EGFR point mutation analysis (**Figure 7a**). Sanger sequencing of purified CTCs from the 7 NSCLC patients' blood samples showed strong molecular correlation with tumor tissues. We also observed the emergence of secondary T790M mutation in serial CTC analyses from a NSCLC patient who received EGFR inhibitor (**Figure 7b-d**). This patient developed resistance to EGFR inhibitor

soon afterwards. These results indicated the potential utility of CTCs as a tool for detecting the emerging resistance to targeted therapies.

Significant progress is currently being made in the area of rapid CTC purification from whole blood samples. Through the development of a user-friendly interface for the 3rd generation Thermoresponsive NanoVelcro Chips we will be able to facilitate CTC characterization. The TR-NanoVelcro assay will produce a number of exciting opportunities for clinical applications. Using Thermoresponsive NanoVelcro Chips one could create patient-specific, CTC-derived cancer lines via *ex vivo* expansion. Such a tool could be used to conduct *ex vivo* testing for sensitivity and resistance to therapy that is patient specific, bringing the field of oncology closer to the goal of personalized care.

Summary of NanoVelcro CTC Assays

The unique advantages of NanoVelcro CTC Assays in contrast to other existing systems are briefly summarized below. First of all, the three generations of NanoVelcro CTC Assays share two common working mechanisms – (i) the combined use of capture agents and embedded nanostructures leads to enhanced affinity between CTCs and NanoVelcro substrates, and (ii) the overlaid PDMS chaotic mixer increases contact frequency between CTCs and NanoVelcro substrates – to achieve desired CTC capture performances. Furthermore, the use of contemporary micro-fabrication techniques in the fabrication of the two key functional components (i.e., nanostructured substrate and PDMS chaotic mixer) guarantees the scalability, reproducibility, and cost-effectiveness to produce these NanoVelcro CTC Assays. Additional advantages include (i) Flexibility: these assays can be employed for both detection and isolation of CTCs with viability and molecular intactness; (ii) Speed and precision: Runtime with semi-automated microscopy allows for optimal pathologic review of all CTCs avoiding contamination. The combined use of 1st-gen NanoVelcro Enumeration Assay and 2nd gen NanoVelcro-LCM technique, takes approximately 4 h to complete the enumeration and isolation of individual CTCs. This is less time than is required for the CellSearch™ assay (4h for enumeration only), and CTC-(HB)Chip (ca. 6-8 h for enumeration only); (iii) Cost efficiency. The current cost of the CellSearch™ assay is approximately $1,200. Performing a NanoVelcro enumeration or isolation assay (including the materials, devices fabrication, surface coating and antibodies) is less than $50; (iv) Sample processing capacity: Given the improved sensitivity of NanoVelcro Assays, we are able to recover CTCs from only 2-mL blood samples. If required, we are able to process up to 5-mL blood in one assay. Multiple units and rounds operating in parallel and in sequence will allow even higher capacity. (v) Simple user interface: The computerized interface for operation and simplified slide-in & click-on chip holder and fluid handler facilitate setup and user-to-user variation making processing and analysis simple. Over the continuous evolution process of the past decade, the

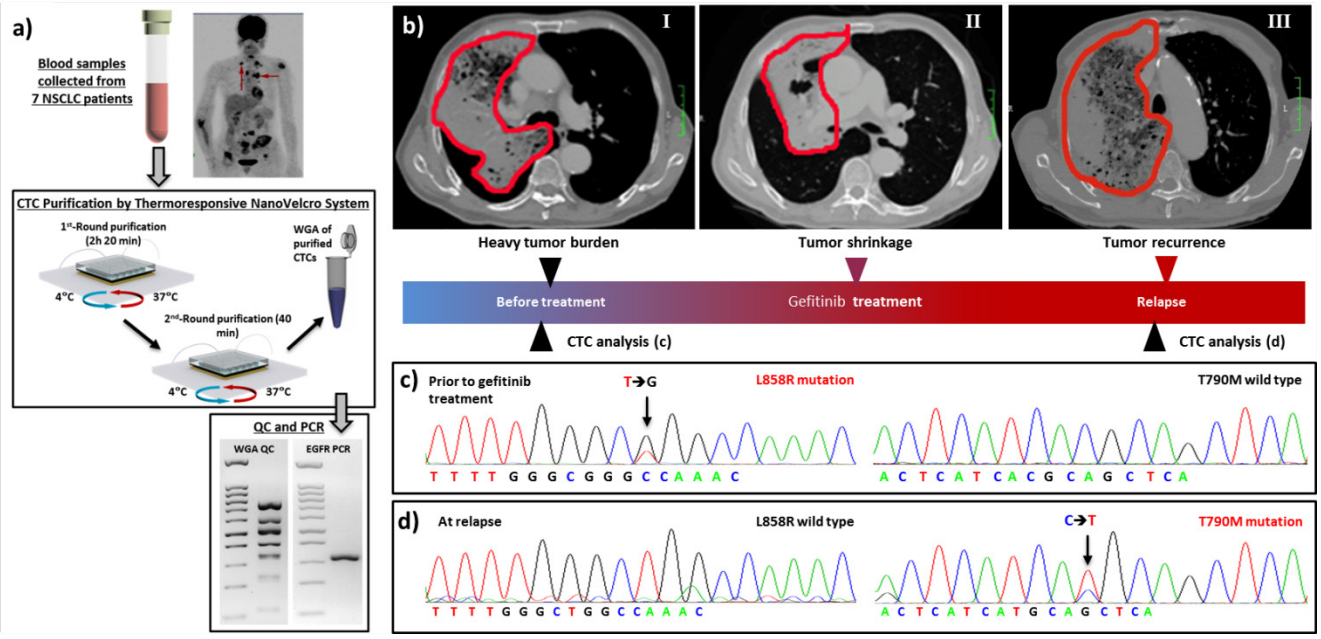


Figure 7. 3rd-Gen NanoVelcro CTC Chips for non-small cell lung cancer (NSCLC) CTC purification, followed by mutational analysis. (a) The workflow demonstrating [83] the use of 3rd-gen NanoVelcro Chip for non-small cell lung cancer CTC purification followed by detection of *EGFR* mutations. (b-d) Longitudinal data showed the mutational analysis of CTCs from a NSCLC patient. (c) L858R mutation was detected in CTCs and tumor tissues before gefitinib (1st-Gen EGFR inhibitor) treatment. Tumor regression was observed soon after initiation of gefitinib, as shown in (b). (d) The secondary T790M mutation was detected later in CTCs when the patient's disease progressed.

research team has accumulated significant research experience and technical knowhow conferring several unique capacities to each generation of NanoVelcro Assay. These capacities include high-resolution fluorescent imaging for morphological analysis of CTCs, LCM technique for single-cell isolation, and temperature-dependent CTC purification. With these unique capacities, NanoVelcro Assays are able to address unmet needs in oncology practice, such as stratification of heterogeneous CTC population (1st-Gen), molecular profiling of CTCs (2nd-Gen and 3rd-Gen), and rapid CTC purification for liquid biopsy (3rd-Gen).

Future scientific and clinical developments

Moving forward, future research endeavors with nanotechnology-enabled CTC assays will be driven by particular needs: (i) acquiring a fundamental understanding of the nanointerfaces between CTCs (e.g., how the underlying physical/chemical properties of any given nanosubstrate affect their CTC-capture performance, as well as the viability and molecular integrity of captured CTCs); (ii) developing new CTC-capture/release mechanisms governed by physiologically compatible stimulations for instant isolation/purification of CTCs with desired viability and molecular integrity to allow for downstream *ex vivo* molecular characterization; (iii) exploiting the growing number of multi-omic analytical technologies (that could result from other research initiatives within NCI Nanotechnology Alliance Program) with single-cell resolution to characterize the heterogeneous CTC pool; (iv) exploring the use of rare-cell culture techniques that will enable *ex vivo* expansion of purified CTCs for in-depth studies (e.g., xenograft models and drug susceptibility testing); (v) studying other types of circulating rare cells (e.g., tumor associated macrophage and stromal cells) and non-cellular particles (e.g., exosomes), which also carry information about the tumor microenvironment.

Following development of these technologic advances, challenges will remain in utilizing these new assays to address unmet needs in the areas of cancer biology and, most importantly, clinical oncology. Research endeavors should be devoted to: (i) performing multi-omic molecular characterizations on CTCs in parallel with tumor tissues from the same patients (including primary and metastatic sites when available) to further refine the CTC-tumor relationship. Such efforts are crucial to the development of CTC-based liquid biopsies. It is conceivable that CTCs may be used as surrogate tumor tissue that will provide relevant information for personalization of cancer treatment; (ii) dissecting CTC subpopulations according to their distinct phenotypes (e.g., molecular fingerprints, morphological characteristics, and behaviors) in order to address the issue of heterogeneity in tumor/CTC pool. For instance, a subpopulation of CTCs with defined small nuclei was discovered to strongly correlate with the presence of visceral metastasis in prostate cancer, offering a new way to detect the onset of the most lethal disease progression events; (iii) conducting analyses on serial CTC samples through monitoring the dynamic change of CTC subpopulations and their multi-omic molecular signatures to better understand the evolution of cancer, which is currently limited by the difficulty of obtaining tumor tissues; (iv) effectively generating and applying CTC-derived cell lines as well as xenograft models to better understand the oncogenic/resistant mechanism, and evaluate a wide range of treatment options that can potentially benefit individual patients. Validation in appropriately powered studies will be needed as these ideas translate directly into the clinical setting. Ultimately, regulatory and commercial efforts will be required to bring these tools to the population at large.

Conclusion and outlook

Early successes in the field of nanotechnology have shown great promise for addressing existing, urgent, and unmet needs in clinical oncology. As the scientific understanding of the dynamic and complex biology of cancer evolves, it has become clear to clinical scientists and cancer biologists that characterizing this dynamic biology will add an important dimension to clinical data. Oncologists practicing cancer care in this evolving biologic environment are already accustomed to handling temporal variation of data. Monitoring the dynamic alterations of biological variables, which themselves follow a distinct and biologically relevant rhythm, is a fundamental part of clinical medicine. Given the limitations of performing serial biopsies or the limited data obtainable in single biomarker panels, to date, this type of dynamic characterization has been possible only in animal models or in limited biomarker panels. The promise that analysis of CTCs and other circulating entities holds is the ability to study the dynamic biology in the system that bears the greatest relevance: the individual patient. In this era of molecular medicine that has brought us beyond the cell to the level of DNA, RNA, and proteins, it has become exceedingly clear that no two patients are identical and no two cancers are identical. Having a non-invasive means of dissecting these differences bridges the gap between the laboratory and the clinic. While these ideas are young, the successes seen in this

field provide ample cause for continued work and fuel the enthusiasm for launching integrated transdisciplinary research in this transformative field.

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Competing Interests

The authors have declared that no competing interest exists.

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