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Title: Microfluidics-Based High-Throughput Circulating Tumor Cell Sorting and Single-Cell Sequencing Technology

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No.**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No.**

3. **Filming location:** Will the filming need to take place in multiple locations? **No.**

Current Protocol Length
Number of Steps: 26
Number of Shots: 56

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

1.1. **Zheyu Liu:** We focus on developing high-performance CTC isolation and analysis methods to advance precision oncology through liquid biopsy.

1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B.roll:2.6*

What are the current experimental challenges?

1.2. **Zheyu Liu:** Current commercial CTC isolation methods have low throughput and efficiency. They are also incompatible with downstream sequencing platforms, which limits biological information recovery.

1.2.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

What research gap are you addressing with your protocol?

1.3. **Zheyu Liu:** This study uses a microfluidic CTC isolation platform to greatly improve the detection rates. We also apply a rare-cell single-cell sequencing chip for deeper liquid biopsy analysis.

1.3.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B.roll:3.8*

Videographer: Obtain headshots for all authors available at the filming location.

Protocol

2. Preparation of Immunomagnetic Beads and Initial HB-Chip Operation for Tumor Cell Isolation

Demonstrator: Zheyu Liu

2.1. To begin, pipette 25 microliters of washed and resuspended streptavidin-modified magnetic beads **[1]** into a tube containing 1 microgram of biotinylated EpCAM *(Epp-Cam)* antibody **[2-TXT].** Incubate the mixture at room temperature while rotating at 20 revolutions per minute for 40 minutes to prepare the immunomagnetic beads **[3]**.

2.1.1. WIDE: Talent pipetting streptavidin-modified magnetic beads into a microcentrifuge tube.

2.1.2. Talent pipetting EpCAM antibody into the tube. **TXT: EpCAM: Epithelial Cell Adhesion Molecule**

2.1.3. Talent placing the tube on a rotator set to 20 revolutions per minute.

2.2. Using a magnetic rack, separate the beads from the supernatant **[1]**. After removing the supernatant, resuspend the beads in 25 microliters of isolation buffer **[2-TXT]**.

2.2.1. Talent placing the tube on a magnetic rack and allowing beads to collect at the side.

2.2.2. Talent adding 25 µL isolation buffer to the bead pellet and gently resuspending it. **TXT: Isolation Buffer: 1% BSA, 2 mM EDTA, D-PBS**

2.3. Pipette 20 microliters of the magnetic bead suspension within 1 to 2 seconds, ensuring no air bubbles are introduced **[1]**. Immediately place the chip vertically on a magnet and allow the beads to settle for 5 minutes without disturbance **[2]**.

2.3.1. Talent drawing 20 microliters of bead suspension into a pipette quickly, ensuring no bubbles form.

2.3.2. Talent positioning the chip vertically against a magnet.

2.4. Collect 4 milliliters of the blood sample into a syringe, ensuring air bubbles are removed **[1]**. Seal the chip's inlet and outlet with liquid to eliminate air bubbles **[2]**. Then insert the sample inlet and outlet tubes into the chip **[3]**.

2.4.1. Talent drawing blood into a syringe while avoiding air bubbles.

2.4.2. Talent sealing the chip's inlet and outlet with liquid. **NOTE**: 2.4.2 and 2.4.3 are combined in one clip

2.4.3. Talent connecting inlet and outlet tubing to the chip.

2.5. Using a syringe pump, inject the sample at a flow rate of 1.5 milliliters per hour **[1]**. After loading the sample, inject 60 microliters of DPBS into the HB*(H-B)* -chip at a flow rate of 0.2 milliliters per hour to wash off unbound cells **[2]**.

2.5.1. Talent setting the syringe pump and starting the injection.

2.5.2. Talent operating syringe pump to inject Dulbecco's phosphate-buffered saline at the set flow rate.

2.6. Remove the magnet **[1]** and manually inject 1.5 milliliters of 5 percent BSA to wash the chip and release captured tumor cells **[2]**.

2.6.1. Talent removing magnet from the chip.

2.6.2. Talent manually injecting bovine serum albumin solution into the chip.

3. Microfluidic-based Circulating Tumor Cells (CTC) purification

3.1. Prepare a 10% MPTS *(M-P-T-S)* solution in ethanol **[1-TXT]**. Immediately introduce 20 microliters of the solution into the HB-chip and incubate at room temperature for 1 hour **[2]**.

3.1.1. Talent measuring and preparing (3-Mercaptopropyl) trimethoxysilane solution in ethanol. **TXT: MPTS: (3-Mercaptopropyl) trimethoxysilane**

3.1.2. Talent pipetting 20 microliters of the solution into the HB-chip inlet.

3.2. Rinse the HB-chip once with anhydrous ethanol **[1]**. Then dry it at 100 degrees Celsius for 1 hour **[2]**.

3.2.1. Talent using a pipette to rinse the chip with anhydrous ethanol.

3.2.2. Talent placing the chip in a drying oven set to 100 degrees Celsius.

3.3. Next, prepare GMBS *(G-M-B-S)* solution in ethanol at a concentration of 0.5 milligram per milliliter **[1-TXT]**. After cooling the chip to 37 degrees Celsius, introduce the GMBS solution and incubate **[2-TXT]**.

3.3.1. Talent preparing GMBS solution in a microcentrifuge tube. **TXT: GMBS: N-γ-maleimidobutyryl-oxysuccinimide**

3.3.2. Talent pipetting GMBS solution into the HB-chip inlet. **TXT: Incubation: RT, 30 min**

3.4. Rinse the HB-chip two times with double-distilled water, followed by two rinses with DPBS **[1]**. Immediately add 15 micrograms per milliliter of streptavidin into the HB-chip and incubate at room temperature for 1 hour or overnight at 4 degrees Celsius **[2]**.

3.4.1. Talent the chip twice with double-distilled water and DPBS. **NOTE**: step 3.4.1 and 3.4.2 are combined in one clip
AUTHORS: Please perform one representative action

3.4.2. Talent pipetting streptavidin solution into the HB-chip inlet.

3.5. After incubation, rinse the HB-chip two times with DPBS saline **[1]**.

3.5.1. Talent performing two Dulbecco's phosphate-buffered saline rinses on the chip.

3.6. Now, prepare the CD45 *(C-D-Forty-Five)* antibody buffer by adding 0.2 percent BSA *(B-S-A)* and 20 micrograms per milliliter biotinylated CD45 antibody to DPBS, and adjust to the final volume **[1]**. Inject 20 microliters of the CD45 antibody buffer into the HB-chip for negative selection of white blood cells **[2]**. After a 1-hour incubation at room temperature, rinse the chip with DPBS **[3]**.

3.6.1. Talent preparing CD45 antibody buffer in a microcentrifuge tube.

3.6.2. Talent pipetting CD45 antibody buffer into the HB-chip inlet.

3.6.3. Talent rinsing the HB-chip with Dulbecco's phosphate-buffered saline.

3.7. Add a blocking solution containing 3 percent BSA and 0.05 percent Tween-20 into the HB-chip **[1-TXT]**.

3.7.1. Talent pipetting blocking solution into HB-chip inlet. **TXT: Incubate and rinse with DPBS before sample loading**

3.8. Aspirate the prepared sample into a syringe, ensuring no air bubbles are present **[1]**. Then seal the chip's inlet and outlet with liquid **[2]**, and connect inlet and outlet tubes to the chip **[3]**.

3.8.1. Talent aspirating sample into syringe while avoiding air bubbles.

3.8.2. Talent sealing chip inlet and outlet with liquid.

3.8.3. Talent attaching inlet and outlet tubes to chip.

3.9. Using a syringe pump, inject the sample at a flow rate of 0.6 milliliters per hour **[1]**. Collect purified tumor cells from the chip outlet for counting and single-cell sequencing **[2]**.

Talent setting and starting syringe pump for injection. **NOTE**: step 3.9.1 and 3.9.2 are combined in one clip

3.9.1. Talent collecting tumor cells from chip outlet into a microcentrifuge tube.

4. Circulating Tumor Cell Capture, Barcoded Bead Loading, and mRNA Preparation Using the HB-Chip

4.1. Pipette 200 microliters of 0.5 percent F-68 *(F-Sixty-Eight)* solution prepared in DPBS, into the chip inlet **[1]**. Perform water bath sonication while holding the chip **[2]**. When bubbles are visible across the microporous region, continue sonication for 30 seconds to remove bubbles from the dual wells **[3]**.

4.1.1. Talent pipetting F-68 solution in Dulbecco's phosphate-buffered saline into chip inlet.

4.1.2. Talent submerging chip in water bath sonicator.

4.1.3. Close-up of bubbles across microporous region during sonication.

4.2. Next, inject 200 microliters of tumor cell suspension into the chip containing 60,000 dual wells **[1-TXT]**.

4.2.1. Talent pipetting tumor cell suspension into chip inlet. **TXT: Shake at 10 rpm/min, 5 min**

4.3. Gently pipette the cell suspension in the chip up and down twice **[1]**, then place on a decolorizing shaker again at for 5 minutes to resuspend uncaptured cells and allow them to settle again **[2]**. Now add 200 microliters of DPBS and 0.5 percent F-68 solution through inlet and aspirate from outlet **[3-TXT]**.

4.3.1. Talent gently pipetting suspension inside chip.

4.3.2. Talent placing chip back on decolorizing shaker.

4.3.3. Talent adding wash solution through inlet and aspirating from outlet. **TXT: Repeat for a total of 3 washes**

4.4. After resuspending the barcoded bead suspension. Immediately inject 200 microliters of suspension into the chip inlet **[1]** and shake at 10 revolutions per minute for 20 seconds **[2]**.

4.4.1. Talent injecting resuspended beads into chip inlet.

4.4.2. Talent placing the chip on a shaker set to 10 revolutions per minute.

4.5. Gently pipette bead suspension twice before placing it back on the shaker **[1-TXT]**

4.5.1. Talent gently pipetting bead suspension in chip. **TXT: Repeat once**

4.6. Now withdraw barcoded bead suspension from outlet **[1]**. Then pipette 200 microliters of 20x Tris-EDTA *(Tris-E-D-T-A)* and 50 millimolar dithiothreitol solution **[2]**. Withdraw liquid from outlet **[3-TXT]**.

4.6.1. Talent withdrawing bead suspension from outlet.

4.6.2. Talent adding Tris-EDTA and dithiothreitol solution into chip. **NOTE**: step 4.6.2 and 4.6.3 are combined in one clip

4.6.3. Talent withdrawing solution from outlet. **TXT: Repeat wash for a total of 4 washes**

4.7. For cell lysis and mRNA *(M-R-N-A)* capture, slowly add 200 microliters of cell lysis buffer into the chip inlet **[1]**. Immediately add 200 microliters of mineral oil to seal the dual wells **[2]**.

4.7.1. Talent pipetting lysis buffer into chip inlet.

4.7.2. Talent adding mineral oil immediately after lysis buffer.

4.8. Remove solution flowing from chip outlet into waste reservoir **[1]**. Then place the chip horizontally and let stand at room temperature for 5 minutes **[2]**.

4.8.1. Talent removing waste solution from outlet.

4.8.2. Close-up of chip resting horizontally on bench.

4.9. Slowly add 200 microliters of 6x saline-sodium citrate solution into inlet **[1]**. Remove the waste liquid and aspirate the remaining solution from chip outlet **[2]**.

4.9.1. Talent adding saline-sodium citrate solution into chip inlet. **NOTE**: step 4.9.1 and 4.9.2 are combined in one clip

4.9.2. Talent removing waste solution from outlet and pipetting out the remaining solution from chip outlet.

4.10. Slowly add 200 microliters of 6x saline-sodium citrate to fill chip **[1]**. Hold a magnet near chip surface and move it slowly from inlet to outlet to gather barcoded beads **[2]**. Quickly aspirate solution and beads into a centrifuge tube containing 6x saline-sodium citrate **[3]**.

4.10.1. Talent filling chip with saline-sodium citrate.

4.10.2. Close-up of magnet being moved along chip surface.

4.10.3. Talent aspirating bead-containing solution into centrifuge tube.

4.11. Wash the barcoded beads three times with 200 microliters of 6x saline-sodium citrate **[1]**, followed by once with reverse transcription buffer **[2]**.

4.11.1. Talent washing beads with saline-sodium citrate. **TXT: Repeat for a total of 3 washes**

4.11.2. Talent washing beads once with reverse transcription buffer.

Results

5. Results

5.1. A uniform distribution of immunomagnetic beads was observed under a magnetic field **[1]**, while minimal residual beads after magnetic removal confirmed successful release **[2]**.

5.1.1. LAB MEDIA: Figure 2C. *Video editor: please highlight the top image*

5.1.2. LAB MEDIA: Figure 2C. *Video editor: please highlight the bottom image*.

5.2. The CTC *(C-T-C)* isolation chip efficiently captured tumor cells from both 1 milliliter and 10 milliliter samples **[1-TXT]**. The addition of the purification chip further increased tumor cell purity **[2]**.

5.2.1. LAB MEDIA: Figure 2D.
TXT: CTC: Circulating Tumor Cell
Video editor: Please highlight the 1mL and 10 mL bars

5.2.2. LAB MEDIA: Figure 2E. *Video editor: Highlight the bars in the "Both" group*

5.3. In peripheral blood samples spiked with minimal LNCaP *(Lin-Cap)* cells **[1],** the CTC sorting system maintained high capture efficiency and purity **[2]**.

5.3.1. LAB MEDIA: Figure 2F. *Video editor: Highlight the green fluorescent cells in the "LNCaP-Calcein AM" panel.*

5.3.2. LAB MEDIA: Figure 2G. *Video editor: Highlight both the red bars and blue dots in the "1 mL" and "10 mL" groups.*

5.4. The single-cell barcoding chip achieved an 85.6% cell **[1]** and 95.7% barcoded bead occupancy ratio **[2]**, resulting in a pairing rate of 81.9% **[3]**.

5.4.1. LAB MEDIA: Figure 3D (left). *Video editor: Please highlight the red bar labeled "Cell"*

5.4.2. LAB MEDIA: Figure 3D (left). *Video editor: Highlight the blue bar labeled "Bead"*

5.4.3. LAB MEDIA: Figure 3D (left). *Video editor: Highlight the green bar labeled "Pairing"*

5.5. Increasing the number of loaded cells improved the microwell occupancy ratio **[1]** without reducing capture efficiency **[2]**.

5.5.1. LAB MEDIA: Figure 3C. *Video editor: Highlight the blue line increasing toward the right side of the graph.*

5.5.2. LAB MEDIA: Figure 3C. *Video editor: Highlight the flat red line across the graph.*

5.6. The integrated CTC isolation and single-cell sequencing workflow produced highly pure tumor cells **[1]** and accurately retained the original tumor cell ratios **[2]**. t-SNE *(T-S-N-E)* analysis distinctly separated PC3*(P-C-Three)*, LNCaP, and Jurkat cells into three clusters, each characterized by unique marker expression **[3]**.

5.6.1. LAB MEDIA: Figure 4B. *Video editor: Highlight the large yellow and blue sections in the "Purified" bar.*

5.6.2. LAB MEDIA: Figure 4B. *Video editor: Highlight the yellow and blue in "Purified" and "Output" bars.*

5.6.3. LAB MEDIA: Figure 4A. *Video editor: Highlight the three distinct color clusters.*

5.7. The Jurkat cell cluster was identified by strong expression of TRBC1*(T-R-B-C-One)*, IGLL1 *(Eye-G-L-L-One)*, CD1E*(C-D-One-E)*, and CD3D *(C-D-Three-D)* **[1]**, confirmed by pathway enrichment for T cell activation **[2]**.

5.7.1. LAB MEDIA: Figure 4D. *Video editor: Highlight the TRBC1, CD3D and CD1E marker plots*

5.7.2. LAB MEDIA: Figure 4E. *Video editor: Highlight the "alpha-beta T cell activation" and "T cell differentiation" boxes.*

5.8. PC3 and LNCaP clusters were distinguished by differentially expressed genes, with PC3 showing high microseminoprotein expression **[1]** and LNCaP expressing NEDD4 *(Ned-Four)* **[2]**.

5.8.1. LAB MEDIA: Figure 4F–G. *Video editor: Highlight the the MSMP plot in 4F*

5.8.2. LAB MEDIA: Figure 4F–G. *Video editor: Highlight the NEDD4 plot in 4F*

- **Streptavidin**

Pronunciation link:
https://www.merriam-webster.com/dictionary/streptavidin
IPA: /strɛpˈtævɪdɪn/
Phonetic Spelling: strep-ta-vi-din

- **Biotinylated**

Pronunciation link:
https://www.merriam-webster.com/dictionary/biotinylated
IPA: /ˌbaɪoʊˈtɪnɪˌleɪtɪd/
Phonetic Spelling: bye-oh-tin-uh-lay-tid

- **EpCAM (Epithelial Cell Adhesion Molecule)**

No confirmed link found
IPA: /ˈɛpkæm/
Phonetic Spelling: Epp-Cam

- **Supernatant**

Pronunciation link:
https://www.merriam-webster.com/dictionary/supernatant
IPA: /ˈsuːpərˌneɪtənt/
Phonetic Spelling: soo-per-nay-tent

- **BSA (Bovine Serum Albumin)**

Pronunciation link:
https://www.merriam-webster.com/dictionary/albumin
IPA: /ˈboʊvaɪn ˈsɪrəm ˈælbjəmɪn/
Phonetic Spelling: boh-vine seer-um al-byoo-min

- **EDTA (Ethylenediaminetetraacetic acid)**

Pronunciation link:
https://www.merriam-webster.com/dictionary/EDTA
IPA: /ˌiːdiːtiːˈeɪ/
Phonetic Spelling: E-D-T-A

- **DPBS (Dulbecco's Phosphate-Buffered Saline)**

No confirmed link found
IPA: /ˌdiːpiːbiːˈɛs/
Phonetic Spelling: D-P-B-S

- **MPTS (3-Mercaptopropyl trimethoxysilane)**

No confirmed link found
IPA: /ɛm-piː-tiː-ɛs/
Phonetic Spelling: M-P-T-S

- **Anhydrous**

Pronunciation link:
https://www.merriam-webster.com/dictionary/anhydrous
IPA: /ænˈhaɪdrəs/
Phonetic Spelling: an-hy-drus

- **GMBS (N-γ-maleimidobutyryl-oxysuccinimide)**

No confirmed link found
IPA: /ʤiː-ɛm-biː-ɛs/
Phonetic Spelling: G-M-B-S

- **Streptavidin** (repeated, already above in #3)

- **CD45**

No confirmed link found
IPA: /ˌsiːˌdiːˈfɔrti faɪv/
Phonetic Spelling: C-D-Forty-Five

- **Tween-20**

No confirmed link found
IPA: /twiːnˈtwɛnti/
Phonetic Spelling: tween-twenty

- **F-68 (Pluronic F-68)**

No confirmed link found
IPA: /ɛfˌsɪkstiˈeɪt/
Phonetic Spelling: F-Sixty-Eight

- **Microporous**

Pronunciation link:
https://www.merriam-webster.com/dictionary/microporous
IPA: /ˌmaɪkroʊˈpɔrəs/
Phonetic Spelling: my-kroh-por-us

- **Tris-EDTA**

No confirmed link found
IPA: /trɪs ˌiːdiːtiːˈeɪ/
Phonetic Spelling: tris-E-D-T-A

- **Dithiothreitol (DTT)**

Pronunciation link:
https://www.merriam-webster.com/dictionary/dithiothreitol
IPA: /daɪˌθaɪoʊˈθriːɪˌtɔl/
Phonetic Spelling: dye-thy-oh-three-ee-tawl

- **mRNA**

Pronunciation link:
https://www.merriam-webster.com/dictionary/mRNA
IPA: /ˌɛmˌɑrɛnˈeɪ/
Phonetic Spelling: M-R-N-A

- **Saline-sodium citrate**

No confirmed link found
IPA: /ˈseɪˌlin ˈsoʊdiəm ˈsaɪtreɪt/
Phonetic Spelling: say-leen so-dee-um sy-trayt

- **Reverse Transcription**

Pronunciation link:
https://www.merriam-webster.com/dictionary/reverse%20transcription
IPA: /rɪˈvɜrs trænˈskrɪpʃən/
Phonetic Spelling: ree-vurs tran-skrip-shun

- **CTC (Circulating Tumor Cell)**

No confirmed link found
IPA: /ˌsiːˌtiːˈsiː/
Phonetic Spelling: C-T-C

- **LNCaP**

No confirmed link found
IPA: /ˈlɪnˌkæp/
Phonetic Spelling: Lin-Cap

- **t-SNE (t-distributed stochastic neighbor embedding)**

No confirmed link found
IPA: /tiː-ɛs-ɛnˈiː/
Phonetic Spelling: T-S-N-E

- **PC3**

No confirmed link found
IPA: /piː-siː-θriː/
Phonetic Spelling: P-C-Three

- **Jurkat**

Pronunciation link:
https://www.merriam-webster.com/dictionary/Jurkat
IPA: /ˈʤɜrkæt/
Phonetic Spelling: jur-kat

- **TRBC1**

No confirmed link found
IPA: /tiː-ɑr-biː-siː-wʌn/
Phonetic Spelling: T-R-B-C-One

- **IGLL1**

No confirmed link found
IPA: /aɪ-ʤiː-ɛl-ɛl-wʌn/
Phonetic Spelling: Eye-G-L-L-One

- **CD1E**

No confirmed link found

IPA: /siː-diː-wʌn-iː/
Phonetic Spelling: C-D-One-E

- **CD3D**

No confirmed link found
IPA: /siː-diː-three-diː/
Phonetic Spelling: C-D-Three-D

- **NEDD4**

No confirmed link found
IPA: /nɛd-fɔr/
Phonetic Spelling: Ned-Four

- **Microseminoprotein (MSMP)**

Pronunciation link:
https://www.merriam-webster.com/dictionary/microseminoprotein
IPA: /ˌmaɪkroʊˌsɛmɪnoʊˈproʊtin/
Phonetic Spelling: my-kroh-sem-ih-no-pro-teen