Chapter 20

Isolation and Characterization of CTCs from Patients with Cancer of a Urothelial Origin

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Abstract

Monitoring of circulating tumor cells' (CTCs) presence has the potential to improve therapeutic management of oncological diseases at an early stage and also to identify patients with increased risk of tumor progression or recurrence before the onset of clinically detected metastasis. Here we describe a new simplified efficient methodology for the separation and in vitro culturing of viable CTCs from peripheral blood by size-based filtration (MetaCell®). The isolation protocol yields preferentially cells bigger than 8 μm enabling further cytomorphological and molecular analysis.

Key words CTCs, Circulating tumor cells, Prostate cancer, Renal, Bladder cancer, Cultivation, In vitro, Gene expression

1 Introduction

CTCs may represent an opportunity to assess cancer spread directly and earlier than established/traditional methods, which classify tumor growth in general. A functional methodology to harvest separated tumor cells from blood provides researchers with a population of viable and proliferating cells to examine gene expression profiles or gene mutations in cancer [1–3].

The examination for CTCs could be useful as well as a complementary cancer screening test, especially for excluding cancer, and including patients with indications for repeated biopsies, e.g. in case of prostate cancer. Serial examination of CTCs enriched from peripheral blood after radical prostatectomy could help in prognosis determination, prospectively [4]. CTCs examination offers an alternative, minimally invasive approach to characterize cancer cell and to study early-stage disease [5, 6].

CTCs are frequently detected in cancer of urothelial origin (CUO) and are also found in patients with clinically localized CUO [6–8]. The analysis of the survival of patients with metastatic CUO suggested that CTCs might have prognostic significance in

Wolfgang A. Schulz et al. (eds.), *Urothelial Carcinoma: Methods and Protocols*, Methods in Molecular Biology, vol. 1655, DOI 10.1007/978-1-4939-7234-0_20,

those with advanced disease. There are multiple approaches to detect CTCs. CTC counts in patients with metastatic CUO could, therefore, be useful for monitoring the response to cancer therapy.

The methodology described here targets viable CTCs captured on a membrane, enriched in a good fitness with a remarkable proliferation potential. Filtration flow of the peripheral blood through the separation membrane is driven by capillarity. The speed of the filtration process depends on the natural blood viscosity. These properties enable setting up in vitro cell cultures from the viable CTCs unaffected by any fixatives, antibodies, or lysing solutions.

In vitro culturing of CTCs is a prerequisite for proliferation tests assessing chemosensitivity of tumors [9]. The protocol described below allows successful culturing of CTCs by use of filtration device (MetaCell®), which enables direct transfer of CTCs captured on the separation membrane to culturing plates (*see* Fig. 1). In the future, CTCs in culture could be used for personalizing oncological treatment and diagnostics.

2 Materials

2.1 Peripheral Blood Collection

1. Monovette tubes (Sarstedt AG & Co., Numbrecht, Germany) containing 1.6 mg EDTA/mL blood as an anticoagulant. Alternatively, Vacuette tubes (Greiner Bio-One) coated with 1.2–2 mg EDTA/mL blood or any similar EDTA—treated tubes can be used.

2.2 Isolation of CTCs

1. Size-based separation device MetaCell® (MetaCell, Ostrava, Czech Republic) (*see* Fig. 2 Meta Cell® filtration tube).
2. Washing fluid: RPMI 1640 medium.

2.3 Incubation and Cultivation of CTCs

1. 6-well plates.
2. RPMI 1640 medium complete (assigned as R+); additives Fetal bovine serum, antibiotics, and Amphotericin B solution.
3. EL-buffer (79217, Qiagen).
4. TrypLE™ Select Enzyme (1X) (Thermofisher Scientific).
5. Cell culture CO_2 incubator.

2.4 CTCs Visualization

1. NucBlue® Live ReadyProbes® Reagent (R37605, Thermofisher Scientific).
2. CellTracker™ Green CMFDA Dye (C2925, Thermofisher Scientific).

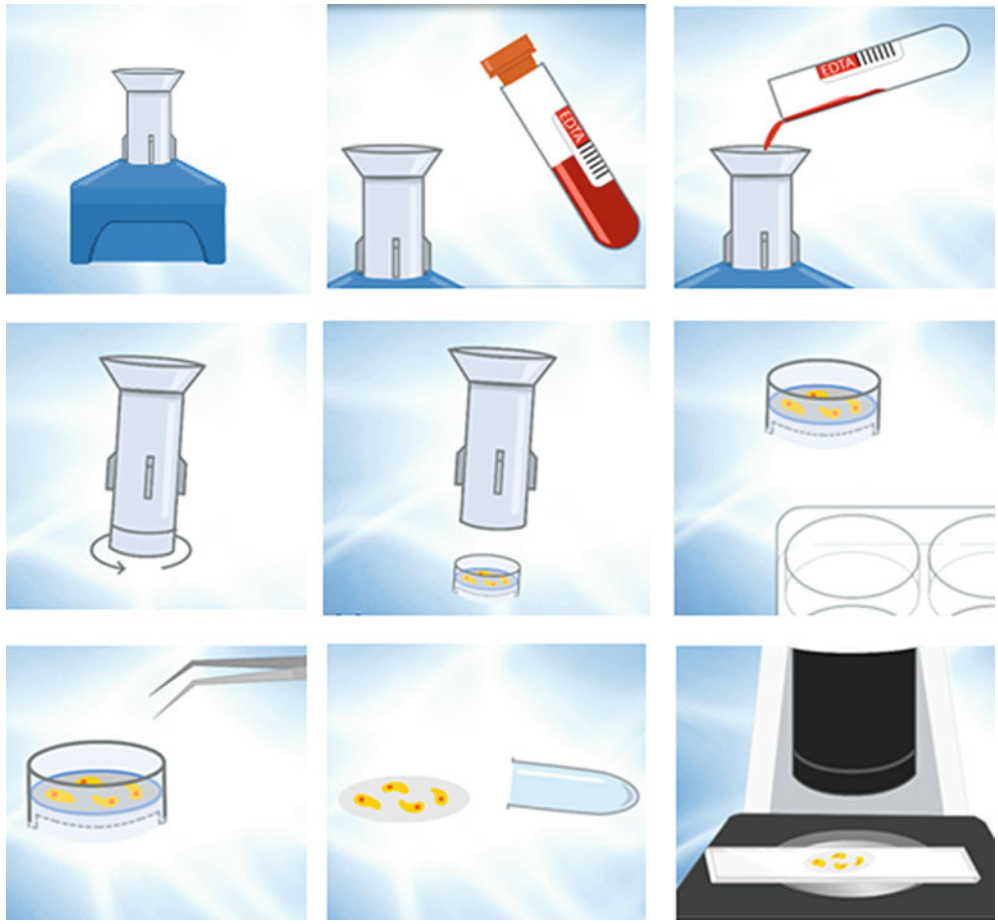

Fig. 1 Filtration procedure as presented by MetaCell is shown in short. Blood is transferred into the separation tube. The filtration starts as soon as the separation membrane touches the absorbent mass placed in the *blue* separation tube holder. After the filtration process the plastic ring with the separation membrane can be removed and placed directly into the culturing wells. After the short incubation period (min. 72 h) the membrane can be taken out of the plastic ring, the cells on the membrane are cytomorphologically evaluated and/or stored for later RNA/DNA analysis in Eppendorf tubes

2.5 Cytomorphological Analysis of CTCs

1. Fluorescence microscope or Inverted fluorescence microscope.

2.6 Isolation of RNA and DNA from CTCs

1. RLT buffer (Qiagen).
2. β-mercaptoethanol; add 100 μL per 10 mL RLT buffer.

2.7 Gene Expression Analysis and Mutational Analysis of CTCs

1. High-Capacity RNA-to-cDNA™ Kit (Thermofisher Scientific) 2. TaqMan® Fast Advanced Master Mix (Thermofisher Scientific)TaqMan® hydrolysis probes (Thermofisher Scientific).

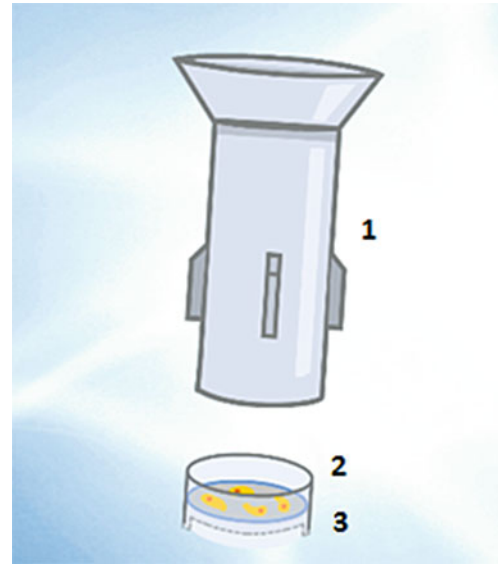

Fig. 2 Part of the filtration set (MetaCell®)—a filtration tube is shown in detail to identify specific parts of the filtration tube used in the protocol description. (1) filtration tube (2) plastic ring—a holder of the separation membrane (3) separation membrane

3 Methods

3.1 Peripheral Blood Collection

1. Peripheral blood is collected into tubes containing EDTA as an anticoagulant (e.g., S-Monovette/Vacuette). The samples are stored at a temperature of 4–8 °C. The isolation procedure should be completed within 24–48 h after the blood withdrawal.

3.2 CTC-Isolation

1. Size-based separation method for viable CTCs-enrichment from unclotted peripheral blood uses MetaCell® filtration tubes within filtration procedure (*see* Fig. 1).
2. MetaCell® tube (*see* Fig. 2) should be treated with UV–light for at least 15 min before use to prevent external contamination.
3. As a standard, 8 mL of blood is transferred into filtration tube. The minimum and maximum volume of the filtered peripheral blood may be adjusted with washing fluid up to 50 mL.
4. After completing the blood transfer, slightly push the plastic column (*see* Fig. 2–No. 1) to create a direct contact between the separation membrane and the absorbent.
5. Control the blood filtration flow, check if the whole blood volume has been filtered (*see* **Note 1**).
6. After blood filtration, the separation membrane (*see* Fig. 2–No. 3) placed in a plastic holder (*see* Fig. 2–No. 3) with captured

cells is washed with RPMI. Use 50% of the starting blood volume for RPMI washing.

7. Repeat the washing step at least twice (*see* **Note 2**).

3.3 CTCs Incubation and Cultivation

1. Remove the tube from the blue holder (*see* Fig. 2) (*see* **Note 3).**
2. Slightly turn and loosen the plastic ring (*see* Fig. 2–No.2) with the membrane (*see* Fig. 2–No. 3).
3. Place the plastic ring (*see* Fig. 2–No. 2–3) with the membrane into the 6-well plate.
4. Add growing medium to the well (*see* **Note 4**).
5. Place the 6-well plate into a CO_2 incubator under standard cell culture conditions (37 °C, 5% atmospheric CO_2) for a minimum of 72 h (incubation) or longer (cultivation) (*see* **Note 5**).
6. If an intermediate CTCs-analysis is intended/necessary, the CTC-fraction can be transferred from the separation membrane (*see* Fig. 2–No. 3) by splashing the plastic ring with the membrane (*see* Fig. 2–No. 2–3) with PBS (1.5 mL) to a cytospin slide (2 slides).

3.4 CTCs Visualization

1. The cells are analyzed by means of vital fluorescent microscopy using unspecific nuclear (NucBlue™) and cytoplasmatic (Cell-tracker™) stain. Basic cytomorphological parameters (*see* Fig. 3) are evaluated by an experienced cytologist/pathologist. As alternative standard hematological staining may be used (May-Grunwald) (*see* **Note 6)**.

3.5 Cytomorphological Analysis

1. The cells captured on the separation membrane are fluorescently stained after the short incubation period (72 h minimum). After the short staining period (15 min) the membrane (*see* Fig. 2–No. 3) with adherent cells is taken out from the plastic ring holder (*see* Fig. 2–No. 2) and the membrane is placed on the microscopic slide.
2. The fluorescently stained cells on the membrane are examined using fluorescence microscopy in two steps: (1) screening at ×20 magnification to locate the cells; (2) observation at ×40/×60 magnification for detailed cytomorphological analysis. Isolated cells and/or clusters of cells of interest are selected, digitized, and the images are then examined by an experienced researcher and/or pathologist.
3. Basic cytomorphological parameters are evaluated by experienced cytologist/pathologist. CTCs are defined as cells with the following characteristics (Fig. 3): (1) with a nuclear size ≥10 μm); (2) irregular nuclear contour; (3) visible cytoplasm, cells size over 15 μm; (4) prominent nucleoli; numerous nucleoli (5) high nuclear-cytoplasmic ratio; (6) observed proliferation, (7) cells invading the membrane pores creating 2D or 3D cell groups.

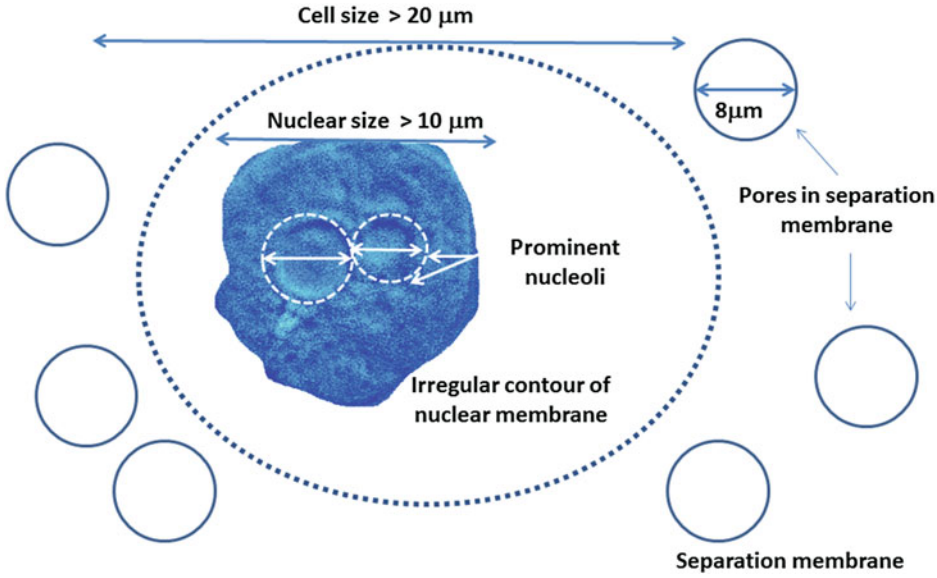

Fig. 3 Cytomorphological parameters of the cancer cells captured on the separation membrane are evaluated based on standard cytomorphological parameters. As the standard parameters were not set for the CTCs officially, we apply cytomorphological criteria reported by MetaCell. Based on these CTCs are defined as cells with the following characteristics: (1) with a nuclear size $\geq$10 µm); (2) irregular nuclear contour; (3) visible cytoplasm, cells size over 15 µm; (4) prominent nucleoli; numerous nucleoli; (5) high nuclear-cytoplasmic ratio; (6) observed proliferation, (7) cells invading the membrane pores creating 2D or 3D cell groups

CTCs separated, cultured, and visualized by fluorescence are shown on Fig. 4–9 (examples are shown for prostate carcinoma (*see* Figs. 4 and 5), bladder carcinoma (*see* Figs. 6 and 7), and renal carcinoma (*see* Figs. 8 and 9)). In all of the cases single CTCs observed are shown in comparison to the proliferating and growing CTC cultures.

3.6 Isolation of RNA and DNA

1. For RNA/DNA isolation, transfer captured cells (including the separation membrane) directly into the RLT buffer with β-mercaptoethanol (600 µL) and store at −20 °C. Standard protocols for RNA or DNA isolation can then be applied. As a rule up to 10–20 ng of RNA are isolated from one membrane.
2. RNA/DNA isolated from the CTC-fraction can be used for molecular analysis according to standard protocols (*see* **Note 7**).

4 Notes

1. In case of blood clotting, please add TrypLE solution, applying ratio Blood: TrypLE = 1:1, maximum volume of TrypLE is 5 mL.

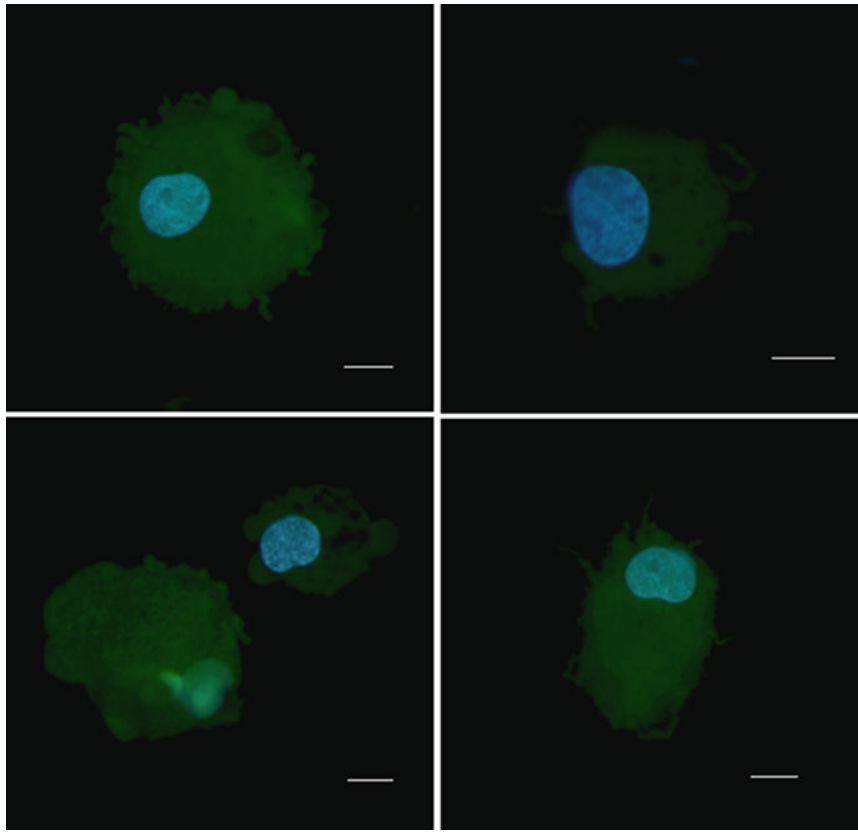

Fig. 4 Single circulating tumor cells isolated from peripheral blood of prostate cancer patient. Bar represents 10 μm

2. If some blood remains on the membrane after the filtration is completed, you may increase the washing solution volume and repeat the washing.
3. You may collect the filtered blood absorbed into the absorbent mass and preserve it for subsequent DNA isolation in dry place.
4. FBS-enriched RPMI medium (10%). Add 1 mL of the media to the bottom of the well first. Add 1 mL of the media to the membrane space over the plastic ring. Add 1 mL of the media to the bottom of the well again. Add 1 mL of the media into the membrane space in the plastic Alternatively, the enriched CTCs fraction can be transferred from the membrane and cultured directly on any plastic surface or a microscopic slide, or the separation membrane may be translocated on a microscopic slide.
5. CellTracker™ solution prepared according to the manufacturer's protocol (max 500 μL) is added to the cultivation well, additionally one drop of NucBlue™ is added directly to

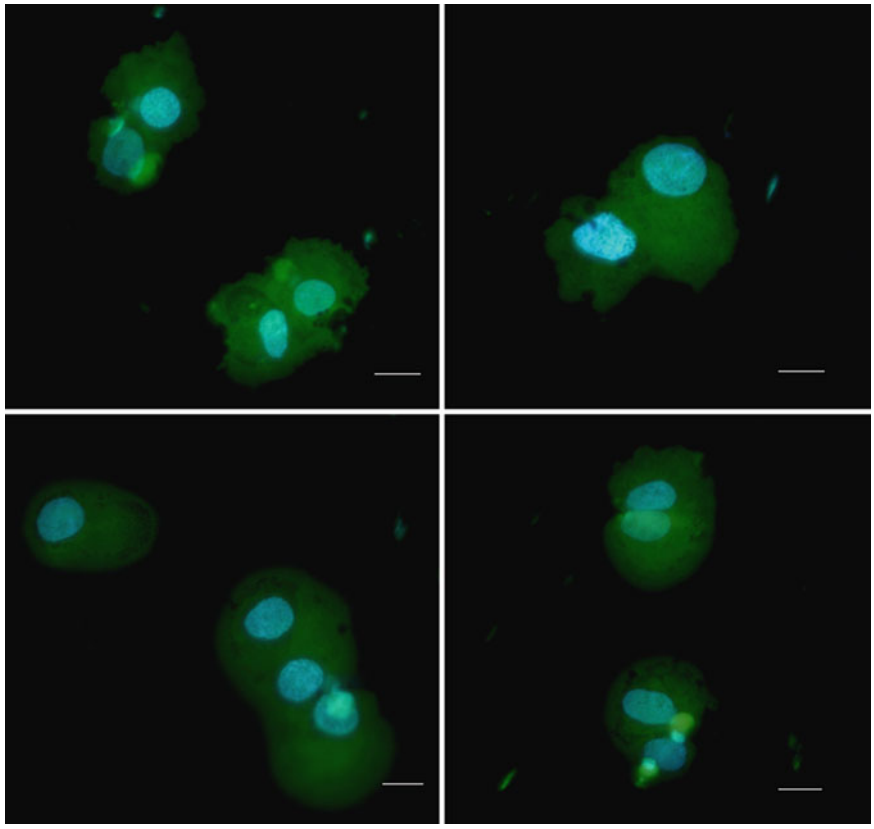

Fig. 5 Circulating tumor cells isolated from peripheral blood of prostate cancer patient are shown as proliferating in a culture. Bar represents 10 μm

the well with captured cells (plastic ring). Cells are stained for a minimun of 15 min.

6. Any commercial test using DNA isolated from the separated cell fraction
7. We usually perform qPCR using probes for highest sensitivity.

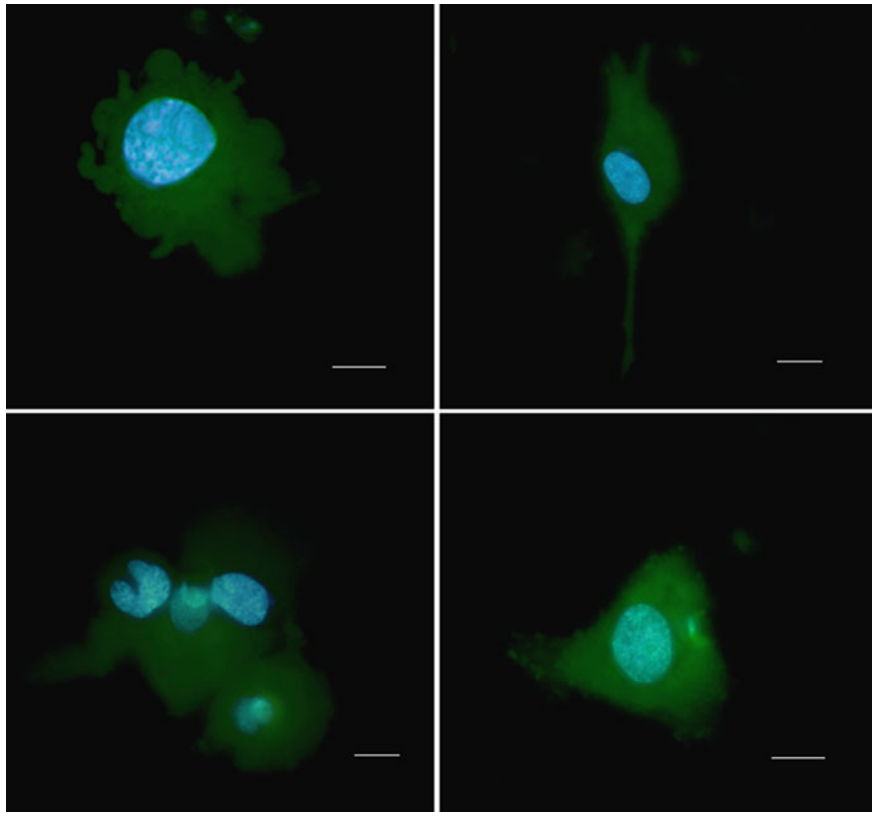

Fig. 6 Single circulating tumor cells isolated from peripheral blood of bladder cancer patient. Bar represents 10 μm

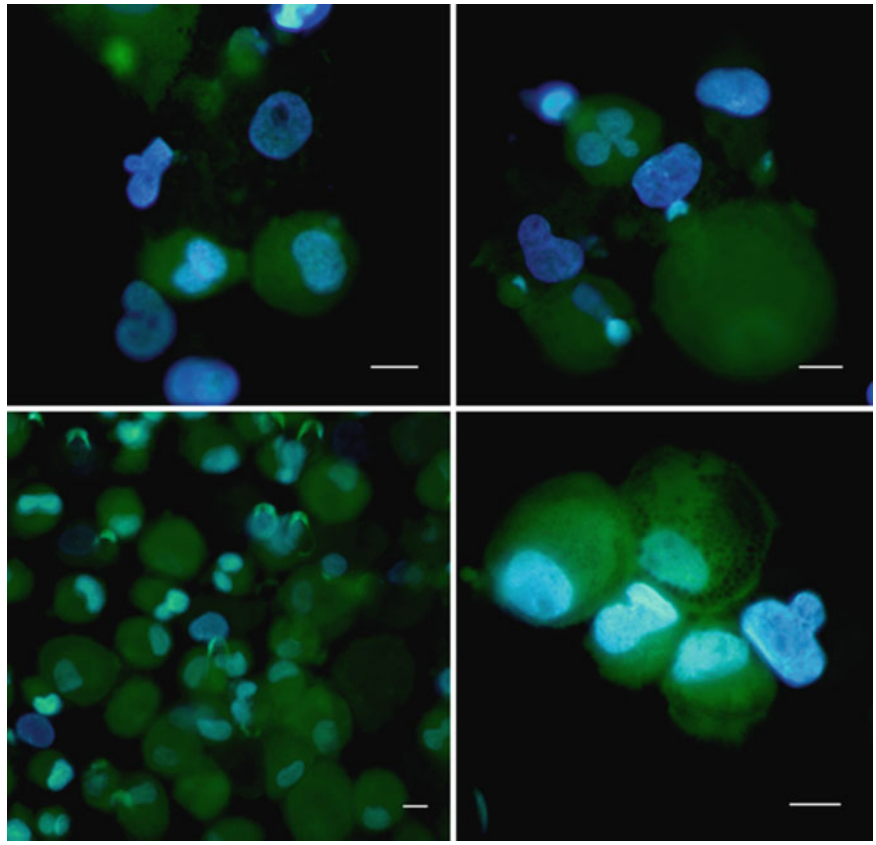

Fig. 7 Circulating tumor cells isolated from peripheral blood of bladder cancer patient are shown as proliferating in a culture. Bar represents 10 μm

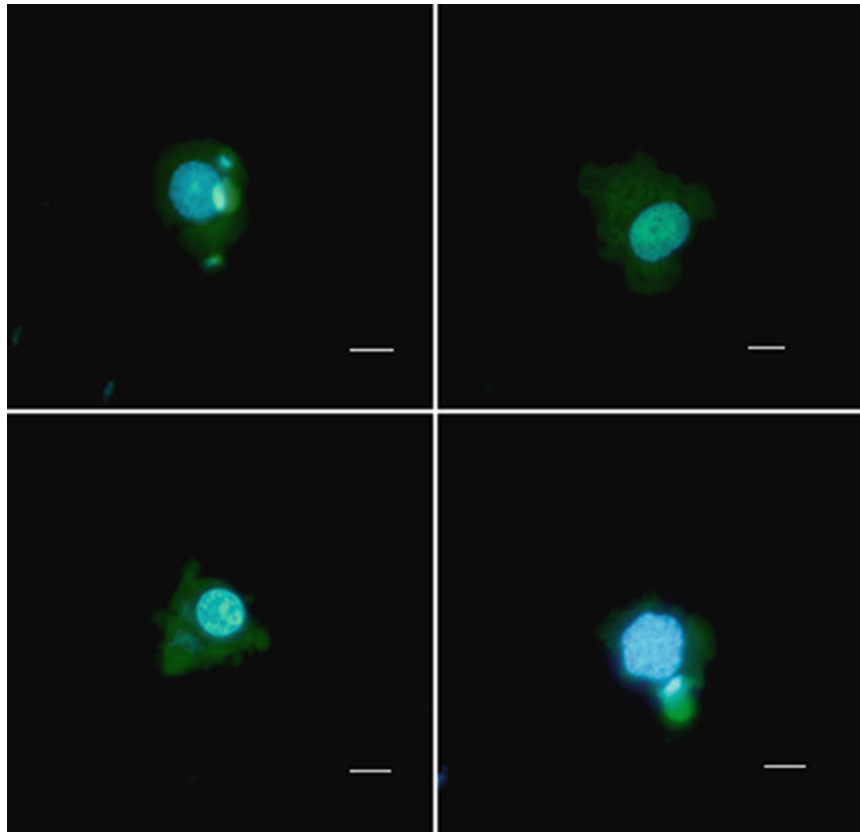

Fig. 8 Single circulating tumor cells isolated from peripheral blood of renal cancer patient. Renal carcinoma CTCs usually exhibit the biggest size (>20 μm) in the comparison with prostate and bladder cancer. Bar represents 10 μm

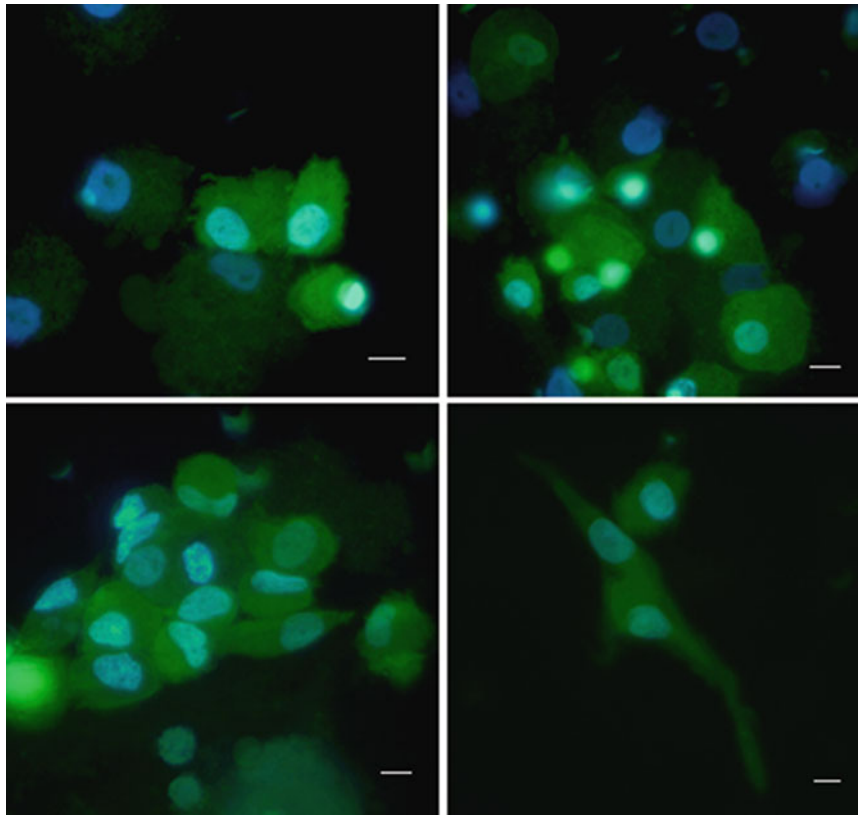

Fig. 9 Circulating tumor cells isolated from peripheral blood of renal cancer patients are shown as proliferating in a culture. The captured cells do exhibit both epithelial and mesenchymal (spindle cell like) morphology. Bar represents 10 μm

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