# Isolation and Characterization of Circulating Tumor Cells from Patients with Cancer

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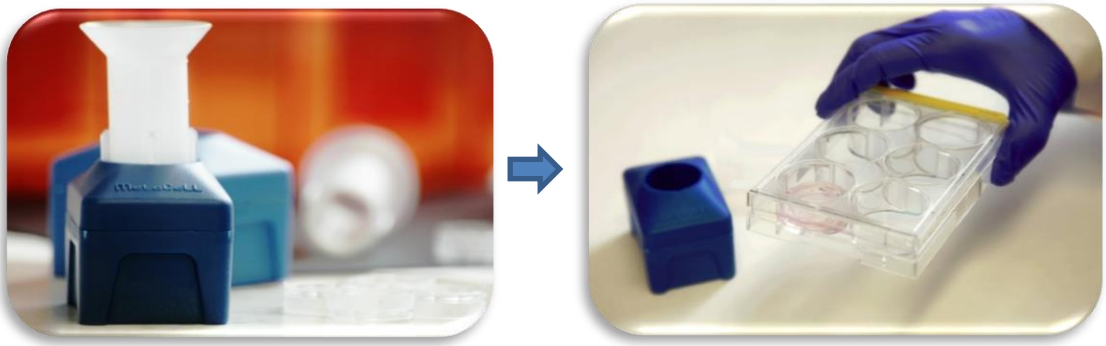
## Abstract

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, Cancer, Cultivation, In vitro, Gene expression



MetaCell

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 expressionprofiles or gene mutations in cancer [1-3].

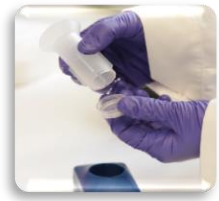
The examination for CTCs could be useful as well as a complementary cancer screening test, especially for excluding cancer, andincluding patients with indications for repeated biopsies, e.g. in case of prostate cancer. Serial examination of

CTCs examination offers an alternative, minimally invasive approach to characterize cancer celland to study early-stage disease [4 - 6]. CTCs are frequently detected in cancer of urothelial origin [6-8].

There are multiple approaches to detect CTCs. CTC counts in patients with metastatic cancercould, 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. Thespeed of the filtration process depends on the natural blood viscosity. These properties enable setting up in vitro cell cultures from theviable CTCs unaffected by any fixatives, antibodies, or lysingsolutions.



In vitro culturing of CTCs is a prerequisite forproliferation tests assessing chemosensitivity of tumors [9].

The protocol described below allows successful culturing of CTCsby 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.

# 1 Materials

*1.1 Peripheral Blood Collection*

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 x 8mL blood volume ).

*1.2* Isolation of CTCs

**Size-based separation device MetaCell® (MetaCell, Ostrava, Czech Republic) (see Fig. 2 Meta Cell® filtration tube).**

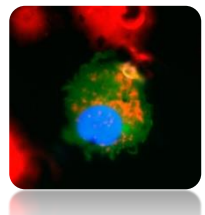
**Washing fluid: RPMI 1640 medium -** https://www.sigmaaldrich.com/CZ/en/product/sigma/r8758

*1.3 Incubation and Cultivation of CTCs*

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

*1.4 CTCs Visualization*

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



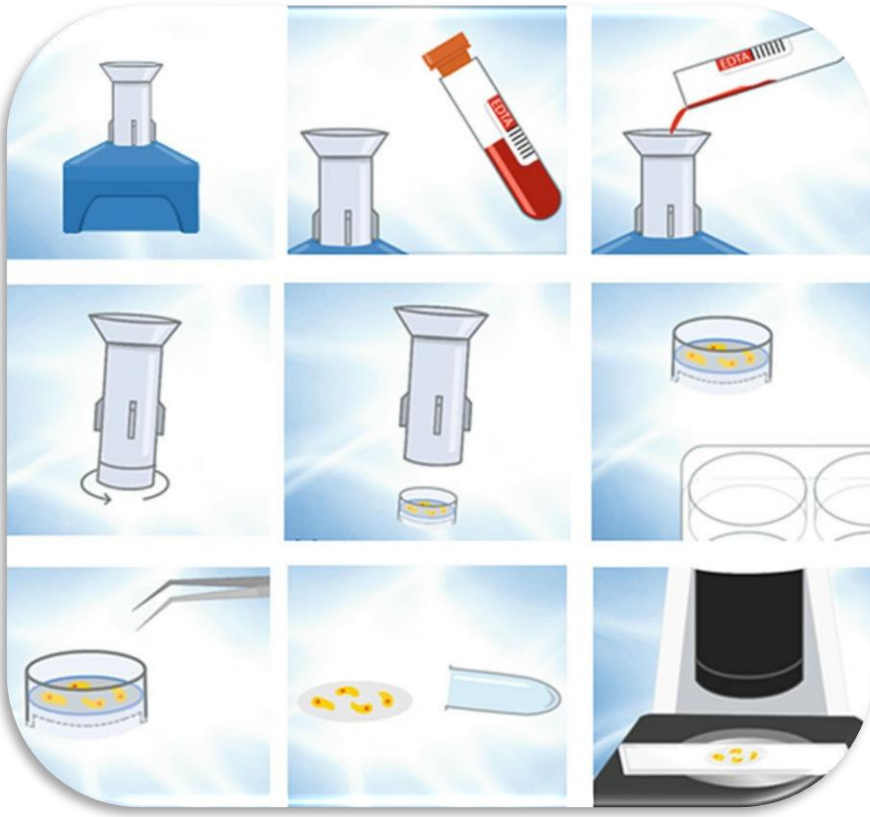



Fig. 1 Filtration procedure as presented by MetaCell is shown in short. Blood is transferred into the separation tube. The filtration starts as soon as the separation membrane touches the absorbent mass placed in the *blue* separation tube holder. After the filtration process the plastic ring with the 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

*1.5 Cytomor-phological Analysisof CTCs*

1. Fluorescence microscope or Inverted fluorescence microscope.

*1.6 Isolation of RNA and DNA from CTCs*

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

*1.7 Gene Expression Analysis and Mutational Analysis of CTCs*

1. High-Capacity RNA-to-cDNA™ Kit (Thermofisher Scien- tific)
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

## 2 Methods

### *2.1 Peripheral BloodCollection*

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 procedureshould be completed within 24–48 h after the blood withdrawal.

### *2.2 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.

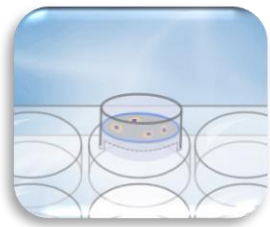

4. After completing the blood transfer, slightly push the plastic column (see Fig. 2–No. 1) to create a direct contact between the separation membrane and the absorbent.

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 capturedcells is washed with RPMI. Use 50% of the starting bloodvolume for RPMI washing.
7. Repeat the washing step at least twice (see Note 2).

### *2.3 CTCs Incubation and Cultivation*

1. Remove the filtration 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).

### *2.4 CTCs Visualization*

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).

*2.5 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 examinedusing fluorescence microscopy in two steps: (1) screening at 20 magnification to locate the cells; (2) observation at 40/60x magnification for detailed cytomorphological analysis.

3. Isolated cells and/or clusters of cells of interest are selected, digitized, and the images are then examined by an experiencedresearcher and/or pathologist.

4. Basic cytomorphological parameters are evaluated by experienced cytologist/pathologist. CTCs are defined as cells withthe following characteristics (Fig. 3): (1) with a nuclear size10 µm); (2) irregular nuclear contour; (3) visible cytoplasm, cells size over 15 µm; (4) prominent nucleoli; numerousnucleoli (5) high nuclear-cytoplasmic ratio; (6) observed proliferation, (7) cells invading the membrane pores creating 2D
or 3D cell groups.

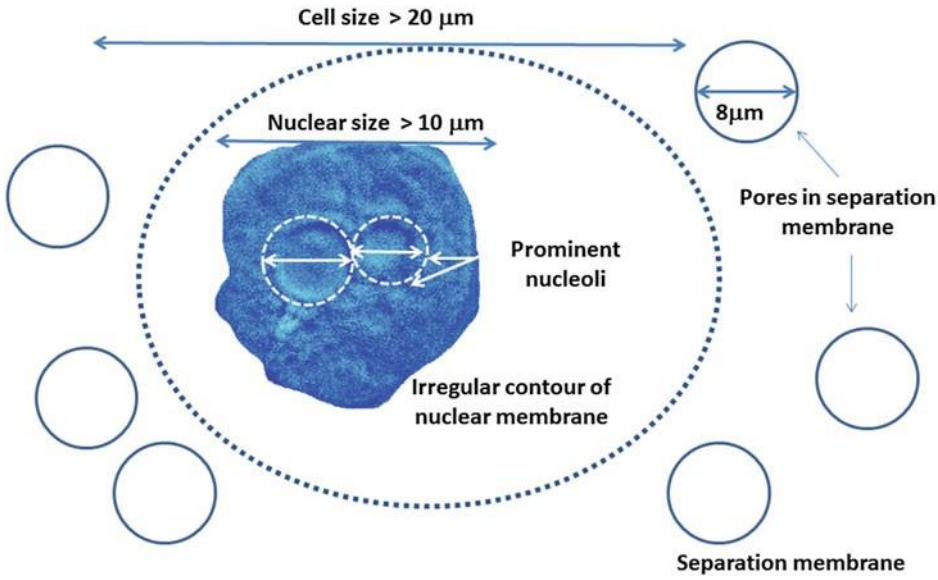



Fig. 3 Cytomorphological parameters of the cancer cells captured on the separation membrane are evaluated based on standard cytomorphological parameters. As the standard parameters were not set for the CTCs offi-cially, we apply cytomorphological criteria reported by MetaCell. Based on these CTCs are defined as cells with the following characteristics: (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

*2.6 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. Asa rule, up to 10–20 ng of RNA is 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).

## 3 Notes

1. In case of blood clotting, please add TrypLE solution, applyingratio Blood:TrypLE 1:1, maximum volume of TrypLE is5 mL.

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 tothe 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 intothe membrane space in the plastic

   Alternatively, the enriched CTCs fraction can be transferred from the membrane andcultured 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 the well with captured cells (plastic ring). Cells are stained for aminimun of 15 min.

6. Any commercial test using DNA isolated from the separatedcell fraction works

7. We usually perform qPCR using probes for highest sensitivity.

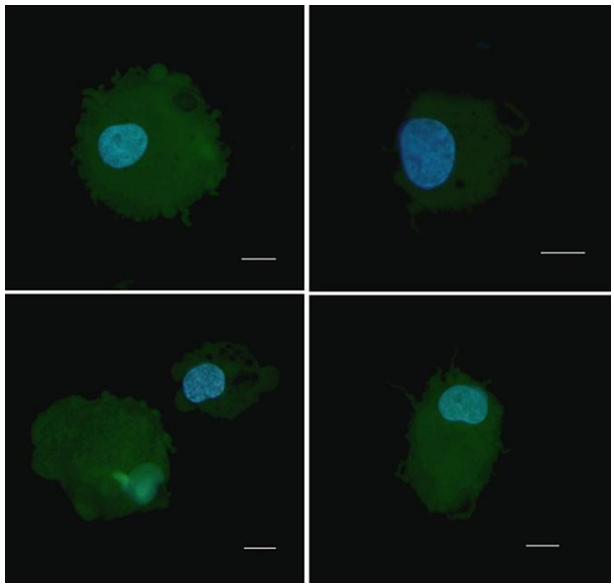


Fig. 4 Single circulating tumor cells isolated from peripheral blood of prostate cancer patient. Bar represents10 µm

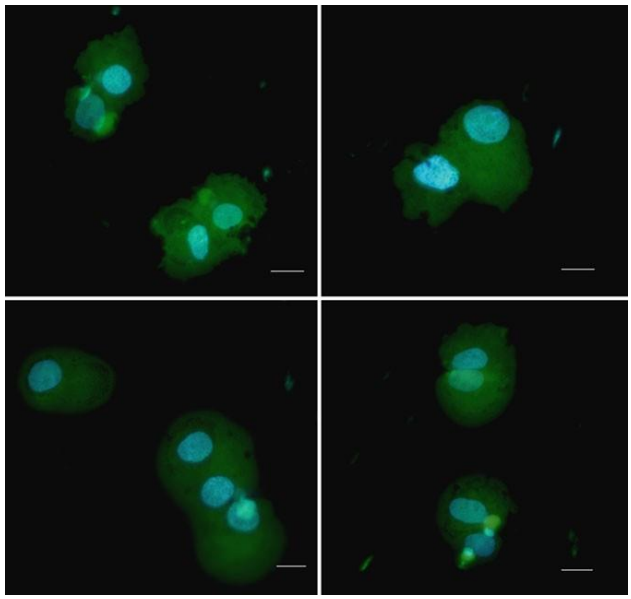


Fig. 5 Circulating tumor cells isolated from peripheral blood of prostate cancer patient are shown as proliferating in a culture. Bar represents 10 µm

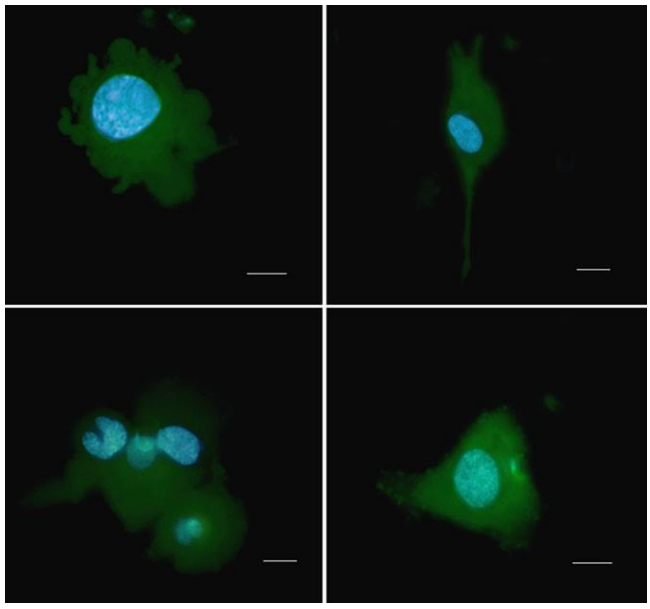


Fig. 6 Single circulating tumor cells isolated from peripheral blood of bladder cancer patient. Bar represents10 µm

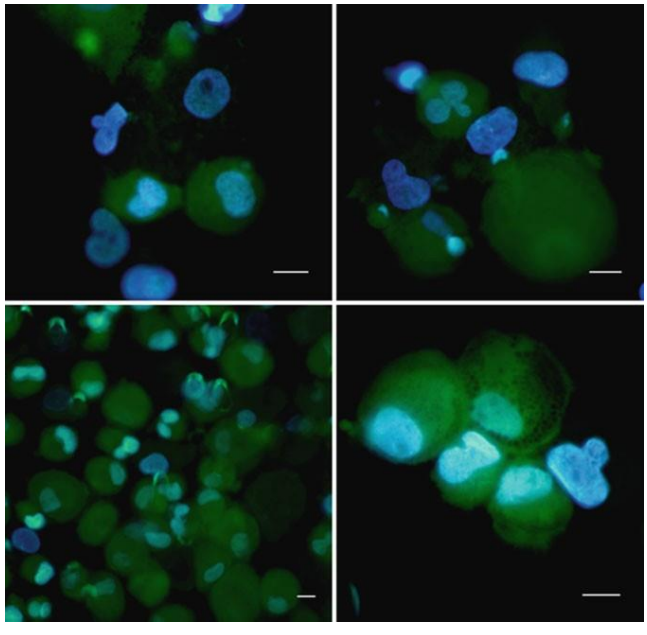


Fig. 7 Circulating tumor cells isolated from peripheral blood of bladder cancer patient are shown as proliferating in a culture. Bar represents 10 µm

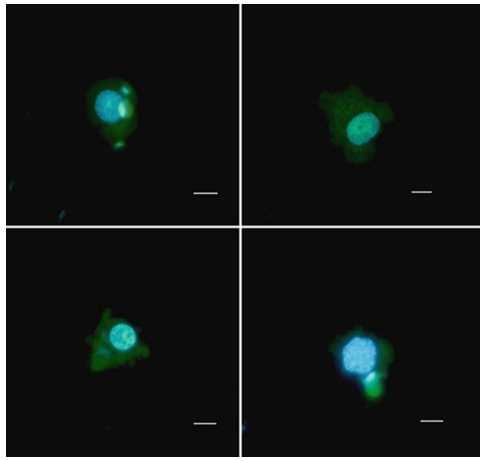


Fig. 8 Single circulating tumor cells isolated from peripheral blood of renal cancer patient. Renal carcinoma CTCs usually exhibit the biggest size (>20 µm) in the comparison with prostate and bladder cancer. Bar represents 10 µm

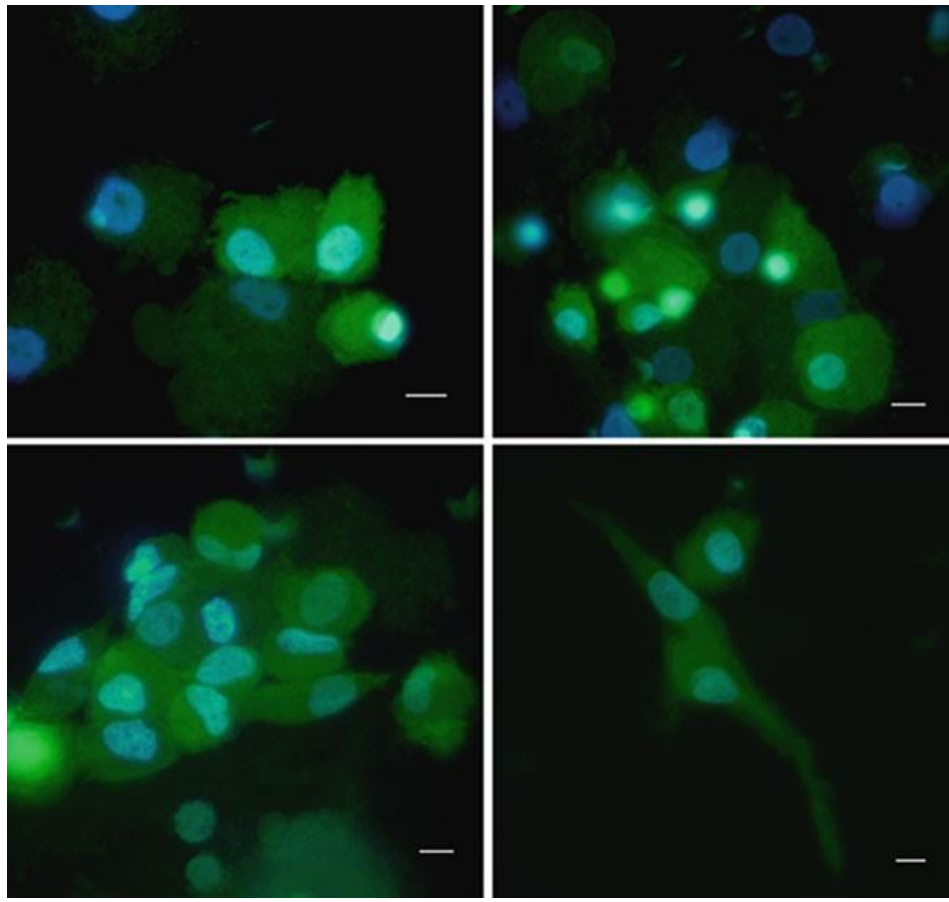


Fig. 9 Circulating tumor cells isolated from peripheral blood of renal cancer patients are shown as proliferatingin a culture. The captured cells do exhibit both epithelial and mesenchymal (spindle cell like) morphology. Bar represents 10 µm

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