

# Circulating Human Prostate Cancer Cells from an Orthotopic Mouse Model Rapidly Captured by Immunomagnetic Beads and Imaged by GFP Expression

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**Abstract.** *Circulating tumor cells (CTCs) are potential precursors of metastasis. They are also of use in diagnosing malignancy and for prognostic purposes. Our laboratory has previously isolated CTCs from orthotopic nude mouse models of human prostate cancer cells where the PC-3 cancer cells express green fluorescent protein (GFP). It was found that orthotopic tumors produced CTCs and not subcutaneous tumors, which may explain why orthotopic tumors metastasize and subcutaneous tumors do not. However, in this previous study, CTCs were observed only after culture. In the present study, using the GFP-expressing PC-3 orthotopic model and immunomagnetic beads coated with anti-epithelial cell adhesion molecule (EpCAM) and anti-prostate specific membrane antigen (PSMA), GFP-expressing CTC were isolated within 15 minutes and were readily visualized by GFP fluorescence. It was possible to immediately place the immunomagnetic-bead-captured GFP-expressing PC-3 CTCs in 3-dimensional sponge cell culture, where they proliferated. The combination of GFP expression and the use of immunomagnetic beads is a very powerful method to obtain CTCs for either immediate analysis or for biological characterization in vivo or in 3-dimensional culture.*

Dissemination of tumor cells into peripheral blood and the presence of circulating tumor cells (CTC) is the first step during the formation of metastases (1-3). CTCs have been isolated and characterized from the blood of cancer patients

by a variety of methods (4). The presence of CTCs in advanced stage prostate cancer patients has been correlated with poor prognosis (5-7). The presence of CTCs predicted survival more reliably than serum prostate-specific antigen (PSA) in prostate cancer patients who underwent resection (8, 9). CTCs have also been shown to predict patient outcome for metastatic breast and metastatic colorectal cancer, as well (8, 10). In addition to prognosis, CTCs can be used to study the biology of a patient's cancer (11, 12).

In previous studies (13, 14), we implanted metastatic human prostate cancer tumor fragments at orthotopic and ectopic sites to determine their ability to deliver viable malignant cells into the circulation. Using PC-3 prostate cancer cells expressing either green fluorescent protein (GFP) or red fluorescent protein (RFP), we demonstrated that viable circulating clones are produced in the orthotopic setting and not when growing subcutaneously. In the previous study, the GFP or RFP-expressing CTCs were detected only after separation and culture. The cultured CTCs had increased metastatic potential compared to parental PC-3 cells upon orthotopic implantation (13).

Recently, Helzer *et al.* (12) used a modified CTC chip to capture CTCs from an orthotopic prostate cancer mouse model. Laser capture microscopy was then used to collect CTCs from the chip. This method also used GFP to visualize the CTCs, but their capture and separation from the chip was tedious and time-consuming.

Magnetic-based separation methods have been used to enrich tumor cells from peripheral blood mononuclear cells (PBMCs). The basic principle of these methods is the use of immunomagnetic beads that are bound to epithelial cell adhesion molecule (EpCAM) antibodies which can recognize two surface glycoproteins (34 kDa and 39 kDa). EpCAM has been found to be expressed on the membrane of a majority of epithelial cancer cell types. The EpCAM antibody-coated magnetic beads bind to antigens on the membrane surface of epithelial cells, which can then be isolated from the samples by

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Figure 1. Noninvasive whole-body image of a nude mouse orthotopically implanted with PC-3-GFP human prostate cancer. The FluorVivo Imaging System (INDEC Biosystems, Santa Clara, CA, USA) was used for noninvasive fluorescence imaging. The primary tumor, as well as peritoneal and abdominal lymph node metastases, and metastasis to the diaphragm, can be seen.

use of a magnetic field (1, 15, 16). As few as five prostate cancer cells were detected per 5 ml of whole blood in model system experiments using anti-EpCAM magnetic particles alone or in combination with anti-PSMA magnetic particles (9).

In the present report, we describe the use of immunomagnetic beads to rapidly and easily isolate and image CTCs from an orthotopic mouse model of GFP-expressing prostate cancer.

## Materials and Methods

**Cell culture.** The PC-3 human prostate cancer cell line, expressing GFP, used in this study has been described previously (17). Except where noted, the cells were grown in RPMI-1640 supplemented with 10% fetal bovine serum and gentamycin (Life Technologies, Inc., Carlsbad, CA, USA) to 70-80% confluence as described previously (13, 18-20).

**Fluorescent orthotopic model of human prostate cancer metastasis in nude mice.** A PC-3 GFP fluorescent orthotopic model of human prostate cancer was used based on surgical orthotopic implantation in the prostate of nude mice (17). Similarly to the parental PC-3-derived tumors, GFP-expressing fluorescent orthotopic tumors exhibit highly aggressive metastatic behavior, in contrast to tumors derived from the same lineage growing subcutaneously (*s.c.*). The orthotopic tumors recapitulate to a significant degree the clinical pattern of metastatic spread of advanced clinical prostate cancer (13, 17). Animal experiments were carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals under NIH Assurance No. A3873-01.

**Immunomagnetic separation and enrichment of human prostate cancer epithelial cells.** Blood of nude mice with orthotopic PC-3-GFP human prostate cancer (0.5-1.0 ml) was obtained by cardiac puncture. Clotted and unclotted blood was put into an EDTA tube (BD, Franklin Lakes, NJ, USA). Erythrocytes were lysed (Buffer EL, Qiagen, Hamburg, Germany) and the CTCs were pelleted by centrifugation and then suspended in 1 ml phosphate-buffered saline (PBS). Immunomagnetic

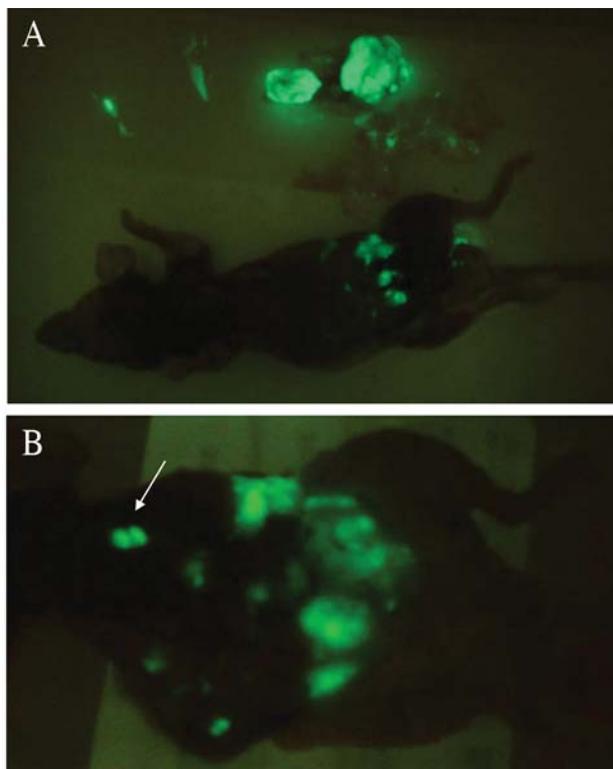


Figure 2. Image of an open nude mouse orthotopically implanted with PC-3-GFP human prostate cancer. In addition to the metastatic sites noninvasively imaged in Figure 1, metastasis to the peritoneum after removal of the primary tumor can be seen in Figure 2A and metastasis to the lung (arrow) can be seen in Figure 2B.

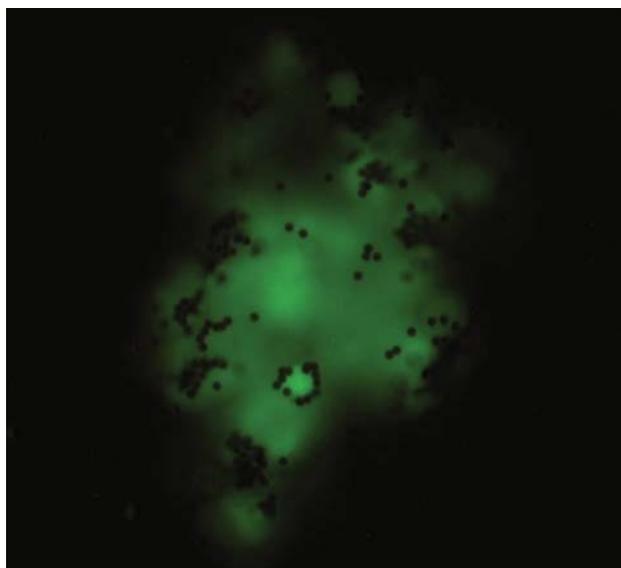


Figure 3. Dissociated primary PC-3-GFP tumor cells captured by immunomagnetic beads. The primary tumor of a mouse orthotopically implanted with the PC-3-GFP human prostate tumor was excised and mechanically dissociated. The dissociated cancer cells were captured by immunomagnetic beads and observed by fluorescence imaging as described in the Materials and Methods for capturing and observing CTCs.

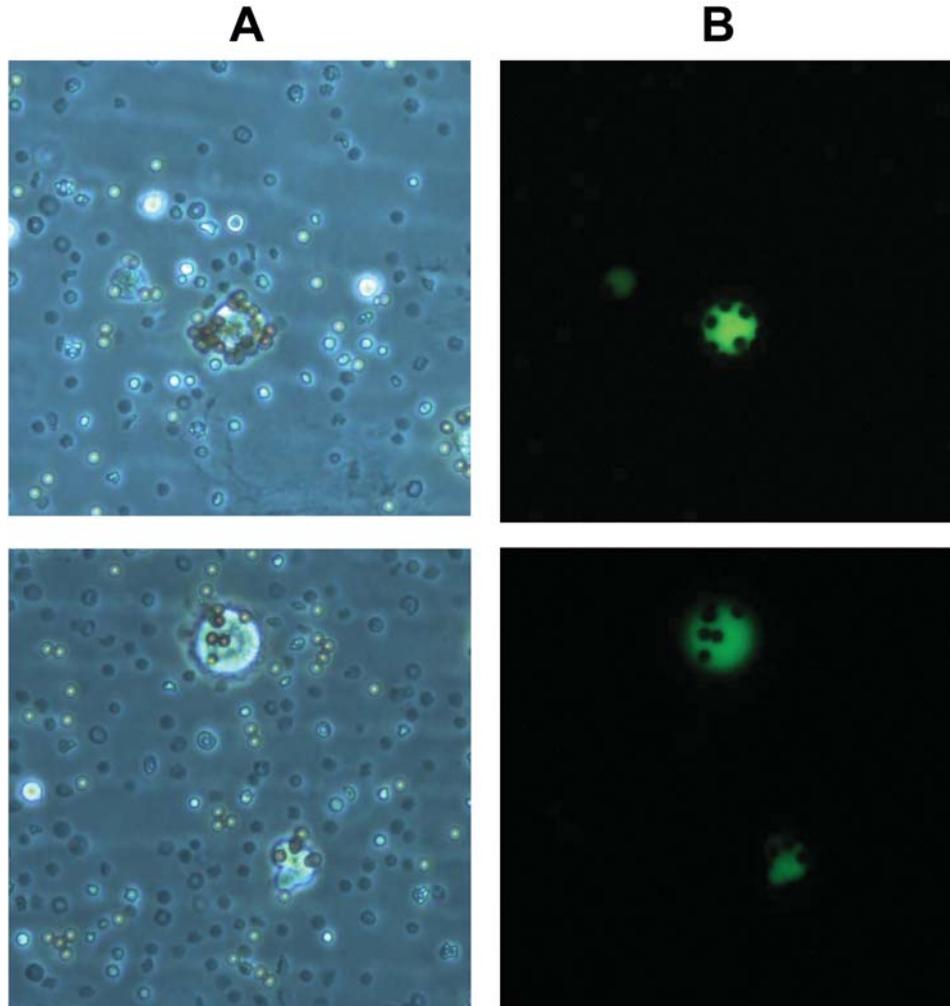


Figure 4. Circulating PC-3-GFP cancer cells captured by immunomagnetic beads. CTCs were harvested by cardiac puncture and captured by immunomagnetic beads as described in the Materials and Methods and observed by fluorescence microscopy using an Olympus IX71 fluorescence microscope (Olympus Corp., Tokyo, Japan). A: Brightfield microscopy. B: Fluorescence microscopy.

beads (AdnaTest ProstateCancer Select Kit, AdnaGen AG, Langenhagen, Germany) were added to the tube according to the manufacturer's protocol. The AdnaTest ProstateCancer Select Kit enables the immunomagnetic enrichment of tumor cells via epithelial and tumor-associated antigens. After 10 minutes of incubation at room temperature, the tube was placed in a Magnetic Particle Concentrator stand (DYNAL, Oslo, Norway), which attaches the beads to the wall of the tube. The attached beads were washed three times with 1 ml PBS. The bead-captured cells were then suspended in PBS or culture medium and observed under fluorescence microscopy.

*Fluorescence imaging.* An Olympus OV100 Small Animal Imaging System (Olympus Corp., Tokyo, Japan) and FluorVivo Small Animal Imaging System (INDEC BioSystems, Santa Clara, CA, USA) were used for brightfield and fluorescence imaging. Experiments were performed in the laboratories of AntiCancer Inc. and at the Third Faculty of Medicine, Charles University Prague.

## Results

*Whole-body fluorescence imaging of primary and metastatic tumors in the orthotopic model of PC-3 human prostate cancer.* Figure 1 shows the extensive primary growth of the PC-3 tumor, as well as its metastasis to peritoneal and abdominal lymph nodes and the diaphragm, observed noninvasively by fluorescence imaging.

*Open fluorescence imaging of primary and metastatic tumors in the orthotopic model of PC-3 human prostate cancer.* Figure 2 shows fluorescence imaging, in the open mouse, of extensive PC-3-GFP tumor growth on the prostate and metastasis to lung as well as to the sites observed noninvasively as described above.

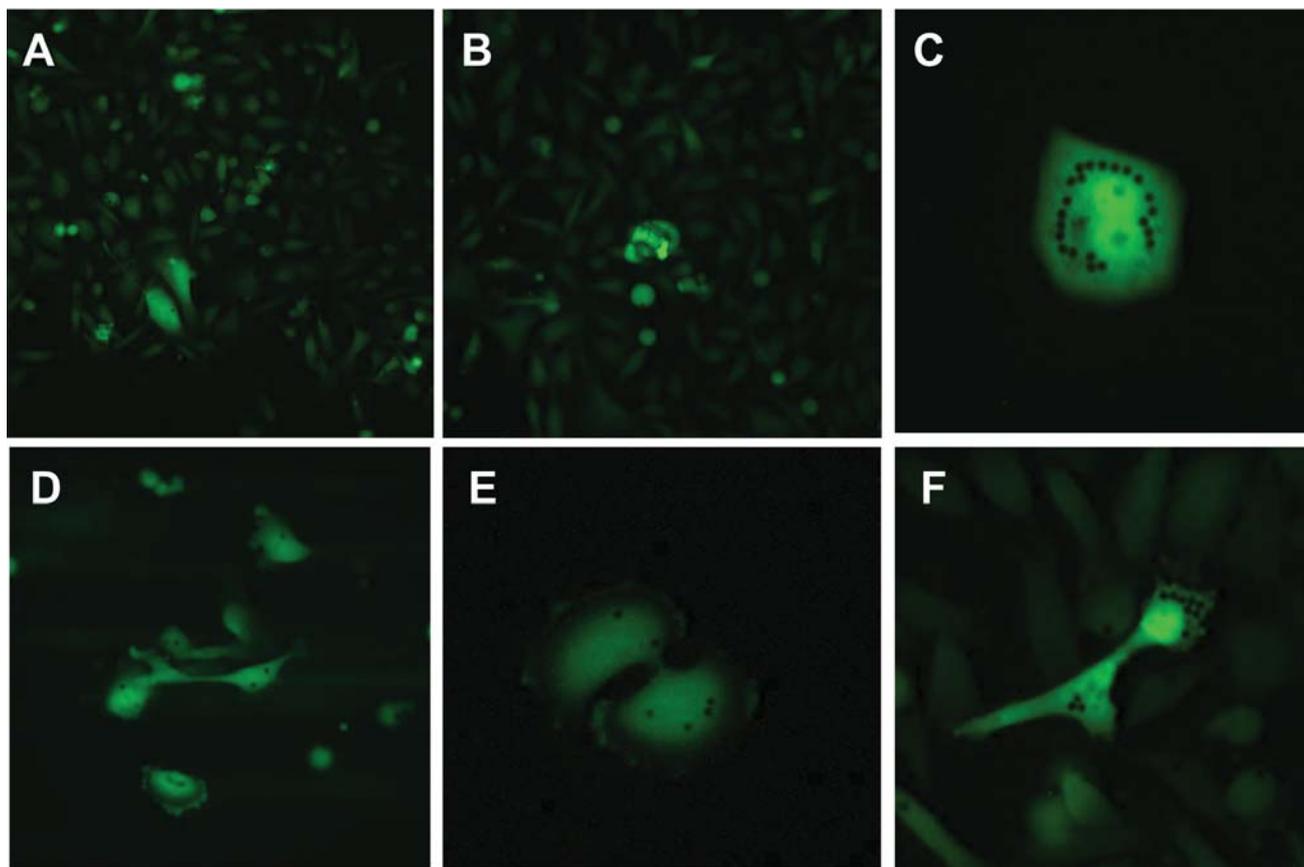


Figure 5. Gelfoam<sup>®</sup> sponge gel cultures of circulating PC-3 cells captured by immunomagnetic beads, growing in RPMI-1640 medium with 10% FBS. A: Three days' culture. B: Four days' culture. C: Four days' culture. Note the immunomagnetic beads bound to the cell which contains two nuclei. D: Two days' culture. E: Four days' culture. F: Four days' culture.

*Capture of disassociated PC-3-GFP primary tumor cells by immunomagnetic beads.* Figure 3 shows PC-3-GFP cancer cells disassociated from the primary tumor captured by the immunomagnetic beads. The captured cancer cells are readily visible by fluorescence microscopy.

*Capture of PC-3-GFP CTC by immunomagnetic beads.* Figure 4 shows PC-3-GFP CTC captured from the circulation of the mouse with an orthotopic tumor by the immunomagnetic beads. The captured CTCs are readily visible by fluorescence microscopy. The CTCs exhibited various morphologies. GFP expression indicates their viability.

*Sponge-gel culture of PC-3-GFP captured by immunomagnetic beads.* Figure 5 shows the immunomagnetic bead-captured PC-3-GFP cells growing in Gelfoam<sup>®</sup>. The PC-3-GFP CTCs thus have the ability to proliferate *in vitro*. Cells of varying morphology were observed in the Gelfoam<sup>®</sup> cultures.

## Discussion

CTCs are potential precursors of metastasis. They are also of use in diagnosing malignancy and for prognostic purposes. Our previous study found that orthotopic tumors produced CTCs in contrast to *s.c.* tumors, which may explain why orthotopic tumors metastasize and *s.c.* tumors do not. However, in the previous study, CTCs were observed only after tedious isolation and culture (13). In the present study, using the GFP-expressing PC-3 orthotopic model and immunomagnetic beads coated with anti-EpCAM and anti-PSMA, GFP-expressing CTC were isolated within approximately 15 minutes after obtaining blood and were readily imaged by GFP fluorescence. It was possible to immediately place the immunomagnetic bead-captured GFP-expressing PC-3 CTCs in 3-dimensional sponge cell culture where they proliferated. The combination of GFP expression and immunomagnetic beads is a very powerful method to obtain living CTCs for either immediate analysis or for biological characterization *in vivo* or 3-dimensional culture.

A central determinant of metastatic efficiency is cancer cell survival during hematogenous dissemination (21). Therefore it is critical to identify a sensitive method for CTC detection and for maintaining the isolated cells alive for further *in vitro* and *in vivo* growth and testing. The results here demonstrate that it is possible to obtain living CTCs within a short time from sampling and that magnetic capture does not influence the survival and proliferative capacity of the CTCs in cell culture.

Recently, patient CTCs were labeled with GFP using a telomerase-dependent adenovirus with the GFP gene (22). Combining the capture and imaging methods described in the present report with such a viral GFP labeling method of CTCs, the clinical evaluation of CTCs can become more effective and widespread.

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None of the authors has any conflicts of interest to declare.

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