Improved methods for detecting and characterizing of tumor cells in disaggregated lymph node metastases.

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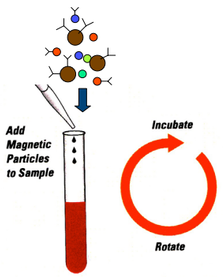
Material and methods:

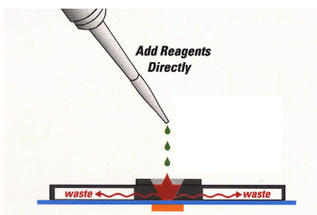
Melanoma lymph node metastases were disaggregated by the use of scalpels and filtered through a 70 um filter to get a cell suspension.

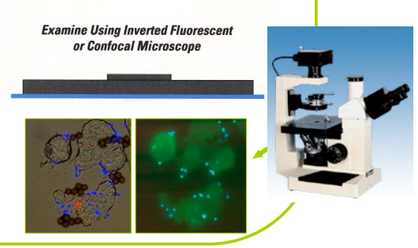
Immunomagnetic beads and fluorescent microspheres were added directly to the cell suspension in a tube and incubated under constant rotation at 4 °C.

After incubation, a fraction of the cell suspension was examined directly in a microscope (see figure 1 and 2) and the rest transferred to EpiSep slides resting on a NeoMag Dock with a magnetic field. Target cells rosetted by immunomagnetic beads are arrested upon contact with the magnetic field area. Excess liquid and non-targeted cells are absorbed in the pads. Fixation and intracellular staining of the captured cells are performed on the slides.

The EpiSep slides are removed from the NeoMag Dock for evaluation in an inverted fluorescent microscope. The slides can be handled and stored for later evaluation similar to conventional immuno-fluorescent slide preparations.







Direct microscopy of cells in supension

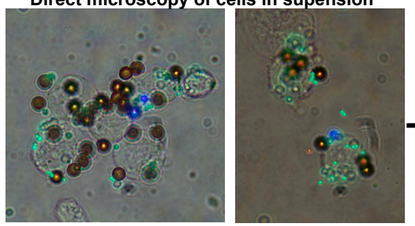
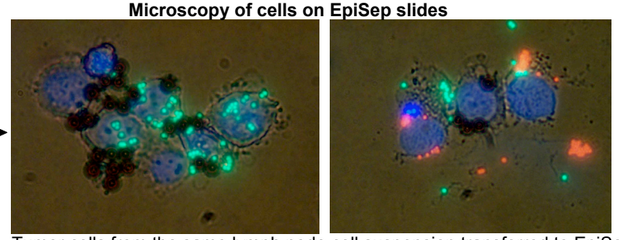


Figure 1: Tumor cells from a lymph node cell suspension viewed directly in a microscope.

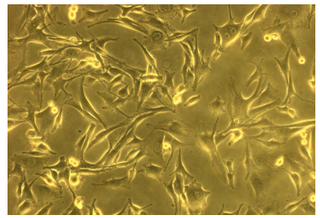
Immunomagnetic beads, **SAM M450 IgG – 9.2.27**
Green fluorescent microspheres – **EP 1**
Blue fluorescent microspheres - **MDR**

Microscopy of cells on EpiSep slides



Tumor cells from the same lymph node cell suspension transferred to EpiSep slides, fixed and stained with DAPI nuclear staining.

Immunomagnetic beads, **SAM M450 IgG – 9.2.27**
Green fluorescent microspheres – **EP 1**
Red fluorescent microspheres – **EGFR**
DAPI nuclear staining



In vitro cell culture from the selected tumor cells.

Direct microscopy of cells in suspension

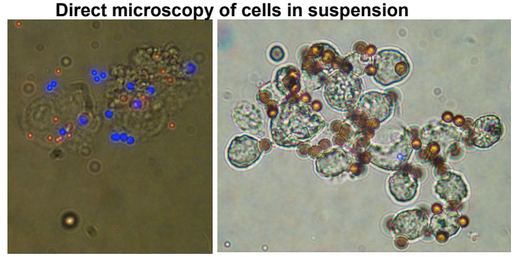
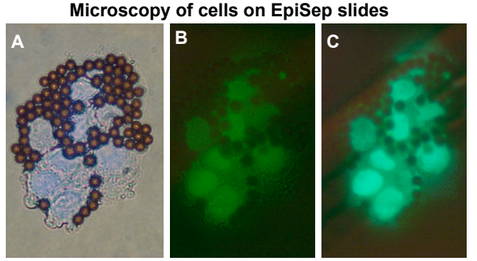


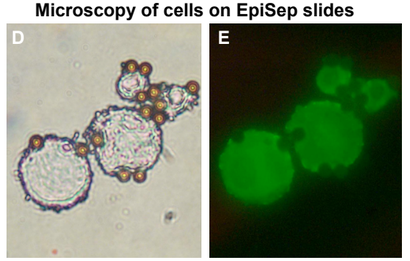
Figure 2: Tumor cells from another lymph node cell suspension as viewed directly in the microscope.

Immunomagnetic beads, **SAM M450 IgG –9.2.27**
Blue fluorescent microspheres - **425.3 (EGFR)**
Red fluorescent microspheres – **376.96**

Microscopy of cells on EpiSep slides

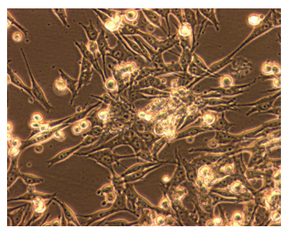


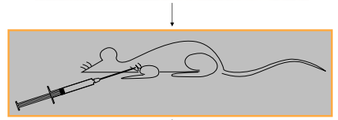
Microscopy of cells on EpiSep slides



Part of the same cell suspension transferred to EpiSep slides for further intracellular staining.

A,B,C: A cell cluster. **A** – rosetted with immunomagnetic beads, **SAM M450 IgG –9.2.27**
B - intracellular staining with **HMB 45/ FITC**
C – in addition **DAPI** nuclear staining
D,E: A cell cluster. **D** - rosetted with immunomagnetic beads, **SAM M450 IgG – 9.2.27**
E - in addition intracellular staining with **MART 1**





Microscopy of cells on EpiSep slides

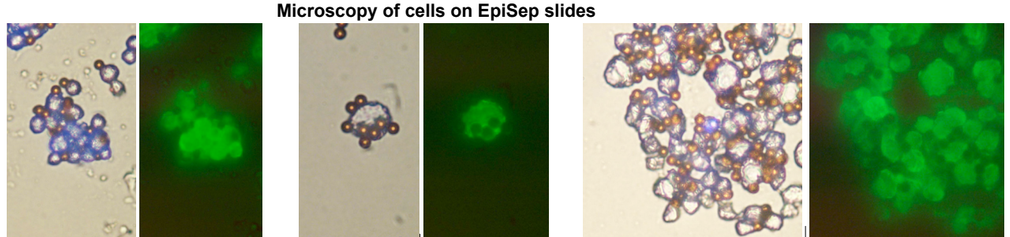


Figure 3: Tumor cells selected from melanoma lymph node metastases, cultured in vitro and injected into the left cardiac ventricle of a nude rat. The rat developed metastases in several organs.

Cells selected from a metastasis in tibia of the rat as viewed in an EpiSep slide. The cells were rosetted with immunomagnetic beads, **SAM M450 IgG 9.2.27** intracellular staining was performed with **MART 1 FITC**

Cells selected from a lung metastases of the same rat as viewed in an EpiSep slide. Rosetted with immunomagnetic beads, **SAM M450 IgG 9.2.27** and intracellular staining was with **HMB45 FITC.**

Cells selected from a brain metastases of the same rat after left cardiac ventricle injection of tumor cells. Labelled with immunomagnetic beads, **SAM M450 IgG 9.2.27** and intracellular staining was performed with **HMB45 FITC.**

Antibodies and beads:

9.2.27- reacts with a 250kD proteoglycan (HMW) melanoma associated antigen

376.96- reacts with a 100kD glycoprotein expressed by human melanoma and carcinoma cells

EP-1- reacts with a HMW melanoma antigen

MDR – reacts with extracellular P-glycoprotein

425.3 – reacts with the EGF receptor

HMB45– reacts with an intra-cytoplasmic antigen expressed in melanomas

MART-1– reacts with a melanoma specific transmembrane/intra cellular antigen (Melan-A).

Sheep anti-mouse(SAM) M450 IgG magnetic beads (Dynal,Invitrogen)

Fluorescent microspheres, 1 um (Molecular Probes, Invitrogen)

INTRODUCTION

The main objective of the study was to develop quick and sensitive methods to detect, isolate and characterize tumor cells present in lymph nodes, core biopsies of suspected malignancies or fine needle aspirats. Such methods may be of significant practical value in the assessment of disease stage, prognosis and possibly in determining optimal therapy. The ability to characterize the isolated tumor cells would make it possible to assess their expression of clinically relevant tumor-associated antigens and biomarkers. Here we present a new approach by combining the advantages of EpiSep XRC technology with our previously described immunomagnetic methods for isolation and characterization of circulating tumor cells from peripheral blood and bone marrow. In this system we used simultaneously immunomagnetic beads and 2-4 types of non-magnetic, fluorescent small microspheres, each type coated with an antibody known to bind cell surface markers relevant for the cancer type studied. The target cell detection is greatly faciliated by the strong fluorescense obtained with the microspheres. This approach allows confirmation of the nature of each, individual detected target cell and also visualizing the expression of tumor specific and prognostically important cell membrane markers, such as EGFr and ErbB2. In addition to visualizing expression of several membrane markers we show that the EpiSep slides makes it possible to also demonstrate the expression of specific intracellular antigens by immunofluorescense. The entire procedure can be completed within 1 -2 hours and can easily be applied to any complex mixture of cells, including FNA's, ascitic and pleural effusions as well as to dissagregated cell suspensions from a variety of solid tumors. The captured cells are pure, viable and can be used for further analysis and in vitro or in vivo culturing. Here we demonstrate the use of the methods on lymph node metastases from melanoma patients.

CONCLUSIONS

We present a simple, rapid and reproducible method for specific ans sensitive detection and characterization of target cells from lymph node metastases. The method can easily be applied to any complex mixture of cells.

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By simultaneous incubation with both immunomagnetic beads and smaller fluorescent microspheres, we can visualize the expression of several tumor specific and prognostically important membrane markers at the same time.

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XRC EpiSep is a cell capture platform that works like a magnetic test strip. Captured cells can be further characterized by intracellular staining, and the EpiSep slides can be stored for later documentation similar to conventional immunofluorescent slide preparations.

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In addition to automatically separating unwanted material, the EpiSep concentrates the desired sample into a small inspection area (0.6 cm^2) in contrast to conventional microscope slides with coverslips, which have an inspection area of 4 cm^2.

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The whole procedure is completed

within 1 - 2 hours.

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ABSTRACT
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Improved Methods for Detecting and Characterizing Tumor Cells in Disaggregated Lymph Node Metastases

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The main objective of the study was to develop improved methods to detect, isolate and characterize tumor cells present in lymph nodes, core biopsies of suspected malignancies or fine needle aspirates. Such methods may be of significant practical value in the assessment of disease stage, prognosis and possibly in determining optimal therapy. The ability to characterize the isolated tumor cells would make it possible to assess their expression of clinically relevant tumor-associated antigens and biomarkers. However, all reported methods have limitations regarding specificity, sensitivity and standardization. Here we show a new approach by combining the advantages of EpiSep XRC technology with our previously described immunomagnetic methods for rapid and simple isolation of circulating tumor cells from peripheral blood and bone marrow. The method is based on the use of immunomagnetic beads and fluorescent microspheres coated with specific antibodies for enrichment of the target cells present in a cell suspension, applying the enriched cell suspension onto a magnetic target cell capture area on the EpiSep slide. Non-target cells and unbound fluorescent microspheres are absorbed by adsorbent pads contained in the EpiSep slide. In this system we used simultaneously 2–4 types of non-magnetic, fluorescent small microspheres, each type coated with an antibody known to bind cell surface markers relevant for the cancer type studied. The target cell detection is greatly facilitated by the strong fluorescence obtained with the microspheres. This approach allows confirmation of the nature of each, individual target cell and also visualizing the expression of tumor specific and prognostically important cell membrane markers, such as EGFR and erbB2. The entire procedure can be completed in less than 2 hours. Here we demonstrate the use of the methods on lymph node metastases from melanoma patients, and in addition to visualizing expression of several membrane markers we show that the EpiSep slides made it possible to demonstrate the expression of specific intracellular antigens by immunofluorescense. The methods can easily be applied to any complex mixture of cells, including FNA's, ascitic and pleural effusions as well as to disaggregated cell suspensions from a variety of solid tumors. The captured cells are pure, viable and can be used for further molecular analysis and in vitro or in vivo culturing.

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