A rare-cell detector for cancer


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Although a reliable method for detection of cancer cells in blood would be an important tool for diagnosis and monitoring of solid tumors in early stages, current technologies cannot reliably detect the extremely low concentrations of these rare cells. The preferred method of detection, automated digital microscopy (ADM), is too slow to scan the large substrate areas. Here we report an approach that uses fiber-optic array scanning technology (FAST), which applies laser-printing techniques to the rare-cell detection problem. With FAST cytometry, laser-printing optics are used to excite 300,000 cells per sec, and emission is collected in an extremely wide field of view, enabling a 500-fold speed-up over ADM with comparable sensitivity and superior specificity. The combination of FAST enrichment and ADM imaging has the performance required for reliable detection of early-stage cancer in blood.

OCCULT tumor cells (OTCs) shed from tumors can travel through the blood stream to anatomically distant sites and form metastatic disease, the major cause of cancer-related death. OTCs are present in circulation in extremely low concentrations, estimated to be in the range of one tumor cell in the background of 10^6–10^7 normal blood cells and are occult to routine imaging and laboratory studies (1). Automated digital microscopy (ADM) using image analysis for recognition of specifically labeled tumor cells has been demonstrated to be the most reliable method currently available for OTC detection (2–5).

However, at the typical scan rate of 800 cells per sec, ADM is too slow to screen for a statistically valid number of OTCs (6). This slow scan rate is a result of two factors. One is the substantial latency associated with stepping the sample under the microscopy objective. This stepping results from the lens’ small field of view. The other factor is the long exposure time that is due to the low level of excitation from broadband illumination sources and the lack of sensitivity of the charge-coupled device detector used for imaging.

Here we report a scanning instrument using fiber-optic array scanning technology (FAST) that can locate OTCs at a rate that is 500 times faster than ADM, with comparable sensitivity and improved specificity. The exposure time is reduced by using a laser source for higher illumination levels and a more sensitive photomultiplier detector. However, our key innovation is providing an optical system with an exceptionally large field of view (50 mm) without a loss of collection efficiency. By collecting the fluorescence in an array of optical fibers that forms a wide collection aperture, the FAST cytometer has a 100-fold increase in field of view over ADM. Although this increase in field of view comes with a reduction in instrument resolution, the resolution is still sufficient for the identification of fluorescently labeled cells. This field of view is large enough to eliminate the need to step the sample under the collection optics, and hence there is no stepping latency. Although other instruments have used brighter sources and photomultiplier detectors (7), the FAST cytometer combines them with a large field of view and achieves a speed-up sufficient for efficient OTC detection in blood.

Materials and Methods

Cell Culture and Treatment. HT29 cells were grown in McCoy’s 5a medium containing 1.5 mM L-glutamine and 10% FBS. For modeling the in vivo situation of decreased epithelial cell adhesion molecule (Ep-CAM) expression of OTCs, HT29 cells were cultured for 3 days in the presence of 30 ng/ml IL-4 (BD Pharmingen) as described by Fleiger et al. (8). IL-4 treatment resulted in a 35% decrease of Ep-CAM expression as determined by direct immunofluorescence staining with anti-Ep-CAM fluorochrome-conjugated antibodies (Miltenyi Biotec, Bergisch Gladbach, Germany).

Immunofluorescent Labeling. Blood samples were processed by using a previously described method (9). Briefly, heparinized blood from human volunteers was spiked with HT29 colorectal cancer cells and subjected to lysis with isotonic ammonium chloride buffer (155 mM NH4Cl/10 mM KHCO3/0.1 mM EDTA, pH 7.4) at room temperature for 5 min. After centrifugation, the remaining white cell pellet was washed and resuspended in PBS, and the total number of living peripheral blood mononuclear cells was counted by using trypan blue exclusion. The cells were attached to custom-designed adhesive slides (Marienfeld, Bad Mergentheim, Germany) at 37°C for 60 min, and the slides then were blocked with cell culture medium at 37°C for 20 min. The deposited cells were fixed in ice-cold methanol for 5 min, rinsed in PBS, and blocked with 20% human AB serum (Nabi Diagnostics, Boca Raton, FL) in PBS at 37°C for 20 min. Slides then were incubated at 37°C for 1 h with a monoclonal anti-pan cytokeratin antibody (Sigma), which recognizes human cytokeratins 1, 4, 5, 6, 8, 10, 13, 18, and 19 in immunoblotting. Subsequently, slides were washed in PBS and incubated with a mixture of Alexa Fluor 488 and R-phycocerythrin-conjugated goat anti-mouse antibody (Molecular Probes) at 37°C for 30 min. After counterstaining with 0.5 μg/ml 4′,6-diamidino-2-phenylindole (Molecular Probes) in PBS at room temperature for 10 min, slides were mounted in ProLong mounting medium (Molecular Probes).

ADM. Coordinates of prospective cells identified by the FAST cytometer were fed into the rare-event imaging system (REIS), a fully automated scanning digital microscopy system. The hardware components of the REIS and the proprietary scanning software have been described in detail elsewhere (4, 10).

Immunomagnetic Enrichment. IL-4-treated and nontreated HT29 cells were spiked into two separate aliquots of 2 ml of peripheral blood. After red blood cell lysis, samples were divided into two parts: the first part was deposited onto adhesive slides as described above. Before cell deposition, the second part was

Abbreviations: OTC, occult tumor cell; ADM, automated digital microscopy; FAST, fiber-optic array scanning technology; Ep-CAM, epithelial cell adhesion molecule; REIS, rare-event imaging system.

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subjected to positive immunomagnetic selection with anti-EpCAM microbeads (Miltenyi Biotec). The immunomagnetic enrichment was performed with MS separation columns (Miltenyi Biotec) according to manufacturer instructions.

**Results**

**Optical System.** The large field of view is enabled by an optical fiber bundle with asymmetric ends. As shown in Fig. 1, the collection end is long (50 mm) and narrow (2 mm), whereas the transmission end is circular (11.3 mm in diameter). In operation, an argon ion laser scans the substrate lying on top of the collection end, and the collected emission is subsequently collimated after the circular aperture. The emission from the fluorescent probes is filtered by using standard dichroic filters before detection in a photomultiplier. The sample moves across the laser scan path on a stage traveling in a direction orthogonal to the laser scan direction. The location of a fluorescing cell is determined accurately by the scan and stage positions at the time of emission. The scanning mechanics enable accurate determination of the emission location (better than 100 μm) for subsequent reviewing.

The laser is scanned at 100 scans per sec by using a galvanometer-rotated mirror, whereas the substrate is moved at 2 mm/sec over it, which produces an exposure rate of 1 cm²/sec⁻¹. The galvanometer can operate at this scan velocity over a 25° scan angle with linear angular time response. An F-Theta field lens transforms the 15.2° actual mirror deflection into linear displacement with a transformation accuracy better than 0.1%, which results in a reproducible distortion over the field of ≈ 20 μm. A second filter analyzes the ratio between the intensities of the fluorescence from different channels to eliminate homogeneous dye aggregates, a common artifact of immunofluorescence staining.

**Measurements.** Detected fluorescent objects are analyzed with software filter operations to differentiate rare cells from false positives. Because the cells are generally smaller than the laser-spot resolution (20 μm), the first filter passes all objects that are below a size threshold (20 μm). A second filter analyzes the ratio between the intensities of the fluorescence from different channels to eliminate homogeneous dye aggregates, a common artifact of immunofluorescence staining.

The FAST capability was tested with samples of HT29 cells spiked in peripheral blood from a healthy donor. The samples were scanned first by the REIS (10), an ADM with image-analysis software optimized for the detection of OTCs. The REIS scan along with visual inspection established the number of tumor cells. The samples then were scanned by the FAST cytometer.

Objects detected by the FAST cytometer were subsequently reexamined under a fluorescence microscope to differentiate rare cells from artifacts for annotation of the FAST image data. The false positives are distinguished from true positives by shape, staining pattern, and the presence of 4′,6-diamidino-2-phenylindole-stained nuclear material. By visual inspection of the microscope images, we determined that the FAST

### Table 1. Data for ADM and FAST cytometer scans of peripheral blood spiked with the HT29 cell line

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sample size, no. of WBCs</th>
<th>REIS OTCs, no. of cells</th>
<th>FAST OTCs, no. of cells</th>
<th>FAST false negatives, no. of cells</th>
<th>FAST sensitivity, %</th>
<th>FAST false positives, no. of objects</th>
<th>FAST specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6 × 10⁶</td>
<td>76</td>
<td>69</td>
<td>7</td>
<td>91</td>
<td>79</td>
<td>1.3 × 10⁻⁵</td>
</tr>
<tr>
<td>2</td>
<td>2 × 10⁶</td>
<td>102</td>
<td>102</td>
<td>0</td>
<td>100</td>
<td>18</td>
<td>6 × 10⁻⁶</td>
</tr>
<tr>
<td>3</td>
<td>4.5 × 10⁶</td>
<td>200</td>
<td>198</td>
<td>0</td>
<td>99</td>
<td>106</td>
<td>2.4 × 10⁻⁵</td>
</tr>
<tr>
<td>Total</td>
<td>13.5 × 10⁶</td>
<td>378</td>
<td>369</td>
<td>9</td>
<td>98</td>
<td>203</td>
<td>1.5 × 10⁻⁵</td>
</tr>
</tbody>
</table>

The sample is scanned first with REIS to determine the total number of OTCs and then scanned with the FAST cytometer. The false negatives are the cells missed by the FAST, and the sensitivity is the ratio of the OTCs detected by the FAST cytometer to the total OTCs. The false positives are artifacts detected by the FAST cytometer that are indistinguishable from OTCs. The specificity is the ratio of false positives detected by the FAST cytometer to the total number of white blood cells (WBCs) in the sample.
Table 2. Scan data for ADM plus the tandem FAST cytometer and REIS for HT29-spiked peripheral blood

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sample size, no. of WBCs</th>
<th>REIS OTCs, no. of cells</th>
<th>Tandem OTCs, no. of cells</th>
<th>Tandem false negatives, no. of cells</th>
<th>Tandem sensitivity, %</th>
<th>Tandem false positives, no. of objects</th>
<th>Tandem specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6 × 10^6</td>
<td>76</td>
<td>65</td>
<td>11</td>
<td>86</td>
<td>13</td>
<td>2 × 10^-6</td>
</tr>
<tr>
<td>2</td>
<td>3 × 10^6</td>
<td>102</td>
<td>101</td>
<td>1</td>
<td>99</td>
<td>5</td>
<td>1.7 × 10^-6</td>
</tr>
<tr>
<td>3</td>
<td>4.5 × 10^6</td>
<td>200</td>
<td>196</td>
<td>4</td>
<td>98</td>
<td>19</td>
<td>4 × 10^-6</td>
</tr>
<tr>
<td>Total</td>
<td>13.5 × 10^6</td>
<td>378</td>
<td>362</td>
<td>16</td>
<td>95</td>
<td>37</td>
<td>3 × 10^-6</td>
</tr>
</tbody>
</table>

The sample is scanned first with the REIS to determine the total number of OTCs. The sample is then scanned with the FAST cytometer, which identifies the locations of possible OTCs, and then REIS scans only those locations. The tandem OTCs are those found in this operation. The false negatives are the cells missed by the tandem operation, and the sensitivity is the ratio of the OTCs detected by the tandem operation to total OTCs. The false positives are artifacts detected by the FAST cytometer that are indistinguishable from OTCs. The specificity is the ratio of false positives detected by the tandem operation to the total number of white blood cells (WBCs) in the sample.

**Discussion**

It is estimated that OTCs are present in circulation at concentrations between 10^-6 and 10^-7 (1). Assuming the lower end of this range, 10^-7, a sample of at least 100 million hematopoietic cells is needed to detect at least one OTC with a high probability (99.995%). An ADM analysis of such a sample size would take 18 h, resulting in 3,000–30,000 objects for a cytopathology examination (2–5). Based on the performance presented here, a FAST prescan of 100 million cells would take 5 min and result in 1,500 objects for subsequent rescanning by ADM. With the improved specificity of the tandem approach, this rescanning with ADM would require subsequent manual examination of only 300 objects. With the tandem approach, the task of screening 100 million hematopoietic cells could be completed within 1 h.

Unlike enrichment approaches, no additional processing is required for FAST cytometry that could result in reduced sensitivity due to cell loss. In addition, unlike alternative techniques for OTC detection such as PCR or fluorescence-activated cell sorting, FAST cytometry allows the cytomorphology of the prospective rare cells to be readily examined at any time.

The availability of a scanning system with the speed and sensitivity of FAST could have a profound impact on the management of cancer patients. First, because tumor cells are estimated to be dispersed into the circulation at the earliest stages of malignant progression, it could be used for the early detection of cancer.
fluorophore much more efficiently than the 488-nm excitation enable dual laser excitation. With this modification, a second rescan time, modifications may be made to the optical path to objects that need to be rescanned by the ADM and hence the methodology for the detection of rare cells at these low concentrations is contradictory because of the absence of reliable information could be used in the image-analysis process to reject false-positive detections.

Although validation of the technology on samples from early- and late-stage cancer patients is still incomplete, preliminary results show that FAST image-analysis filters are adequate for detecting OTCs in cancer patient peripheral blood, although these OTCs are not nearly as uniform in shape as are tumor cell lines. Because the resolution of its scan is much lower than that of ADM, FAST is insensitive to morphological variation, and consequently, the sensitivity and specificity of the FAST assay is more independent of OTC heterogeneity than an ADM assay, which relies on morphological analysis for filtering.

OTCs constitute the link between primary and metastatic lesions, and after the removal of known primary or metastatic lesions, they constitute the actual target of therapy. Consequently, a better understanding of these cells will provide critical information about the metastatic potential, drug sensitivity, and development of therapy resistance in metastasizing tumors. The simplicity of the FAST approach provides a technology base for a low-cost, robust, clinical diagnostic instrument that could be used for the early diagnosis of cancer and for monitoring the efficacy of anticancer therapy for solid tumors. FAST cytometry could become as essential and ubiquitous as the Pap smear for cancer diagnosis.