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Laser-guidance based cell detection for identifying malignant cancerous cells without any fluorescent markers

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Abstract

Laser guidance technique employs the optical forces generated from a focused Gaussian laser beam incident on a biological cell to trap and guide the cell along the laser propagation direction. The optical force, which determines the guidance speed, is dependent on the cellular characteristics of the cell being guided, such as size, shape, composition and morphology. Different cell populations or subpopulations can be detected without any fluorescent markers by measuring their guidance speeds. We found that cell guidance speeds were sensitive enough to monitor the subtle changes during the progression of mouse fibroblast cells from normal to cancerous phenotype. The results also demonstrated that this technique can effectively distinguish mouse mammary cancerous cells with different metastatic competence. Laser guidance technique can be used as a label-free cell detection method for basic cell biological investigation and cancer diagnosis.

Keywords

Cancer diagnostics; Cell detection; Laser guidance; Optical force

Introduction

Rapid and efficient techniques for identifying and distinguishing cells based on their morphological differences play an important role in experimental cell biology, immunology and biomedical research (Dainiak et al. 2007). These differences include structural and compositional alternations that are intrinsic to various tissue types or induced by disease or medication. Cellular function related biological science necessitates the isolation and purification of particular cell types from a mixture of various cell types. Identification of particular cell types in a cell population can lead to improvements in many medically relevant problems including cancer diagnosis (Mohamed et al. 2004), stem cell characterization (Chan and Lieu 2009), and function assessment of genetically modified cells (Vanvlasselaer 1995). Conventional cell sorting technique, flow cytometry, evaluates cellular features through fluorescence markers and purifies the heterogeneous cell suspension into fractions containing a single cell type (Ibrahim and van den Engh 2007).

Although markers provide biological assay with a high degree of specificity, introducing exogenous fluorescent markers will cause toxicity or immunogenicity, perturb the cell's native microenvironment, influence cell properties and viability, and subsequently compromise the clinical use of the cells (Wei et al. 2007). Additionally, appropriate markers may be not available for every cell type, and some markers are difficult to successfully introduce to the cells (Buckman et al. 2001). Therefore, techniques and instruments to detect cells accurately and consistently without using fluorescence-based cell markers are critical to clinical applications. In this report, we developed a label-free cell detection technique based on optical force to maintain the structural and functional integrity of the cells after cell-type identification.

The optical forces, which are generated from the momentum change induced by the interaction between laser beam and cell, have been employed in many label-free cell detection configurations (Kim et al. 2007; MacDonald et al. 2004; Sun et al. 2007). These techniques applied optical force to physically separate different cell types, but cannot achieve high sensitivity for cell subtype identification with small morphological differences. In our laser-guidance based cell detection technique, the laser beam is weakly focused; therefore optical forces can trap a cell into the beam axis and propel it along the axis with relevant guidance speeds. Size, shape, composition, internal morphology, and surface membrane properties all contribute to the cell's optical properties, which influence the optical forces experienced by a cell to a laser beam. Consequently, cells with morphological differences exhibit different guidance speeds determined by the optical forces. Our laser guidance technique integrates the microscopic interactions among photons and molecules throughout the entire cell to affect a single macroscopic outcome: guidance speeds, which provides a highly sensitive cell detection method.

Many diseases, especially cancer, are often curable with an early treatment but can become fatal otherwise. The primary basis for pathology evaluation remains morphological change in suspect tissue, but tissue biopsy can only be performed in later stages of disease with a noticeable collection of malignant cells. Therefore, precursory cancer detection requires a highly sensitive method to identify a small portion of diseased cells from a large cell population. During the cell's progression from a fully mature, post-mitotic state to a replicating, motile, and immortal cancerous phenotype, cell's optical properties change with the malignant transformation. The cell speeds resulted from laser guidance can be measured as an inherent and label-free cell marker and provided information about its state. With laser guidance technique, the normal cell line and its counterpart malignant cancerous cell line were used to compare differences in their guidance speeds. Two mouse mammary carcinoma cell lines were also investigated to distinguish cancerous cells at different metastatic stages.

Materials and methods

Laser-guided cell detection system

The laser-guided cell detection system, shown schematically in Fig. 1, was composed of a tuneable Ti-Sapphire laser (3900S, Spectra Physics) operated in TEM₀₀ mode with the wavelength of 800 nm. A spatial filter assembly constructed by a focusing lens, a pinhole (diam. = 25 μ m) and a collimated lens was used to produce a clean and spatially uniform Gaussian beam. The beam was then reflected by a right-angled prism to form a vertical guidance. Using a plano-convex lens, the collimated beam was then focused into the chamber with a cell feeding mechanism. The power of the beam inside the chamber was experimentally determined to be 150 mW for cell viability. The beam waist width was experimentally measured as 4.2 μ m using knife-edge scanning method. The cell guidance chamber composed of two bolt-nut mounted parts provided a watertight guidance channel

with cross-section of $3 \times 3 \text{ mm}^2$ to minimize the convection force. The laser guidance process was recorded using a long-working-distance objective and a high-resolution CCD camera with three IR filters to remove artifacts from guidance beam. The illumination source was a collimated green LED. The CCD camera was mounted on a 3D translational stage to allow the center of the CCD to be aligned to the laser guidance region. Cell images were captured using the Matrox Imaging frame grabber at the rate of 30 frames/s. For each image, the cell's centroid was determined digitally and used as the cell location. The guidance speed was calculated from the positions of the cell in two separate frames and the time intervals between those two frames.

Theoretical simulation

Theoretical simulation of optical forces not only is the key to understand laser guidance technique, but also essential to evaluate its feasibility for cell detection. By providing the visualized picture of optical forces generation from light reflection and refraction, geometric optical method was introduced by Ashkin (1998) for the single-beam optical trap on a sphere of diameter much larger than the wavelength. However, the laser beam was assumed in Ashkin's method to be composed of identical rays focused at one point, which is opposed to a Gaussian beam with various ray densities and ray directions in different beam's regions. In laser guidance regions near the focal point, the light rays no longer propagate along straight lines even in the free space, thus Ashkin's method cannot provide an accurate simulation for laser guidance phenomenon. To calculate cell guidance speeds, the formula was modified based on Ashkin's theory by using TEM₀₀ mode Gaussian beam to describe the focused trapping beam. The intensity of each ray is determined by the Gaussian beam's profile, and its direction is considered to be perpendicular to the Gaussian beam's wavefront. The axial optical force was given by Ma et al. (2009)

$$F_z(z) = \frac{4n_1 P r^2 \theta_m}{c} \int_0^{\theta_m} \frac{1}{\omega^2(z)} \exp \left[-2 \left(\frac{r \sin \theta}{\omega(z)} \right)^2 \right] \cdot Q(\theta) \cos i \cdot \sin \theta d\theta \quad (1)$$

where P is the power of laser beam, $\omega(z)$ is the beam width, r is the cell's radius, and c is the speed of light. The incident angle is expressed as $i = \theta + \alpha$, and angle α can be calculated by $\alpha = \arcsin(r \sin \theta / R_\omega)$, where R_ω represents the curvature of Gaussian beam's wavefront. The upper limit of the angle θ_m is set to $\pi/2$ only when the beam width is larger than the cell's radius. When a cell is guided into a region where beam width is smaller than cell's radius, the upper limit of the angle θ_m is calculated by $\theta_m = \arcsin[\omega(z)/r]$. The parameter $Q(\theta)$ is a dimensionless factor given by

$$Q(\theta) = \cos(i - \theta) + R \cos(i + \theta) - \frac{T^2 [\cos(i + \theta - 2\gamma) + R \cos(i + \theta)]}{1 + R^2 + 2R \cos 2\gamma} \quad (2)$$

where R and T are the Fresnel coefficients for reflection and transmission, and refractive angle can be obtained from $\gamma = \arcsin(n_1 \sin i / n_2)$, where n_1 , n_2 are the refractive indices of medium and cell, respectively.

While a cell is levitated by axial optical force in the cell-suspension medium, the resultant force from the buoyancy force and the gravity force is negligible comparing to the guidance force. For a typical cell being guided by axial optical forces, it can attain its terminal speed of motion in the order of microseconds, and hence the drag force balance the guidance force with no time comparing to the measurement time scale at millisecond (Ma et al. 2008). Therefore, the guidance force is considered to be equal to the drag force at each measurement position. Consequently, the guidance speed can be theoretically calculated based on the formula $v = F_z(z) / 6\pi\mu r$ with the parameters: wavelength 800 nm, laser power

150 mW, beam waist 4.2 μm , refractive index of media 1.33, and viscosity of media at 30°C 0.798 mPa s.

The maximum guidance speeds were theoretically calculated with different cell's diameters (Fig. 2a, the value of cell's refractive index was given as 1.36) and different cell's refractive indices (Fig. 2b, the value of cell's diameter was given as 10 μm) respectively. The simulation results demonstrated that different cell types or subtypes with different sizes and refractive indices can be distinguished by their maximum guidance speeds. We found that 1% size variation around mean value (10 μm) only induced 0.25% change in the guidance speeds, but 1% refractive index variation around mean value (1.36) induced 100% change in the speeds. The cell refractive index attributable to optical interaction of laser beam with cellular organelles provided fundamental biophysical information about the chemical composition and organizational structure of cells. The variation of refractive indices among cell types dominated the change of cell guidance speeds, thus variation of cell size within one cell type can be negligible.

Cell preparations

Laser guidance technique has been verified to detect different commercial cell lines by measuring their guidance speeds (Sridharan et al. 2005). To demonstrate its potential in cancerous cell identification, WEHI-164 cancerous cells were used to compare with normal 3T3-L1 cells. To further explore its capability to detect malignant cancerous cells within the same cell population, two mouse mammary carcinoma cell lines, 4T1 and 4TO7, were investigated to distinguish cancerous cells at different metastatic stages.

3T3-L1, WEHI-164, 4T1 and 4TO7 cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) with 2 mM L-glutamine, 1.5 g sodium bicarbonate/l, 4.5 g glucose/l, 10% (v/v) fetal bovine serum (FBS), and 1% (v/v) penicillin/streptomycin. These cell lines were maintained in the culturing medium at 37°C in a humidified 5% CO₂ incubator. Before laser guidance experiments, the cells were suspended using 0.25% trypsin/EDTA, centrifuged for 5 min at $\sim 1,000\times g$, then resuspended in phosphate buffered saline at about 5,000 cells/ml.

Results and discussions

A typical cell guidance procedure began with the preparation of the guidance chamber and cell suspension. The cell suspension was loaded into a 50 μl microsyringe coupled with a hollow fiber, which was sealed into the cell-guidance chamber. Cells were injected through the hollow fiber into the media-filled chamber. When two or more cells located in the guidance region, the following cells can be considered as a micro-lens to locally focus the laser beam and propel the cell in front. The measured guidance speeds in the multi-cell guidance event were inaccurate due to the acceleration, thus the rate of cell feeding was controlled to maintain single-cell guidance. In Fig. 3, a cell was being guided by a laser beam and travelling upward along the beam axis in the vertical direction.

To verify our hypothesis, two cell lines, 3T3-L1 mouse fibroblasts and WEHI-164 mouse fibrosarcomas, were statistically compared by their maximum guidance speeds using laser guidance technique, shown in Fig. 4a. WEHI-164 cells are malignant cancerous cells derived from fibrous connective tissue and characterized by basic fibroblast morphology. The significant differences on guidance speeds between 3T3-L1 fibroblasts and WEHI-164 fibrosarcomas demonstrated not only successful detection of different cell types, but also the potential of this technique to distinguish cancerous cells versus normal cells. The cell consists mainly of an aqueous solution of salts and proteins leading to a mean refractive index of approx. 1.36 (Curl et al. 2005). Cell nuclei give visible contribution to the total

phase shift of the cell with a relatively high refractive index (about 1.38) in the phase measurement. The mitochondria contain a large volume of thin membranes which are folded or arranged in tubes, which induces a mean refractive index of 1.4. However, a large number of mitochondria are needed to contribute to overall refractive index of a whole cell (Beuthan et al. 1996). As normal cells transform into malignant cancerous phenotype, the progressive changes can be observed as increased nuclear-cytoplasmic ratio, increased number and size of nucleoli, and increased number of mitochondria. All of these morphological changes contribute to a higher refractive index comparing with the normal ones (Choi et al. 2010) which gives the reason for the increase of guidance speeds.

To study the capability of laser guidance technique for cancer diagnostics, two mouse mammary carcinoma cell lines with different metastatic competence, 4TO7 and 4T1, were used to compare their differences in guidance speeds. The 4TO7 primary tumors can continuously seed distant sites, but fail to colonize these sites following their arrival. On the other hand, 4T1 cells are highly tumorigenic and invasive. They can spontaneously metastasize from primary tumor in the mammary gland to multiple distant sites including lymph nodes, blood, liver, lung, brain and bone (Yang et al. 2004). To initiate a metastatic colony at distant sites, a metastatic cancerous cell needs to acquire the ability of interaction with the local microenvironment, migration, invasion, resistance to apoptosis, and angiogenesis. The metastatic transformation results in major phenotypic changes that affect cell surface receptor expression, cytoskeletal function, growth factor and cytokine secretion, proteolytic enzyme production, and the glycosyltransferase and glycosidase repertoire (Bacac and Stamenkovic 2008). These combined changes alter the way in which the transformed cells respond to a laser beam during the laser guidance process. Therefore, shown in Fig. 4b, two mammary cancerous cell lines with different metastatic competence can be significantly differentiated using laser guidance technique without any cellular markers.

Conclusions

The laser guidance was generated by the optical force that integrated multiple intrinsic features of the cell, such as size, shape, composition, morphology, and membrane properties. Our results demonstrated that cell guidance speeds can serve as a sensitive indicator of the state of cellular development during the disease differentiation. Since this technique is based solely on cell's inherent optical properties, there is no need for preparation with fluorescent dyes or magnetic beads. Subsequently, pure samples of isolated cells can be investigated without artificial corruption from standard genomic or proteomic techniques, which are often impeded by the scarcity of the cells of interest. Tumors are composed of a heterogeneous collection of cells with different levels of individual gene expression; therefore, the predominant cell type or its metastasis may display subtle genotypic and phenotypic differences. The ability to accurately detect cancerous cells at different metastatic stages demonstrated that laser guidance technique has great application potential in clinical cancer diagnostics. It has been proposed as a highly sensitive and label-free cell sorting technique to identify and separate heterogeneous cancerous cells, which will be cultured to establish an engineered tissue construct for the study of cell-heterogeneity mediated regulation of microenvironments during cancer development.

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Fig. 1. Laser-guided cell detection system. The *red beam* indicated the laser beam for cell guidance, the *green beam* indicated the collimated illumination light, and *yellow tube* indicated the cell guidance chamber

Fig. 2. Maximum guidance speeds calculated using geometric optical method with different cell's sizes (**a**) or refractive indices (**b**)

Fig. 3.
Sample frames of a typical cell guidance by a laser beam propagating upward

Fig. 4. Our laser-guided cell detection system can effectively distinguish the normal cell line and its cancerous counterpart (a), or distinguish two cancerous cell lines with different metastatic competence (b) without any cell markers ($N = 20$, $P < 0.001$)