An approach to cell membrane diagnosis: laser-induced “reformation” of the murine erythroleukaemia cell from a spherical to planar membrane

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Abstract. Detection of the cytoplasmic domain of transmembrane oncoproteins attracts primary attention in cancer diagnosis, since the diagnostically marked extracellular domain is shed off along with increased malignancy. The shedding problem severely inhibits the conventional cell diagnosis, like immunomicroscopy from outside the cells. If detection on the cytoplasmic surface of each cell membrane becomes as extensive as the detection on the whole cell membrane and as efficient as that done within seconds, it will be in high demand. We propose here a new application of femtosecond laser, where a single cell is ruptured as a laser-induced cavity expands in cytosol and the whole cell membrane is thus projected over a flat surface in a short period of time (< 10 s). Using this configuration, we achieved projection of the cytoplasmic surface of the cell membrane for 57% of murine erythroleukemia cells irradiated, including 34% of them at full extension of the membrane. The inside-out projection was confirmed by immunostaining both D-actinin in murine erythroleukemia cells and an oncoprotein c-erbB2 in human adenocarcinoma cells. This projection requires only a small area (< 1000 μm²) and a short irradiation interval on a microscope slide, thus leaves room for a high throughput serial process.

1. Introduction

The oncoprotein c-erbB2 (or HER2; a transmembrane tyrosine kinase receptor) is overexpressed in human breast cancer, and is an established prognostic and predictive marker [1]. In addition to inspection, molecules of the c-erbB2 protein over the cell surface are a therapeutic target of the well-known anti-breast-tumor reagent, Herceptin (or Trastuzumab) [2]. However, the extracellular domain of c-erbB2 is lost by enzymatic cleavage (or shedding) [1-3], which makes the diagnosis from outside the cell less conclusive (shedding problem hereafter; Scheme 1, Panel 1). Furthermore, the very oncoprotein once truncated acquires malignant feature of elevated metastatic incidences [3]. These are the persistant theme, where loss of function phenotype looms in cancer diagnosis, and the hunt for the truncated oncoprotein becomes crucial [2].
Fortunately, the cytoplasmic domain of c-erbB2 remains intact after shedding of the extracellular domain [3, 4]. This leaves the possibility that the amount of truncated c-erbB2 expressed on the cell membrane can be measured from inside. Our purpose is to establish a method by which the cell membranes are projected inside-out onto a flat surface, thereby exposing and manifesting (projecting hereafter) the cytoplasmic domain of membrane proteins, which can be detected in the cases either with or without shedding (Scheme 1, Panel 2). This projection would overcome the shedding problem and open to a new biopsy. Here we propose “cell membrane diagnosis” and demonstrate it.

Several methods have been reported on attempts at projecting the cytoplasmic surface of the cell membrane, but more extensive and efficient methods are awaited. Sectioning of frozen or embedded cells makes the cells expose their internal membrane surface [4, 5]; however, the sectioning depth and its variations restrict extensive and accurate detection of the whole membrane, that is, poor projection. Strip of the cell membrane by attaching and drawing two flat surfaces is another method [6], although efficient projection depends on elaborate alignment between the surfaces at pinching cells. If cells are cut open one by one, the projection can gain high reproducibility and high throughput. To realize this, one can choose femtosecond laser (FSL) [7]. We anticipated that FSL irradiation to each cell is rapid, secure, and selective, although knowing little about the phenomenon when the laser energy is emitted in the micro-environment inside the free globular cell membrane. We characterized this phenomenon, and pursue a novel application of FSL to the shedding problem.

In our model, the highest energy of the FSL focus causes two contrasting modes of breakdown in a cell, ablation and cavitation [7]. Ablation has been widely used as spatially-limited cell cleavage [8] and temporal-resolving organelle disruption [9]. Not yet biomedical use of cavitation at the FSL focus has been reported. We thus designed exploitation of FSL-induced cavitation for projecting the cell membrane. We expected a cavity (bubble) occurs in cytosol, grows upon impact propagation, pressurizes the membrane from inside, and then break down to project the inner surface.

Scheme 1. The shedding problem (panel 1). Comparison between detection of the membrane proteins from outside and detection after projection on a flat surface (panel 2).

Figure 1. Panel 1 shows steps of fixation, irradiation, and projection of a cell. Panel 2, the real sequence of events observed. Pane 3, the area measurement before and after irradiation.

2. Materials and methods
2.1. **Cell preparation.** Cells from murine erythroleukemia (MEL) and human adenocarcinoma RERF-LC-KJ (KJ) were purchased from HSRRB Osaka. MEL cells were maintained in DMEM (Sigma) containing 10% fetal calf serum (Biological Culture Biologicals) in an atmosphere of 5% CO₂ at 37°C. For fix MEL cells, suspension of the cells (8.0 x 10⁵ cells/ml) was applied onto an amino-group-modified slide glass (MAS coat, MATUNAMI), and incubated for 1 hr at 37°C. An unfixed fraction of MEL cells were washed off the slide glass with a fresh culture medium. KJ cells were maintained in RPMI1640 medium (Sigma). KJ cells were fixed as in the case of MEL cells, except that the cells were once harvested with 0.25% trypsin solution (Nacalai Tesque) at 37°C and washed twice with RPMI1640 medium.

2.2. **FSL irradiation and Cell membrane projection.** Irradiation to cells was carried out with an upright microscopy (BX51WI, Olympus) equipped with a titanium sapphire laser oscillator (MaiTai model 409, Spectra Physics; laser power 450-600 mW, wavelength 800nm, repetition rate 80 MHz, pulses width <100 fs). The beam was directed towards an objective lens with the numerical aperture of 1.20, which formed a 1μm focal spot in diameter. The focal spot was positioned and directed into cytosol with a precisely-controlled XYZ stepping stage (Hakuto). In projection, the FSL pulses were applied (for the duration mention in the results) into the cytosol of cells mounted on the slide glass. Time-lapse bright-field images were simultaneously obtained through the same objective lens.

2.3. **Immunofluorescence microscopy.** The MEL cell membranes remaining on the slide glass after projection were subsequently incubated with blocking buffer (10% BSA in PBS) for 1 hr, and then with 5 μg/ml anti-α-actinin primary antibody (MAB1682, Chemicon) for 2 hr. After incubation with primary antibody, the membranes were washed three times with blocking buffer and incubated with 7.5 μg/ml anti-mouse-IgG antibody coupled with Cy3 fluorescent dye (AP124C, Chemicon) for 1 hr. After removal of the secondary antibody, the membranes were washed three times with PBS. The membranes were further double-stained with membrane-staining dye DiO (5 μM, D275, Molecular Probes) for 20 min. After incubation with DiO, the membranes were washed three times with PBS, and observed with a fluorescence microscope. Staining and observation were carried out at room temperature. KJ cell membranes were immunostained with anti-c-erbB2 primary antibody (Histofine HER2 kit, Nichirei Bioscience) in place of anti-α-actinin primary antibodies as in MEL cell immunostaining.

3. **Results and Discussion**

3.1. **Cell membrane projection by FSL irradiation.** To check first whether FSL pulses generate caviation in cytoplasm, each MEL cell was captured stably on an aminated surface, and then irradiated gradually from the bottom to the center of the cell (along vertical direction) (Fig. 1a). As the laser focus approached to the cytoplasm of the captured cell, an air bubble was generated, and expanded inside the cell (Fig. 1, Panels 1b and 2b). This phenomenon caused cell rupture, and seemed to project the cell membrane onto the substrate surface (Fig. 1, Panels 1c and 2c). It took 5-10 sec for the cell projection to complete, and was very rapid. The cells projected onto the substrate were in most cases transformed into a planar shape, while the contours of the cells became larger after projection. We hypothesized that this transformation was due to unfolding from its spherical shape (Fig. 1 Panel 3a) to an extended sheet spread over a flat surface (Fig. 2, Panel 3c). We verified this possibility next.

3.2. **Characterization of projected cell membranes.** We measured the surface area of the ruptured cell, and estimated how well the area represented a single layer of the cell membrane. We cut off along the contour of each irradiated cell, and weighed the enclosed area. The areas obtained were normalized by those calculated for the original membranes before the irradiation, which were approximated to a sphere. The histogram in Panel 1 of Figure 2 shows a distribution of the number of cells against the net
increase in the contour area. This distribution suggested morphologically distinct three groups after irradiation. Indeed, back-tracing of images of the same group revealed that most of Group II and III corresponded with collapsed globular membranes (Fig. 2, Panel 2c) and projected flat membranes (Fig. 2, Panel 2d), respectively. Group I was a rare case, where almost all the cell bodies disappeared due to ablation or were detached off the substrate surface (Fig. 2 Panel 2b). By improved control of FSL irradiation (intensity, draw speed, and bulk osmosis), we could resolve the groups, and obtain a single large group of projected flat membranes.

To confirm that projected surfaces were the originally cytoplasmic side of the MEL cell membrane, we immunostained α-actinin, a clear indicator of the MEL cell inner surface (α-actinin is a membrane skeleton protein anchored on the cytoplasmic side of the cell). MEL cells with and without irradiation were treated with anti-α-actinin antibody, and then secondary antibody labelled with Cy3. The cell projected on the substrate surface was extensively stained with anti-α-actinin antibody (Fig. 2, Panels 2i and 2j). This antibody is shown colocalized with a membrane lipid staining dye, DiO (Fig. 2, Panels 2f and 2g). However, the cell without irradiation showed a negligible level of nonspecific staining by anti-α-actinin antibody (Fig. 2, Panel 2h) that is on the outer surface but not the inner surface. These observations suggested the MEL cell membrane after the irradiation was successfully projected with its cytoplasmic surface up (inside out).

Figure 2. MEL cells projected. Panel 1 shows the number of cells sorted by the ratio of increase in cell membrane areas after FSL irradiation. Three major groups were observed in the distribution. Panel 2, the representative images of the cell before irradiation and the cells which were dropped in the three groups in Panel 1. The observation was carried out with bright field (first row), fluorescent membrane staining by DiO (second row), and fluorescent immunostaining by anti-α-actinin antibody (third row).

3.3. Single cell membrane diagnosis of cancer phenotypes. Then, by the FSL-assisted projection, we addressed the shedding problem with the KJ cell, as a surrogate human cancer diagnostic model (Scheme 1). For KJ cells, c-erbB2 molecules are once overexpressed, and then 80 percent of them undergoes shedding to lose their extracellular domains. Only 20 percent retains the domain [4]. This leads to the speculation that inspection of c-erbB2 on the KJ cell from outside (e.g. immunostaining) is less decisive, and indicates a tested pseudonegative, unless the cells are projected. Immunostaining on KJ cells was carried out except that anti-c-erbB2 antibody, which specifically recognizes the cytoplasmic domain of the c-erbB2 oncoprotein, was used in place of anti-α-actinin antibody. As expected, projected KJ cells showed significant staining (Fig. 3d), while the cell without irradiation showed minimum staining, that is, a potential risk of malpractice (Fig. 3c). The projection reported
here was demonstrated to resolve the shedding problem. FSL irradiation toward a single cell was enough to obtain a confirming signal of the overexpressed c-erbB2 over the whole cell membrane, leading to progress in nanomedicine.

![KJ cells projected as a shedding model (also see Scheme 1). Immunological detection of c-erbB2 on the cytoplasmic surface of the KJ cell membrane is snapped. The KJ cell without irradiation (a, c) and the projected membrane of an irradiated KJ cell (b, d) are observed both in bright field (a, b) and in fluorescent immunostaining by anti-c-erbB2 antibody (c, d).](image)

4. Conclusions
FSL irradiation was demonstrated applicable in a single cell membrane diagnosis, where the cell membrane is projected within a short period of time onto a small flat area. Although successful exposure was achieved in 57% of the irradiated cells, there is room for improvement in the rate of successful exposure. The reproducibility of exposure of the cytoplasmic surface was confirmed high according to two independent immunostainings of the membrane proteins. The method in this study [10] can be a basis of cell membrane diagnosis, and may become integrated and automated into a lab-on-a-chip, an ideal bedside gadget.

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