Laser-induced fusion of human embryonic stem cells with optical tweezers

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(Received 3 February 2013; accepted 27 June 2013; published online 15 July 2013)

We report a study on the laser-induced fusion of human embryonic stem cells (hESCs) at the single-cell level. Cells were manipulated by optical tweezers and fused under irradiation with pulsed UV laser at 355 nm. Successful fusion was indicated by green fluorescence protein transfer. The influence of laser pulse energy on the fusion efficiency was investigated. The fused products were viable as gauged by live cell staining. Successful fusion of hESCs with somatic cells was also demonstrated. The reported fusion outcome may facilitate studies of cell differentiation, maturation, and reprogramming. © *2013 AIP Publishing LLC*. [http://dx.doi.org/10.1063/1.4813822]

Cell fusion, a process by which two or multiple cells merge to form a single entity, plays an important role in numerous biological events and applications such as tissue regeneration.[1,2] Cell fusion has also been found to contribute to nuclear reprogramming[3–5] and other *in vivo* transplantation experiments.[6] Indeed, techniques of cell fusion *in vitro* have been investigated since the discovery of such multinucleated cells as skeletal muscle and tumors.[7,8] Most existing *in vitro* methods for inducing cell fusion are either virally or chemically induced and notably are mass production techniques, which lead to heterogeneity and reduced spatiotemporal resolution at the single-cell level. By contrast, electrofusion is a technique for targeting individual cells with potential specificity and precision;[9] however, it requires complicated geometries of the electric field and usually needs to be integrated with a micro-fluidic chip.[10] Another potential solution is the use of a pulsed laser to perturb the cell membrane and subsequently induce fusion.[11] The engineering approach of using optical tweezers with a continuous wave laser to trap and transport cells,[12,13] followed by cutting cells by using optical scissors with a pulsed laser, can achieve specific cell fusion efficiently.[14,15]

Recent stem cell research has discovered that stem cell could reprogram somatic cell through cell fusion. In the current paper, we report the use of optical tweezers and pulsed UV laser to accomplish efficient fusion of human embryonic stem cells (hESCs) and also hESC with somatic cell. Results indicated that two or multiple hESCs could be fused by pulsed UV laser irradiation with a high efficiency (>50%). This targeting laser-induced cell fusion could be an efficient tool for studying stem cell differentiation, maturation, and reprogramming.

Our experiments were conducted on a cell manipulation system[16] with a holographic optical trapping (HOT) system (BioRyx 200, Arryx) and laser scissors (LSS, Arryx) [Fig. 1]. A 1064 nm continuous-wave (Nd: YAG laser) was emitted from a laser source (V-106C-3000 OEM J-Series, Spectra Physics) and transmitted by an optical fiber and HOT device, which could split the beam and simultaneously create multiple optical tweezers. The laser beam was directed by the dichroic mirror and focused by an objective (Nikon 60× water immersion, NA = 1.2) installed on an inverted microscope (ECLIPSE Ti-U, Nikon) to act as three-dimensional optical tweezers. A pulsed diode-pumped, passively Q-switched solid-state laser (FTSS 355-50, CryLas) emitting at a wavelength of 355 nm was used as laser scissors for cutting the cell membrane. The laser beam was expanded with a telescope and then passed through a rotatable linear polarizer (10LP-UV, Newport) guided by reflective mirrors. The beam was finally directed to fill the back of the same microscope objective via another dichroic mirror. The laser scissors had a diffraction limited spot diameter of 0.36 μm. The focus controller (TDC001, Thorlabs) guided the position of one lens in the telescope, which then adjusted the height of the cutting spot relative to the image plane.

The microscope was customized by installing a two-dimensional X–Y motorized stage (ProScan, Prior Scientific, resolution 15 nm), on which an environment-controlled chamber (Chamlide TC-L Live Cell Instrument, Seoul, Korea) was placed, providing a 37 °C temperature and 5% CO_2 concentration. The fluorescence illuminator (X-Cite® 120PC Q, Lumen Dynamics) generated an excitation light that passed through filter combinations housed in a filter cube and excited the fluorescence in the fluorescence protein labeled cells. All the fluorescence images were captured by a cooled color charge-coupled device (CCD) camera (QImaging, Canada).

The hESC was cultured on a commercially available serum-free, feeder-independent system (mTeSR1, STEMCELL Technologies) and supplemented with BD Matrigel™ hESC-qualified Matrix (BD) as suggested by the manufacturer. Primary human dermal fibroblasts (HDFns, Gibco) were cultured on a 24-well culture plate (BD Falcon) and maintained in Dulbecco's Modified Eagle Medium with 20% fetal bovine serum.

Prior to the fusion experiment, the single cells were obtained with 0.05% trypsin–EDTA treatment. After proper

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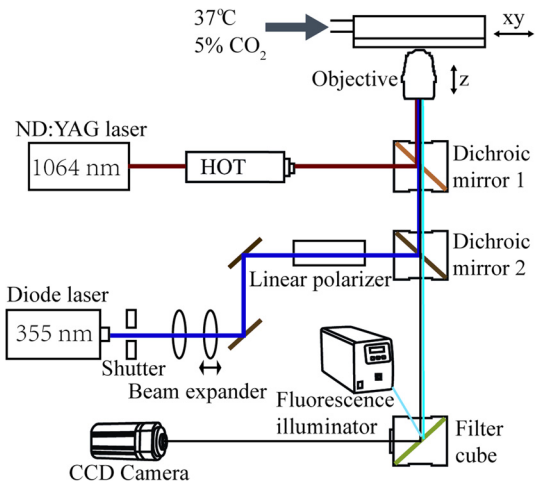

FIG. 1. Schematic of setup for laser-induced cell fusion.

neutralization, the cells suspended in mTeSR1 were stored temporarily at 4 °C.

In the fusion experiment, we first attempted to induce the fusion of two suspending hESCs [Fig. 2]. After enzymatic dissociation, green fluorescence protein (GFP) labeled and unlabeled hESCs were mixed and suspended with 200 μl mTeSR1 in a 35 mm glass-bottom dish (In Vitro Scientific). An additional 500 μl mTeSR1 was loaded into the dish rim to compensate for evaporation of the medium. The dish was kept inside the environment-controlled chamber, under conditions of 37 °C and 5% CO_2 in air. The objective was adjusted to focus on the cells, and the glass bottom was scanned line by line by controlling the X-Y motorized stage. One GFP labeled cell and one unlabeled cell were trapped and manipulated by optical tweezers to form a cell pair, and then transferred to a spot for laser scissor cutting [Fig. 2(a)]. The laser scissors functioned at a frequency of 10 Hz, and each pulse had a duration time of 1 ns. To avoid unnecessary damage by the UV pulsed laser, only 5 pulses were emitted per treatment. The laser scissors cut the cell membrane at the point of contact between the two cells. Upon cutting with the laser scissors, cytoplasmic GFP transferred from the GFP labeled cell to the unlabeled cell immediately [Fig. 2(b)], indicating the start of cell fusion. The contact point on which the fusion initiated was then enlarged, and a tunnel was formed at the contact area during the first minute [Fig. 2(c)]. The two spherical cells gradually became a larger, single elliptical cell while their cytoplasm is continued to mix. The fused cell gradually rounded after approximately 15 min [Fig. 2(d)] and became spherical after 30 min [Fig. 2(e)]. The dark shadow in the fused cell corresponded to the nucleus of the original unlabeled cell. The mixing of their cytoplasm continued until the nucleus of the unlabeled cells became invisible as the GFP level inside the fused cell became unified after approximately 55 min [Fig. 2(f)].

We also induced fusion of two hESCs attached to the cover glass to illustrate that laser induced cell fusion is likewise applicable to adherent cells. After cell pairing, two cells could naturally bind to the extracellular matrix while maintaining their cell pair shape [Fig. 3(b)]. Cell fusion was then induced by cutting the boundary of two adjacent cell membranes with laser scissors before the two cells could completely attach to the cover glass.

As a step further, we examined whether this technique could be applied to induce multiple cell fusion. As shown in Figure 3, a GFP labeled hESC was fused with two unlabeled hESCs under manipulation of multiple optical tweezers. The cell-cell junctions of the GFP labeled hESC and the two unlabeled hESCs were cut by pulsed laser. The GFPs transferred from the green cell to the adjacent transparent cells after every cut, and the three cells exchanged their cytoplasm [Fig. 3(e)]. The results demonstrated a successful multi-cell fusion through laser-induced engineering approach.

After the fusion experiments, the dish was placed in a large incubator (CelCulture®, ESCO) and the fused cells were cultured in mTeSR1 at 37 °C and 5% CO_2.

The fusion efficiency of hESCs was analyzed at different energy range of the UV laser irradiation. Here, a successful fusion was defined as the merging of two cells into a single cell within the first hour. The fusion efficiency was evaluated based on the percentage of the successful fusions over the total fusion operations. Figure 4 is a plot of fusion efficiency versus laser energy. In our experiment, the energy threshold for inducing cell fusion was determined to about 14.47 μJ per pulse. Above 14.45 μJ, the fusion efficiency increased with laser energy. The maximum fusion efficiency that could be achieved was 62.2%, with about 18 μJ of pulse energy. The fusion efficiency decreased with further energy

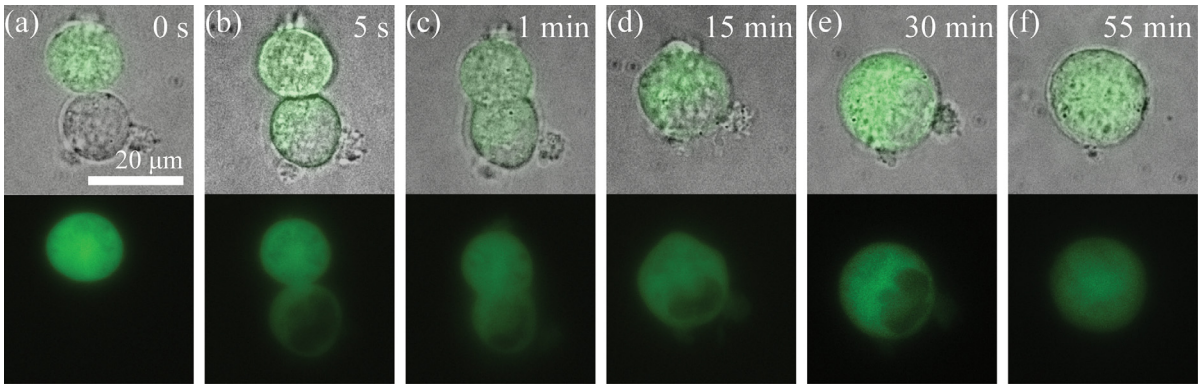

FIG. 2. Laser-induced cell fusion of two hESCs in suspension. (a) A GFP labeled cell (green) and an unlabeled cell (transparent) formed a cell pair before laser cutting. (b) After laser cutting, GFP transfer from green cell to transparent cell. (c)–(f) Time lapse images showing fusion process (c) 1 min, (d) 15 min, (e) 30 min, and (f) 55 min after laser cutting. Scale bar, 20 μm.

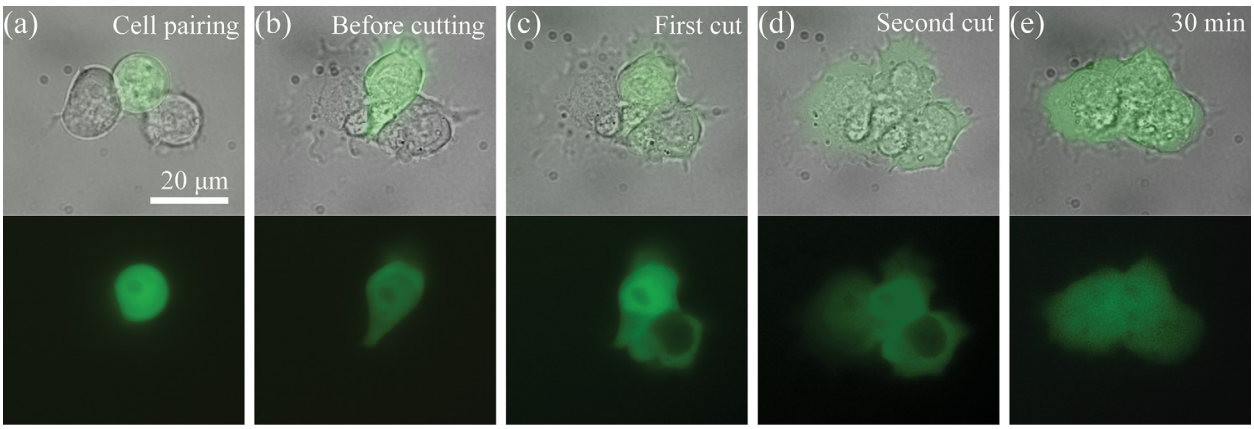

FIG. 3. Laser-induced cell fusion of three adherent cells. (a) Three cell formed cell pairs. (b) Three cells attached to cover glass in cell pair shape. (c) A GFP labeled cell (middle) fused with unlabeled cell (right) after the first cut. (d) The bi-nucleated hESC was then fused with another unlabeled cell (left) by the second cut. (e) 30 min after laser cutting, 3 cells became one single cell. Scale bar, 20 μm.

increment. The use of higher laser energy was observed to cause more damage to the fused cell instead of prolonging survival. Therefore, we fused cells with laser pulse energy of 16 μJ and evaluated fused cell viability. Long-term viability was examined by incubating fused cell in culture medium with 0.5 μM of SYTOX Orange dye for 10 min. SYTOX Orange dye can easily penetrate apoptotic cells with compromised plasma membranes, but will not penetrate healthy cell membranes. 90% fused cell could survived longer than 2.5 h and 40% fused cells could survive longer than 10 h ($n = 20$) when the fused cells were incubated in mTeSR1 at 37 °C and 5% CO_2. Figure 5(a) shows no apoptosis was detected in a fused cell 12 h after cell fusion. The fused cells had two nuclei, as indicated by the specific nuclear fluorescent protein [Fig. 5(c)]. The results indicated that viable multinucleate cells could be obtained if optimal laser energy was used.

We finally induced the fusion of hESCs with somatic cells, HDFns [Fig. 6]. The mCherry fluorescence protein (mCherry FP) labeled HDFns were mixed with unlabeled hESCs immediately after enzymatic dissociation [Fig. 6(a)]. After laser cutting the membranes, the two cells started to fuse, as indicated by the transfer of the red fluorescence from HDFn to the unlabeled hESC [Fig. 6(b)]. Similar to the hESC fusion, the dark area representing the nuclei of the hESC was immediately observed after laser cutting. The area moved toward the HDFns during the first minute and then disappeared when the cytoplasm was mixed further [Fig. 6(c)]. The fusion was completed within approximately 10 min [Fig. 6(d)], and the fused cell attached to the cover glass exhibited morphology similar to surrounding cells [Fig. 6(e)]. Results demonstrated the effectiveness of laser-induced cell fusion technology on fusing stem cells with somatic cells.

In conclusion, we have demonstrated a targeting laser-induced cell fusion technique using pulsed UV laser at 355 nm to fuse two and multiple stem cells and also stem cell with somatic cell. Viable multinucleate cells were created after laser-induced stem cell fusion. This specific and accurate technique can be used for creating multinucleated mature cells by fusion stem cells or progenitors to facilitate the investigation on cell fusion-driven differentiation and maturation.

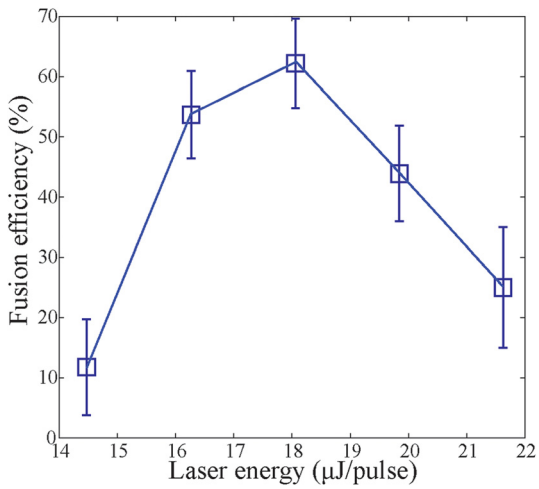

FIG. 4. Fusion efficiency data for the cutting of $n > 200$ cell pairs using pulsed UV laser. Each point represents the mean ± standard error of at least 4 experiments.

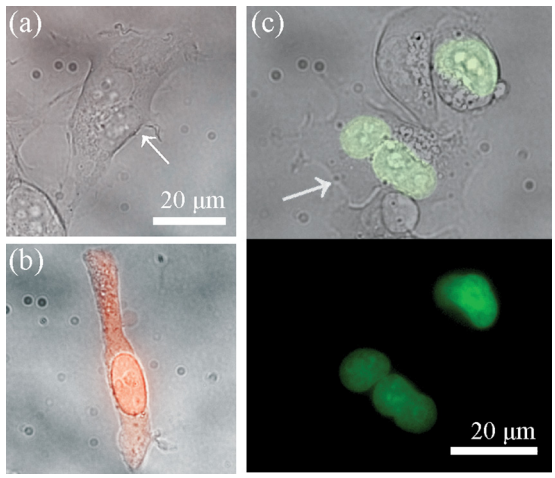

FIG. 5. (a) The fused cell (arrow) was viable and no apoptotic signal was detected, as indicated by negative SYTOX Orange staining 12 h after fusion. (b) One hESC fixed by 4% paraformaldehyde was positively stained with SYTOX Orange staining. (c) The fused cell has two normal nuclei structures. Arrows indicate fused cells. Scale bar, 20 μm.

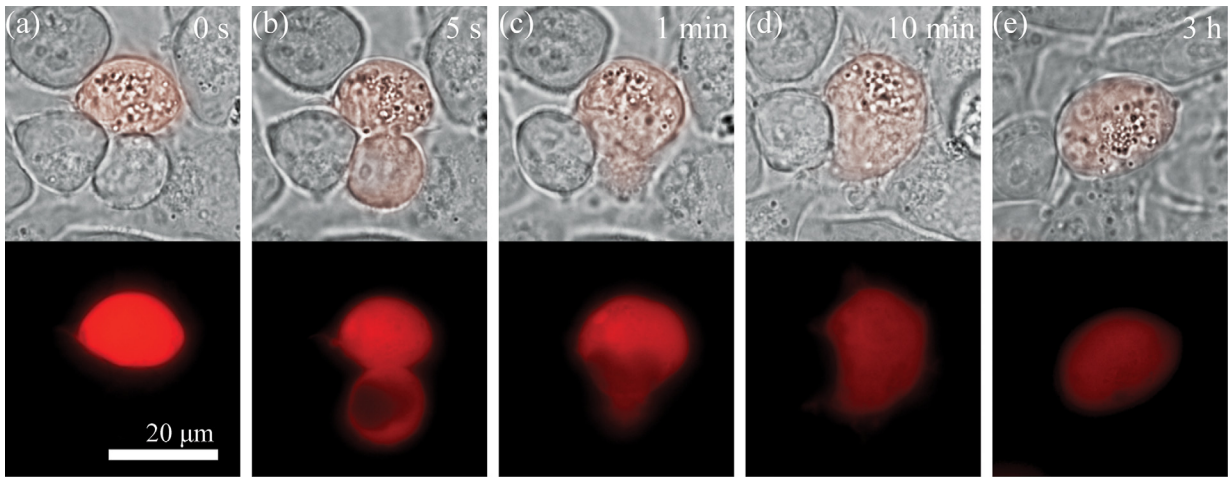

FIG. 6. Laser-induced fusion of hESC and HDFn. (a) Image of a mCherry FP labeled HDFn (red); (b) The red HDFn was fused with a transparent hESC after pulsed laser cutting. (c) The fused cell gradually rounded up during the first minute. (d) 10 min after laser cut cell pair. (e) 3 h after laser cutting, the fused cell exhibited morphology similar to surrounding cells. Scale bar, 20 μm.

The work was supported by grants from the Theme-based Research Scheme (T13-706), the Research Grants Council of the Hong Kong Special Administrative Region, China (Reference No. CityU 120310), and University Grants Council (UGC) Special Equipment Grant (SET_CityU 01).

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