Use of a Right Triangle Chip and Its Engraved Shape as a Transferrable x-y Coordinate System from Light Microscopy to Electron Microscopy

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ABSTRACT

A right triangle chip was prepared by cutting a rectangular polystyrene plate (0.3 mm²) that was produced by the ejection of melted polystyrene into a metal mold. The chip was attached on the inside bottom of a culture dish. In light microscopy, the x-y coordinates of several HeLa single-cells were registered by referring to the x-y axes that were defined as the 2 lines forming the right angle. After fixation of these cells and resin embedding, the epoxy block containing cell specimen was peeled off from the culture dish by heating at 145°C for 5 min. Then the right triangle chip was separated by heating again at 145°C for 3 min. The engraved right triangle was used as an x-y coordinate system in electron microscopy. The target HeLa cells registered initially in light microscopy could be found shortly in electron microscopy. The right triangle chip and its engraved shape could be used as an x-y coordinate system transferrable from light microscopy to electron microscopy.

Keywords : x-y Coordinate System, Single-cell Experiment, Correlative Light-electron Microscopy

1. Introduction

Recently much interest has been paid to viable single-cell experiments and consequently to time-lapse microscopic observation during culture for long time.1-3 The term “single-cell” means an isolated single cell and a target single cell in multi-cell system. During usual culture, culture dishes are occasionally observed with the naked eyes or under a light microscope to check the culture condition. In those cases, it is not necessary to find definite target cells in a dish at every observation. In the time-lapse microscopic observation of specific target single-cells, however, it is essential to find respective target cells shortly at every observation. To enable such rapid finding, an appropriate location marker such as grid lines or letters should be necessary.

To meet this requirement, several types of culture dishes with those location markers were developed and now are commercially available. Those dishes, however, are still inconvenient for the observation of small cells such as embryonic stem cells with 10–15 µm in diameter, because the grid lines are too thick to determine the x-y coordinates of the target single-cells. Another problem is that thick grid lines and large letters might also become the obstacles against cell observation.

Previously we developed a disposable x-y coordinate micro-chip and demonstrated its feasibility in time-lapse observation of single ES cells.4 The micro-chip could be attached with an adhesive tape on the bottom of any type of a culture dish from outside. The x-y coordinate of every cell in the dish could be determined by referring to this micro-chip by means of an automatic stage control system. The micro-chip was placed in the periphery of a dish bottom and therefore it did not become an obstacle against microscopic observation of the central area of the dish. It was essential to make a very thin line on the micro-chip to be used as the standard x-y axes. Its thickness should be no thicker than 2 µm. Our idea was to make a small level gap of 2 plastic plates by mold injection method. Finally we found that the gap line looked as a line with no thicker than 2 µm under microscopic observation. Such a successful system was named as the sugiwaculture system.3

More recently, there has arisen an intense need of continued observation from light microscopy to electron microscopy. Pioneering works about this subject by Haraguchi et al. has been called correlative light-electron microscopy (CLEM).5 Observation targets in CLEM were, for instance, green fluorescent protein (GFP),6 GFP-labelled protein,7 engineered peroxidase,8 and adhesion protein markers.9 In summary, CLEM uses a special glass plate with location markers such as grid lines and/or letters on the bottom. That is typically a 35 mm culture dish with a gridded cover slip attached on the dish bottom center. The gridded cover slips are commercially available. A lattice pattern is formed with grid lines printed or laser processed on the cover slip. The lattice unit size is e.g. 500 µm × 500 µm and the width of the surrounding grid line is 40–50 µm. When cells are observed with an inverted fluorescent microscope, it should happen to occur that target cells with a size of no greater than 15 µm are hidden behind a grid line. However such a problem may be avoided by using a cover slip without grid lines at cell observation area. The production of such a cover slip is technically possible though it is not yet commercially available. The present idea of an x-y coordinate system could avoid this problem by a simpler technology without laser processing machines.

Another and more important point was the transfer of the xy-address information from light microscopy to electron microscopy. Alphabets and numbers indicating the address of each lattice unit...
can be used only in light microscopy. They cannot be transferred to the sample for electron microscopy. Therefore it is necessary to make hand drawings of position and shape of the cells of interest during the observation with a fluorescent microscope. Hand drawing means freehand sketches and notes describing what the experimenter noticed just during microscopic observation. Careful and speedy observation are necessary but more exact image information of photograph might not be necessary. In electron microscopic view, the target cells should be found by referring to the information of hand drawings. Therefore it was necessary to develop a light-electron microscopy transferrable address marker.

Our idea was to use a right triangle plastic chip and its engraved shape. We expected that the sharp line of a plastic chip and its engraved shape may be used as an x-y coordinate system in both light and electron microscopy. Such an idea is based on the idea of the coordinate micro-chip in sugawaculture system.4

2. Experimental

2.1 Culture of HeLa cells

The medium for HeLa cells (HeLaM) was prepared by mixing 44.5 ml of DMEM (Sigma), 5 mL of 10% FBS (Biological Industries), 0.5 ml of (×100) non-essential amino acids (Gibco), and 50 µl of 2-mercaptoetanol. HeLa cells stored at −152°C were thawed at 37°C and suspended in 500 µl of HeLaM in a 50 ml tube. The cells were collected by centrifugation and suspended in 1 ml of HeLaM in a vial. Then the supernatant was removed by centrifugation at 1500 rpm at 4°C for 5 min and 4 ml of HeLaM was added to suspend the cells again. Two ml of this cell suspension was added to a gelatin coated culture dish (10 cm²) made of polystyrene. The gelatin coating was performed beforehand by adding a phosphate buffer saline (PBS) containing 0.1% gelatin in a culture dish, placing the dish still for 5 min, and replacing the PBS solution by 8 ml of HeLaM.

When HeLa cells in the dish became 70–80% confluent after culture for 4–5 d, the cells were washed twice with PBS. Then 0.3 ml of trypsin-EDTA solution (0.25% trypsin, 1 mM EDTA) was added to the dish to collect cells. After reaction at 37°C for 5 min under 5% CO₂; 3 ml of HeLaM was added to the dish to suppress the trypsin reaction. The suspended cells were gently homogenized by pipetting and transferred in a 15 ml tube to apply centrifugation at 1500 rpm at 4°C for 5 min. The collected cells were suspended in 2 ml of HeLaM and dispensed in dishes (10 cm²) at 3 × 10^5 cells/dish and then cultured at 37°C under 5% CO₂.

2.2 Coordinate chip

A right triangle chip depicted in Fig. 1a was prepared by cutting a polystyrene plate (0.3 mm²) that was produced by pressure ejection of melted polystyrene into a metal mold. The right triangle chip was disinfected with 70% ethanol and attached on the inside of the culture dish bottom (3.5 mm²) with cyanoacrylate adhesive. The dish was fully rinsed with distilled water and disinfected with 70% ethanol and then coated with gelatin. The 2 sides at the right angle were defined as x-y axes. The right angle corner was registered as the origin (Fig. 1b).

2.3 Femtoinjection of an injection marker into HeLa cells

The x-y coordinates of target single-cells of HeLa were registered by means of the sugawaculture system. Then a solution of 1 mg/ml Dextran Texas-Red 70000 (DTR) was injected into a HeLa cell using a single-cell manipulation supporting robot (SMSR) (Fig. 1c).

Figure 1. Schematic diagram of the preparation of an epoxy block containing cell specimens. A right triangle was attached on the inside of the bottom of a culture dish (a). Cells were cultured and their locations were registered as x-y coordinates referred to the x-y axes illustrated in (b). DTR was femtoinjected into the nucleus of HeLa cells (c). A polyethylene capsule containing 100% EPON 812 was placed upside down onto the dish bottom to incubate at 60°C for 48 h (d). The epoxy block was peeled off from the dish bottom (e) and then the right triangle chip was separated from the epoxy block (f).

Figure 2. Embedded chip and its engraved shape. A polyethylene tube containing epoxy resin was peeled off from the dish bottom. The chip was embedded in the surface layer of the block (a) and then this chip was removed. The resulting engraved shape was observed by light microscopy (b). O, Ox, Oy: origin, x axis, and y axis.

2.4 Fixation and resin embedding

After the femtoinjection, the HeLaM in the dish was replaced by PBS for rinsing and then by DMEM containing 2.5% glutaraldehyde and 2% paraformaldehyde for fixation. The dish was incubated at 20–25°C for 1 h. After rinsing with PBS three times, 2 ml of 0.1 M PBS containing 1.5% OsO₄ was added to the dish for incubation at 20–25°C for 1 h. Then the dish was washed with distilled water three times followed by dehydration with 30%, 50%, 90%, and 100% ethanol. After that, ethanol was replaced by 10%, 30%, 50%, 70%, and 90% EPON 812 ethanol solutions in this order. Finally 90% EPON 812 ethanol solution was replaced by 100% EPON 812 for incubation at 20–25°C overnight. About 80% of the volume of a 1 ml polyethylene capsule (BEEM Capsule, inside diameter 8 mm) was filled with 100% EPON 812 and the capsule was placed upside down onto the dish. Then the incubation was continued at 60°C for 48 h to allow the EPON to polymerize (Fig. 1d).
2.5 Separation of epoxy block from a culture dish

A dish in which HeLa cells and a coordinate chip were embedded in EPON 812 was incubated at 60°C for 3 d. The dish was observed with a fluorescent microscope to confirm that the DTR injected cells were found around the x-y coordinate system registered initially before incubation at 60°C. Then the dish was placed on a hot plate heated at 145°C for 5 min and the epoxy block was peeled off from the dish bottom (Fig. 1e) (Fig. 2a).

2.6 Separation of a coordinate chip from the epoxy block

The epoxy block was placed again on a hot plate heated at 145°C for 3 min and then the coordinate chip was separated from the epoxy block with a tweezers (Fig. 1f). The engraved shape of the chip looked as a right angle and expected to be used as the x-y axes as well (Fig. 2b). In fact, the DTR injected cells could be found around the same x-y coordinates as registered initially.

2.7 Electron microscopy

The epoxy block containing cell specimens was fixed on an ultramicrotome (Leica, Ultracut-S) and trimmed with a razor blade. Then sections with a thickness of 95 nm were made with a diamond knife, and then stained with uranyl acetate followed by lead citrate. The section was observed with a scanning electron microscope (SEM) (JEOL, JSM-7800F) under the condition of 3 kV electron beam. The original SEM images were reversed black and white.

3. Results and Discussion

3.1 Influence of fixation and resin embedding on the x-y axes in light microscopy

Two ml of HeLaM containing 1.0 × 10^5 cells was dispensed in each of 5 culture dishes and the dishes were incubated at 37°C under 5% CO2. Culture time was 48h for 4 dishes (dish1–dish4) and 72h for the rest one (dish5). Then DTR was introduced into the nucleus of target HeLa cells. The fluorescence in the nucleus indicates successful introduction of DTR.

We observed that a DTR introduced target cell stayed at its original position even after fixation and resin embedding (Fig. 3a). Then we concluded that those treatments caused no effect on the cell position. The relative positions of nearby cells and their shapes were supporting information for the confirmation of the originally registered cell position. We also observed that a target cell disappeared after those treatments (Fig. 3b). In such a case, however, the relative positions of nearby cells and their shapes were essential information for the confirmation that the cell-disappeared position was the originally registered cell position. In our study, every case of cell disappearance in every dish (Table 1) was such a case.

On the other hand, if a target cell and its several nearby cells disappeared simultaneously, it should be difficult to confirm the registered cell position. We observed that a target cell disappeared after those treatments (Fig. 3b). In such a case, however, the relative positions of nearby cells and their shapes were essential information for the confirmation that the cell-disappeared position was the originally registered cell position. In our study, every case of cell disappearance in every dish (Table 1) was such a case.

Table 1. Number of locations that were initially registered as target cell locations and confirmed as the same locations after cell fixation and resin embedding. L-cell: number of locations of target cells initially registered by referring to a right triangle chip. L-cell+: number of locations where initially registered cells finally remained. L-cell±: number of locations that were initially registered and finally confirmed by the relation among nearby cells irrespective of the target cell remained or disappeared.

| No. | Dish | L-cell | L-cell+ | L-cell± |
|-----|------|--------|---------|---------|
| 1   | 14   | 13     | 7       | 13      |
| 2   | 14   | 9      | 14      |
| 3   | 10   | 6      | 10      |
| 4   | 9    | 7      | 9       |
| 5   | 11   | 8      | 11      |

Table 2. Comparison of cell locations registered by light microscopy and confirmed as the same positions by electron microscopy.

| Cell No. (i) | xA(x, y) | yA | xB(x, y) | yB | xA-xB | yA-yB | OAi (µm) | OBi (µm) | AiBi (µm) |
|--------------|---------|----|---------|----|-------|-------|----------|----------|-----------|
| 1            | 760     | 443| 860     | 495| 100   | 52    | 880      | 992      | 113       |
| 2            | 704     | 764| 766     | 841| 62    | 77    | 1039     | 1138     | 99        |
| 3            | 550     | 544| 579     | 565| 29    | 21    | 774      | 810      | 36        |
| 4            | 1168    | 619| 1150    | 687| -18   | 68    | 1322     | 1339     | 70        |
| 5            | 612     | 355| 664     | 449| 52    | 94    | 708      | 801      | 107       |
3.2 Coordinates of cells determined by electron microscopy

The x-y coordinates of 5 cells initially registered by light microscopy are listed in Table 2. The x-y coordinates in μm unit of the first example were A1 (760, 443). Referring to these data, the corresponding cell was looked for in the view of SEM. The x-y axes of a fixed cell in epoxy section (d). The x-y coordinate system that is transferrable from light microscopy to electron microscopy. In sharp contrast to usual gridded plates, no image of target cells is hidden by this coordinate system. The present coordinate system is expected to be a simple and useful tool for CLEM.

original registered cell position. In fact, however, there was no such a difficult case in our study.

4. Conclusion

A right triangle chip and its engraved shape was feasible as an x-y coordinate system that is transferrable from light microscopy to electron microscopy. In sharp contrast to usual gridded plates, no image of target cells is hidden by this coordinate system. The present coordinate system is expected to be a simple and useful tool for CLEM.

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