Preparation of 2-NBDG solution Dissolve 2-NBDG in KRB (pH 7.35–7.40). The concentration of 2-NBDG and glucose should be determined according to the purpose of the experiment (see PROCEDURE). ▲ CRITICAL Do not freeze the 2-NBDG solution, as it will precipitate when thawing.

EQUIPMENT SETUP

Culture dish and superfusion chamber Mark random scratches beforehand on the outside of the oval glass bottom of the culture dish with a diamond knife for later immunocytochemical identification of the cells. Although a round glass-bottom culture dish can be used, the medium flow is smoother in an oval one. Prepare a plastic plate with a leaf-shaped hole, as the glass part of the dish is too shallow to obtain a stable medium flow (**Fig. 2**). This plate can be readily made using the culture dish by removing its glass bottom. Attach the plate tightly to the plastic part of the culture dish with vacuum grease just before experiment (see PROCEDURE). Thus, the depth of the glass part of the dish is doubled. A silicone rubber plate with an oval hole can be used instead of the plastic plate (**Fig. 2**). In this case, small projection portions made on the silicone plate (slashed part in **Fig. 2**) will help precise positioning of the cover glass.

Attach a square small cover glass (10 × 11 mm^2) to the plastic plate with a small amount of vacuum grease to cover the central part of the oval glass bottom (**Fig. 2**). The cover glass helps to smooth flow while decreasing the volume of superfusate, assuring rapid change of the solution, and also helps to prevent optical noise owing to fluctuation of the medium surface level. When only the central part of the bath is covered, drugs can be directly dropped onto the small gap upstream of the cover glass. In addition, local temperature at the region of interest below the cover glass can be easily checked during the experiment. ▲ CRITICAL Adjust the position, angle and height of the inlet and, especially, the outlet needle carefully (**Fig. 2**) to obtain smooth laminar flow.

Place a round, flat-surface heating glass stage on the microscope stage. A plastic holder plate (14 × 14 × 0.9 cm^3) with an opening (approximately 35 mm) in the central part should be fixed on the heating stage. The inner diameter of the opening must be determined by the outer diameter (sometimes tapered) of the dish used. Mount the culture dish into the plastic holder plate so that the glass bottom of the culture dish directly touches the heating glass stage (37 °C). Long-WD objective lenses (see EQUIPMENT) are required owing to the thickness of the heating glass stage.

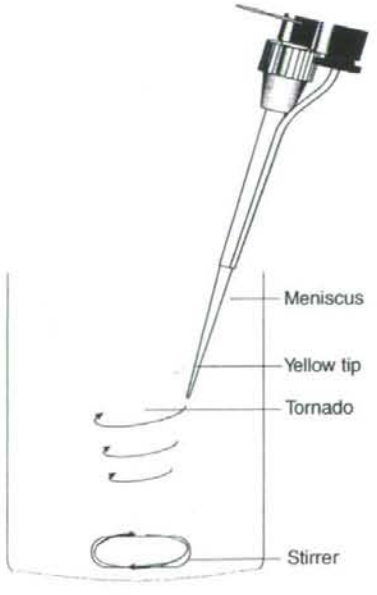

Figure 3 | Dissolving fura-2/AM in DMSO into Krebs Ringer bicarbonate buffer (KRB).

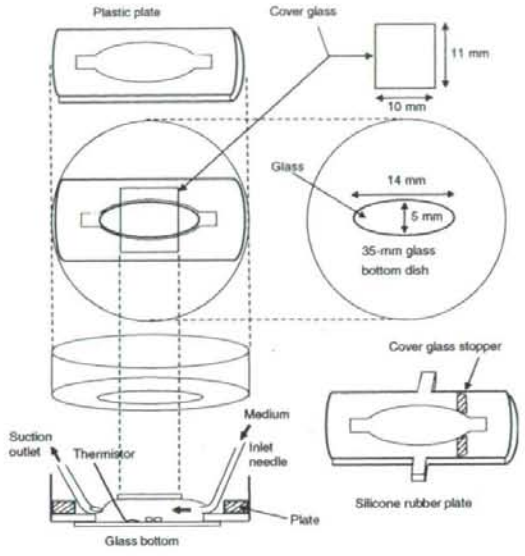

Figure 2 | A culture dish–based chamber for live-cell imaging. A plastic (or silicone rubber) plate is attached to the glass-bottom culture dish to make a bath for superfusion. The central part of the bath is covered by a cover glass to promote smooth flow and rapid exchange of superfusate.

Check the temperature of the superfusate at the region of interest by inserting a very thin thermistor probe between the cover glass and the glass bottom before (or sometimes during) the experiment. An extra dish with no cells should be prepared for just this purpose. Constant temperature at the area of interest (36.5–37.5 °C) indicates that the whole chamber system is working properly.

Use a low-pulsation peristaltic pump and a vacuum pump for the delivery and the removal of the superfusate, respectively. Substitution of the polyethylene tube with a stainless steel pipe of very thin internal diameter helps to decrease dead volume from the superfusate bottle to the dish. Adjust the vacuum pressure with a screw valve and/or three-way valves at 30–40 kPa so that cells are left on the dish. A simple delivery system using hydrostatic pressure can be used instead of a peristaltic pump. ▲ CRITICAL The shape of the end of the outlet needle for removing superfusate is critical to achieving a stable flow. The main consideration is that the hole at the end should be opened in the upward direction (**Fig. 2**). Bend a stainless steel needle (20G, 0.90 × 70 mm) smoothly so as not to interfere with smooth medium flow inside the needle. Then enlarge the hole at the end of the needle obliquely to the axis as large as is possible using sandpaper so that the needle has an elongated hole on the very tip. When the position and the angle of the needle are adequate, superfusate is sucked constantly from the hole together with air, making a constant sucking sound. We have designed a height-, angle- and rotation-adjustable small holder for the needle for this purpose (**Fig. 4**); this is available from Narishige Scientific Instruments, Tokyo, Japan (see **Table 1**).

PROCEDURE

Preparation for superfusion ● TIMING 5 min

1| Take the culture dish out from the CO_2 incubator, and gently absorb the culture medium outside the glass bottom part of the dish. Then wipe the medium left on the plastic floor of the dish completely using a cotton swab, leaving medium only on the glass bottom part.

2| Attach the plastic plate with a leaf-shaped hole (**Fig. 2**) tightly to the culture dish with silicone vacuum grease.

BOX 2 | $[Ca^{2+}]_I$ MEASUREMENT COMBINED WITH MEASUREMENT OF 2-[*N*-(7-NITROBENZ-2-OXA-1,3-DIAZOL-4-YL)AMINO]-2-DEOXY-D-GLUCOSE (2-NBDG) UPTAKE IN PANCREATIC ISLET CELLS

REAGENTS

- Fura-2/AM (Dojindo, cat. no. 343-05401)
- DMSO (Dojindo)
- Ca^{2+}-free Krebs Ringer bicarbonate buffer (KRB) (see REAGENT SETUP below)
- $CaCl_2$ (0.1 M solution) (Wako)
- Glucose (Wako)
- Eagle's minimum essential medium containing kanamycin (60 μg ml^{-1}) (Nissui, cat. no. 1) and 5.6 mM glucose (MEM)
- EGTA (0.1 M solution) (Sigma, cat. no. E-4378)
- BSA (Fraction V, pH of 1% aqueous solution is 5.2) (Sigma, cat. no. A-4503)
- Tolbutamide (Sigma, cat. no. T0891)
- Guinea pig anti-swine insulin antibody (Dako, cat. no. N1542)
- Rabbit anti-porcine glucagon antibody (Dako, cat. no. L1813)
- Rhodamine-conjugated goat anti-rabbit IgG (Cappel)
- Rhodamine-conjugated goat anti-guinea pig IgG (Chemicon)
- Sodium phosphate buffer (PB)
- Paraformaldehyde (Nakalai)

EQUIPMENT

- Non-coated glass-bottom dishes
- Dichroic mirror and barrier filter (Nikon DM400) (BA510 LP)
- Excitation filter Hamamatsu MC340- and 380-nm excitation filters

REAGENT SETUP

Preparation of islet cells (i) Isolate islets of Langerhans from 8–12-week-old Harlan Sprague–Dawley rats by collagenase digestion under nembutal anesthesia[25]. (ii) Transfer the islets into ice-cold Ca^{2+}-free KRB containing 5.6 mM glucose. (iii) Centrifuge at 800 r.p.m. for 30–60 s (in a himac CR5B from Hitachi High Technologies, Tokyo, Japan) at room temperature (22–28 °C) and wash sediments with KRB containing 0.1 mM Ca^{2+}, 0.1% BSA and 5.6 mM glucose (repeat three times). A small addition of Ca^{2+} at this point will mitigate cellular damage by EGTA treatment in the next stage. (iv) Dissociate the islet into single cells by incubation for 15–17 min at room temperature in 200 μl Ca^{2+}-free KRB containing 1 mM EGTA, 0.1% BSA and 5.6 mM glucose. Make a 0.1 M EGTA stock solution for this purpose (adjust pH to 7.4 by 1 N HCl). (v) Triturate 8–12 times using a yellow tip. (vi) Transfer the dissociated islets into 10 ml of MEM supplemented with 10% FBS (MEM-FK). (vii) Centrifuge at 800 r.p.m. for 3 min (in a himac CR5B from Hitachi High Technologies, Tokyo, Japan) at room temperature, discard the supernatant by suction pipette and re-suspend the cells in 200 μl of MEM-FK. (viii) Plate a small amount of single cells (such as 30 μl) on a culture dish in the center of the oval glass bottom (**Fig. 2**). (ix) Leave the cells in a CO_2 incubator (5% CO_2, 37 °C) for 20 min until cells adhere to the glass bottom. (x) Add 0.5–1.0 ml of culture medium slowly. Cells can be maintained in the CO_2 incubator for up to 2 d. However, Ca^{2+} responsiveness to glucose stimulation is obtained from a maximal number of healthy β-cells during the several hours after plating.

Ca^{2+}-free KRB (mM) NaCl, 129; KCl, 4.7; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 5.0; HEPES, 10; pH 7.35.

Preparation of fura-2/AM solution

1. Dissolve 10 μl 1 mM fura-2/AM in DMSO solution into 10 ml KRB containing 2.8 mM glucose and 1 mM Ca^{2+} (final concentration of fura-2/AM, 1 μM), as described.

Note: Use DMSO that has been kept dried, since moisture absorption interferes with dissolution of fura-2/AM (in DMSO) into aqueous solution. Detergents to promote dissolution such as Cremophor EL (C5135, Sigma) are not necessary. The volume of fura-2/AM-containing KRB should be determined according to the chamber volume and the total dead volume in the inlet tubing.

2. Stir vigorously 10 ml KRB solution in a small glass vial (such as 12.5 ml) using a very small stirrer magnet so that a stable, tornado-like vortex appears in the central part of the solution (**Fig. 3**). Use a high-performance magnetic stirrer.

Note: If a low-performance stirrer is used, the position of the vortex may move right or left unstably, and dissolution will be unsuccessful.

3. Push out the fura-2/DMSO solution continuously and as slowly as possible from a yellow tip. Hold the tip almost perpendicularly along, but slightly outside, the vortex wall. When dissolution is successful, fura-2/AM in DMSO disappears spirally into the KRB solution similar to a pale smoke or is almost unseen. Never suck the KRB into the tip by releasing pushing force.

Note: If water leaks into the tip, small oil droplets will be seen coming out from the tip into the KRB when fura-2/AM is pushed out, and the tip end will be polluted by white deposit. These droplets are due to undissolved fura-2, indicating unsuccessful dissolution. Discard the tip and try again from the beginning in such a case.

4. When subdivided into aliquots, keep fura-2/AM at below −20 °C in a tightly sealed box containing hygroscopic material to prevent moisture absorption during frequent use.

Preparation of glucose responsiveness experiment

1. Make KRB containing 1 mM Ca^{2+} as a superfusate during measurement. Make 100 ml of 0.1 M $CaCl_2$ stock solution to add Ca^{2+} to KRB. Prepare KRB containing 2.8 and 16.8 mM glucose.
2. Adjust pH of KRB stock solution to 7.3 with NaOH, because it will shift to 7.35–7.40 by the day of experiment.

Note: pH exceeding 7.4 may produce unsuccessful results.

BOX 2 | CONTINUED

3. Prepare tolbutamide in KRB by dissolving 1 M stock solution of tolbutamide (in DMSO) into KRB containing 2.8 mM glucose in a manner similar to that depicted in **Figure 3** (final concentration of tolbutamide, 200 μM).

PROCEDURE

Measurement of $[Ca^{2+}]_i$ in response to glucose stimulation: *fura-2/AM loading* ● TIMING 30 min

1. Prepare 10 ml of KRB (depending upon the total dead volume in the inlet tubing) containing 2.8 mM glucose and 1 μM fura-2/AM for each culture dish.
2. Superfuse the islet cells with the fura-2/AM in KRB at a flow rate of 0.3 ml min^{-1} for 30 min at 37 °C.
3. Wash fura-2/AM solution with KRB containing 2.8 mM glucose at 37 °C. This protocol is both easy and time saving. Indeed, because only islet cells strongly adhered to the glass bottom are left on the dish after the loading, cells of interest can be searched for using the microscope immediately after fura-2/AM loading, which contributes to obtaining responses from cells still in a healthy condition.
4. As an alternative to the above method of loading fura-2/AM by superfusion, fura-2/AM can be loaded by exchanging culture medium with KRB containing 2.8 mM glucose and 1 μM fura-2/AM, and incubated for 30 min at 37 °C in a humidified atmosphere containing 5% CO_2 (in a CO_2 incubator).

Measurement of $[Ca^{2+}]_i$ in response to glucose stimulation: $[Ca^{2+}]_i$ measurement ● TIMING 1 h

5. Superfuse KRB containing 2.8 mM glucose. Start searching the area of interest without waiting additional post-loading minutes for the hydrolysis of AM residue, as the searching process is usually time consuming.
6. Capture transmitted light images of cells of interest.
7. Start imaging the ratio F340/F380. Fura-2 fluorescence is detected every 5 or 10 s at 500–520-nm wavelength following excitation at 340-nm (F340) and 380-nm (F380) wavelengths. The ratio image is obtained using an Argus 50. (See Troubleshooting section below.)
8. Exchange superfusate to KRB containing 16.8 mM glucose for 5–10 min and then return to KRB containing 2.8 mM glucose.
9. After the ratio recovers to baseline for about 10 min or so, check responsiveness of β-cells to tolbutamide by superfusing KRB containing 200 μM tolbutamide for a brief period within 30 s.

Measurement of 2-NBDG uptake ● TIMING 30–60 min

10. After finishing Ca^{2+} imaging, start measurement of 2-NBDG uptake in a manner similar to that described for MIN6 cells. To discriminate β-cells from other cell types, KRB containing 200 μM 2-NBDG and 2.8 mM glucose is loaded for 1 min. Quenching of fura-2 fluorescence is not required since the 2-NBDG fluorescence is strong enough in comparison with fura-2 fluorescence, and the 2-NBDG uptake is evaluated by the relative increase in the fluorescence.

ANTICIPATED RESULTS

A raw example of measurement of $[Ca^{2+}]_i$ and subsequent 2-NBDG uptake in pancreatic islet cells is shown in **Figure 6**. After loading fura-2/AM by superfusion, a region of interest is selected and a transmitted light image is captured (**Fig. 6a**). Cells to be monitored are then selected on the fluorescent image (**Fig. 6b**). Information on the $[Ca^{2+}]_i$ response to glucose stimulation (**Fig. 6c**) and the subsequent uptake of 2-NBDG by a brief superfusion (**Fig. 6d,e**) were used to evaluate the divergent uptake of heterogeneous islet cells in combination with later immunocytochemistry (**Fig. 6f**). Since islet cells consist of a heterogeneous population of cells, including glucose-responsive insulin-secreting β-cells and glucose-unresponsive glucagon-secreting α-cells as well as somatostatin-secreting δ-cells, later immunocytochemical identification of cell type is essential (see **Box 3**).

TROUBLESHOOTING

Problem: Extraordinary large ratio in $[Ca^{2+}]_i$ imaging.
Possible reason: Unhealthy cells may show a large ratio. Especially important, islet cells do not remain healthy for many hours in KRB containing 2.8 mM glucose. In addition, care should be taken to prevent high-temperature degradation of the coating materials of the emission filter, which can be continuously exposed to heat radiation from the xenon lamp during the experiment despite the heat-insulating lens.
Solution: Carry out experiments using freshly prepared cells. To retard degradation of the coating materials of the filter, empty the home filter position and use two heat-insulating lenses in the pathway from the lamp. The condition of the filters should be checked by making a calibration curve for $[Ca^{2+}]_i$.

▲ CRITICAL STEP A toothpick and a small bent spatula can be used to spread the grease and press the plate, respectively. If you use too much grease, it comes out from the gap between the dish and the plate and interferes with medium flow. If you use too little, the superfusate leaking into the gap will interfere with smooth exchange of the superfusate during drug application, which is especially problematic when high concentrations of 2-NBDG are applied.

3| Cover the central part of the oval glass bottom with a square cover glass with a small amount of vacuum grease (**Fig. 2**).

4| Mount the dish on the plastic holder on the inverted microscope stage and start superfusion immediately with KRB containing 5.6 mM glucose. Paint a small mark on the rim of the dish so that it coincides with the mark on the holder. This makes it possible to reproduce the angle of the dish when immunocytochemistry is conducted later.

BOX 3 | IMMUNOCYTOCHEMICAL IDENTIFICATION OF ISLET CELLS AFTER MEASUREMENT OF 2-[*N*-(7-NITROBENZ-2-OXA-1,3-DIAZOL-4-YL)AMINO]-2-DEOXY-D-GLUCOSE (2-NBDG) UPTAKE

REAGENTS

- Guinea pig anti-swine insulin antibody (Dako, cat. no. N1542)
- Rabbit anti-pig glucagon antibody (Dako, cat. no. L1813)
- Rhodamine-conjugated goat anti-guinea pig IgG (Chemicon)
- Rhodamine-conjugated goat anti-rabbit IgG (Cappel)
- Sodium phosphate buffer (PB)
- Paraformaldehyde (Nakalai)

EQUIPMENT

- Dichroic mirrors and filters: Nikon DM575 (EX 510–560, BA 590 LP)

Immunocytochemistry ● TIMING **5 h–4.5 d**

1. At the end of the measurement of 2-NBDG uptake, capture transmitted light images of the analyzed cells at ×20, ×10, ×4 and ×2 for immunocytochemical identification of their location. Low-magnification lenses are especially convenient for finding the analyzed region in reference to the scratches previously made under the glass bottom of the culture dish.
2. Fix cells in cold 0.1 M PB containing 2% paraformaldehyde overnight at 4 °C. Alternatively, pour the cold fixative on the cells and leave cells for up to 1 h at room temperature.
3. Wash three times (rotate the dish gently and wait for 5 min between each washing) with 0.01 M PBS. Cells may be treated with 1% BSA.
4. React cells with primary antibodies, such as guinea pig anti-pig insulin antibody (1:100) or rabbit anti-pig glucagon antibody (prediluted), at room temperature for 1 h or at 4 °C overnight.

Note: Can be left up to 3 d at 4 °C.

5. React with rhodamine-conjugated secondary antibody (1:100–1:500) at room temperature for 1 h.
6. Wash three times with PBS.
7. Mount the dish on the plastic holder on the microscope stage so that the mark on the rim of the dish coincides with the mark on the holder.
8. Show the previous low-magnification image of the cells on the PC monitor and affix tiny triangles made of opaque tape on both ends of individual major scratches observed on the monitor screen. Then find the same scratch pattern by microscope and adjust the field of view so that the identical pattern is seen on the monitor using the opaque triangles as guides. Finer adjustment can be made by comparing the current image with the previous image. Repeat the process in higher-magnification views.
9. Examine rhodamine fluorescence.

ANTICIPATED RESULTS

See **Figure 6f**.

Measurement of 2-NBDG uptake ● TIMING **30–60 min**

5| Select area of interest and capture a transmitted light image.

6| Demarcate cells. Select an area for evaluating changes in background fluorescence.

7| Adjust the gain of the detection system and capture fluorescent images. A transmitted light image with no ND filter is useful for identifying locations of cells on the monitor screen by the intrinsic fluorescence of the cells. Strong ND filters are used when the fluorescence of cells is saturated after loading 2-NBDG.

8| Superfuse MIN6 cells with glucose-free KRB for 15 min.

9| Change superfusate to D-glucose-free KRB containing 50–600 μM 2-NBDG for 15–120 s, and then wash.

▲ CRITICAL STEP The concentration of 2-NBDG and the loading period are to be determined according to the purpose of the experiment, the type of GLUTs of interest and the signal-to-noise ratio of the detection system. For the signal-to-noise ratio and the superfusion technique used in the present system, the shortest practical loading period is 15 s, and the smallest concentration is 50 μM. When 600 μM 2-NBDG is used, a loading period longer than 120 s should be avoided because of the nonlinear increase in the 2-NBDG fluorescence over time[9]. With a system requiring a long loading period (e.g., tens of minutes),

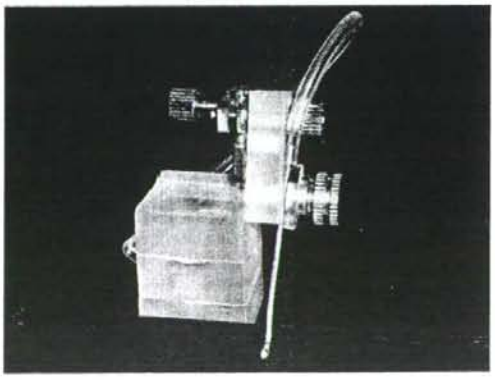

Figure 4 | A custom-made holder for the outlet needle (Narishige). Height, rotation angle and lean angle of the needle are freely adjustable without using a screwdriver.

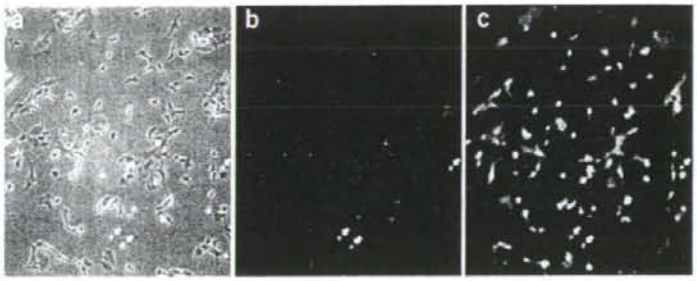

Figure 5 | Measurement of 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2-NBDG) uptake into MIN6 cells. (**a**) Transmitted light image (×20). Arrows indicate debris of dead cells. Raw fluorescent images measured at 540-nm wavelength with no neutral density (ND) filter (**b**) before and (**c**) after loading Krebs Ringer bicarbonate buffer (KRB) containing 600 μM 2-NBDG for 15 s. Note that no increase in the fluorescence intensity is seen for the debris (arrows). The field of view in the fluorescent images (**b**) and (**c**) was narrowed by the diaphragm in the fluorescent light path.

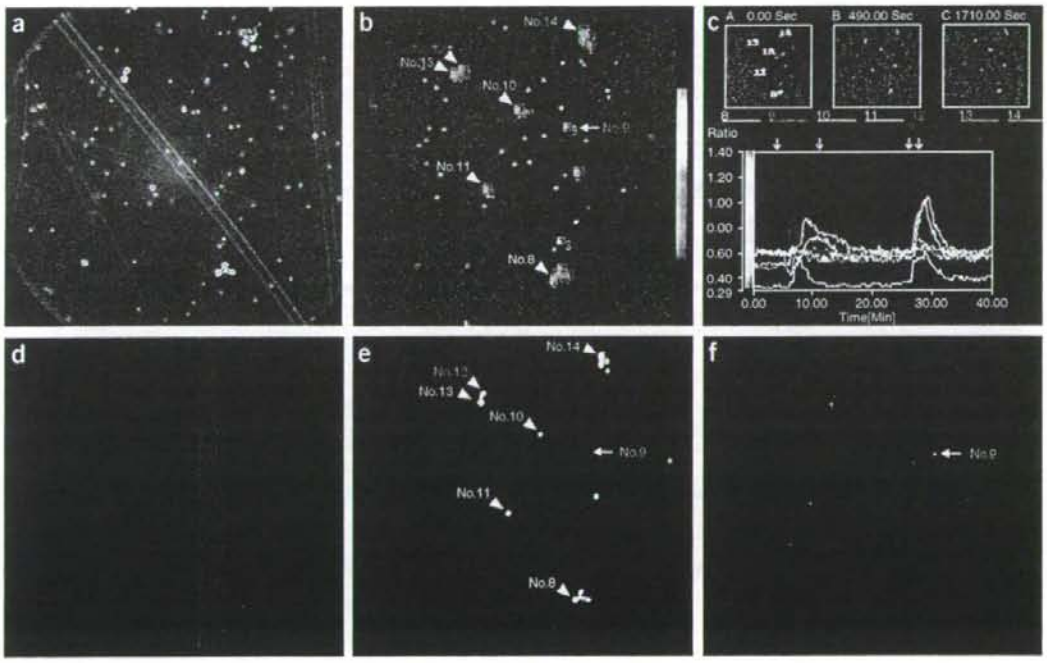

Figure 6 | Measurement of $[Ca^{2+}]_i$ in response to glucose stimulation and subsequent 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2-NBDG) uptake in living pancreatic islet cells followed by immunocytochemical identification. (**a**) Transmitted light image (×20). Scratches made under the glass bottom of the dish are visible. (**b**) Raw fluorescent image examined at 500–520-nm wavelength for 380-nm excitation after loading of 1 μM fura-2/AM by superfusion. Areas demarcated by squares were tentatively selected for monitoring $[Ca^{2+}]_i$ responses before starting the measurement. Of these, the responses of cells indicated by colored numbers are exemplified (see below). (**c**) A window of the Argus 50 for $[Ca^{2+}]_i$ analysis. Seven areas can be analyzed on one page of the Argus 50 (seven colors are assigned automatically). Areas showing increases (no. 8 and nos. 10–14) and no increase (no. 9) in $[Ca^{2+}]_i$ are expressed as the change in fura-2 fluorescence ratio (340 or 380 nm) (lower panel). Small downward arrows above the lower panel indicate periods of superfusing Krebs Ringer bicarbonate buffer (KRB) containing 16.8 mM glucose (between the two left arrows) and 200 μM tolbutamide (between the right arrows). The response times to tolbutamide coincided for cells responding to high glucose, whereas the response times to glucose varied, indicating accuracy of the superfusion and differences in intracellular metabolism after glucose uptake among cells. Upper insets are provided by the software for checking locations of cells and changes in the fluorescence at different time points. Fluorescent images measured at 540-nm wavelength (**d**) before and (**e**) after loading 200 μM 2-NBDG for 1 min. Cells incorporating 2-NBDG are β-cells[9], and uptake of 2-NBDG into cell no. 9 was undetectable during 1 min loading. (**f**) Immunocytochemistry of glucagon. Rhodamine-conjugated secondary antibody was used. Cell no. 9 (arrow) was clearly immunopositive for glucagon. Rhodamine fluorescence was examined with a 590-nm longpass filter (excitation wavelength 510–560 nm). Note that the location of the cells incorporating abundant 2-NBDG can be identified by their vague fluorescence. In (**a**–**e**), cells were superfused continuously with KRB containing 2.8 mM glucose at 37 °C, and the KRB was supplemented as indicated. In these original pictures, no subtraction or shading correction was done. Contrast was adjusted for clarity only in panels (**d**) and (**e**). Note that some cells were lost and some not seen in the original transmitted light image entered from upstream during superfusion. Some of these data were first published in ref. 9.

some investigators use a very low concentration of 2-NBDG (10 μM)[13]. For a long loading period, time-dependent extinction should also be considered. In MIN6 cells, the intensity of 2-NBDG fluorescence (200 μM loaded for 60 s) was linearly decreased to 87.7, 70.9 and 56.6% 15, 30 and 60 min after uptake, respectively, under dark condition (K. Yamada and N. Inagaki, unpublished data). Care should be taken that the actual loading period of 2-NBDG from the beginning of superfusion to complete washing is constant for all of the cells compared. Differences in the local temperature within the area of interest should be within 1 °C (e.g., 36.5–37.5 °C) to minimize temperature-dependent variance of the uptake[9].

? TROUBLESHOOTING

10| When 2-NBDG uptake is evaluated in the presence of glucose, skip Step 8 and change the superfusate to KRB containing 2-NBDG and glucose. The glucose concentration added to the KRB should be determined according to the purpose of experiment. To test inhibition of 2-NBDG uptake by D-glucose, superfuse cells with KRB containing 11.2 mM D-glucose and compare the 2-NBDG uptake with that in the absence of D-glucose. The uptake of 2-NBDG (600 μM loaded for 2 min) in the presence of 11.2 mM glucose was significantly inhibited by 52.5 ± 6.3% compared with uptake in the absence of glucose[9].

11| After washing for 5 min, capture fluorescent images.

12| Calculate the relative fluorescence intensity of each cell before and after loading 2-NBDG by subtracting the corresponding background fluorescence from the fluorescence intensity of the cells.

13| Calculate the net increase in the fluorescence intensity for each cell by subtracting the relative fluorescence intensity before from that after loading 2-NBDG.

14| To test whether the 2-NBDG uptake occurs through GLUTs, superfuse cells with KRB containing 10 μM cytochalasin B 5 min before the 2-NBDG loading. In MIN6 cells, the increase in fluorescence by loading 200 μM 2-NBDG for 15 s was almost completely inhibited in the presence of cytochalasin B[9].

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reasons	Solution
9	Variable results in 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2-NBDG) fluorescence in quantitative measurements or high background fluorescence due to 2-NBDG	Unstable superfusion in the chamber	Check for smooth superfusion by dropping a small amount of dye such as pontamine sky blue (Brilliant Blue 6B, CI-24420, Tokyo Chemical Industry, Tokyo, Japan) using an extra dish without cells. Adjust the position of the outlet needle so that it slightly protrudes into the glass bottom part. Adjust the inlet angle and direction finely so that a smooth, laminar flow is obtained. A constant sound of sucking of the superfusate from the outlet needle indicates a stable flow. Measure the temperature of each region of interest with a thin thermistor probe to confirm that the temperature difference is within 1 °C. An area meeting this criterion can be marked on the cover glass. Measurement of the local temperature also helps in finding irregular flow
		Pollution by 2-NBDG fluorescence from the forceps used for placing and removing the plastic plate on the glass-bottom culture dish, or other materials such as the thermistor probe	Every time a dish is exchanged, the plate and forceps used should be rinsed thoroughly. Make several additional plates to save time. Each time vacuum grease is taken from the tube, use a new disposable toothpick so that the grease itself is not polluted by 2-NBDG fluorescence. Similar care should be taken for other parts
		Variance of uptake specific to the particular cell types of interest	See ANTICIPATED RESULTS

ANTICIPATED RESULTS

An example of the raw measurements of 2-NBDG uptake in MIN6 cells is shown in **Figure 5**. The fluorescence of MIN6 cells, which was only slightly discernible before loading, was remarkably increased by a brief (15 s) superfusion of 600 μM 2-NBDG in the absence of glucose. In the transmitted light image, cells with an abnormal round shape are easily distinguished from normal cells exhibiting irregular shape[24]. Abnormal cells and areas where multiple cells are overlapping are to be excluded from the

analysis. MIN6 cells exhibit a relatively homogeneous uptake of 2-NBDG. However, when other cell types are used, care should be taken to determine whether the variable uptake of 2-NBDG is due to intrinsic differences in the glucose uptake of individual cells (**Box 2**, **Box 3** and **Fig. 6**).

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Corrigendum: A real-time method of imaging glucose uptake in single, living mammalian cells

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In the version of this article initially published, on p. 754 under "Reagents," "N-(7-nitrobenz-2-oxa-1,3-diazol-4yl-)amino chloride" should have been "4-Chloro-7-nitrobenzofurazan". The error has been corrected in the HTML and PDF versions of the article.

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ORIGINAL ARTICLE

Evaluation of a new medium for the enumeration of total coliforms and *Escherichia coli* in Japanese surface waters

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coliforms, EC-Blue-10, *E. coli*, enumeration, MPN, water.

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Abstract

Aim: A new medium, EC-Blue-10, containing chromogenic and fluorogenic substrates, KNO_3 and sodium pyruvate has been developed for the rapid simultaneous detection and enumeration of total coliforms and *Escherichia coli* in water.

Methods and Results: Two evaluations of EC-Blue-10 were carried out. Firstly, EC-Blue-10 was compared with Colilert-MPN for 96 water samples using MPN for total coliforms and *E. coli*. Secondly, the detection of coliforms and *E. coli* were compared using 2400 tubes of EC-Blue-10 and Colilert-MPN. The regression coefficients between EC-Blue-10 and Colilert-MPN for total coliforms and *E. coli* were 0·91 and 0·89, respectively. For the detection results, the Cohen's kappa values between the two media were 0·79 for coliforms and 0·72 for *E. coli*.

Conclusions: EC-Blue-10 is almost same as Colilert-MPN for the detection of coliforms and *E. coli* in surface waters. Further evaluation for EC-Blue-10 is needed to verify in different geographical areas.

Significance and Impact of the Study: EC-Blue-10 is useful method for the rapid and simultaneous detection of total coliforms and *E. coli* in water sample.

Introduction

Total coliforms and *Escherichia coli* are important indicators of the sanitary quality of drinking water. The standard test for the coliform group is either the multiple-tube fermentation technique (Grasso *et al.* 2000) or the membrane-filter technique (Bernasconi *et al.* 2006). Since traditional multiple-tube fermentation and membrane-filter methods require a minimum of 24 h incubation followed by a confirmation procedure lasting 24–48 h, there is a requirement for rapid test methods for the emergency testing of drinking water supplies. During recent decades new chromogenic or fluorogenic, defined substrate methods based on β-galactosidase for total coliforms or β-glucuronidase for *E. coli* and ready-made culture media have been introduced (Edberg and Edberg 1988). Many chromogenic media based on β-galactosidase for total coliforms use *o*-nitrophenol-β-D-galactopyranoside (ONPG) or chlorophenol red-β-D-galactopyranoside (CPRG) as a substrate. The results of studies comparing media containing ONPG or CPRG and 4-methylumbelliferyl-β-D-glucuronide (MUG) with standard methods (Edberg *et al.* 1988; Buckalew *et al.* 2006) provide critical information confirming the accuracy of the defined substrate technology (DST) method, its comparability to a standard method, and its applicability for use. ONPG could be replaced successfully by 5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside (X-Gal) (Manafi and Kneifel 1989). However, there are few studies testing commercially available liquid media that use X-Gal and MUG for the simultaneous determination of total coliforms and *E. coli* in water (Manafi and Kneifel 1989; Geissler *et al.* 2000; Hörman and Hänninen 2006). We have developed a new medium using X-Gal and MUG for rapidly and simultaneously detecting total coliforms and *E. coli* in water. EC-Blue-10 is a new medium in a special

plastic disposable tube sterilized by electron beam. After incubation at 35°C for 24 h, the development of a blue-green colour in an initially light yellow-coloured solution demonstrates the presence of coliforms and fluorescence at 366 nm in the same tube demonstrates the presence of *E. coli*. Since a DST method has been used as a Japanese standard method since 1992 (Japan Water Works Association [JWWA] 2001), it was decided to use this DST method to confirm the validity of EC-Blue-10 for rapidly and simultaneously detecting coliforms and *E. coli* in temperate humid climate zone waters sampled in Japan.

Materials and methods

EC-Blue-10

EC-Blue-10 consists of a granulated medium in a special plastic bottle sterilized by electron beam. The medium was developed primarily for the rapid growth of *Enterobacteriaceae* (Kodaka *et al.* 1995) and contains the following ingredients g l^{-1}: Trypticase peptone (Becton, Dickinson and Company, Sparks, MD, USA) 5·0, NaCl 5·0, K_2HPO_4 4·0, KH_2PO_4 1·0, KNO_3 1·0, sodium pyruvate 1·0, sodium dodecyl sulphate (SDS) 0·1, MUG 0·1, X-Gal 0·1, isopropyl-β-D-thiogalactopyranoside 0·1 and pH 7·1 ± 0·2.

Effect of media for the detection of coliform bacteria from chlorinated water sample

Escherichia coli American Type Culture Collection (ATCC, VA, USA) 11775, *Citrobacter freundii* ATCC 8090, *Enterobacter cloacae* ATCC 13047 and *Klebsiella pneumoniae* ATCC 13883 were used in this study. Each coliform bacterium was suspended in sterilized phosphate buffer saline (PBS) solution prepared to make 20 000 CFU 400 ml^{-1} in 500 ml Erlenmeyer flask. The bacteria suspension was kept in the water bath at 20°C until mixing with chlorine solution. The chlorine solution was prepared to a target concentration of 0·3 mg l^{-1} (0·3 ppm) in 400 ml sterilized PBS solution in 500 ml Erlenmeyer flask. The chlorine solution was kept in the water bath at 20°C until mixing with the bacterial suspension. The 400 ml bacterial suspension and 400 ml chlorine solution were mixed promptly in 1000 ml sterilized brown Erlenmeyer flask with stopper. This mixed solution was stirred by stirring bar and 100 ml mixed solution was taken at 15, 30, 60, 120 and 300 s after adding sodium hypochlorite (NaOCl) into 300 ml sterilized Erlenmeyer flask containing 0·5 ml of 1 mol l^{-1} of sodium thiosulfate. Each 50 ml sample was diluted with 450 ml sterilized PBS solution. About 10 ml of diluted sample was inoculated into 10 tubes of EC-Blue-10, Colilert-MPN, Lactose Broth with bromothymol blue (LB: Nissui Pharmaceutical Co. Ltd, Tokyo, Japan) and Brilliant Green Lactose Bile broth (BGLB: Nissui Pharma.). These cultures were incubated at 36 ± 1°C for 48 h. During the 48-h incubation, the positive reaction was observed at 20, 24, 28 and 48 h. This experiment was done three times for each strain. The comparison for detection of each coliform from chlorinated water samples was carried out to sum up the positive results from the data of three experiments.

Influence of heterotrophic bacteria for coliforms and *E. coli* detection

Each coliform bacterium (*E. coli* ATCC 11775, *Cit. freundii* ATCC 8090, *Ent. cloacae* ATCC 13047 and *Kl. pneumoniae* ATCC 13883) was suspended in 250 ml sterilized PBS solution to make 20 CFU ml^{-1}. Three heterotrophic bacteria [*Flavobacterium odoratum* Japan Collection of Micro-organisms (JCM, Saitama, Japan) 7458, *Acinetobacter calcoaceticus* JCM 6842 and *Pseudomonas aeruginosa* from JWWA] were mixed and suspended in 250 ml PBS solution to make 60 000 CFU ml^{-1}. Each coliform bacterium and the heterotrophic suspension were mixed in equal volumes. The mixed bacterial suspension was inoculated into 10 tubes of two media and incubated at 35°C. The positive reaction was observed at 20, 24, 28 and 48 h. The experiments were done in duplicate for each coliform bacterium.

Evaluation procedure for untreated and chlorinated natural water

The natural water samples were collected by eight waterwork stations following the instructions issued by JWWA between September (average temperature: 24·4°C at Tokyo) and October (average temperature: 20·1°C at Tokyo) 1998. Ten-litre water samples were collected in sterilized bottles and were kept in the dark and at a cool temperature (5°C) until examination. The examinations were carried out within 48 h of collection. Each water sample was tested in duplicate on the same day. The water samples were put into sterilized 3000 ml Erlenmeyer flasks and stirred with a magnetic stirrer at 20 ± 1°C. The following procedure was carried out in accordance with the instructions of JWWA. NaOCl solution was added to give a concentration of 0·2 mg l^{-1} for lake-waters and 0·5 mg l^{-1} for river-waters. The sampling time after the addition of NaOCl was 20, 60, 180, 300 and 1800 s. Each water sample was then put into sterilized 300 ml Erlenmeyer flasks containing 1 mol l^{-1} of sodium thiosulfate. Total coliforms and *E. coli* in each of the 16 untreated and 80 chlorinated natural water samples (total 96 water samples) were then estimated using EC-Blue-10 and Colilert-MPN (Colilert, IDEXX Laboratories, KK, Tokyo,

Japan) by the five-tube, five-dilution MPN method. Each medium was incubated at 36 ± 1°C. The results were read at 24 h for total coliforms and *E. coli*. The presence of coliforms using EC-Blue-10 was identified by the development of a blue-green colour in an initially light yellow coloured solution and the presence for *E. coli* was identified by the development of fluorescence at 366 nm in the same vessel. For Colilert-MPN, the development of a yellow colour indicated the presence of coliforms and fluorescence on exposure to long-wavelength UV light denoted the presence of *E. coli*.

Isolation and identification

The presence of total coliforms was confirmed by identifying the bacterial isolate(s) to species level from at least one positive EC-Blue-10 and Colilert-MPN tube per row according to the method of Edberg *et al.* (1988). The isolation of bacteria was carried out by streaking onto Levine–Eosin Methylene Blue agar (L-EMB, Becton, Dickinson and Company). Colonies with a typical green metallic sheen, representative of each morphology present, were picked and re-streaked on XM-G agar (the agar medium containing 5-bromo-6-chloro-3-indoxyl-β-D-galactopyranoside (Magenta-Gal) and 5-bromo-4-chloro-3-indoxyl-β-D-glucuronic acid, cyclohexylammonium salt (X-Gluc), Nissui Pharma.) and incubated for 24 h at 35°C. Presumptive identification for coliforms and *E. coli* was confirmed by Magenta-Gal and X-Gluc reactions on XM-G agar, respectively. Bacterial isolates were inoculated onto plate count agar to confirm the purity of cultures. The isolates for identification were selected after due consideration of geographical differentiation, sampling time and colony morphology on L-EMB and XM-G agar. Gram-negative rods were identified by API 20E system (bioMerieux Japan Ltd, Tokyo, Japan) and ID TEST EB-20 (Nissui Pharma.) (Kodaka *et al.* 2004). The identification of all isolates was also confirmed by standard methods.

Tests for microbial and physical properties

The tests were done according to the Japanese Standard Methods for Examination of Water (JWWA 1993). The standard plate count (SPC) using plate count agar incubated at 36 ± 1°C for 24 ± 2 h and the heterotrophic plate count (HPC) using PYG agar (g l^{-1}: peptone 2·0, glucose 0·5, yeast extract 1·0, agar 15, pH 7·0 ± 0·1) incubated at 20 ± 1°C for 7 days were carried out before adding NaOCl. The residual chlorine was measured using the *N,N*-diethyl-*p*-phenylenediamine method. The turbidity was measured with a turbidimeter (ANA-7S Tokyo, Koden, Tokyo, Japan) comparing the sample with a kaolin turbidity standard solution [1 mg of kaolin in 1000 ml of distilled water has a turbidity of approx. one nephelometric turbidity units (NTU)]. The water temperature was measured with Celsius liquid-in-glass thermometer. The pH was measured with a pH meter (HM-60V, TOA Electronics, Tokyo, Japan) with a glass electrode.

Statistical analysis

Total results for the 96 sets of MPN data were calculated as $\log_{10}$ MPN of total coliforms and *E. coli* 100 ml^{-1} of water samples. Statistical calculations were carried out with the MICROSOFT EXCEL 2000 statistics package. The statistical analysis consisted of regression analysis and paired *t*-test for the MPN data. The chi-square test and Cohen's kappa for homogeneity of presence/absence results were compared using 2400 tubes of EC-Blue-10 and Colilert-MPN, respectively. All statistical analyses were performed with a level of significance of 0·05. The data were also analysed according to the ISO 17994 (2004) for the establishment of equivalence between EC-Blue-10 and Colilert-MPN methods, prescribes calculation of 100-times the logarithmic (ln) difference. The evaluation of equivalence is based on the mean and the expanded uncertainty derived from the standard uncertainty of the mean.

Results

Effect of media for coliform bacteria from chlorinated water sample

No difference between 24 and 48 h for positive reactions were observed among four media. Therefore, we have compared the cultures for positive reaction at 24 h incubation. A total of 30 tubes were examined at each chlorine treatment time. For the detection of *E. coli* ATCC 11775 after chlorine treatment for 15, 30, 60, 120 and 300 s, positive X-Gal tubes of EC-Blue-10 were 6, 4, 9, 3 and 0, respectively, and positive ONPG tubes of Colilert-MPN were 11, 4, 11, 1 and 1, respectively. Positive MUG tubes of EC-Blue-10 were 6, 4, 9, 3 and 0, respectively, and positive MUG tubes of Colilert-MPN were 10, 2, 11, 1 and 1, respectively. The positive gas production tubes of LB were 19, 15, 18, 4 and 3, respectively. The positive gas production tubes of BGLB were 16, 10, 16, 3 and 0, respectively. For the detection *Cit. freundii* ATCC 8090, positive X-Gal tubes of EC-Blue-10 were 29, 29, 21, 7 and 1, respectively, and positive ONPG tubes of Colilert-MPN were 30, 30, 25, 7 and 5, respectively. The positive gas production tubes of LB were 30, 30, 13, 12 and 4, respectively. The positive gas production tubes of BGLB were 27, 12, 3, 3 and 0, respectively. For the detection of *Ent. cloacae* ATCC 13047, positive X-Gal tubes of EC-Blue-10 were 15, 7, 4, 2 and 0, respectively, and positive ONPG

tubes of Colilert-MPN were 6, 3, 4, 1 and 0, respectively. No positive tubes for gas production in LB and BGLB were observed. For the detection of *Kl. pneumoniae* ATCC 13883, positive X-Gal tubes of EC-Blue-10 were 27, 27, 26, 9 and 12, respectively, and positive ONPG tubes of Colilert-MPN were 3, 5, 3, 1 and 1, respectively. The positive gas production tubes of LB were 25, 29, 28, 20 and 23, respectively. The positive gas production tubes of BGLB were 19, 21, 23, 14 and 12, respectively. MUG reaction in each medium was not observed with *Cit. freundii* ATCC 8090, *Ent. cloacae* ATCC 13047 and *Kl. pneumoniae* ATCC 13883.

Heterotrophic bacterial influence to detect coliforms and *E. coli*

The heterotrophic bacteria at 10^4 CFU level found in these samples appeared neither to interfere with coliforms and *E. coli* detection nor to account for the differences between EC-Blue-10 and Colilert-MPN in coliforms and *E. coli* detection. Mixtures of the heterotrophic bacteria did not result in false-negative analyses.

Untreated and chlorinated natural water samples

A total of 96 MPN tests for each water sample and each NaOCl exposure time were carried out using EC-Blue-10 and Colilert-MPN. The results of the regression analyses for total coliforms are shown in Table 1. Median $\log_{10}$ MPN 100 ml^{-1} ± standard deviation (SD) for total coliforms with EC-Blue-10 and Colilert-MPN were 2·11 ± 1·32 and 2·23 ± 1·38, respectively. The regression coefficient, slope and intercept between EC-Blue-10 and Colilert-MPN were 0·91, 0·96 and –0·0012, respectively (Table 1). The results of the regression analyses for *E. coli* are shown in Table 1. Median $\log_{10}$ MPN 100 ml^{-1} ± SD for *E. coli* with EC-Blue-10 and Colilert-MPN were 0 ± 0·91 and 0 ± 0·89, respectively. The regression coefficient, slope and intercept between EC-Blue-10 and Colilert-MPN were 0·89, 0·85 and 0·084, respectively. The means of MPN results for total coliforms and *E. coli* with EC-Blue-10 and Colilert-MPN were not statistically significantly different ($P > 0·05$) by paired *t*-test. For all comparisons, the slope and intercept values, as determined by linear regression analysis, were close to 1·00 and 0·00, respectively. The presence/absence results using 2400 tubes of EC-Blue-10 and Colilert-MPN were compared. For the coliform test, the results were 959 positive tubes of EC-Blue-10 and 925 positive tubes of Colilert-MPN (Table 2). For the *E. coli* test 208 tubes were positive using EC-Blue-10 and 217 tubes were positive using Colilert-MPN (Table 2). These results indicated no significant difference between the two media using the chi-square test ($P > 0·05$). The substantial agreements between the two kits obtained using Cohen's kappa were 0·79 for total coliforms and 0·72 for *E. coli*. Table 3 shows statistical evaluations of the equivalence of the two methods for total coliforms and *E. coli* according to ISO 17994 (2004). Samples were excluded from calculations when both methods gave zero (0, 0). The expanded uncertainty was derived from the standard uncertainty of the mean by using the coverage factor $k = 2$. The evaluation for results of the comparison and the confidence interval of the expanded uncertainty around the mean was calculated by computing the lower limit (x_L) and upper limit (x_H). The x_L and x_H for total coliforms and *E. coli* were –3·0 and 51·6 and –44·9 and 44·3, respectively. Assuming that the maximum acceptable deviation (D) has been chosen as D = 10%. The means of relative difference for total

Table 1 Parameters of each test for total coliforms and *Escherichia coli* from water samples*

	Total coliforms		*E. coli*	
Parameters	EC-Blue-10	Colilert-MPN	EC-Blue-10	Colilert-MPN
No. of tested samples	96	96	96	96
No. of positive samples	93	87	37	41
Median ($\log_{10}$ MPN/100 ml)	2·11	2·23	0	0
SD ($\log_{10}$ MPN/100 ml)	1·32	1·38	0·91	0·89
95% Confidence limit	0·26	0·28	0·18	0·18
Regression coefficient	0·91		0·89	
Slope	0·96		0·85	
Intercept	–0·0012		0·084	
t†	1·75		–0·17	
df	95		95	

*Include 16 untreated and 80 chlorinated water samples.
†Paired *t*-test at the significance level ($P = 0·05$).

Table 2 Comparison of presence/absence results from 2400 tubes for coliforms and *Echerichia coli*

	Coliforms				*E. coli*			
Test kit	Pr*	Ab†	*k*‡	*P*§	Pr*	Ab†	*k*‡	*P*§
EC-Blue-10	959	1441	0·79	0·62	208	2192	0·72	0·10
Colilert-MPN	925	1475			217	2183		

*Pr, presence.
†Ab, absence.
‡*k*, Cohen's kappa value.
§*P*, *P* value by chi-square.

Table 3 Statistical evaluation of the equivalence of the two MPN methods for total coliforms and *Escherichia coli* according to ISO 17994 (2004)

	No. of Samples	n_0*	n†	Mean relative difference	SD	Expanded uncertainty range x_L	Expanded uncertainty range x_H	One-sided evaluation
Total coliforms	96	1	95	24·3	133·0	−3·0	51·6	Inconclusive
E. coli	96	47	49	−0·3	156·0	−44·9	44·3	Inconclusive

*n_0, number of samples excluded because of zero.
†n, number of samples retained for analysis.

Table 4 Statistical evaluation of P/A results according to ISO 17994 (2004)

	*n*A*	*n*B†	x^2‡
Coliform	147	101	8·53
E.coli	49	63	1·75

**n*A, the number of samples where EC-Blue-10 was positive and Colilert-MPN negative.
†*n*B, the number of samples where EC-Blue-10 was negative and Colilert-MPN positive.
‡x^2, Poisson index of dispersion.

coliforms and *E. coli* were 24·3 and −0·3, respectively. The evaluations for total coliforms and *E. coli* in accordance with one-sided evaluation of ISO 17994 (2004) were both 'inconclusive' because the data were insufficient for decisions. Table 4 shows statistical evaluation of P/A results for coliform and *E. coli* according to ISO 17994 (2004). The values of the Poisson-index of dispersion (x^2) for coliform and *E. coli* were 8·53 and 1·75, respectively. EC-Blue-10 and Colilert-MPN methods were considered to be 'different' for coliform, however both methods were considered to be 'not different' in accordance with evaluation for two P/A methods of ISO 17994 (2004).

Bacterial isolates

Table 5 shows species of Gram-negative isolated from untreated and chlorinated water samples. The total number of isolates for identification from EC-Blue-10 and Colilert-MPN were 41 and 46, respectively. The coliforms (excluding *E. coli*) that were isolated from water samples were *Cit. amalonaticus*, *Ent. agglomerans*, *Ent. cloacae*, *Ent. intermedium*, *Kl. Pneumoniae* and *Serratia marcescens*. There were mixed cultures of total coliforms present in both EC-Blue-10 and Colilert-MPN tubes.

Relation between coliforms and bacterial counts

Table 6 shows the microbiological and physical properties of each sample during the experiments. The HPC ranged from 3550 to 140 500 CFU ml^{-1} with PYG agar and the SPC ranged from 1305 to 141 000 CFU ml^{-1} with plate count agar. No relationship was noticed between SPC, HPC and coliforms.

Table 5 Species of Gram-negative identified

Species	% of all isolates identified by EC-Blue-10	Colilert-MPN
Coliforms		
Citrobacter amalonaticus	5	2
Enterobacter agglomerans	13	1
Ent. cloacae	1	9
Ent. intermedium	3	1
Escherichia coli	40	42
Klebsiella pneumoniae	3	9
Serratia liquefaciens	1	2
Ser. marcescens	10	1
Noncoliforms		
Aeromonas caviae	1	2
Morganella morganii	5	2
Providencia alcalifaciens	1	2
Pseudomonas aeruginosa	8	1
Ps. fluorescens	3	4
Ps. putida	5	15
Proteus vulgaris	1	7

Total isolates for identification from EC-Blue-10 and Colilert-MPN were 41 and 46, respectively.

Discussion

Statistical evaluations of the equivalence of the EC-Blue-10 and Colilert-MPN methods for total coliforms and *E. coli* according to one-sided evaluation of ISO 17994 (2004) were 'inconclusive' for total coliforms and *E. coli*. About 25 additional samples for total coliforms and about 1000 additional samples for *E. coli* would have been sufficient numbers to reach firm decisions. The ONPG test with the ONPG peptone-water medium is preferable for growth of the organisms (Lowe 1962). EC-Blue-10 contains biological material such as peptone for the enhanced growth for bacteria, whereas Colilert-MPN is minimal medium for bacteria. Two ingredients of

Table 6 Microbial and physical properties of each water sample during the experiments

Sampling areas	No. of coliforms isolated		No. of *E.coli* isolated		SPC* (CFU ml^{-1})	HPC† (CFU ml^{-1})	Free Cl^-(mg l^{-1})	Total Cl^-(mg l^{-1})	Temp. (°C)	NTU‡	pH
	EC- Blue-10	Colilert- MPN	EC-Blue-10	Colilert-MPN							
A-lake	25	17	4	4	1305	2563	0·1	0·2	21·1	0·6	7·3
B-river	31	38	12	16	21700	120500	0·2	0·4	20·5	2·9	7·4
C-river	26	20	6	6	141000	140500	0·3	0·5	20·1	0·5	7·4
D-lake	46	35	14	5	1118	3550	0·1	0·2	20·8	2·0	8·6
E-river	32	34	8	11	2503	26575	0·2	0·5	20·0	0·8	7·3
F-river	30	26	11	10	16275	71000	0·4	0·5	19·6	2·6	7·3
G-river	33	34	4	7	1973	6700	0·1	0·3	20·1	1·5	7·6
H-river	43	47	13	25	19950	71000	0·1	0·5	20·3	1·1	7·5
Mean	33	31	9	11	25728	55299	0·2	0·4	20·5	1·5	7·6
SD	8	10	4	7	47408	54313	0·1	0·1	0·5	0·9	0·4

Sixteen untreated and 80 chlorinated water samples were tested.

*SPC, Standard plate count.

†HPC, Heterotrophic plate count.

‡NTU: Nephelometric turbidity units.

EC-Blue-10 are also different from Colilert-MPN. Firstly, EC-Blue-10 contains sodium pyruvate as nonenzyme peroxide-degrading compound to increase the detection of chlorine-stressed coliform bacteria (Sartory 1995). Secondly, the KNO_3 in EC-Blue-10 is important for bacteria, as it allows energy production during nitrate respiration (Hadjipetrou and Stouthamer 1965). Bacteria, commonly considered part of the total coliform group were isolated from both EC-Blue-10 and Colilert-MPN tubes. *E. coli* was isolated from tubes with both positive-colour and -fluorescence. *E. coli* was primarily isolated from water samples, followed by *Ent. agglomerans*, *Kl. pneumoniae* and *Serratia marcescens*. Every isolate was inoculated into both media to confirm the reactions. No different reactions were observed between EC-Blue-10 and Colilert-MPN. There did not appear to be a significant difference in the distribution of bacterial species in either medium. The HPC on most samples were higher than the SPC. Only one sample, C-river, had almost the same microbiological count (Table 6). The results of heterotroph interference study and the results in Table 6 support the notion that heterotrophic bacteria do not interfere with the detection or enumeration of total coliforms and *E. coli* by the EC-Blue-10. After the addition of NaOCl, total coliforms were detected from the H-river water sample using both methods. The microbial and physical properties of this water sample were not significantly different from the other water samples (Table 6). We have not investigated why total coliforms were detected after the addition of NaOCl. There was a concern that bacteria other than *E. coli* might exhibit fluorescence. No false-negative results were observed in this study. However, we did find false-positive results, with β-glucuronidase positive *Staph. warneri* being isolated from EC-Blue-10 and pyoverdin positive *Ps. putida* being isolated from Colilert-MPN. *Staph. warneri* could be resistant to 0·1 g SDS l^{-1} in EC-Blue-10 (Kodaka *et al.* 1995). We agree with Edberg *et al.* (1988) that each test was limited by design to drinking water distribution samples and the user should first establish the efficacy of the test in each water sample. A weak fluorescent reaction for MUG test can be read in the aqueous phase of the medium. A disadvantage of EC-Blue-10 was that it was difficult to read a weak-positive blue colour, because the base colour of EC-Blue-10 is light yellow. However, the medium in EC-Blue-10 was developed primarily for the rapid growth of *Enterobacteriaceae* (Kodaka *et al.* 1995). If coliforms were present in the water sample, they could grow sufficiently. Therefore, it would be very rare to observe a weak reaction and if a weak reaction was observed, it could be confirmed by comparison to the EC-Blue-10 comparator.

In conclusion, EC-Blue-10 gave results that were almost statistically equivalent to the DST method currently accepted by the Ministry of Health, Labour and Welfare of Japan. Therefore, the EC-Blue-10 is as useful as the DST method for the detection of coliforms and *E. coli* in temperate humid climate zone water. However, the water samples tested were very limited in this evaluation of EC-Blue-10 and therefore, it is recommended that a more extensive evaluation of EC-Blue-10 be undertaken.

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Microscopy AND Microanalysis

An Instant Cell Recognition System Using a Microfabricated Coordinate Standard Chip Useful for Combinable Cell Observation with Multiple Microscopic Apparatuses

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Abstract: Disposable coordinate standard (CS) chips were fabricated by the ejection of melted polystyrene into a metal mold. The CS chip surface was divided into four parts different in height and width. The edge lines of these parts could be recognized as straight lines 2 μm in width in the microscope view and used as the X and Y axes for the culture dish. The CS chip was attached on the bottom of a culture dish outside. Then the dish was set on the microscope stage and moved by means of a motorized automatic stage. The X-Y coordinates of many single-cells in a culture dish were registered, respectively. Once registered, any single-cell could instantly be brought to the center of the microscope view even after displacing the dish from the stage for a while and setting it again on the stage. Therefore, experimenters can easily search any single-cell in any culture dish on any microscope at any time. Such a system is remarkably useful for various modes of single-cell experiment and named "Suguwaculture," which means "instantly" ("sugu" in Japanese) + "recognizable" ("wakaru" in Japanese) + "culture" (during culture).

Key words: instant cell recognition, coordinate standard chip, X-Y coordinates registration, combinable observation on multiple microscopes, Suguwaculture, single-cell experiment, high throughput microinjection, microscopic image analysis

Introduction

Recently, an enormous amount of information has been accumulated about genomes of various species, and many efforts have been made to cluster it and to give annotations to respective genes (Ashburner et al., 2000; Overbeek et al., 2005). To certify the annotated functions of respective genes, it is necessary to apply the genes of interest to target viable cells and to analyze their responses. Therefore, much interest has now been paid to single-cell experiments. The term "single-cell" means an isolated single cell (one egg cell of vertebrates or insects, one blood cell, one somatic cell separated from tissue by enzymatic treatment, etc.) and a particular target single cell in multicell system (one epidermis cell in skin, one liver cell in a small chip of liver, one leaf cell in a small chip of leaf, etc.) as well. In single-cell experiments, the microscopic observation of each single-cell and its manipulation should be repeated many times every day. Experimenters must continue time-consuming and labor-intensive work, because it is difficult to find each target cell of interest among many other cells within a short time.

Formerly, we developed a single-cell manipulation supporting robot (SMSR) (Matsuoka et al., 2005; Matsuoka & Saito, 2006) to realize higher throughput microinjection. Microinjection includes not only the insertion of a micropipette into a target cell but also various associated work such as X-Y stage manipulation, microscope focus adjusting, the selection and transportation of a cell, the exchange of a micropipette, and the record of the microscopic images of the cell. This work may be classified into two categories: actions performed by microscopic observation (on-microscope actions) and actions performed by taking the eye off the microscope (off-microscope actions) (Matsuoka et al., 2006). Frequent

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changes of these on-microscope and off-microscope actions are annoying and time-consuming. The concept of SMSR is to let an operator concentrate his/her attention only on the microinjection by facilitating other associated actions.

To realize this concept, an X-Y coordinates registration system for each cell was essential. Formerly, various plates with multiple microwells or multiple pores were devised (Meek & Pantano, 2001; Yang et al., 2002; Maruyama et al., 2005; Park et al., 2005; Retting & Folch, 2005; Yasukawa et al., 2005). There are commercially available microscope slides and culture dishes with special scale bars or grid patterns for X-Y coordinates registration (Grid seal, Asahi Techno Glass-IWAKI, Chiba, Japan; Cellocate, Eppendorf, Hamburg, Germany) and stage control systems for time-lapse measurements (AQUA-COSMOS, Hamamatsu Photonics, Hamamatsu, Shizuoka, Japan; MetaMorph, Molecular Device, Downingtown, PA). The slide for a laser-scanning cytometer (LSC) should be the most elegant one (Kamentsky & Kamentsky, 1991) and can be applied to multiple LSCs such as LSC101 (Olympus, Tokyo, Japan), iCyte and iCys (CompuCyte, Cambridge, MA). Those devices seemed to be useful for the X-Y coordinates registration and were applied to some cases of single-cell experiments. However, they were designed for the specific apparatus and not supposed to be used in other apparatuses. That was one reason that a novel device common to various apparatuses was necessary. Another and more critical reason was that those slides and dishes should contact directly with test samples (cells and tissues). Many cell scientists are nervous about their material and surface treatment, because they could influence the growth and physiological properties of test samples. Therefore, it was an intense need to develop a novel X-Y coordinates registration system in which any type of dishes could be used.

To meet this requirement, we previously developed a coordinate glass chip by a photo-resist process, but its cost was too high to be used as disposable chips. Then we intended to develop a more convenient chip at low cost. This study reports a successful result of the production of a plastic chip made by the melted polymer ejection method.

Materials and Methods

Fabrication of the Coordinate Standard Chip

The coordinate standard (CS) chip was produced by the pressure ejection of melted polymer into a metal mold. The Young's modulus, the fracture toughness, and the cost of various polymers were compared, and finally polystyrene was selected as the best one. The metal mold was machined according to the suggestions and advice of several experts. An adhesive tape made of transparent film was pasted on the chip. The CS chip was packaged with plastic double sheet at one chip per partition.

The Suguwaculture System

A microscope used in this study was an inverted microscope IX-71 (Olympus, Tokyo, Japan). Microscopic images were captured with an ORCA-ER CCD camera (Hamamatsu Photonics) and analyzed by AQUA-C Imaging (Hamamatsu Photonics). A cross reticule was set in an eyepiece of the IX-71. An automatic stage system was a part of the SMSR (Matsuoka et al., 2005). A single-cell manager previously developed for SMSR was updated to fit the present system. The system was also combined with another microscope system, for example, the inverted laser scanning microscope (LSM510 system [Axiovert and LSM510], Carl Zeiss, Jena, Germany) for high quality image analysis.

Microinjection Experiments

Fluorescein dextran (MW 70,000) (D-1823, Invitrogen-Molecular Probes, OR) was used to demonstrate consecutive microinjection into multiple mouse embryonic stem (ES) cells. The fluorescent dye (5 $\mu g/\mu l^{-1}$) was microinjected by pressure.

Culture of Mouse ES Cells

Feeder free mouse ES cells were provided by H. Niwa (Center for Developmental Biology, Riken Institute, Kobe) and cultured at 37°C in the absence of feeder cells in Glasgow minimal essential medium (GMEM) (G5141; Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal calf serum (FCS) (04-001-1A; Biological Industries, Belt-Haemek, Israel), 1 mM sodium pyruvate, 0.1 mM 2-mercaptoethanol, 1× nonessential amino acids (NEAA) (11140, Invitrogen-Gibco, Grand Island, NY), and 1000 $U ml^{-1}$ of leukemia inhibitory factor (LIF) (ESG1107; Chemicon International, Temecula, CA) on gelatin (TYPE B from Bovine Skin, G9391; Sigma-Aldrich)-coated polystylen culture dishes (35 mmϕ) (3000-035; Asahi Techno Glass-IWAKI, Chiba, Japan) (Smith, 1991). A cell suspension (2 ml) containing 5000–10,000 ES cells was placed on the bottom of the culture dish and covered with mineral oil and then incubated at 37°C.

Culture of Tobacco Cells

Cultured tobacco cell line BY-2 derived from *Nicotiana tabacum* L. cv. Bright Yellow-2 (Kato et al., 1972) was cultured in Linsmaier-Skoog medium (30 $g l^{-1}$ sucrose, 200 $mg l^{-1}$ potassium phosphate monobasic, 100 $mg l^{-1}$ myo-inositol, 1 $mg l^{-1}$ thiamine hydrochloride, 4.41 $g l^{-1}$ Murashige and Skoog Plant salt Mix [2633022; MP Biomedicals, Illkirch, France]) supplemented with 0.2 $mg l^{-1}$ 2,4-dichlorophenoxyacetic acid (2,4-D) (LSD medium) at 28°C in the dark with rotation at 130 rpm. The cultured cells were transferred into a fresh medium every 7 days. After a 4-day culture since the last transfer, the BY-2 cells were used in subsequent experiments.

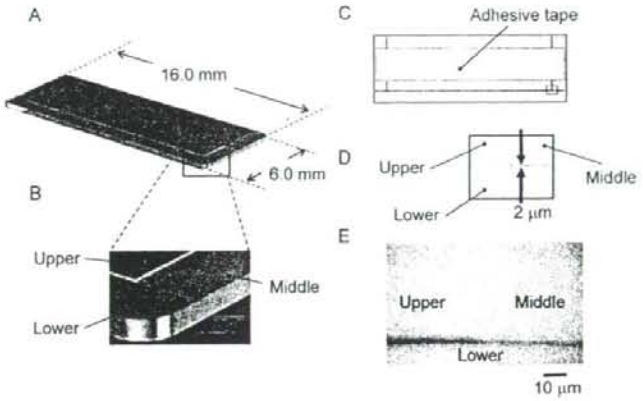

Figure 1. Surface structure and size of a CS chip. Three sizes are provided: 16 mm × 6 mm × 0.3 mm **(A)**, 16 mm × 10 mm × 0.3 mm, and 40 mm × 23 mm × 0.3 mm. Adhesive tape is pasted on the top surface of the chip **(B,C)**. Microscopic view of the edge lines formed on the top surface **(D,E)**.

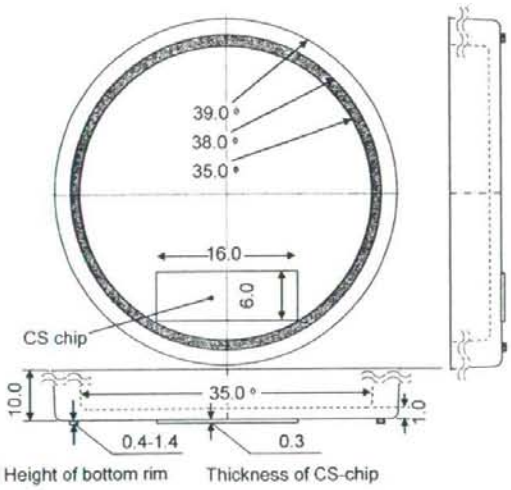

Figure 2. CS chip attached on a 35 mmϕ culture dish (3000-035, Asahi Techno Glass-IWAKI) (Figure unit: mm).

Results and Discussion

A plastic CS chip was fabricated as depicted in Figure 1. The chip size was 16.0 mm × 6.0 mm. The chip surface was divided into four parts different in height and width (Fig. 1A). The edge lines of these parts could be recognized as straight lines 2 μm in width in the microscope view (Fig. 1D,E). The chip was attached on the bottom of a culture dish outside (Fig. 2). The culture dish depicted in Figure 2 is a popular one used in our laboratory. The thickness of the CS chip was made no thicker than 0.3 mm because the height of bottom rim of commercially available culture dishes ranges from 0.4 to 1.4 mm.

Then the dish was set on the microscope stage (Fig. 3). The stage can be driven by handling a joystick controller. A foot switch was used to register the X-Y coordinates. The software developed previously for SMSR was properly upgraded and applied to the present system. Figure 4 depicts how to register the X-Y axes fixed to respective culture dishes. A culture dish is set in the dish holder on the automatic stage (Fig. 4). The coordinates fixed to the automatic stage are U-V axes. The experimenter drives the joystick controller so that the point P of the CS chip comes to the microscopic view center (MVC). The point P is registered as the origin by clicking the foot switch. Then the experimenter drives the joystick controller so that the point Q of the CS chip comes to MVC. The line PQ is registered as the X-axis by clicking the foot switch. The Y-axis is defined automatically as a line that passes the origin (P) at right angle to the X-axis.

The cell coordinates registration follows the X-Y axes registration. The experimenter selects any one cell and drives the joystick controller so that the cell (e.g., $Cell_1$ in Fig. 5) comes to MVC. By clicking the foot switch, the X-Y coordinates of the cell are registered and at the same time the U-V coordinates are calculated automatically according to the formulas depicted in Figure 5. In the same way, the cell search and the registration of its X-Y coordinates are performed successively. The number of the cell is registered consecutively and displayed in the column on "Cell number" (Fig. 6). The relative position of the registered cell is plotted in a circle representing the culture dish. When different kinds of cells are concerned, they can be differently

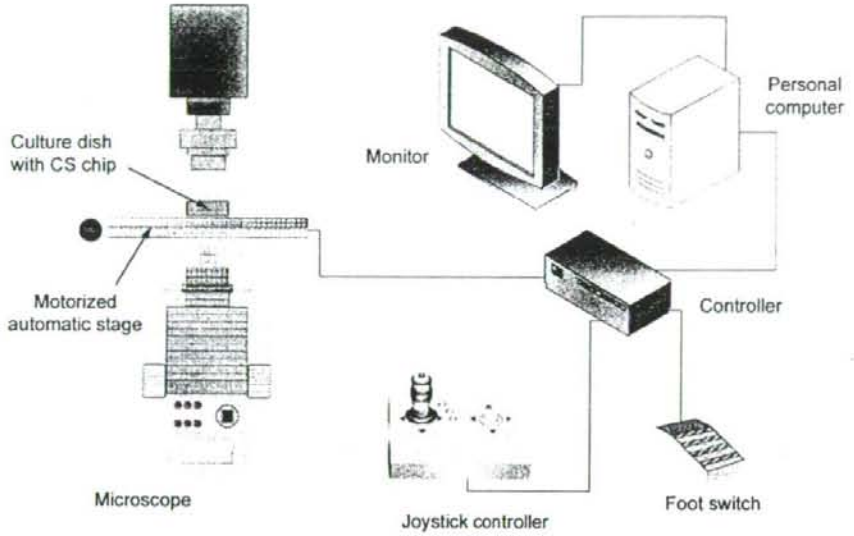

Figure 3. Schematic diagram of a Suguwaculture system.

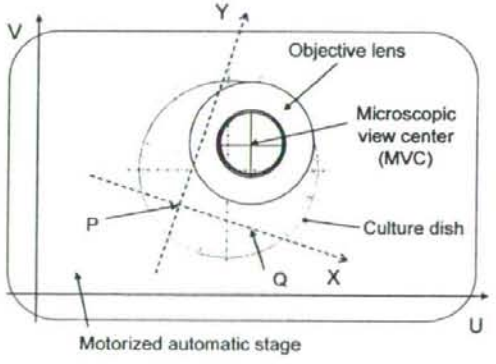

Figure 4. Realtive position of a culture dish, an automatic stage, and an objective lens. A culture dish with a CS chip is set on the microscope stage, and the points P and Q are adjusted at the MVC in this order to register the origin and the X axis. Microscopic view (center area of the objective lens) is illustrated on the nonscale.

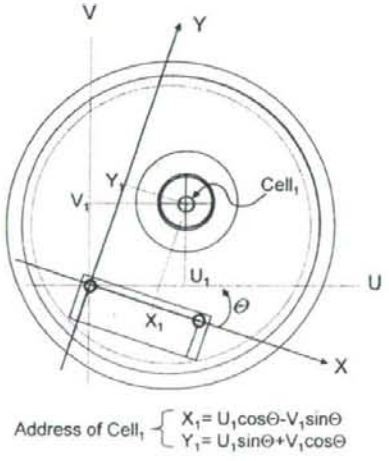

Figure 5. Registration of the X-Y coordinates of a cell. (U_1, V_1) is the distance from the origin automatically measured from the driving distances of two pulse motors. The motors drive the stage in the U and V directions, respectively. X_1 and Y_1 are calculated automatically from U_1 and V_1 according to the formula.

marked in the "Cell kind" column. The "Remarks" column is used to record the conditions and results of successive experiments.

When a single-cell experiment such as microinjection or image capture is to be performed successively, an experimenter can call $Cell_i$, $Cell_{i+2}$ . . . $Cell_{i+n}$ to the MVC consecutively only by clicking the foot switch. Alternatively, the experimenter can select $Cell_i$ directly by placing the mouse pointer at the ith "Cell number" (Fig. 6) and clicking the mouse. Such a rapid cell search function was remarkably useful for various single-cell experiments.

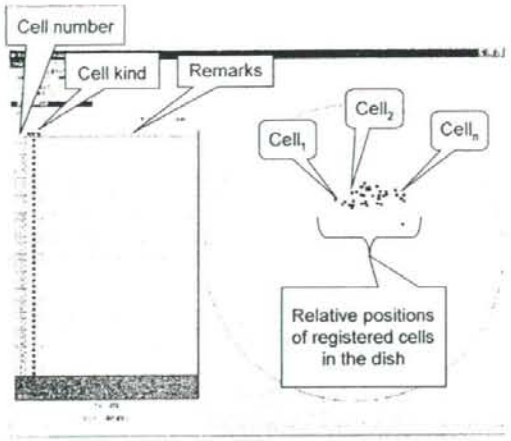

Figure 6. Typical image of a TV monitor displaying the list and the site map of registered cells.

Even when the dish has to be displaced from the automatic stage, and after a while, it is set on the stage again, this rapid cell search function can be used only by the registration of the X-Y axes first before single-cell experiments. Such cases include, for instance, the simultaneous use of multiple culture dishes, the cell culture in a separate incubator, and the image capture with different microscopic apparatuses (Fig. 7).

Figure 8 illustrates the cell images just called to the MVC. The size of a mouse embryonic stem cell is no greater than 20 μm in diameter (Fig. 8A) and its center was registered as the cell position (X_i, Y_i). In the case of tobacco cultured cells line BY-2 (Fig. 8B), the cell size was 50×100 μm^2 and the cell center was registered as the cell position (X_j, Y_j). Figure 8C illustrates the consecutive calls of mouse ES cells for the cell registration (a) and microinjection of a fluorescent dye (b) with IX-71, and the cell observation (c) and fluorescent microscopic image capture (d) with the LSM510 system. These cells could be called precisely to the registered cell positions, respectively, and microinjection could be performed rapidly at 200 cells/h (b). Figure 8D demonstrates the consecutive observation of the growth process of single-cells for 6 days. Except for everyday observation time, the dish was placed in an incubator throughout. Three images (a, b, c) were captured from different dishes. Even in case (c), where three single cells lined within the same view, the target cell (indicated by the arrow) could be called to the MVC instantly and easily distinguished from other neighbor cells.

Conclusions

The disposable CS chip has realized high performance of the Suguwaculture system. With this system, the X-Y coordinates data of single-cells can be used in common in time-lapse experiments using multiple apparatuses. The CS chip

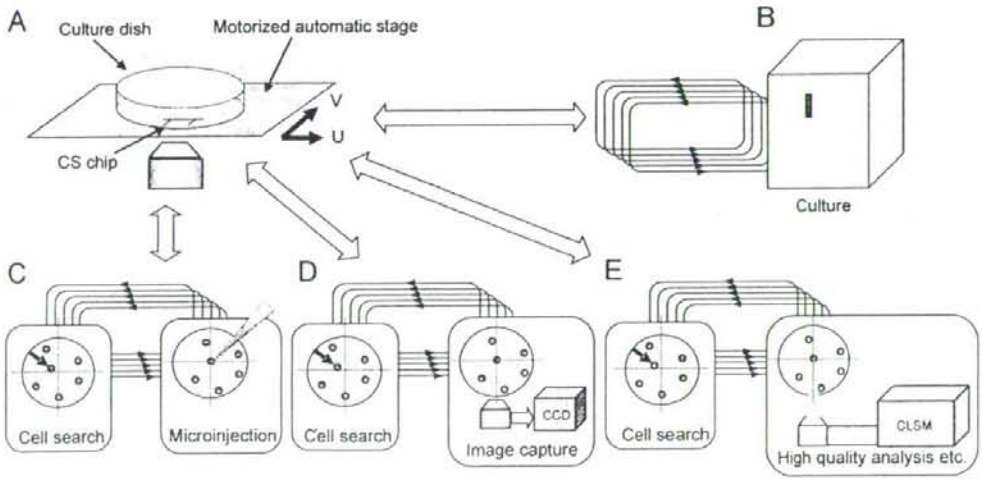

Figure 7. Time-lapse single-cell experiments using multiple apparatuses demonstrated by the Suguwaculture system. **A:** Single-cell experiments such as cell registration and cell manipulation on the automatic stage of an inverted microscope. **B:** Culture in an incubator. **C:** Microinjection. **D,E:** Time-lapse image captures with other optical apparatuses.