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Functional Control of Target Single Cells in ES Cell Clusters and Their Differentiated Cells by Femtoinjection

Hideaki Matsuoka, Mikako Saito and Hisakage Funabashi

*Department of Biotechnology and Life Science,
Tokyo University of Agriculture and Technology
Japan*

1. Introduction

Microinjection is well recognized as an ideal but technically difficult method for the direct introduction of any molecules into single cells (King, 2004; Matsuoka et al., 2006). The difficulty depends on the cell size, intracellular structure, and the physical properties of the cell surface. Egg cells are an easy target because of their large size of 100–200 μm . Blastocysts are also a large target for the injection of embryonic stem (ES) cells (Fig. 1 (A)). For blastocysts, the tip diameter of an injector is 5 μm or greater. On the other hand, fibroblasts are as small as 20–30 μm but comparatively easy targets because their cell surface appears to be firm. Moreover they appear to be resilient against deep insertion of an injection capillary. By contrast, plant cells are larger than fibroblasts but much more difficult targets. Plant cells usually have large vacuoles in the intracellular space and therefore microinjection should be performed into a thin space between the cell membrane and the vacuole. Typical examples are tobacco cultured cells, BY-2, with a size of 40×80 μm (Fig. 1 (B)) and rice protoplasts with a diameter of 30–50 μm . By comparison, ES cells are extraordinarily difficult targets because of their small size (15–20 μm) (Fig. 1 (C)) and sticky cell surface. In fact, microinjection of a plasmid vector into ES cells was not successfully performed by a microinjection expert until our experiment performed in 2005 (Matsuoka et al., 2005).

Before then, microinjection speed was no higher than 10 cells per h and the success rate was 7–8%. In the case of ES cells, the success rate was only 0.2%. Therefore, it was essential to increase the throughput of the method for the purpose of single-cell studies. Thus, we developed a useful robot that could support the microinjection operator; a single-cell manipulation supporting robot (SMSR) (Matsuoka et al., 2005). Using the SMSR, the injection speed increased to 100 cells per h and its success rate reached as high as 10%, even when operated by non-specialist personnel. The quantity of DNA ejected from the injection capillary was estimated to be no greater than 50 fg, and consequently the quantity of DNA actually introduced into a cell was in the fg range. Moreover, when an enhanced green fluorescent protein (EGFP) expression vector was injected into ES cells, the EGFP expression intensity changed in line with the varied concentration of the vector in the injection capillary. This method has been termed femtoinjection because it has enabled semi-quantitative injection at the femtogram level (Matsuoka et al., 2007).

In this chapter we describe the potential applications of femtoinjection to single-cell analysis in ES cell studies. The single cell indicates an isolated cell as well as a particular target cell within a cluster of cells.

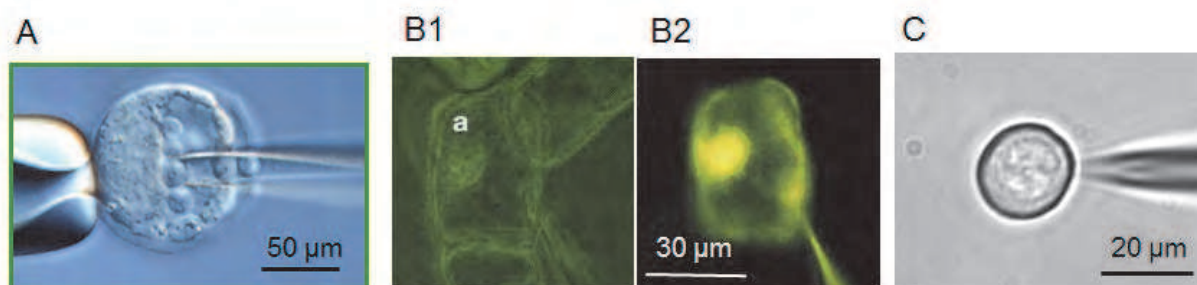


Fig. 1. Microinjection into various cells. (A) Injection of mouse ES cells into a blastocyst; (B) injection of a fluorescent dye (lucifer yellow) into the cytosol of a tobacco cultured cell, BY-2, showing phase-contrast image (B1) and fluorescence image (B2); (C) injection into a mouse ES cell.

2. Why is femtoinjection important?

One of the important objectives of bio-research is to understand the functional role of biomolecules that impact on cellular life as a whole. Many biological functions such as enzymatic activities, signal transduction, and transcriptional and translational activities have been individually proven by employing conventional molecular biological methods. However, in many cases, these methods require destruction of the cells and thus it is difficult to observe the final impact on a living cell. Moreover, in many cases, investigational assays are carried out *in vitro* which provides a completely different environment to that inside a cell.

For instance, the concentrations of macromolecules such as proteins and nucleic acids inside living cells are extremely high (Zimmerman & Trach, 1991), and under such specific conditions biomolecules behave differently, when compared with *in vitro* experiments. This is known as the macromolecular crowding effect, and it has been shown that a wide variety of intracellular processes such as protein folding, the association of ribosomes, and the binding of polymerase to DNA are affected by this effect (Hancock, 2004; Zimmerman & Minton, 1993; Zimmerman & Trach, 1988). Furthermore, the localization of biomolecules, which creates the gradation of concentrations and separated reactions in a cell, must be considered as an essential factor for investigating the original activities, despite being very difficult to reconstruct *in vitro*. Moreover, molecules do not work alone but interact with other molecules, some of which may be unknown factors, leading to sequential reactions.

Thus, conventional methods are indeed limited for the investigation of the functional roles in a living cell and so the development of a new method has been required. One of the most straightforward methods is the microinjection of molecules of interests into a living cell. In fact, microinjection was used to obtain specific data about their functional roles in living cells. However, the performance of ordinary microinjection was insufficient because it was difficult to produce quantitative data. Thus, we have long been engaged in the development of a high-performance microinjection method as described below.

3. SMSR and “suguwaculture”

The SMSR was designed according to the experience of microinjection experts. The most important functions are the operation modes of an injection capillary. The speed and timing of a push–pull motion is dependent on the cell size, the physical properties of the cell surface, and the experience of the operator. Thus, the operational parameters can be adjusted to optimum values for different conditions. The most recent version of the SMSR is shown in Fig. 2.

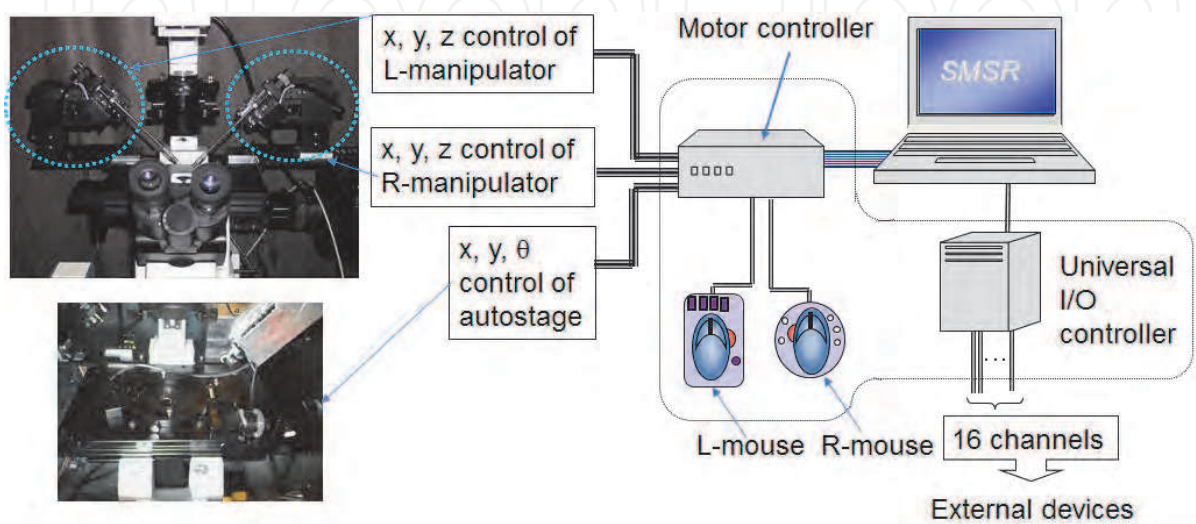


Fig. 2. Schematic of the SMSR.

Microinjection includes not only the insertion of a micropipette into a target cell but also various associated operations such as X–Y stage manipulation, microscope focus adjustment, selection and transformation of a target single cell, exchange of a micropipette, and recording of microscopic images. These operations may be classified into two categories: operations performed by observation with a microscope (on-microscope operations) and operations performed without a microscope (off-microscope operations). Frequently changing between on-microscope and off-microscope operations is annoying and time-consuming. The concept of SMSR is to enable the operator to concentrate their attention only on the microinjection by facilitating associated operations.

To realize this concept, an X–Y coordinate registration system for each target cell was essential. We developed a disposable coordinate standard (CS) chip by ejecting melted polystyrene into a metal mold (Yamada et al., 2008). The chip size was 16.0 mm × 6.0 mm, and the chip surface was divided into four parts of different height and width (Fig. 3 (A), (B)). An adhesive tape was pasted onto the chip (Fig. 3 (C)) so that it could be attached to a culture dish simply by removing a cover sheet and placing it beneath the dish. The edges of these parts could be recognized as straight lines 2 μm in width under the microscope (Fig. 2 (D), (E)). The culture dish depicted in Fig. 2 (F) is a popular one and one which is used in our laboratory. The thickness of the CS chip is no thicker than 0.3 mm because the height of the bottom rim of commercially available culture dishes is in the range 0.4–1.4 mm.

Many cell scientists are concerned about the dish material and its surface treatment, because these factors can influence the growth and physiological properties of the test samples (cells and tissues). Once they are able to obtain successful results using a particular type of dish, they will continue to use dishes of the same type. Therefore, it is extremely important

that the CS chip can be applied to any type of dish. Moreover the CS chip must not contact the test samples directly. Our new CS chip meets these requirements and cannot be replaced by other devices such as multiple microwells and multiple pore plates, some of which are already commercially available.

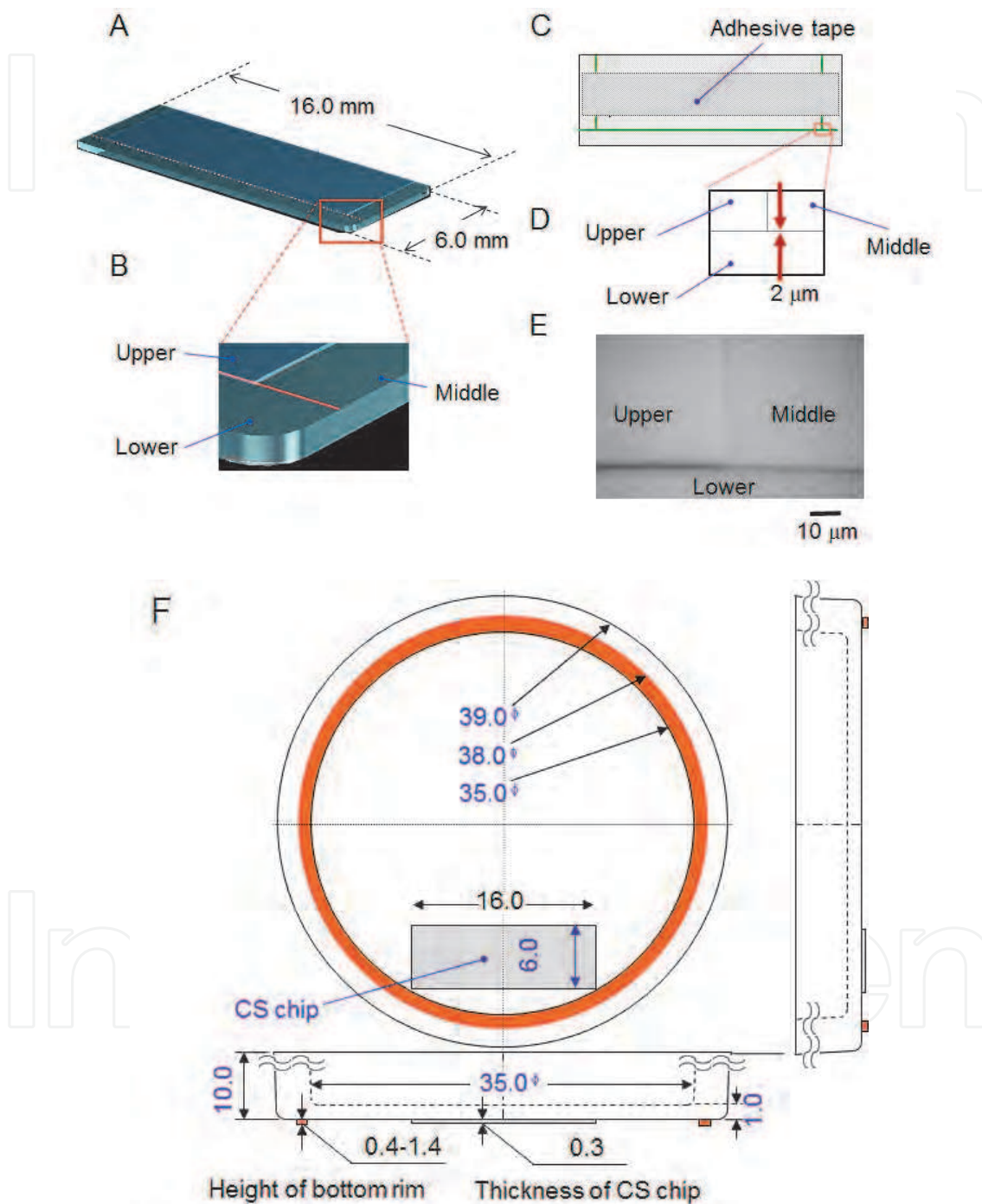


Fig. 3. Coordinate standard chip. Reproduced from *Micros. Microanal.* (2008) 14, 236-42 with permission from Cambridge University Press.

A standard protocol for single-cell experiments such as microinjection or image capture is as follows. Initially, the X-Y coordinates of 50-100 target single cells per culture dish are

consecutively registered, after which an operator can signify $Cell_1$, $Cell_2$, ..., and $Cell_n$ in the relevant order to the microscopic view center simply by clicking a foot switch. Alternatively, an operator can select a particular cell number, $Cell_i$, directly by selecting the i -th "Cell number" with a mouse. If the dish is removed from the automatic stage and replaced some time later, the rapid cell search function can be used because of the registered X-Y axes. Such applications include, for instance, the simultaneous use of multiple culture dishes, cell culture in a separate incubator, and image capture with a different microscope (Fig. 4). Therefore, operators 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. We have named this system "Suguwaculture", which means instantly (*sugu* in Japanese) recognizable (*wakaru* in Japanese) culture.

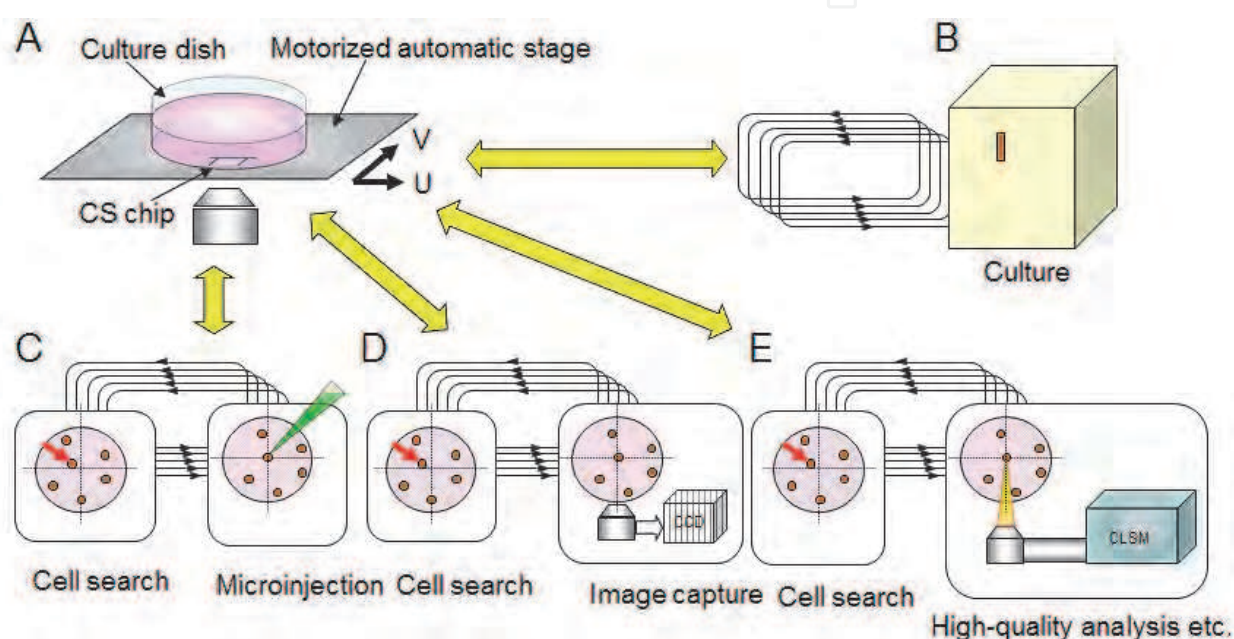


Fig. 4 Time-lapse single-cell experiments with multiple apparatus using the Suguwaculture system. (A) single-cell experiments such as cell registration and cell manipulation on the automatic stage of an inverted microscope, (B) culture in an incubator, (C) microinjection, (D), and (E) time-lapse image captures with other optical apparatus. Reproduced from *Micros. Microanal.* (2008) 14, 236-42 with permission from Cambridge University Press.

4. What can be done by femtoinjection?

4.1 Semi-quantitative introduction of macromolecules of any size

DNA solution in a glass capillary was ejected into a micro water drop and the quantity of DNA in the water drop was determined by quantitative real-time polymerase chain reaction. The ejected quantity was influenced by the diameter of the capillary tip and the intensity and period of pressure. The optimum conditions of the tip diameter, the pressure strength, and the pressure application period were typically 0.5–0.8 μm , 0.70 kgf/cm^2 , and 30 ms, respectively. For DNA concentrations of 10, 50, and 100 $\text{ng}/\mu\text{l}$ under optimum conditions, the ejected quantity changed accordingly within the range 5–50 fg. Therefore, the quantity of DNA actually introduced into an ES cell was estimated to be no greater than the fg level (Matsuoka et al., 2007).

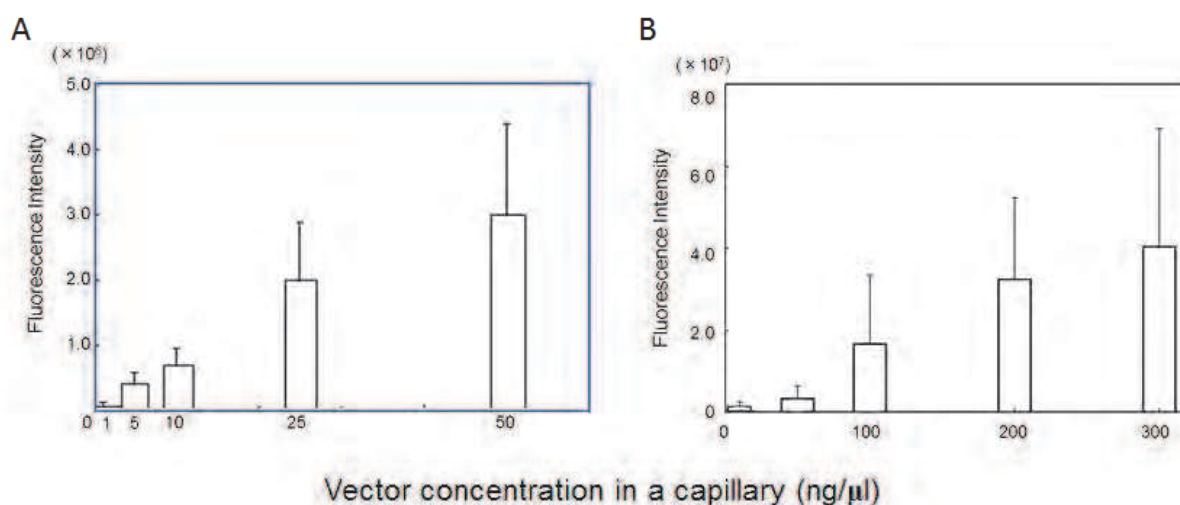


Fig. 5. Semi-quantitative relationship between the *EGFP* expression intensity and the vector concentration in a capillary in the range 1–50 ng/ μ l (A), and a higher range from 10–300 ng/ μ l (B). Error bar indicates mean \pm SD (n=10).

An EGFP expression vector was injected into ES cells to visualize the gene expression. The fluorescence of EGFP appeared after 24 h of culture not only in the injected cell but also in the neighboring cells that were daughter cells or their further descendent cells. The fluorescence intensity was averaged over the whole area of the target single cell or the single cluster containing the initial target cell. Thus, the calculated intensity of EGFP fluorescence was thought to be proportional to the quantity of the gene successfully introduced into the cell. In fact, the EGFP fluorescence intensity increased as the vector concentration increased from 1 to 300 ng/ μ l (Fig. 5) (Saito & Matsuoka, 2010)

If the average of multiple cells is used, methods such as lipofection and electroporation might show a semi-quantitative introduction performance. By comparison, we investigated the performance of lipofection using an EGFP expression vector (pCAG-EGFP). Mouse ES cells were suspended in a solution containing pCAG-EGFP (5 μ g DNA/50 μ l medium) and a cell fusion-inducing agent, lipofectamine 2000 (15 μ l/35 μ l medium). After incubation at 37°C for 24 h, fluorescent cell colonies were selected arbitrarily for microscopic image analysis. The fluorescence intensity was integrated and averaged over each colony. Thus, the obtained values were then averaged to quantify the gene expression intensity. Then the concentration of pCAG-EGFP or lipofectamine was varied from 1/100 to 100 times the initial concentrations. As depicted in Fig. 6, the optimum condition could be determined but it was difficult to find a proper range in which a quantitative or semi-quantitative relationship could be satisfied.

4.2 Semi-quantitative introduction of multiple factors

Control of the relative expression intensity of multiple genes is of great importance. To demonstrate such semi-quantitative control, we investigated a particular case of two genes: *EGFP* and *DsRed*. We prepared pCAG-EGFP, a *DsRed* expression vector (pCAG-*DsRed*), and a composite vector, pCAG-EGFP-IRES-*DsRed*, in which both genes were constructed in tandem (Fig. 7 (A)). When a solution containing pCAG-EGFP and pCAG-*DsRed* at the same concentration (5 ng/ μ l) was injected into an ES cell, the fluorescence intensity of EGFP appeared to be much higher than that of *DsRed* (Fig. 7(B)). In the case of pCAG-EGFP-IRES-*DsRed*, the fluorescence image was similar (Fig. 7 (C)).

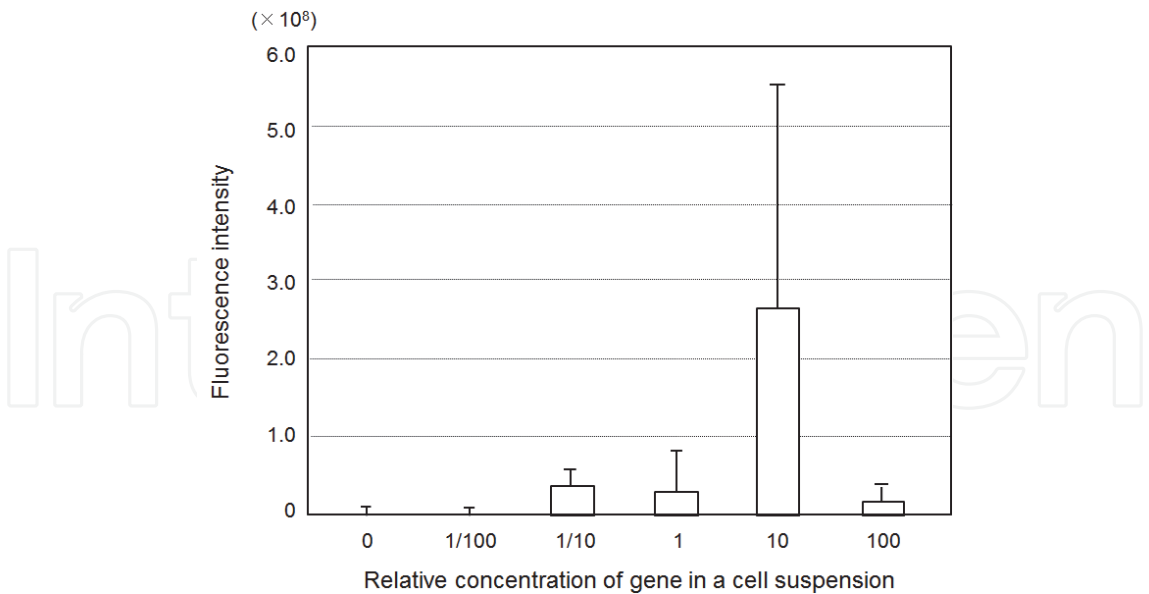


Fig. 6. Relationship between the *EGFP* expression intensity and the vector concentration in a cell suspension for lipofection. Error bar indicates mean \pm SD (n=20).

The molecular size of EGFP protein is 25.4 kDa and the EGFP can pass through the nuclear membrane, because the cut-off size of the nuclear membrane is thought to be 50 kDa. Therefore, EGFP distributes both in the cytosol and the nucleus. By contrast, DsRed needs to form a tetramer to become a fluorescent protein. The molecular size of the DsRed tetramer (DsRedT4) is 103.6 kDa. The tetramer is likely to be formed immediately in the cytosol, because fluorescent DsRed was distributed predominantly in the cytosol. Assuming that the fluorescence intensities of the respective fluorescent units, EGFP and DsRedT4, are the same, the relative fluorescence intensity of EGFP to DsRed should be 4:1 if the gene copy number is the same.

In fact, however, the intensity of EGFP expression was roughly 10 times higher than that of DsRed in the case of either separate vectors (Fig. 7 (D)) or the composite vector (Fig. 7 (E)). Next we mixed pCAG-EGFP and pCAG-DsRed at a ratio of 1:10 and injected the mixture into ES cells. Consequently the gene expression intensity of both genes was the same (Fig. 7 (F)). This result demonstrates that it is feasible to control the relative expression intensity of multiple genes easily as desired by regulating a ratio of the mixture.

4.3 Temporal and spatial control of injection in a cell colony

In order to analyze the functional role of a single cell in a cluster or colony of multiple cells, it is necessary to introduce a gene or other marker into a particular target single cell and then continue microscopic observation. In the case of the ES cell, an isolated single cell grows to be a colony with a diameter of approximately 50 μ m after culture for 72 h. Therefore, we prepared such colonies of ES cells and demonstrated temporal and spatial control of femtoinjection into a target single cell.

The first case was the injection of a fluorescent dye, Dextran-Alexa 488 (1 μ g/ μ l, MW 70 kDa). The success of physical introduction into only a single cell was confirmed by the diffusion pattern of the dye (Fig. 8 (A)). The culture was continued for a further 42 h. Then injection of the same dye into two single cells was performed. The physical introduction of the dye was successful at this time too (Fig. 8 (B)). Such an injection into any single cell located at the surface of a colony was possible.

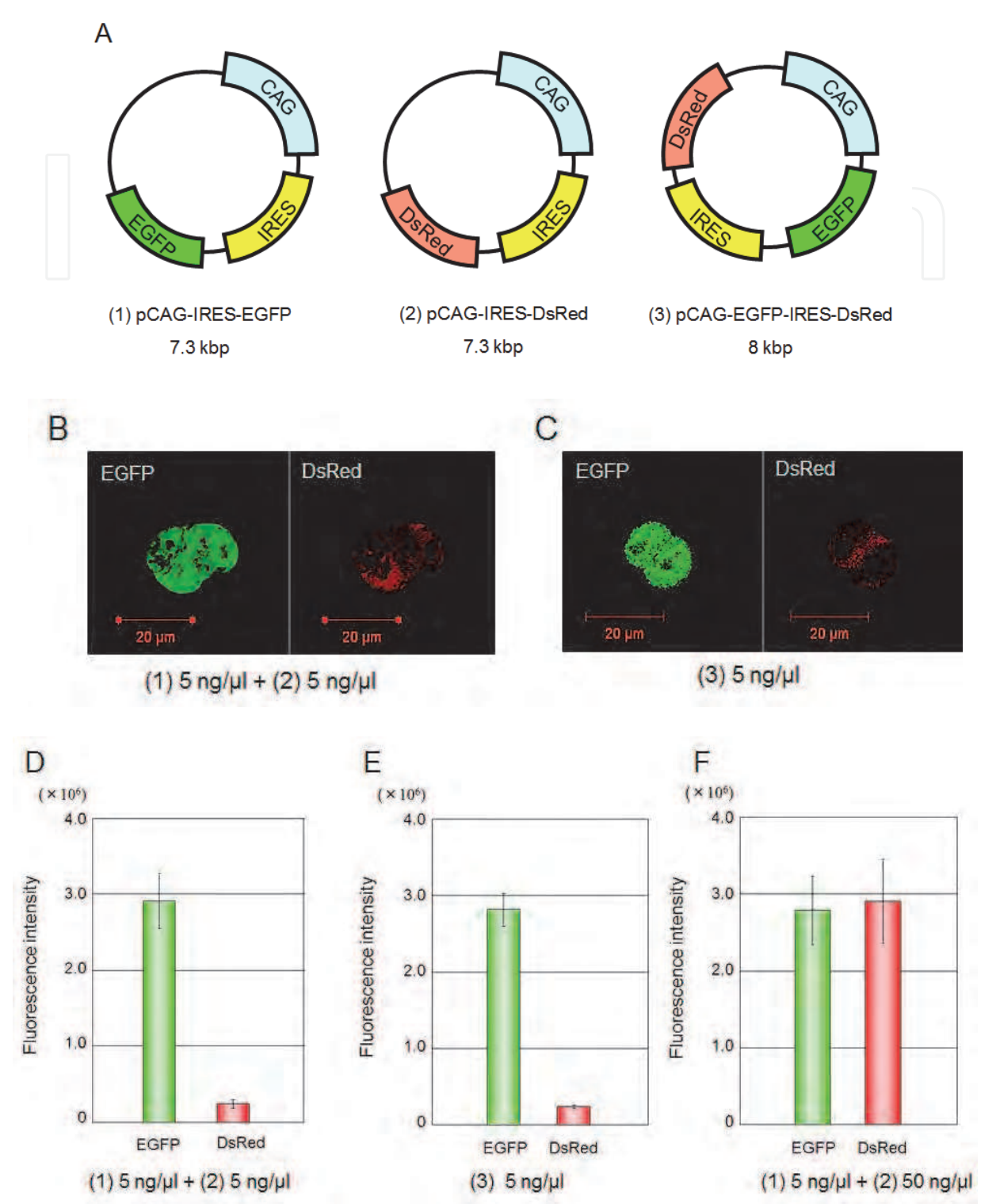


Fig. 7. Quantitative relationship in simultaneous introduction of EGFP and DsRed expression vectors. (A) Constructs of vectors; (B), (C) Examples of fluorescent images of EGFP and DsRed, respectively; (D), (E), (F) Relative intensities of EGFP and DsRed fluorescence. Error bar indicates mean \pm SD (n=10).

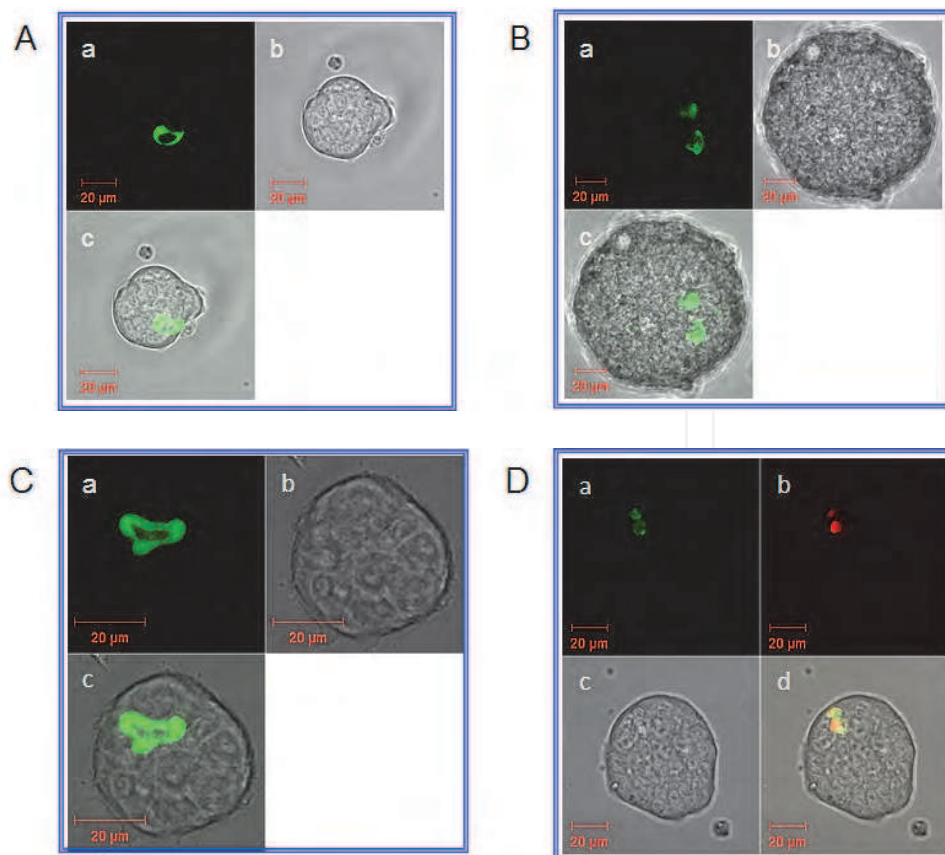


Fig. 8. Injection of Dextran-Alexa 488 (A, B) or Dextran-Alexa 488 and pCAG-DsRed (C, D) into a target single cell of an ES cell colony. Injection was performed after culture for 72 h. (A, C) Immediately after injection; (B, D) 42 h after injection. (A–C) (a) Dextran-Alexa 488, (b) phase-contrast image, (c) superposition of a and b; (D) (a) Dextran-Alexa 488, (b) DsRed, (c) phase-contrast image, (d) superposition of a, b, and c.

The next case was the simultaneous injection of a gene expression vector together with a fluorescent dye. A solution containing pCAG-DsRed (50 ng/μl) and Dextran-Alexa 488 was injected into a single cell in a colony (Fig. 8 (C)). The diffusion pattern of Dextran-Alexa 488 became a marker of the success of physical introduction. After incubation for 24 h, the expression of DsRed was observed in the same cell in which the diffusion of Dextran-Alexa 488 was observed (Fig. 8 (D)). This indicates the physical and physiologic success of injection.

4.4 Spatial control of injection site in a cell: cytosol or nucleus

When a plasmid vector is introduced into a cell, the vector has to be transported to the nucleus. Therefore, its expression efficiency will be enhanced if the vector is introduced directly into the nucleus rather than into the cytosol. The nucleus is located in the center of a cell. The direct injection into the nucleus requires a deeper insertion of an injection capillary. Such an insertion, however, seems to decrease the likelihood of cell viability. Next we investigated the possibility of non-lethal insertion into the nucleus of ES cells.

Dextran-Alexa 488 was injected into single cells of a DsRed-expressing ES cell line. This cell line shows red fluorescence only in the cytosol, because DsRedT4 cannot pass through the nuclear membrane (Fig. 9 (A)). For the same reason, Dextran-Alexa 488 cannot either.

Consequently if Dextran-Alexa 488 is introduced into the cytosol, the cytosol will become yellow. On the other hand, if the dye is introduced into the nucleus, the cytosol will remain red and the nucleus will become green. Indeed, two patterns were observed as depicted in Fig. 9 (B) and (C). These results indicate direct injection into the cytosol and nucleus, respectively.

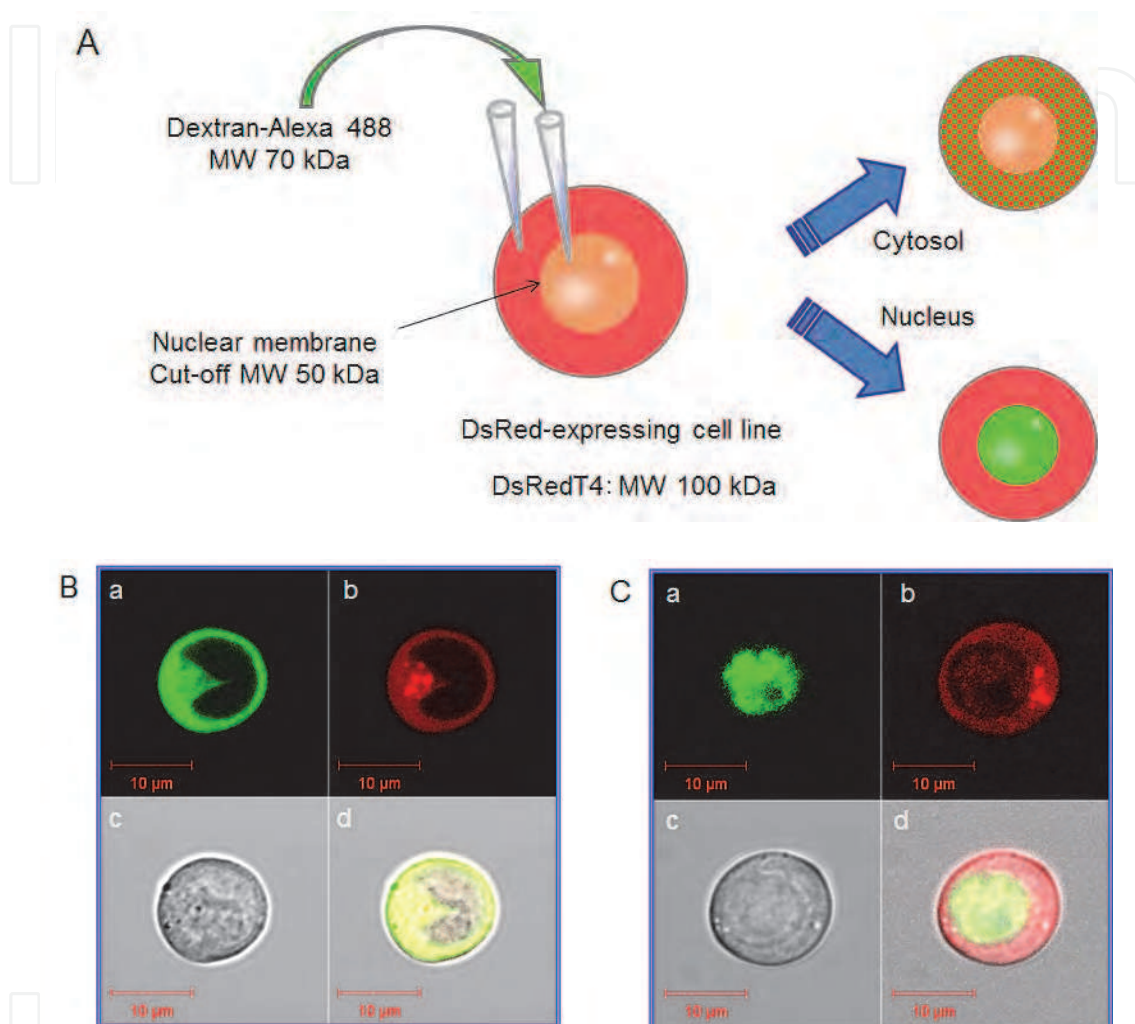


Fig. 9. Injection of Dextran-Alexa 488 into a single cell of a DsRed-expressing ES cell line. (A) Experimental protocol; (B) example of introduction into the cytosol; (C) example of introduction into the nucleus. (a) Dextran-Alexa 488, (b) DsRed, (c) phase-contrast image, (d) superposition of a, b, and c.

Next, we investigated the effect of direct injection on the expression efficiency of an injected vector. The yellowish green fluorescent protein Venus was selected as a demonstrative gene production because Venus fluorescence was expected more stable and intense than EGFP. The Venus-expression vector and Dextran-Fluorescein (MW 70 kDa) were injected into DsRed-expressing ES cells. The results are summarized in Fig. 10.

Injection was performed into 306 single cells, and 180 cells maintained viability. Dextran-Fluorescein was observed in 158 of 180 cells. This indicates that the success rate of physical introduction of the fluorescent dye was 52% (158/306). In these 158 cells, 117 injections were into the cytosol, whereas 41 were injected into the nucleus. The expression rate of the *Venus*

gene was no higher than 27% (31/117) in the case of cytosolic injection. By contrast, the rate reached 83% (34/41) for nucleic injection. These results strongly suggest that the direct introduction of a vector into the nucleus is effective for the enhancement of gene expression efficiency in spite of a high risk of loss of cell viability due to the deep insertion.

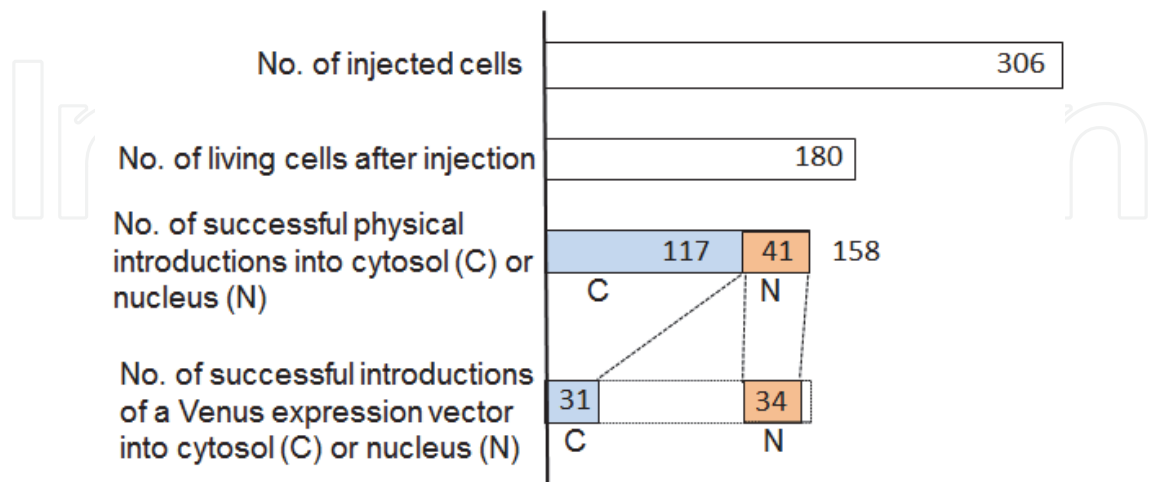


Fig. 10 Comparison of gene expression efficiencies attained by injection into the cytosol and nucleus.

5. Analysis of a DNA nuclear targeting sequence as a potential enhancer of the expression of a femtoinjected exogenous gene in ES cells

In order to enhance the expression efficiency of an injected gene, it is effective to inject it directly into the nucleus, as described above. However, deep insertion into the nucleus is likely to cause serious damage to the cell. Therefore, an alternative method is required to promote transportation of the gene toward the nucleus. A simple and promising approach is to add a specific sequence into a vector, using a so-called DNA nuclear targeting sequence (DTS) (Dean, 1997; Dean et al., 1999a, 1999b) (Fig. 11).

A DTS is a specific sequence recognized by transcriptional factors. Therefore, the injected vector is expected to form a complex with the specific or general transcriptional factor. Since the transcriptional factors are imported into the nucleus by an intrinsic active transport system, the vector–DTS complex may also be imported into the nucleus via the same system. Consequently the transport of the vector into the nucleus is expected to be promoted to result in the enhancement of its expression.

For instance, the 72 base pairs of the simian virus 40 (SV40) promoter/enhancer sequence (SV40-DTS) was reported to be a DTS (Dean, 1997), which enhanced the nuclear import so that the efficiency of the gene expression increased compared to without the DTS. Since this sequence has the binding site of ubiquitously expressed transcriptional factors, it has been suggested that the introduced vector DNA forming a complex with these transcriptional factors passes through a nuclear pore with the help of the importin family which recognizes nuclear localization signals of transcriptional factors (Miller & Dean, 2009; Miller et al., 2009). Although it has been revealed that SV40-DTS functions as a DTS in various types of cells, the function in ES cells remains unclear, probably due to the difficulty of the injection of a vector into the cytoplasm.

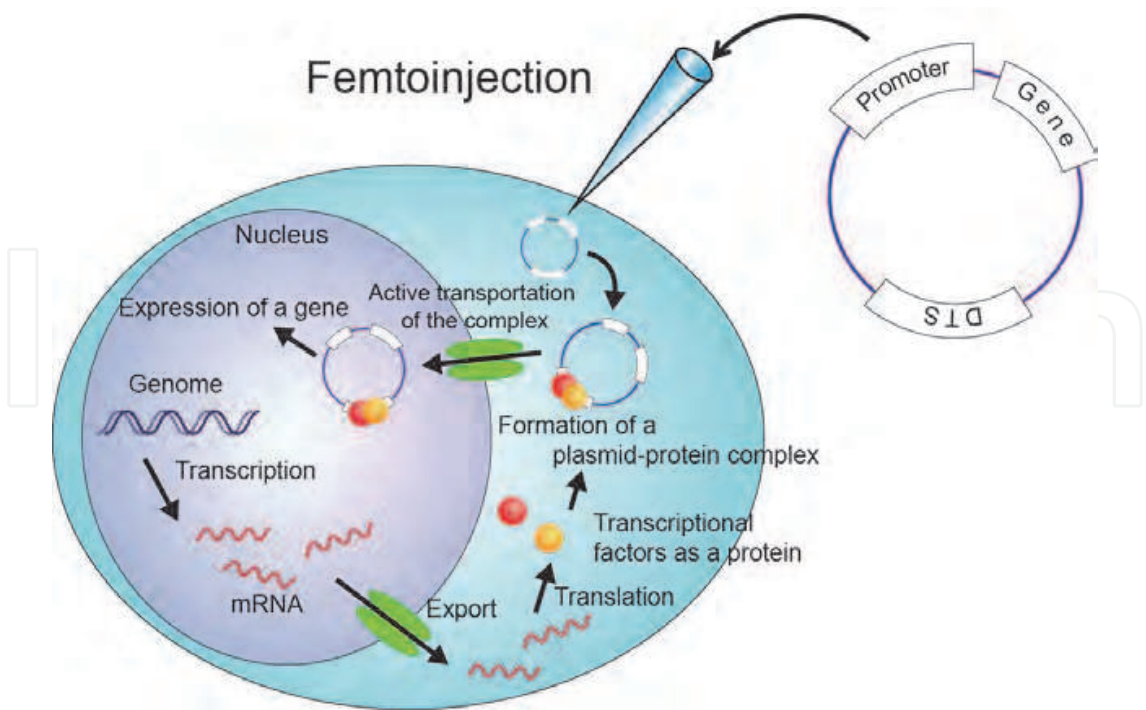


Fig. 11. The enhancement of gene expression efficiency of femtoinjected plasmid by utilizing protein life dynamics. The femtoinjected plasmid is drugged into a nucleus via the intrinsic transportation system recognizing a nuclear localization signal of transcriptional factors after forming a plasmid-protein complex. Thus the plasmids are actively delivered in nucleus, expressing the gene efficiently. Reproduced from *Biochem. Biophys. Res. Commun.* (2010) 400), 554-8 with permission from Elsevier.

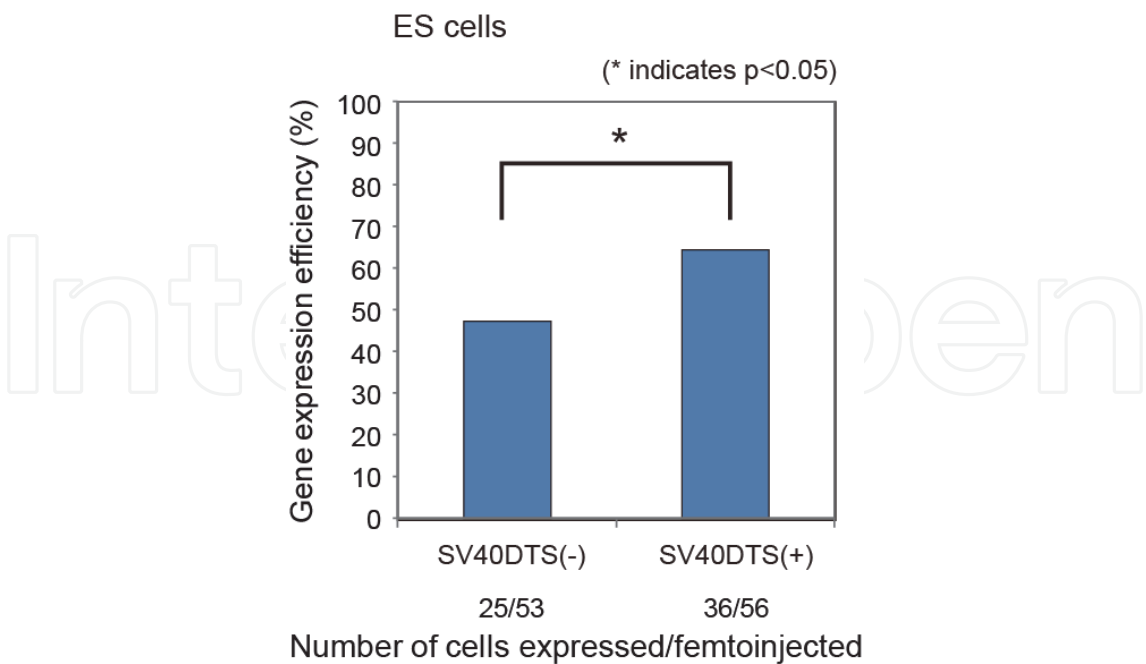


Fig. 12. Effect of SV40-DTS on the gene expression efficiency in ES cells. The plasmids containing SV40-DTS express a probe gene (EGFP) efficiently in ES cells. Reproduced from *Biochem. Biophys. Res. Commun.* (2010) 400), 554-8 with permission from Elsevier.

However, with femtoinjection, it is now feasible to investigate the effect of a DTS on the efficiency of gene expression in ES cells (Funabashi et al., 2010). An EGFP expression vector (SV40-DTS positive vector or control vector encoding no DTS) and Dextran-Texas Red (MW 40 kDa) were injected into ES cells. According to the protocol described in section 4.4, the injected site was determined (i.e., cytosol or nucleus). The MW of Dextran-Texas Red is similar to the cut-off size of the nuclear membrane (MW ca. 50 kDa), Dextran-Texas Red can hardly diffuse into the nucleus. The efficiency of gene expression was defined as “the number of cells in which EGFP expression was observed per number of cells in which the EGFP gene was femtoinjected into the cytoplasm determined by the observation of Dextran-Texas Red”. As shown in Fig. 12, comparison of gene expression efficiency between the SV40-DTS positive vector-injected ES cells and ES cells injected with the control vector clearly revealed the effect of SV40-DTS. This was the first report to identify the role of DTS in ES cells (Funabashi et al., 2010).

Inspired by the success of the investigation, we hypothesized that a sequence recognized by ES cell-specific transcriptional factors should function as an ES cell-specific DTS. Oct3/4 binding Sox2 regulatory region 2 (SRR2) has been reported to be a distal enhancer for Sox2 expression where the Oct3/4 and Sox2 complex is bound (Tomiooka et al., 2002). As both Sox2 and Oct3/4 are well-known ES cell-specific transcriptional factors for maintaining pluripotency (Masui et al., 2007; Niwa et al., 2000; Niwa, 2007), it is expected that the 81 bp of SRR2 (Fig. 13) functions as a DTS (termed SRR2-DTS) and that it promotes the transportation of the plasmid giving rise to the efficiency of gene expression.



Fig. 13. Sequence of Sox2 regulatory regions 2 (SRR2). SRR2 is an 81 bp sequence containing Oct3/4 and Sox2 binding sequences. Reproduced from *Biochem. Biophys. Res. Commun.* (2010) 400, 554-8 with permission from Elsevier.

In the case of MIN6 cells (mouse insulinoma cells (Miyazaki et al., 1990)) which express neither Sox2 nor Oct3/4, no difference is observed in terms of the gene expression efficiency regardless of the types of introduced plasmids as shown in Fig. 14 (A), whereas ES cells femtoinjected with the SRR2-DTS-positive plasmid exhibit higher gene expression efficiency than ES cells injected with the SRR2-DTS-negative plasmid (Fig. 14 (B)). This result supports our hypothesis that the enhancement of gene expression observed is due to the expression of these ES cell-specific transcriptional factors.

The observed phenomenon can possibly be explained as follows. The mRNAs of ES cell-specific transcriptional factors Oct3/4 and Sox2 were exported to the cytoplasm, and then translated into proteins. These proteins met the femtoinjected plasmids which had SRR2-

DTS forming the complex on the sequence. This complex was drugged into the nucleus via the nucleic pore transportation system in which the nuclear localization signals on these transcriptional factors were recognized by importin family members. This active transportation of the plasmid resulted in the higher gene expression efficiency. Indeed, it has been reported that Oct3/4 and Sox2 are actively transported into the nucleus by importin $\alpha 1/\beta 1$ complex and possibly by importin $\beta 1$, respectively (Yasuhara et al., 2007, 2009), in ES cells. It has also been suggested that these importins will function work on the SV40-DTS depending transportation (Dean, 1997; Dean et al., 1999; Lam & Dean, 2010; Miller et al., 2009). Thus, both sequences function as DTSs in ES cells. In particular, it has been suggested that SRR2-DTS functions as an ES cell-specific DTS.

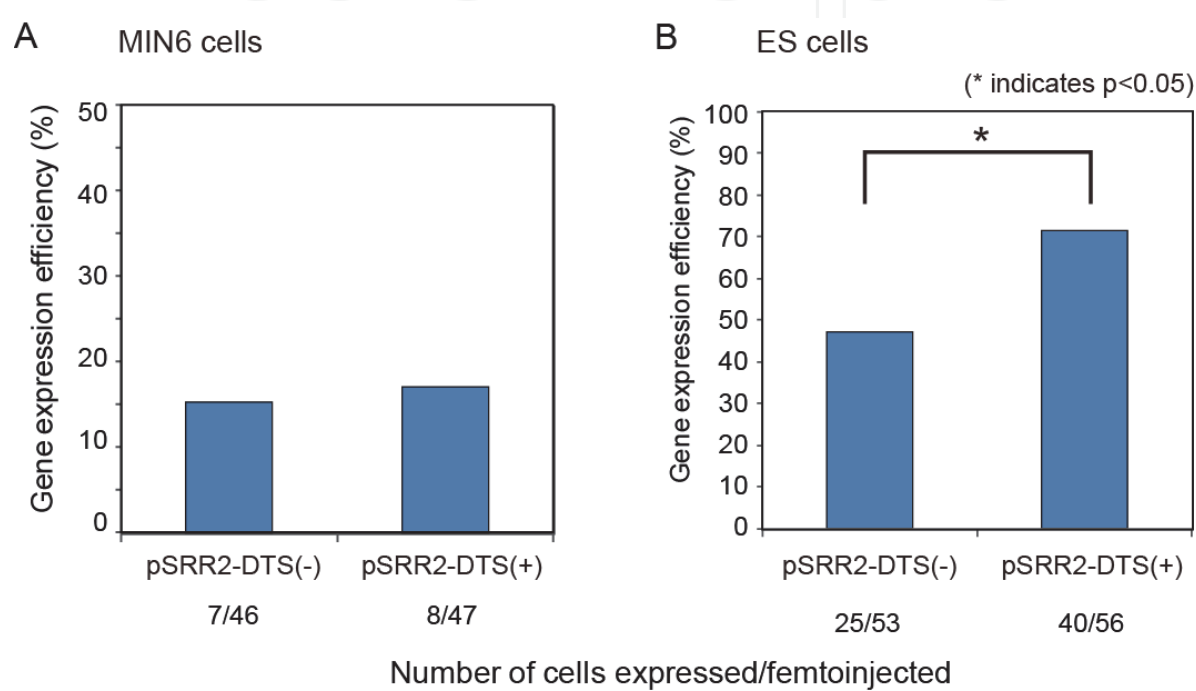


Fig. 14. Effect of SRR2 region on the gene expression efficiency in MIN6 cells (A) and ES cells (B). There was no effect of SRR2 sequence on the gene expression efficiency in the case of MIN6 cells while clear enhancement was observed in case of ES cells. Reproduced from *Biochem. Biophys. Res. Commun.* (2010) 400, 554-8 with permission from Elsevier.

Femtoinjection has enabled injection into living single ES cells of all substances including ions, fluorescent dyes, and nucleic acids, which range from low molecular weight chemicals to macromolecules, as long as they are soluble. On the other hand, it is not yet feasible to aim for high resolution injection sites such as small organelles in mammalian cells. The intrinsic transport system inside living cells such as the protein life dynamics utilized here is expected to help the distribution of introduced substances to the place where one desire to send for.

6. Femtoinjection assay for differentiation-inducing factors based on a morphological indicator

With regard to quantitative gene expression control, how to maintain the undifferentiated state of ES cells is of particular interest. Several genes such as *Oct3/4*, *Sox2*, and *Nanog* are well

recognized as undifferentiated cell markers (Masui et al., 2007; Niwa, 2007). However, it is also recognized that none of them alone is an exclusive marker. Rather the undifferentiated state seems to be maintained by an appropriate balance of their expression intensities. To prove this supposition, the simultaneous control of expression intensities of these genes is thought to be an effective method. Femtoinjection is particularly useful for this purpose.

First, we varied the expression intensity of Oct3/4 alone by femtoinjection of Oct3/4 expression vector into ES cells (Matsuoka & Saito, 2010). As a non-invasive marker of differentiation, we introduced the perimeter-to-radius ratio (PR ratio) as a novel morphologic indicator (Fig. 15). Generally ES cells are circular but change into a morphologically complex shape once differentiated. The PR ratio can sensitively reflect such a change. If the cell shape is a true circle, the PR ratio is 6.28; however, it becomes greater as the cell shape becomes more complex.

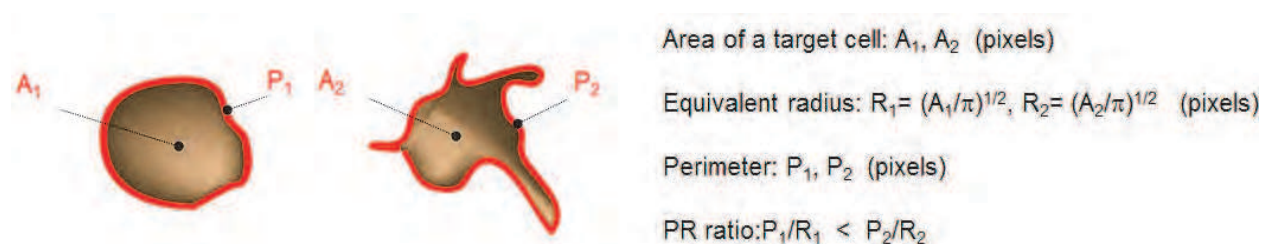


Fig. 15. Definition of PR ratio for quantitative expression of morphologic complexity. Reproduced from *ECS Transactions* (2009) 16, 9-14 with permission from The Electrochemical Society.

As shown in Fig. 16, an Oct3/4 expression vector containing an EGFP reporter gene (pCAG-Oct3/4-IRES-EGFP) was injected into single cells of mouse ES cells. As a control, an EGFP expression vector was injected into single cells in the same manner. At the same time, no injection control cells were prepared and observed in parallel. After injection, cell morphology was recorded every 24 h and the PR ratio was determined. In some cases, the PR ratio increased at 24 h but returned to the initial level at 48 h or later. In other cases, it increased continuously throughout the observation period for 72 h. Both with and without injection control, the PR ratio was 7.3–8.0 until 72 h. Based on these results, we defined the differentiated state as follows: PR ratio became greater than 8.0 and remained at this level at 72 h. According to this definition, the differentiation rate was determined as (number of differentiated cells/number of EGFP positive cells at 24 h) \times 100%.

Figure 17 shows an example of injection of pCAG-Oct3/4-IRES-EGFP at a concentration of 600 ng/ μ l in the injection capillary. At a concentration of 300, 600, or 1200 ng/ μ l, the differentiation rate was 2/6 (33%), 5/7 (71%), and 6/7 (86%), respectively. Though the number of test samples is small, this result suggests that the increase in Oct3/4 expression intensity is effective for the promotion of differentiation of ES cells.

For the up-regulation of a target gene expression or a specific protein effect, the introduction of the over-expression vector is thought to be a useful method, as demonstrated above. The introduction of mRNAs or target proteins is thought to be a more direct way to cause the up-regulation effects. On the other hand, the down-regulation of a target gene expression may be produced by the introduction of siRNA or shRNA. The introduction of inhibitors against the molecules of interests including antibodies and dominant negatives may also be an effective way to produce the down-regulation effects. In either case, the quantitative introduction of respective factors is essential for the regulation.

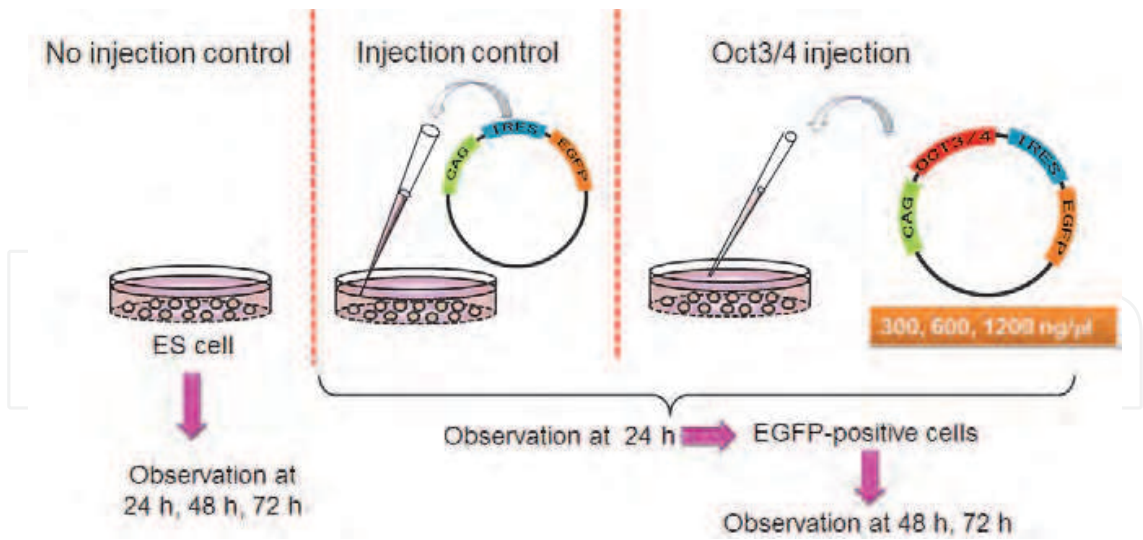


Fig. 16. Protocol for femtoinjection of Oct3/4 expression vector.

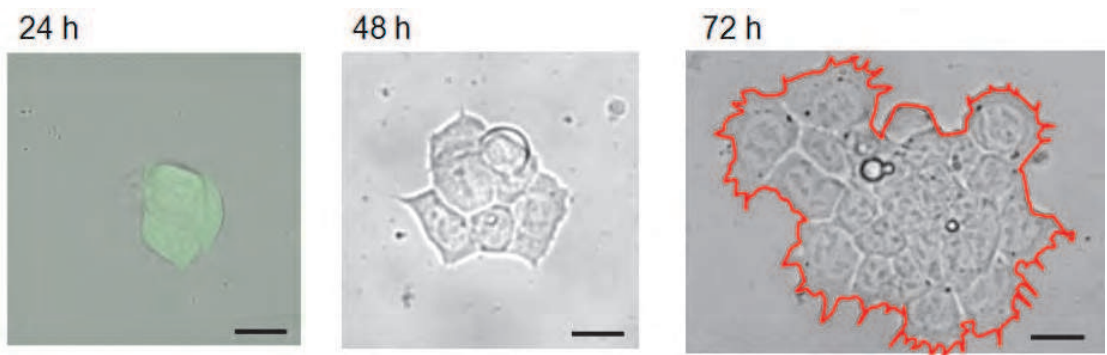


Fig. 17. Morphologic changes in the ES cell colony induced by injection of Oct3/4 expression vector. The concentration of pCAG-Oct3/4-IRES-EGFP in the injection capillary was 600 ng/μl. Scale bar indicates 20 μm. Reproduced from *ECS Transactions* (2009) 16, 9-14 with permission from The Electrochemical Society.

7. Marking vibrating muscle fiber cells with non-harmful fluorescent dyes

We have previously cultured mouse ES cells and tried to prepare muscle fibers. In the course of the study, it was interesting to find that the polyamines spermine could induce remarkable growth of multi-layer muscle fibers (Fig. 18) (Sasaki et al., 2008). The growth process was so unique that we have been deeply engaged in gene expression and functional analyses. With regard to the pulsation function, it should be of fundamental significance to analyze how to acquire synchronously pulsation potency. Therefore, we considered it necessary to analyze the cell-to-cell communication in a cell sheet during synchronous pulsation and during rest. Thus, we intended to challenge the injection into single cell in muscle fiber during its pulsation as well as when rest.

Femtoinjection into a particular target cell in an ES cell colony was demonstrated in section 4.3. Such injection into a target single cell is also possible in a cell cluster composed of differentiated cells of different sizes and shapes, as long as the outline of a target single cell can be observed microscopically. In the present case of pulsating cells, however, it seemed to be almost impossible. Next we re-examined various parameters relevant to injection

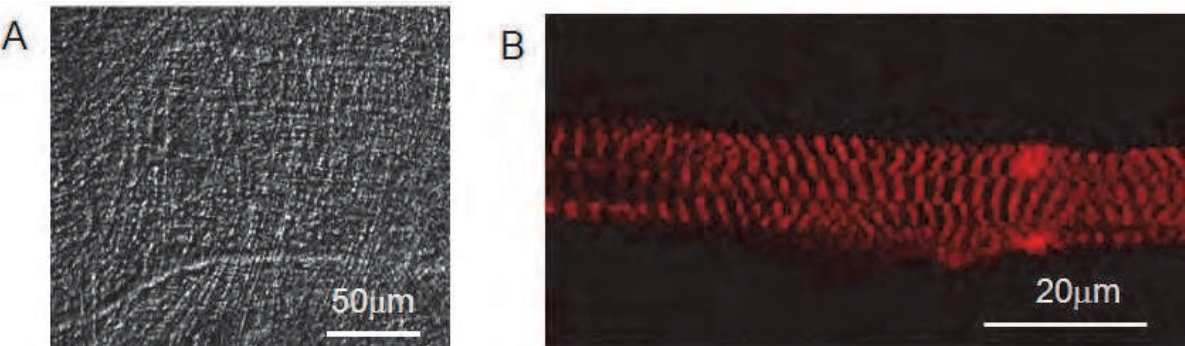


Fig. 18. Muscle fiber prepared from mouse ES cells with spermine treatment. (A) Multilayer sheet of contractile muscle fiber; (B) fluorescence image of striated muscle fibers stained using an antibody to α -actinin that was detected with an Alexa Fluor-labeled secondary antibody.

performance and finally succeeded in injecting a pulsating single cell in the muscle fiber, as described below.

We aimed to suppress the pulsation intensity by cooling the muscle fiber for a while and to then perform injection, before warming it to room temperature. To confirm the cooling effect, it was necessary to monitor the pulsation intensity. The optical intensity at a particular position in the muscle fiber was measured continuously and its relative change was quantified to express the pulsation intensity at the measured position. Figure 19 shows

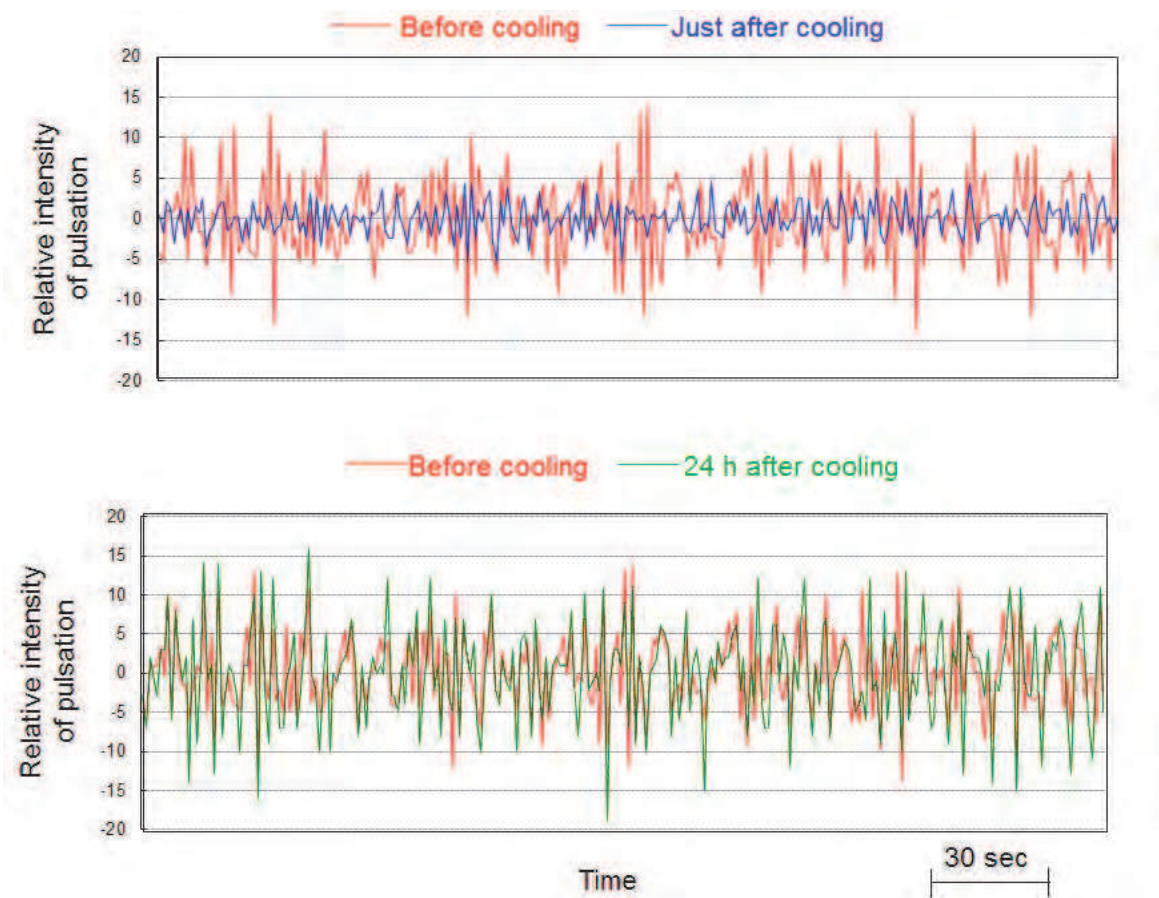


Fig. 19. Influence of cooling on the pulsation of a muscle fiber.

a record obtained during the temperature variations. The cells cultured at 37°C were rapidly cooled to 4°C. The pulsation intensity decreased to between a half and one-third. Then the cells were warmed gradually to room temperature. After 24 h from the start of warming, the cells recovered their initial pulsation intensity to the original level. Therefore, cooling to 4°C for a short period of time had no obvious effect on the intrinsic pulsation function.

Another point to be considered with regard to muscle fibers is the physical properties of the cell membrane. Then the tip parameters were modified to adapt to muscle fiber. Finally the tip diameter of an injection capillary was made smaller and the tip length was made shorter than for capillaries used for ES cells. Using this capillary, a solution of Alexa 488 (1 µg/µl, MW 643) and/or Dextran-Texas Red (10 µg/µl, MW 3,000) was injected into the target single cells simultaneously. Dextran-Texas Red stayed within a small area around the injection point that was supposedly within the target cell (Fig. 20). Therefore, only the injection into the target cell was considered successful. On the other hand, Alexa 488 distributed to a wider area (approximately 200 µm×100 µm). However, the diffusion area was not a simple circular pattern but an irregular one. Such an irregular diffusion pattern was unexpected. The present results are still preliminary but suggest a heterogeneous distribution of gap junctions with different opening diameters.

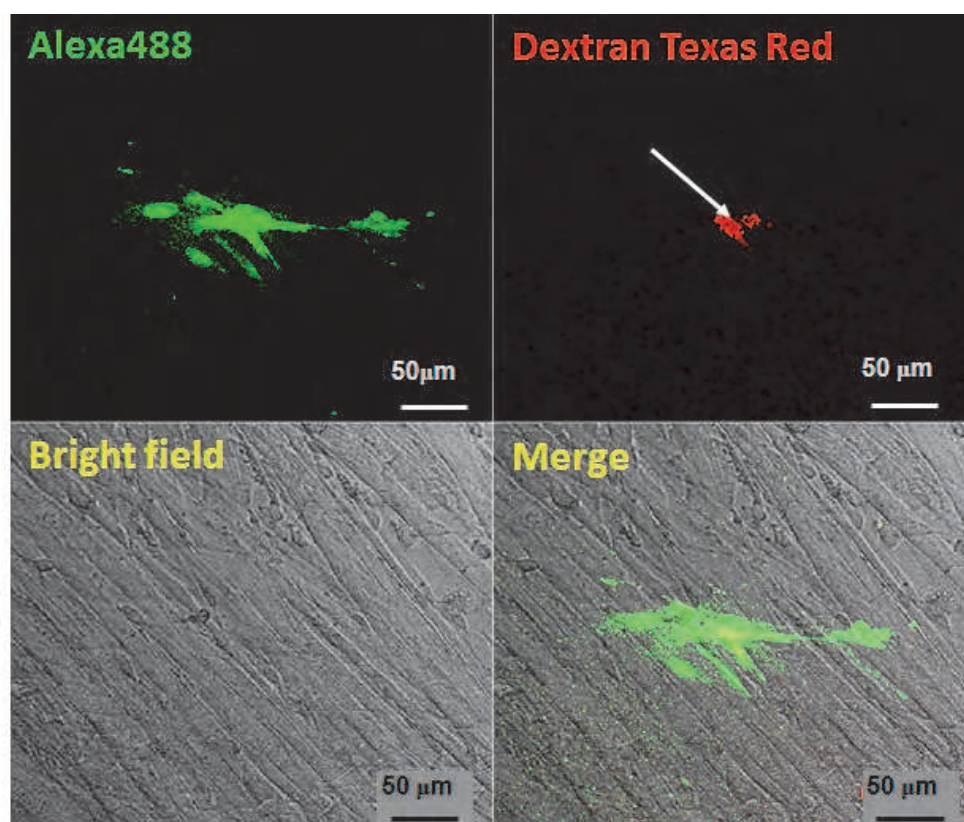


Fig. 20. Femtoinjection of Alexa 488 and Dextran-Texas Red into a single pulsating cell of a muscle fiber.

8. Automatic injection system

SMSR is a useful machine but its further improvement is required to produce a fully automatic system. To accomplish this, it is essential to detect a stop signal for the injector

when the injector tip has just penetrated the cell membrane. Next we devised an electric circuit to measure the intracellular potential sensitively without a high degree of electrical noise. The measured potential change was thought to be a useful control signal for the injector drive. In order to measure the electrical potential and at the same time introduce a marker dye into a target cell, a double barrel capillary was used (Matsuoka et al., 2006).

A glass capillary with a θ -shaped cross section (outer diameter 1 mm) was heated and pulled to make the tip diameter smaller than 1 μm using a laser puller. One channel of the capillary was filled with a solution of 0.5 M KCl. A Ag/AgCl wire was then inserted into the capillary to produce a potential measuring electrode (MeaE) (Fig. 21). The reference electrode for MeaE was a commercially available Ag/AgCl electrode (RE). The other channel was filled with a solution containing 0.5 M KCl and 1 mM lucifer yellow. A Pt wire was inserted into the capillary to produce a dye-introducing electrode (IntE). The counter electrode (CE) for the IntE was prepared by filling a glass tube with 3% agar gel containing 0.5 M KCl.

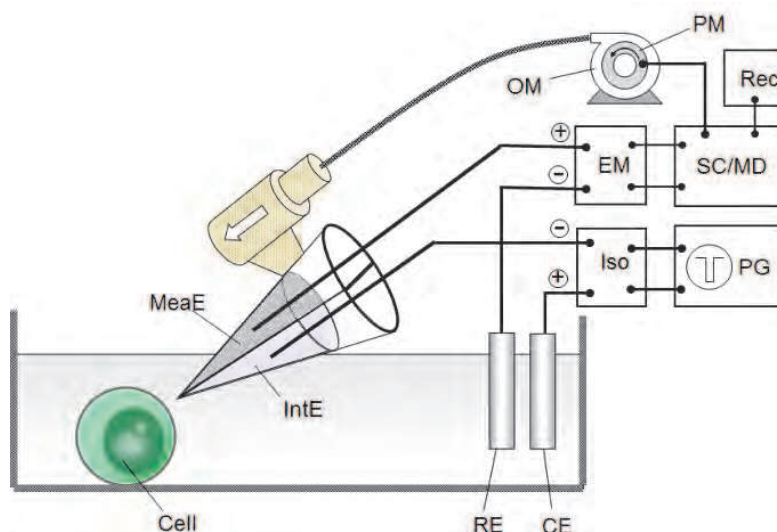


Fig. 21. Schematic diagram of an automatic microinjector positioning system. MeaE: potential measuring electrode, IntE: dye-introducing electrode, RE: reference electrode for MeaE, CE: counter electrode for IntE, Iso: isolator, PG: pulse generator, EM: electrometer, SC/MD: signal converter and pulse motor driver, PM: pulse motor, OM: oil pressure manipulator, Rec: recorder. Reproduced from *Bioelectrochemistry* (2006) 69, 187-92 with permission from Elsevier.

A microinjector was propelled manually and carefully penetrated into a mouse ES cell. The output potential of the MeaE changed sharply to -16 mV (Fig. 22 (A)). It returned to the initial level when removed from the cell. The value of -16 mV is defined as the intracellular potential and is equivalent to the cell membrane potential if the intracellular space is assumed to be homogeneous. The mean and standard deviation (SD) of the intracellular potential for 35 sample ES cells were -16.3 mV and 5.6 mV, respectively (Fig. 22 (B)). A steady intracellular potential is available but the level is variable from cell to cell. Therefore, an absolute value cannot be used as the sign of cell membrane penetration.

Instead, we intended to use the differential of potential change as a stop signal for the pulse motor (Fig. 23). Thus, the microinjector was correctly positioned in the cell without losing cell viability. Its success rate was 73% for ES cells. After positioning the microinjector in the cell, lucifer yellow was introduced by electrophoresis via the IntE. Under this condition, the

rate of viable cells became 16%. Such a loss of viability was thought to be due to an electrical potential that was inevitably loaded onto the cell membrane during the electrophoretic introduction of lucifer yellow.

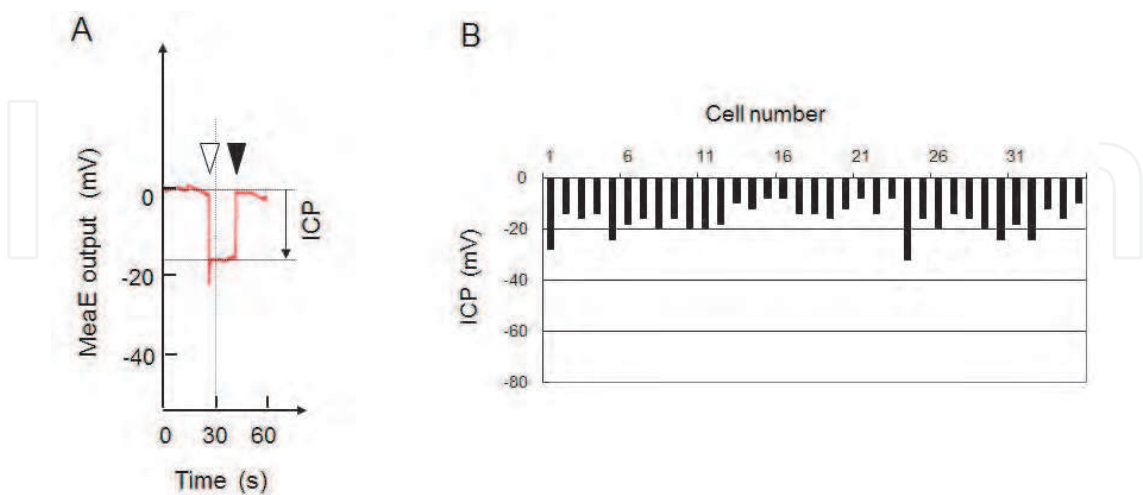


Fig. 22. Intracellular potential (ICP) of mouse ES cells. (A) ICP change profile obtained during the penetration of an injector into the cell (white triangle) and the removal of the injector from the cell (black triangle); (B) steady-state value of ICP from 35 cells. Reproduced from *Bioelectrochemistry* (2006) 69, 187-92 with permission from Elsevier.

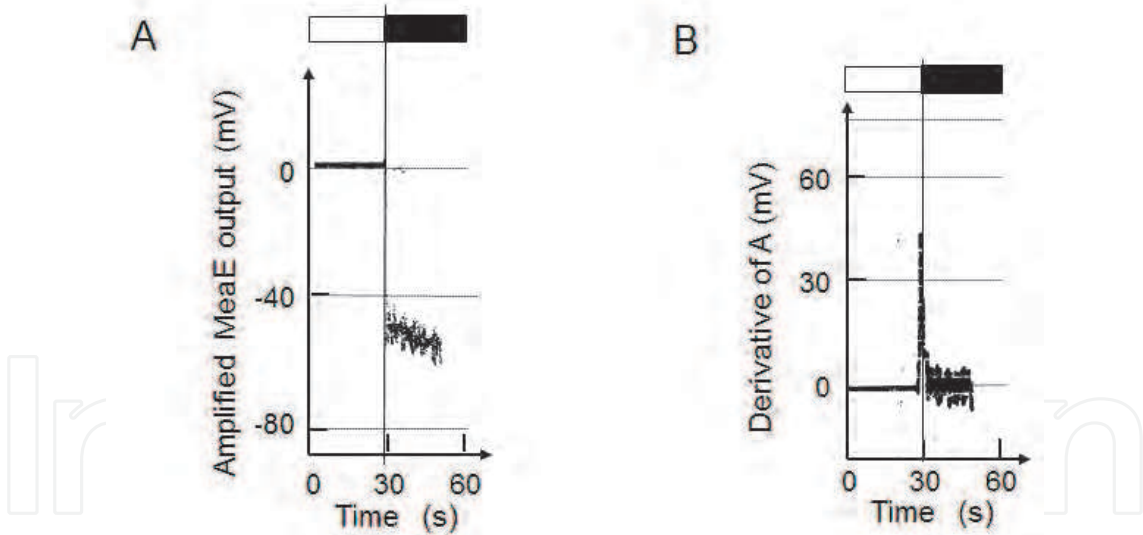


Fig. 23. Potential change (A) and its differential (B) during the penetration of an injector through the cell membrane. White box: an injector is moving during this period; black box: the injector stops and stayed there during this period. The ordinate of (B) indicates the differentiated and properly amplified value of (A). Reproduced from *Bioelectrochemistry* (2006) 69, 187-92 with permission from Elsevier.

9. Conclusion

As a result of the development of SMSR, femtoinjection is now a practical methodology. Femtoinjection has enabled the semi-quantitative introduction of any size of

macromolecules, simultaneous introduction of multiple factors, temporal and spatial control of injection in a cell colony, and spatial control of the injection site whether into the cytosol or nucleus. The results described in this chapter provide some understanding of the significance of single-cell studies. Technological progress will further improve the spatio-temporal precision. Consequently this method can provide us with novel insight into cell functions, especially in ES cell studies, with regard to how to maintain the undifferentiated state and how to produce specific functional cells.

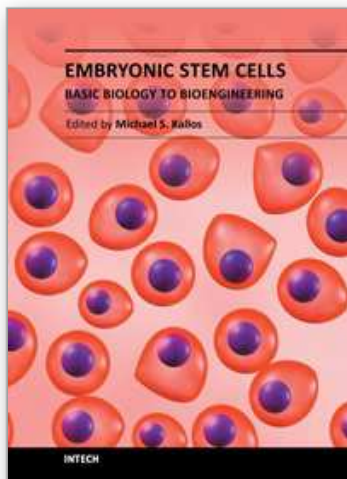
10. Acknowledgments

The studies described in this chapter were funded from CREST of Japan Science and Technology Agency and Grants-in-Aid for Scientific Research, from the Ministry of Education, Culture, Sports, Science, and Technology. We thank Soichiro Shimoda, Meiri Shibusawa, Masakazu Ozaki, Hajime Mizukami, Marina Kawazoe, Makoto Takatsu, and Yohei Yamada for their contribution to these studies. We appreciate kind donation of mouse ES cell lines by H. Niwa (RIKEN, CDB), MIN6 by J. Miyazaki (Osaka University), and Venus expression vector by A. Miyawaki (RIKEN, BSI).

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Embryonic Stem Cells - Basic Biology to Bioengineering

Edited by Prof. Michael Kallos

ISBN 978-953-307-278-4

Hard cover, 478 pages

Publisher InTech

Published online 15, September, 2011

Published in print edition September, 2011

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How to reference

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Hideaki Matsuoka, Mikako Saito and Hisakage Funabashi (2011). Functional Control of Target Single Cells in ES Cell Clusters and Their Differentiated Cells by Femtoinjection, *Embryonic Stem Cells - Basic Biology to Bioengineering*, Prof. Michael Kallos (Ed.), ISBN: 978-953-307-278-4, InTech, Available from: <http://www.intechopen.com/books/embryonic-stem-cells-basic-biology-to-bioengineering/functional-control-of-target-single-cells-in-es-cell-clusters-and-their-differentiated-cells-by-femt>

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