1 Application of visualization techniques for cell and tissue engineering

3 Running title: Visualization and cell/tissue engineering

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15 Key words: Cell and tissue engineering; Visualization; Fluorescent microscopy;
16 Fluorescent protein; Two-photon microscopy; Protein production; Stem cells

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1 **Abstract**

2

3 Visualization has been an indispensable technique in the biological field. The
4 advantage of visualization is to perform non-disruptive analyses with high spatio and
5 temporal resolution. Using these properties, visualization has been employed for cell
6 and tissue engineering research, including therapeutic protein production, cell and
7 organelle manipulation, and stem cell technology. For cell assessment and manipulation,
8 two-photon microscopy based on femtosecond laser is becoming a major tool because
9 of its high depth resolution, low cell damages, and depth of penetration into tick
10 specimen. Non-disruptive and single cell observation/manipulation technique is a
11 powerful tool for stem cell research. In this review, we discuss recent developments in
12 cell and tissue engineering in relation to the revolution in visualization techniques.

13

1 **Introduction**

2
3 The importance of cell culture techniques has been increasing for production
4 of therapeutic proteins such as **therapeutic antibody**, development of artificial organs,
5 and evolution of tissue engineering. One of the most important features of cell culture
6 techniques is assessment of cells. A lot of types of techniques have been employed for
7 the evaluation of cells in cell and tissue engineering field; ELISA for measuring protein
8 secretion (1), mRNA level and expression level of some marker proteins for
9 differentiation (2), and flow cytometry for protein productivity (3, 4). Although these
10 methods are conventional and reliable, non-disruptive and non-time-consuming
11 techniques are required for tissue engineering research. Flow cytometry is a powerful
12 method for this purpose, because it can analyze and sort living cells for further analyses,
13 and evaluate thousands of cells in a few seconds. However, biological analyses should
14 ideally be performed ‘in dish’ or ‘in vivo’. The microscopy-based visualization
15 techniques can achieve them.

16 Visualization is one of the fundamental and important techniques in cell
17 biological field. Compared with other biological methods, such as biochemical or
18 genetic methods, visualization technique has some advantages; non-disruptive
19 evaluation, and highly spatio-temporal resolution. In addition, the development of
20 optical technologies enables us to observe and evaluate samples as well as manipulate
21 cells and tissues.

22 In this review, we discuss recent developments and applications of
23 visualization techniques for cell and tissue engineering.

1 **Live cell imaging**

2
3 High temporal resolution is one of the major advantages of visualization
4 techniques. To take advantage of this benefit, live cell imaging is a basic and
5 indispensable technique. Maintenance of cells in optimum conditions, e.g. temperature,
6 humidity, pH, and culture medium, is an important prerequisite for successful live cell
7 imaging. Optimum temperature and humidity conditions are achieved using an on-stage
8 incubator. To decrease the signal-noise ratio, a medium without phenolred is often used.
9 The pH is stabilized by on-stage incubator with a CO₂ chamber or adding HEPES into
10 the medium. In addition, reduction of light-toxicity is another key point for live cell
11 imaging. In the view of fluorescent bleaching and light-toxicity, the intensity of
12 excitation light source should be minimized. **The cell division processes can be**
13 **visualized by current techniques (FIG. 1).**

14 In most cases, in order to perform live cell imaging, the cells should be
15 visualized by some methods. Phase contrast microscope and differential interference
16 contrast (DIC) microscope have been used for observation of living cells without any
17 staining. In addition, fluorescently-labeled molecules are becoming a major tool for
18 living cell visualization with the development of fluorescent microscopy. There are
19 several methods for visualization of living cells using fluorescence; staining cells with
20 small molecules such as Hoechst 33342 and DiOC₆, using fluorescently-labeled
21 antibodies against cell-surface antigens, and expressing fluorescent proteins, such as
22 green fluorescent protein (GFP). GFP has several advantages for living cell
23 visualization. GFP was first reported in 1962 by Shimomura and colleagues (6). After
24 cloning its gene from *Aequorea victoria* in 1992 (7), GFP has been widely used in
25 various biological fields as a useful tool. For example, GFP has enabled us to analyze
26 not only subcellular localization of proteins but also dynamics and mobility of labeled
27 molecules in living cells. Fluorescence recovery after photobleaching (FRAP) and

1 fluorescence correlation spectroscopy (FCS) are representative methods for the mobility
2 analyses of fluorescently-labeled molecules. Especially, FRAP can be performed using
3 conventional laser scanning microscope without any specific equipments. In 2000,
4 Misteli et al. analyzed chromatin dynamics by FRAP using GFP-tagged histone H1 (8),
5 and then, this method has been widely used by cell biologists. FRAP is suitable for
6 analyzing the dynamics of molecules with relatively slow mobility, such as core
7 histones (9). In addition, it was shown that posttranslational modification of histone
8 proteins affects chromatin dynamics by FRAP (10, 11). **GFP expression can be also**
9 **used for assessment of transfection efficiency (12).** Luminescence proteins, such as
10 luciferases, have been reported as a good reporter gene, however, the one clear and
11 critical advantage of fluorescent proteins is that they enable us to visualize the
12 molecules in living cells without any substrates. Thus, various variants, such as yellow,
13 cyan, and ultramarine (13), were produced from GFP by genetic modification. Red
14 fluorescent proteins were also cloned from coral (14) and sea anemone (15). A lot of
15 researchers have tried modifying fluorescent proteins, for example, to improve
16 maturation and stability (16), to produce bright (17) or monomeric (18) proteins, and for
17 resistant to oxidative conditions (19). In addition, photoconversion fluorescent proteins
18 have been also developed (20-22). These proteins change their color by irradiation with
19 light of a specific wavelength. They can be used not only for localization/dynamics
20 analysis of proteins but also for cell/organelle marking and mobility analysis of proteins.
21 Live cell imaging combined with a phototoxic red fluorescent protein, KillerRed, opens
22 a door to perform spatially and temporally specific knockdown analyses. KillerRed
23 produces reactive oxygen species (ROS) by irradiation with strong green light, and then
24 inactivates fused and/or interacted proteins (23). Recently, the combined method
25 revealed the spatio- and temporal-specific function of chromosomal proteins responsible
26 for cell division (24, 25). Moreover, the application of apoptosis induction for tumor
27 cells in mice exhibited the possibility of photodynamic therapy of cancer (26).

Cell line assessment for biochemical engineering

Visualization techniques have been employed in cell and tissue engineering field. Chinese hamster ovary (CHO)-dihydrofolate reductase (DHFR) gene amplification is one of the most frequently used systems in industrial field for therapeutic protein production using mammalian cells (27). To apply the gene amplification system to industrial processes, one of the most critical points is efficiency of gene amplification. Cells can be resistant to methotrexate (MTX), a dihydrofolate analogue and inducer of dhfr gene amplification, by various way including gene amplification, membrane-permeability mutation (28), and expression of DHFR with low or no sensitivity to MTX (29). The step-wise selection can be used to obtain a highly productive cell pool (30). In addition, the locus of the delivered gene also affects on gene amplification efficiency (31). Yoshikawa et al. analyzed the chromosomal locus of the amplified dhfr gene in MTX-resistant CHO cell pools and clones by fluorescent in situ hybridization (FISH), one of the visualization techniques for gene locus. The dhfr gene in highly productive cell pools or clones is mainly amplified at the telomeric region (1, 32). Theoretically, the candidates for highly productive cell clones can be assessed at the preliminary stage by this method before time-consuming step-wise selection with MTX. Moreover, gene targeting into “highly amplification region” enable us to obtain highly productive cell pools easily. Omasa et al constructed a CHO genomic bacterial artificial chromosome (BAC) library from exogenous dhfr gene-amplified CHO cells (33), and analyzed specific chromosomal sequence adjacent to exogenous dhfr-amplified region based on imaging (34).

Quality control of proteins is another important aspect of therapeutic protein production. Proteins are synthesized by ribosomes on rough endoplasmic reticulum (ER), folded in the ER, processed in the Golgi apparatus, and secreted from cell surface. During these steps, most important and critical stages is quality control in the ER, since

1 non-folding proteins is excluded from ER and degraded (ER-associated degradation,
2 ERAD). Thus, evaluation of the status of cargo proteins in the ER is one of the
3 important factors for effective protein production. However, it has been difficult to
4 evaluate the situation of cargo proteins in the ER in living mammalian cells. Recently,
5 single molecule observation based on total internal reflection fluorescence microscopy
6 (TIRFM) was applied for this purpose. Nagaya et al. revealed that glycosylation of
7 cargo proteins affects on their mobility in the ER by transient immobilization on the ER
8 membrane using TIRFM and fluorescent correlation spectroscopy (FCS) (35).
9 Glycosylation patterns are the most important stages to assess cargo protein folding by
10 the molecular chaperon calnexin (36). These results suggest that the mobility of cargo
11 proteins in the ER reflects the maturation of proteins in the ER lumen. Therefore,
12 observation of cargo protein dynamics in the ER lumen can be used for evaluation of
13 cell lines and optimization of culture conditions for therapeutic protein production, such
14 as temperature, osmotic pressure, and culture medium.

15

16 **Cell and organelle manipulation by two-photon microscope**

17 Recently, laser scanning microscopy has been employed not only for the
18 observation of specimen but also for the manipulation of tissue, cells, and/or organelles.
19 Two-photon excitation fluorescence microscopy with a near-infrared ultrashort laser has
20 several advantages for research in the biological field; high spatial resolution, deep
21 penetration into thick samples, and reduced photon-induced damages. Further, the
22 benefit of multi-photon excitation for cell and organelle manipulation is induction of
23 localized structural modification and/or ablation in living cells and tissues by non-linear
24 absorption. Thus, the influence of heat to the surrounding areas is minimized.

25 Organelles and intracellular structure, such as mitochondria, and actin fibers,
26 can be disrupted or dissected by near-infrared ultrashort laser irradiation (37-39). The
27 differences between photobleaching of the fluorophore and intracellular ablation after

1 focusing femtosecond laser pulses could be distinguished. Watanabe and colleagues
2 investigated the difference between bleaching and ablation using fluorescently-labeled
3 mitochondria and nucleus. The organelles were first labeled with fluorescent proteins in
4 living cells, and irradiated by femtosecond laser at various energies. Then, the
5 organelles were re-stained with organelle-specific fluorescent dye for living cells, such
6 as MitoTracker and Hoechst33342, and then evaluated. The results of re-staining clearly
7 indicated that the highly irradiated organelle and/or structure were disrupted (FIG. 2) (38,
8 40, 41). After disruption of mitochondria, both intracellular and intercellular calcium
9 wave was observed (FIG. 3). In general, laser irradiation at high energy causes cell
10 damages, and sometimes causes cell death. Although a HeLa cell was irradiated and its
11 mitochondrion was ablated by two-photon excitation, the cell could divide into two
12 daughter cells (40). This result indicates that site-specific irradiation by focusing
13 ultrashort lasers does not result in critical damage to living cell activities. Femtosecond
14 laser surgery was applied to sever individual dendrites in sensory neurons and axons of
15 motor neurons in living *Caenorhabditis elegans* roundworms to study the functional and
16 regenerative response to nerve damage (42). Another application of nanosurgery is cell
17 transfection, or the creation of a small perforation in the cellular membrane and the
18 subsequent injection of foreign materials, typically DNA. Tirlapur and König reported
19 the targeted transportation of plasmid DNA vector encoding GFP into CHO cells by
20 focusing femtosecond laser pulses (43)

21 Two-photon microscopy based on femtosecond laser techniques can be also
22 used for cell purification. A single living adherent cell could be isolated from
23 collagen-coated culture plate using femtosecond laser-induced shockwave (44).
24 Although single cell isolation is achieved by flow cytometry, the cell morphology can
25 not be evaluated by this method. Recent developments in molecular biology techniques
26 enable us to quantify the mRNA level isolated from single cell (45). We can assess cell
27 characterization by combination of single cell isolation techniques using two-photon

1 microscopy and quantitative RNA analysis. These techniques can be also applied for
2 stem cell technologies.

3 Another advantage of two-photon microscopy is visualization in thick
4 specimen such as whole organisms and tissues. The depth of focus of two-photon
5 excitation is deeper than that of single-photon excitation, since light sources with longer
6 wavelength are used for two-photon excitation. The hematopoietic cells and neurons in
7 living mice were visualized using two-photon microscopy (46, 47). The fluorescent
8 probes for tumor imaging have been developed (48). Two-photon microscopy allows
9 larger penetration depth compared with confocal microscopy, however, aberration
10 effects due to inhomogeneous samples distorts the focal spot and reduce the spatial
11 resolution and decrease image brightness and/or contrast (49, 50). Wavefront
12 compensation with adaptive optics can restore a diffraction limited focus within an
13 inhomogeneous sample that introduces wavefront aberrations and enable us to image
14 deep inside samples and increase in image brightness and/or contrast.

15 Considering its low cytotoxicity, visualization techniques using two-photon
16 excitation might be suitable for diagnostic use. Nonlinear microscopy, such as second
17 harmonic generation, third harmonic generation, nonresonant fourwave mixing,
18 stimulated parametric emission, coherent anti-Stokes Raman scattering, and stimulated
19 Raman scattering have become invaluable tools in biological and medical applications
20 with minimal invasiveness. Ultrashort laser pulses in the near-infrared region have
21 potential applications in imaging and manipulation of intracellular organelles.

22 23 **Visualization for stem cell researches**

24 Stem cell science or technology is one of the most noteworthy fields today. A
25 major problem of stem cell research is that only a small number of stem cells are
26 available. In the case of mouse hematopoietic cells, the percentage of hematopoietic stem
27 and progenitor cells is less than 0.2% of the total bone marrow cells (51). In general, it

1 is difficult to obtain a sufficient number of cells for analysis or evaluation by
2 conventional methods such as biochemical analyses, e.g. western blotting and
3 immunoprecipitation. Visualization techniques might resolve these problems, since
4 microscopy is non-destructive method, and requires only a few numbers of cells for
5 analysis (Table 1). Another advantage of visualization for stem cell field is for “in situ”
6 analysis. Multicellular organisms should be developed from single fertilized cells.
7 During development, some cells asymmetrically divided to generate diversity. In 1994,
8 it was shown that a cytoplasmic protein, numb, regulates asymmetric cell division in
9 *Drosophila* cells by determining its in situ localization using microscopy (52). After
10 asymmetric division of a stem cell, one daughter cell should be stem cell and the other
11 is differentiated cell. Visualization provided us clear evidence of stem cell
12 differentiation and cell division. Scaffidi and Misteli clearly demonstrated asymmetric
13 differentiation of cells after cell division by immunofluorescence using the stem cell
14 marker anti-SSEA-1 (53). The “cell marking technique” using photoconversion
15 fluorescent proteins, such as paGFP (20) and Kaede (21), might provide clear and direct
16 evidence of asymmetric cell division. These photoconversion fluorescent proteins
17 change their color by excitation at a specific wavelength. In the case of Kaede, the
18 emission peak shifts from 518 nm (green) to 572 nm (red) by UV irradiation (21).
19 Because specific cells can be irradiated, we can monitor the fate of “red cells” by
20 imaging. Arimura et al., applied this technique to organelle. They analyzed plant single
21 mitochondrial fusion and fission using live cell imaging (54).

22 Non-destructive and prompt cell assessment are another important aspects for
23 cell and tissue engineering including stem cell engineering, since quality control of
24 living cells is indispensable for further use. Flow cytometry has both these features, and
25 this technique has been widely used for cell assessment in stem cell research. However,
26 it generally requires cell staining using antibodies against cell surface markers, and is
27 not suitable for analyzing adhesion cells. To address these problems, imaging-based

1 stem cell assessment techniques have been developed. Conventional microscopy, such
2 as phase-contrast microscopy and wide-field fluorescent microscopy, also provides us
3 useful information regarding cell differentiation and situation (5, 55-59). Phase-shifting
4 laser microscope enables us to measure the three-dimensional cell morphology without
5 requiring specimen fixation and staining (60), which is one of the key parameters for the
6 assessment of cell differentiation and situation. Using this technique, Tokumitsu et al.,
7 reported the discrimination of normal cells from malignant cells (61). Thus,
8 visualization techniques, including image analysis, have been employed in stem cell
9 researches, although there are a lot of points to improve such as automation, validation,
10 and accuracy, especially for clinical use.

11

12 **Future directions**

13 Recent developments in microscopic techniques including devices, fluorescent
14 dyes, and image analyses have contributed to the development of cell and tissue
15 engineering. With the development of ES/iPS/stem cell technologies, the experimental
16 scale will be much smaller because we can obtain only a small number of cells.
17 Visualization can address this problem. However, it is generally difficult for biologist
18 alone to achieve further development of microscopic techniques. Further cooperation
19 with the researchers in another field, such as physicist and chemist, might be
20 appreciated.

21

1 **Acknowledgements**

2

3 This research was supported by a Grant-in-Aid for Scientific Research on Priority Areas
4 from Japanese Ministry of Education, Science, Culture, Sports, and Technology and
5 BIRD and SENTAN grant to S.M. and H.F. from the Japan Science and Technology
6 Agency.

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References

1. **Yoshikawa, T., Nakanishi, F., Itami, S., Kameoka, D., Omasa, T., Katakura, Y., Kishimoto, M., and Suga, K.:** Evaluation of stable and highly productive gene amplified CHO cell line based on the location of amplified genes, *Cytotechnology*, **33**, 37-46 (2000).
2. **Hoen, P.A.C., Hirsch, M., de Meijer E.J., de Menezes, R.X., van Ommen, G.J., and den Dunnen, J.T.:** mRNA degradation controls differentiation state-dependent differences in transcript and splice variant abundance, *Nucleic Acids Res.*, **39**, 556-566 (2011).
3. **Zeyta, M., Borth, N., Kunert, R., and Katinger, H.:** Optimization of sorting conditions for the selection of stable, high-producing mammalian cell lines, *Biotechnol. Prog.*, **15**, 953-957 (1999).
4. **Yoshikawa, T., Nakanishi, F., Ogura, Y., Oi, D., Omasa, T., Katakura, Y., Kishimoto, M., and Suga K.I.:** Flow cytometry: an improved method for the selection of highly productive gene-amplified CHO cells using flow cytometry, *Biotechnol. Bioeng.*, **74**, 435-442 (2001).
5. **Gambe, A.E., Ono, R.M., Matsunaga, S., Kutsuna, N., Higaki, T., Higashi, T., Hasegawa, S., Uchiyama, S., and Fukui, K.:** Development of a multistage classifier for monitoring system of cell activity based on imaging of chromosomal dynamics, *Cytotechnology*, **71A**, 286-296 (2007).
6. **Shimomura, O., Johnson, F. H., and Saiga, Y.:** Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusan, *Aequorea*, *J. Cell. Comp. Physiol.*, **59**, 223-239 (1962).
7. **Prasher, D.C., Eckenrode, V.K., Ward, W.W., Prendergast, F.G., and Cormier, M.J.:** Primary structure of the *Aequorea victoria* green-fluorescent protein, *Gene*, **111**, 229-233 (1992).

- 1 8. **Misteli, T., Gunjan, A., Hock, R., Bustin, M., and Brown, D.T.:** Dynamic binding
2 of histone H1 to chromatin in living cells, *Nature*, **408**, 877-881 (2000).
- 3 9. **Kimura, H., and Cook, P.R.:** Kinetics of core histones in living human cells: little
4 exchange of H3 and H4 and some rapid exchange of H2B, *J. Cell Biol.*, **153**,
5 1341-1353 (2001).
- 6 10. **Contreras, A., Hale, T.K., Stenoien, D.L., Rosen, J.M., Mancini, M.A., and**
7 **Herrera, R.E.:** The dynamic mobility of histone H1 is regulated by cyclin/CDK
8 phosphorylation, *Mol. Cell. Biol.*, **23**, 8623-8636 (2003).
- 9 11. **Higashi, T., Matsunaga, S., Isobe, K., Morimoto, A., Shimada, T., Kataoka, S.,**
10 **Watanabe, W., Uchiyama, S., Itoh, K., and Fukui, K.:** Histone H2A mobility is
11 regulated by its tails and acetylation of core histone tails, *Biochem. Biophys. Res.*
12 *Commun.*, **357**, 627-632 (2007).
- 13 12. **Higashi, T., Nagamori, E., Sone, T., Matsunaga, S., and Fukui, K.:** A novel
14 transfection method for mammalian cells using calcium alginate microbeads, *J.*
15 *Biosci. Bioeng.*, **97**, 191-195 (2004).
- 16 13. **Tomosugi, W., Matsuda, T., Tani, T., Nemoto, T., Kotera, I., Saito, K., Horikawa,**
17 **K., and Nagai, T.:** An ultramarine fluorescent protein with increased photostability
18 and pH insensitivity, *Nat. Methods*, **5**, 351-353 (2009).
- 19 14. **Matz, M.V., Fradkov, A.F., Labas, Y.A., Savitsky, A.P., Zeraisky, A.G.,**
20 **Markelov, M.L., and Lukyanov, S.A.:** Fluorescent proteins from
21 nonbioluminescent Anthozoa species, *Nat. Biotechnol.*, **17**, 969-973 (1999).
- 22 15. **Merzlyak, E.M., Goedhart, J., Shcherbo, D., Bulina, M.E., Shcheglov, A.S.,**
23 **Fradkov, A.F., Gaintzeva, A., Lukyanov, K.A., Lukyanov, S., Gadella, T.W., and**
24 **Chudakov, D.M.:** Bright monomeric red fluorescent protein with an extended
25 fluorescence life time, *Nat. Methods*, **4**, 555-557 (2007).
- 26 16. **Nagai, T., Ibata, K., Park, E.S., Kubota, M., Mikoshiba, K., and Miyawaki, A.:**
27 A variant of yellow fluorescent protein with fast and efficient maturation for

- cell-biological applications, *Nat. Biotechnol.* **20**, 87-90 (2002).
17. **Kremers, G.J., Goedhart, J., van den Heuvel, D.J., Gerritsen, H.C., and Gadella, T.W.:** Improved green and blue fluorescent proteins for expression in bacteria and mammalian cells, *Biochemistry*, **46**, 3775-3783 (2007).
18. **Shaner, N.C., Campbell, R.E., Steinbach, P.A., Giepmans, B.N., Palmer, A.E., and Tsien, R.Y.:** Improved monomeric red, orange, and yellow fluorescent proteins derived from *Discosoma* sp. Red fluorescent protein, *Nat. Biotechnol.* **22**, 1567-1572 (2004).
19. **Suzuki, T., Arai, S., Takeuchi, M., Sakurai, C., Eabana, H., Higashi, T., Hashimoto, H., Hatsuzawa, K., and Wada, I.:** Development of cysteine-free fluorescent protein for the oxidative environment, *PLoS One*, **7**, e37551 (2012).
20. **Patterson, G.H., and Lippincott-Schwartz, J.:** A photoactivatable GFP for selective photolabeling of proteins and cells, *Science*, **297**, 1873-1877 (2002).
21. **Ando, R., Hama, H., Yamamoto-Hino, M., Mizuno, H., and Miyawaki, A.:** An optical marker based on the UV-induced green-to-red photoconversion of a fluorescent protein, *Proc. Natl. Acad. Sci. USA.*, **99**, 12651-12656 (2002).
22. **Habuchi, S., Ando, R., Dedecker, P., Verheijen, W., Mizuno, H., Miyawaki, A., and Hofkens, J.:** Reversible single-molecule photoswitching in the GFP-like fluorescent protein Dronpa, *Proc. Natl. Acad. Sci. USA.*, **102**, 9511-9516 (2005).
23. **Bulina, M.E., Chudakov, D.M., Britanova, O.V., Yanushevich, D.B., Chepurnykh, T.V., Merzlyak, E.M., Sckrob, M.A., Lukyanov, S., Lukyanov, K.A.:** A genetically encoded photosensitizer, *Nat. Biotechnol.*, **24**, 95-99 (2006).
24. **Serebrovskaya, E.O., Gorodnicheva, T.V., Ermakova, G.V., Solovieva, G.V., Zaqaynova, E.V., Chudakov, D.M., Lukyanov, S., Zarsky, A.G., and Lukyanov, K.A.:** Light-induced blockage of cell division with a chromatin-targeted phototoxic fluorescent protein, *Biochem. J.*, **435**, 65-71 (2011).
25. **Matsunaga, S., Takata, H., Morimoto, A., Hayashihara, K., Higashi, T.,**

- 1 **Akatsuchi, K., Mizusawa, E., Yamakawa, M., Ashida, M., Matsunaga, T.M.,**
2 **Azuma, T., Uchiyama, S., and Fukui, K.:** RBMX: a regulator for maintenance and
3 centromeric protection of sister chromatid cohesion, *Cell Rep.*, **1**, 299-308 (2012).
- 4 26. **Shirmanova, M.V., Serebrovskaya, E.O., Lukyanov, K.A., Snopova, L.B.,**
5 **Sirotkina, M.A., Prodanetz, N.N., Bugrova, M.L., Minakova, E.A., Turchin, I.V.,**
6 **Kamensky, V.A., Lukyanov, S.A., and Zaqaynova, E.V.:** Phototoxic effects of
7 fluorescent protein KillerRed on tumor cells in mice, *J. Biophotonics*, in press
8 (2012).
- 9 27. **Omasa, T.:** Gene amplification and its application in cell and tissue engineering, *J.*
10 *Biosci. Bioeng.*, **94**, 600-605 (2002).
- 11 28. **Assaraf, Y.G. and Schimke, R.T.:** Identification of methotrexate transport
12 deficiency in mammalian cells using fluoresceinated methotrexate and flow
13 cytometry, *Proc. Natl. Acad. Sci. USA.*, **84**, 7154-7158 (1987).
- 14 29. **Haber, D.A., Beverley, S.M., Kiely, M.L., and Schimke, R.T.:** Properties of an
15 altered dihydrofolate reductase encoded by amplified genes in cultured mouse
16 fibroblasts, *J. Biol. Chem.*, **256**, 9501-9510 (1981).
- 17 30. **Omasa, T., Itami, S., Kameoka, D., Katakura, Y., and Suga, K.:** Selection and
18 stability for recombinant CHO cell line expression human GM-CSF in gene
19 amplification, p. 29-33. In Nagai, K. and Wachi, M. (ed.), *Animal Cell Technology:*
20 *Basic and Applied Aspects*, Kluwer Academic Publishers, Netherland (1996).
- 21 31. **Kündig, C., Leblanc, E., Papadopoulou, B., and Ouellette, M.:** Role of the locus
22 and of the resistance gene on gene amplification frequency in methotrexate resistant
23 *Leishmania tarentolae*, *Nucleic Acids Res.*, **18**, 3653-3659 (1999).
- 24 32. **Yoshikawa, T., Nakanishi, F., Ogura, Y., Oi, D., Omasa, T., Katakura, Y.,**
25 **Kishimoto, M., and Suga, K.:** Amplified gene location in chromosomal DNA
26 affected recombinant protein production and stability of amplified genes, *Biotechnol.*
27 *Prog.*, **16**, 710-715 (2000).

- 1 33. **Omasa, T., Cao, Y., Park, Y.C., Takagi, Y., Kimura, S., Yano, H., Honda, K.,**
2 **Asakawa, S., Shimizu, N., and Ohtake, H.:** Bacterial artificial chromosome library
3 for genome-wide analysis of Chinese hamster ovary cells, *Biotechnol. Bioeng.*, **104**,
4 986-994 (2009).
- 5 34. **Park, J.Y., Takagi, Y., Yamatani, M., Honda, K., Asakawa, S., Shimizu, N.,**
6 **Omasa, T., and Ohtake, H.:** Identification and analysis of specific chromosomal
7 region adjacent to exogenous dhfr-amplified region in Chinese hamster ovary cell
8 genome, *J. Biosci. Bioeng.*, **109**, 504-511 (2011).
- 9 35. **Nagaya, H., Tamura, T., Higa-Nishiyama, A., Ohashi, K., Takeuchi, M.,**
10 **Hashimoto, H., Hatsuzawa, K., Kinjo, M., Okada, T., and Wada, I.:** Regulated
11 motion of glycoproteins revealed by direct visualization of a single cargo in the
12 endoplasmic reticulum, *J. Cell Biol.*, **180**, 129-143 (2008).
- 13 36. **Helenius, A., and Aebi, M.:** Role of N-linked glycans in the endoplasmic reticulum,
14 *Annu. Rev. Biochem.* **73**, 1019-1049 (2004).
- 15 37. **König, K.:** Laser tweezers and multiphoton microscope in life science, *Histochem.*
16 *Cell. Biol.*, **114**, 79-92 (2000).
- 17 38. **Watanabe, W., Arakawa, N., Matsunaga, S., Higashi, T., Fukui, K., Isobe, K.,**
18 **and Itoh, K.:** Femtosecond laser disruption of subcellular organelles in a living
19 cells. *Opt. Express*, **12**, 4203-4213 (2004).
- 20 39. **Heisterkamp, A., Maxwell, I.Z., Mazur, E., Underwood, J.M., Nickerson, J.A.,**
21 **Kumar, S., and Ingber, D.E.:** Pulse energy dependence of subcellular dissection by
22 femtosecond laser pulses, *Opt. Express*, **13**, 3690-3696 (2005).
- 23 40. **Shimada, T., Watanabe, W., Matsunaga, S., Higashi, T., Ishii, H., Fukui, K.,**
24 **Isobe, K., and Itoh, K.:** Intracellular disruption of mitochondria in a living HeLa
25 cell with a 76-MHz femtosecond laser oscillator, *Opt. Express*, **13**, 9869-9880
26 (2005).
- 27 41. **Watanabe, W., Matsunaga, S., Higashi, T., Fukui, K., and Itoh, K.:** In vivo

- 1 manipulation of fluorescently-labeled organelles in living cells by multiphoton
2 excitation, *J. Biomed. Opt.*, **13**, e031213 (2008).
- 3 42. **Yanik, M.F., Cinar, H., Cinar, H.N., Chisholm, A.D., Jin, Y., and Ben-Yakar, A.:**
4 Nanosurgery: Functional regeneration after laser axotomy, *Nature*, **432**, 822 (2004).
- 5 43. **Tirlapur, U.K. and König, K.:** Targeted transfection by femtosecond laser, *Nature*,
6 **418**, 290-291 (2002).
- 7 44. **Hosokawa, Y., Takabayashi, H., Miura, S., Shukunami, C., Hiraki, Y. and**
8 **Masuhara, H.:** Nondestructive isolation of single cultured animal cells by
9 femtosecond laser-induced shockwave, *Appl. Phys. A*, **79**, 795-798 (2004).
- 10 45. **Sucher, N.J., Deitcher, D.L., Baro, D.J., Warrick, R.M., and Guenther, E.:**
11 Genes and channels: patch/voltage-clamp analysis and single-cell RT-PCR, *Cell*
12 *Tissue Res.*, **302**, 295-307 (2000).
- 13 46. **Bousso, P.:** Real-time imaging of T-cell development, *Curr. Opin. Immunol.*, **16**,
14 400-405 (2004).
- 15 47. **Maschio, M.D., Beltramo, R., De Stasi, A.M., and Fellin, T.:** Two-photon calcium
16 imaging in the intact brain, *Adv. Exp. Med. Biol.*, **740**, 83-102 (2012).
- 17 48. **Zhang, S., Hosaka, M., Yoshihara, T., Negishi, K., Iida, Y., Tobita, S., and**
18 **Takeuchi, T.:** Phosphorescent light-emitting iridium complexes serve as a
19 hypoxia-sensing probe for tumor imaging in living animals, *Cancer Res.*, **70**,
20 4490-4498 (2010).
- 21 49. **Booth, M.J.:** Adaptive optics in microscopy, *Philos. Transact. A Math. Phys. Eng.*
22 *Sci.*, **365**, 2829-2843 (2007).
- 23 50. **Botcherby, D.E., Watanabe, T., Srinivas, S., Booth, M., and Wilson, T.:**
24 Image-based adaptive optics for two-photon microscopy, *Opt. Lett.*, **34**, 2495-2497
25 (2009).
- 26 51. **Matsuura, S., Yan, M., Lo, M.C., Ahn, Y.E., Weng, S., Dangoor, D., Matin, M.,**
27 **Higashi, T., Feng, G.S., and Zhang, D.E.:** Negative effects of GM-CSF signaling

- 1 in amurine model of t(8;21)-induced leukemia, *Blood*, **119**, 3155-3163 (2012).
- 2 52. **Rhyu, M.S., Jan, L.Y., and Jan, Y.N.:** Asymmetric distribution of numb protein
3 during division of the sensory organ precursor cell confers distinct fates to daughter
4 cells, *Cell*, **76**, 477-491 (1994).
- 5 53. **Scaffidi, P., and Misteli, T.:** In vitro generation of human cells with cancer stem
6 cell properties, *Nat. Cell Biol.*, **13**, 1051-1061 (2011).
- 7 54. **Arimura, S., Yamamoto, J., Aida, J.P., Nakazono, M., and Tsutsumi, N.:**
8 Frequent fusion and fission of plant mitochondria with unequal nucleoid distribution,
9 *Proc. Natl. Acad. Sci. USA.*, **101**, 7805-7808 (2004).
- 10 55. **Kato, R., Kaga, C., Kunimatsu, M., Kobayashi T., and Honda, H.:** Peptide
11 array-based interaction of solid-bound peptides and anchorage-dependent cells and
12 its effectiveness in cell-adhesive peptide design, *J. Biosci. Bioeng.*, **101**, 485-495
13 (2006).
- 14 56. **Fujita, S., Ota, E., Sasaki, C., Takano, K., Miyake, M., and Miyake, J.:** Highly
15 efficient reverse transfection with siRNA in multiple wellsof microlitter plates, *J.*
16 *Biosci. Bioeng.*, **104**, 329-333 (2007).
- 17 57. **Takagi, M., Kitabayashi, T., Koizumi, S., Hirose, H., Kondo, S., Fujiwara, M.,**
18 **Ueno, K., Misawa, H., Hosokawa, Y., Masuhara, H., and Wakitani, M.:**
19 Correlation between cell morphology and aggrecan gene expression level during
20 differentiation from mesenchymal stem cells to chondrocytes, *Biotechnol. Lett.*, **30**,
21 1189-1195 (2008).
- 22 58. **Tokumoto, Y., Ogawa, S., Nagamune, T., and Miyake, J.:** Comparison of
23 efficiency of terminal differentiation of oligodendrocytes from induced pluripotent
24 stem cells versus embryonic stem cells in vitro, *J. Biosci. Bioeng.*, **109**, 622-628
25 (2010).
- 26 59. **Kim, M.H., Sonoi, R., Yamada, K., Inamori, M., and Kino-oka, M.:** Analysis of
27 locality of early-stage maturation in confluent state of human retinal pigment

- 1 epithelial cells, J. Biosci. Bioeng., **113**, 778-781 (2012).
- 2 60. **Takagi, M., Kitabayashi, T., Ito, S., Fujiwara, M., and Tokuda, A.:** Noninvasive
- 3 measurement of three-dimensional morphology of adhered animal cells employing
- 4 phase-sifting laser microscope, J. Biomed. Opt., **12**, e054010 (2007).
- 5 61. **Tokumitsu, A., Wakitani, S., and Takagi, M.:** Noninvasive discrimination of
- 6 human normal cells and malignant tumor cells by phase-sifting laser microscopy, J.
- 7 Biosci. Bioeng., **109**, 499-503 (2010).
- 8

FIG. 1

FIG. 2

FIG. 3

Tables

Table 1 The number of cells required for analysis

Methods	Cell number	Types of analysis
Biochemistry	$> 10^4$	Destructive
Flow cytometry	$>10^3$	Non-destructive
Molecular biology	$>10^1$	Destructive
Microscopy	$1-10^1$	Non-destructive