Journal of Bioscience and Bioengineering
VOL. 115 No. 2, 122–126, 2013

www.elsevier.com/locate/jbiosc

REVIEW

Application of visualization techniques for cell and tissue engineering

Tsunehito Higashi,[1,§,*] Wataru Watanabe,[2] and Sachihiro Matsunaga[1]

Department of Applied Biological Science, Faculty of Science and Technology, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 278-8510, Japan[1] and Electronics and Photonics Research Institute, National Institute of Advanced Industrial Science and Technology, 1-1-1 Umezono, Tsukuba, Ibaraki 305-8568, Japan[2]

Received 23 July 2012; accepted 16 September 2012
Available online 18 October 2012

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

[**Key words:** Cell and tissue engineering; Visualization; Fluorescent microscopy; Fluorescent protein; Two-photon microscopy; Protein production; Stem cells]

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

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

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

LIVE CELL IMAGING

High temporal resolution is one of the major advantages of visualization techniques. To take advantage of this benefit, live cell imaging is a basic and indispensable technique. Maintenance of cells in optimum conditions, e.g., temperature, humidity, pH, and culture medium, is an important prerequisite for successful live cell imaging. Optimum temperature and humidity conditions are achieved using an on-stage incubator. To decrease the signal-noise ratio, a medium without phenol red is often used. The pH is stabilized by on-stage incubator with a CO_2 chamber or adding HEPES into the medium. In addition, reduction of light-toxicity is another key point for live cell imaging. In the view of fluorescent bleaching and light-toxicity, the intensity of excitation light source should be minimized. The cell division processes can be visualized by current techniques (Fig. 1).

In most cases, in order to perform live cell imaging, the cells should be visualized by some methods. Phase contrast microscope and differential interference contrast (DIC) microscope have been used for observation of living cells without any staining. In addition, fluorescently-labeled molecules are becoming a major tool for living cell visualization with the development of fluorescent microscopy. There are several methods for visualization of living cells using fluorescence; staining cells with small molecules such as Hoechst 33342 and $DiOC_6$, using fluorescently-labeled antibodies against cell-surface antigens, and expressing fluorescent proteins, such as a green fluorescent protein (GFP). GFP has several

* Corresponding author. Tel.: +81 11 706 6958; fax: +81 11 706 7824.
E-mail address: thigashi@med.hokudai.ac.jp (T. Higashi).
§ Present address: Department of Cellular Pharmacology, Graduate School of Medicine, Hokkaido University, Nishi 7, Kita 15, Kita-ku, Sapporo, Hokkaido 060-8638, Japan.

1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2012.09.008

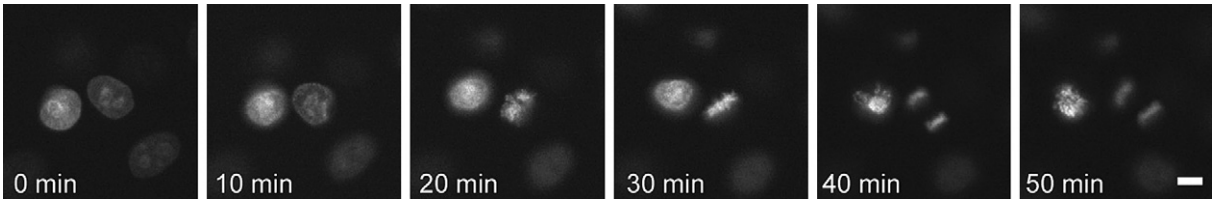

FIG. 1. Chromosomal dynamics in living HeLa cells. Time-lapse imaging was performed using HeLa cells stably expressing EGFP-histone H1 (5). The images were captured every 10 min using IX71 fluorescent microscope (Olympus, Tokyo, Japan) equipped with ×40 objective lens (Olympus, PlanFLN, NA = 1.30). Bar: 10 μm.

advantages for living cell visualization. GFP was first reported in 1962 by Shimomura and colleagues (6). After cloning its gene from *Aequorea victoria* in 1992 (7), GFP has been widely used in various biological fields as a useful tool. For example, GFP has enabled us to analyze not only subcellular localization of proteins but also dynamics and mobility of labeled molecules in living cells. Fluorescence recovery after photobleaching (FRAP) and fluorescence correlation spectroscopy (FCS) are representative methods for the mobility analyses of fluorescently-labeled molecules. Especially, FRAP can be performed using conventional laser scanning microscope without any specific equipments. In 2000, Misteli et al. analyzed chromatin dynamics by FRAP using GFP-tagged histone H1 (8), and then, this method has been widely used by cell biologists. FRAP is suitable for analyzing the dynamics of molecules with relatively slow mobility, such as core histones (9). In addition, it was shown that posttranslational modification of histone proteins affects chromatin dynamics by FRAP (10,11). GFP expression can be also used for assessment of transfection efficiency (12). Luminescence proteins, such as luciferases, have been reported as a good reporter gene, however, the one clear and critical advantage of fluorescent proteins is that they enable us to visualize the molecules in living cells without any substrates. Thus, various variants, such as yellow, cyan, and ultramarine proteins (13), were produced from GFP by genetic modification. Red fluorescent proteins were also cloned from coral (14) and sea anemone (15). A lot of researchers have tried modifying fluorescent proteins, for example, to improve maturation and stability (16), to produce bright (17) or monomeric (18) proteins, and for resistant to oxidative conditions (19). In addition, photoconversion fluorescent proteins have been also developed (20–22). These proteins change their color by irradiation with light of a specific wavelength. They can be used not only for localization/dynamics analysis of proteins but also for cell/organelle marking and mobility analysis of proteins. Live cell imaging combined with a phototoxic red fluorescent protein, KillerRed, opens a door to perform spatially and temporally specific knockdown analyses. KillerRed produces reactive oxygen species (ROS) by irradiation with strong green light, and then inactivates fused and/or interacted proteins (23). Recently, the combined method revealed the spatio- and temporal-specific function of chromosomal proteins responsible for cell division (24,25). Moreover, the application of apoptosis induction for tumor 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 analog 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 non-folding proteins are excluded from ER and degraded (ER-associated degradation, ERAD). Thus, evaluation of the status of cargo proteins in the ER is one of the important factors for effective protein production. However, it has been difficult to evaluate the situation of cargo proteins in the ER in living mammalian cells. Recently, single molecule observation based on total internal reflection fluorescence microscopy (TIRFM) was applied for this purpose. Nagaya et al. revealed that glycosylation of cargo proteins affects on their mobility in the ER by transient immobilization on the ER membrane using TIRFM and FCS (35). Glycosylation patterns are the most important stages to assess cargo protein folding by the molecular chaperon calnexin (36). These results suggest that the mobility of cargo proteins in the ER reflects the maturation of proteins in the ER lumen. Therefore, observation of cargo protein dynamics in the ER lumen can be used for evaluation of cell lines and optimization of culture conditions for therapeutic protein production, such as temperature, osmotic pressure, and culture medium.

CELL AND ORGANELLE MANIPULATION BY TWO-PHOTON MICROSCOPE

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