CELLULAR IMAGING-BASED BIOLOGICAL ANALYSIS FOR CANCER DIAGNOSTICS AND DRUG TARGET DEVELOPMENT

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ABSTRACT

Cellular imaging may be defined as a multidisciplinary discipline that detects and analyzes cellular macromolecules with the help of microscopy and computer programming. Cellular imaging approaches allow a highly specific and sensitive inquiry for the nature of important biomolecules, whereas molecular biology techniques often lose the spatial information of biomolecules due to homogenization of cells. Many current developments such as confocal laser scanning microscopy, multiphoton, and fluorescence resonance energy transfer (FRET) advanced the understanding of molecular and functional systems in cells. In parallel, sophisticated probe technologies greatly enhanced optical sensitivity and molecular specificity at the single cell level. With the help of reliable image analysis techniques, molecular data from normal and diseased cells are generated in high throughput manners to investigate the biomarkers and drug target candidates. In this report, some recent technologies in cellular imaging are outlined and image analysis applications in molecular diagnostics and drug target development are discussed.

1. INTRODUCTION

Biomedical imaging is one of the most important tools in health care that help clinicians to perform diagnosis and treatment of human diseases. X-ray, radioisotope imaging, computed tomography (CT), and magnetic resonance imaging (MRI) offer structural and anatomical information at the tissue levels (*in vivo*). Screening for any early stage diseases and cell-based diagnosis requires biological information at the cellular and molecular biology levels. Cellular imaging techniques in diagnosis of malignant tumors deal with tissue or cells taken out of the body (*ex vivo*). Cellular imaging investigates functional and molecular changes in cells as well as morphological changes during the development of diseases or during the therapy. Cellular imaging provides a direct evidence of cell type-specific and subcellular information of a certain biomolecule, whereas traditional molecular biology techniques cannot due to the homogenization of cells. In addition, unlike flow cytometry, cellular imaging allows the interesting cells to be readily examined at any time.

Pathologists can grade the malignancy of the diseased tissue based on microscopic impression of histological features such as nuclear shape, chromatin texture, and tissue architecture. Recently, microscopic detection of biomolecules in tissue has become important to investigate functional and molecular causes of the disease. Since diseases that may appear similar morphologically can behave differently, the status of biological molecules may provide us more reliable information for assessing the malignancy of a disease. Thus, an accurate measurement of biological molecules is likely becoming quite critical in clinical decision making.

Cellular images can be analyzed through, for example, visual recognition of histopathological hallmarks or visual discrimination of immuno-staining intensity levels to estimate the amount of biomolecules existed in the cell. Although these visual tasks are considered as effective and fairly reproducible, there are numerous cellular imaging applications where manual methods prove to be inaccurate and prohibitively inefficient. For instance, a few rare cancer cells in millions of normal cells in the histological slides can be more efficiently found by a fiber-optic array scanning technology [1].

Cellular imaging technologies can generate a massive amount of biological data that contain temporal and spatial information for multiple molecular species within cells throughout tissues or in culture. Such quantitative data may support a mathematical model of cellular functions to better understand the mechanisms of disease processes [2].

2. MICROSCOPIC IMAGING MODALITIES AND PROBE TECHNOLOGY

A microscope is an optical instrument designed to magnify the image of the object, separate the details in the image, and render the details visible [3]. Besides commonly used bright-field and epi-fluorescence microscopes, two advanced fluorescence microscopes outlined in this report are confocal microscope and multiphoton microscope. In confocal microscopy, a focused laser spot excites fluorophores in labeled cells by raster scanning. Fluorescent emission signals are refocused but only signals originated from in focus planes can go through the pinhole placed in front of the detector. The removal of out of focus signals offers the optical sectioning capability as well as the increase of the resolution [4]. In a typical multiphoton microscopy, the two photons generated by pulsed lasers are focused at a scanning spot. The total energy of two photons is absorbed by fluorophores within a femtosecond time scale that results in the excitation of the fluorophore and consequently the emission of fluorescence signals. The confined excitation of the fluorophore offers inherent optical sectioning capability. Furthermore, the absence of the pinhole in the optical path increases the sensitivity significantly compared to confocal microscopy. The absorption of two photons enables the use of

longer wavelength excitation, which penetrates deeper into samples [5,6].

The critical components that convert the optical signals into digital form are charge-coupled devices (CCD) and photo-multiplier tubes (PMT). CCDs are used for transmission light and epi-fluorescence microscopes. PMTs are for scanning microscopy such as confocal and multiphoton microscopes.

Chromogens are light diffracting substances commonly prepared with substrates for colorimetric applications. Chromogen substrate systems can react with enzyme labels such as horseradish peroxidase, alkaline phosphatase, and β-D-galactosidase [7]. Specific enzymes are bound with target molecules in immuno-reaction complexes in the proximity of which chromogens precipitate. This localized precipitation of chromogen enables the detection of biomolecules under the light microscope. The use of chromogen substrate system is limited to two targets located in different subcellular compartments.

The principle of fluorescence microscopy lies on the property of a fluorophore that can be excited by a photon of a certain wavelength and emits a photon of a longer wavelength. The spectral range of fluorophores is within visible range between 500 and 670 nm, although some probes can be used in the near infrared region. Multiple targets may be detected by using multiple fluorophores simultaneously in the same cell. Fluorophores have been developed to probe individual cells, organelles, trafficking of biomolecules, pH, calcium, and redox status. They are also engineered to report ionic and nonionic second messengers and report various kinase activities [8]. However, spectral properties of fluorophores limit the use of greater than three dyes simultaneously and fluorescence of dyes also tends to fade away quickly over time. Inorganic semiconductor nanocrystals, "quantum dots" (QDs) can be excited by a single-wavelength light source and emit fluorescence whose wavelength depends directly on the size of the dot. QD conjugates with biomolecules are well suited for multicolor or multiplex applications because of their narrow (typically 20 to 40 nm) emission spectra and anti-photobleaching properties [9]. Probes explained so far are used to label biomolecules that do not have inherent signaling molecules. However, some proteins are genetically encoded to emit fluorescent signals to investigate their activities in live cells.

Green fluorescent proteins (GFP) offer a fluorescent label that can be fused into a host protein without affecting native function due to its small size [10]. For example, GFP can signal physiological activation by integrating to a host protein that translocates from cytoplasm to nucleus upon stimulation. Several mutated variants of GFP with different spectral characteristics, enabling the multiple target detection, have used to measure translocation and activation of many proteins.

Fluorescence resonance energy transfer (FRET) is a physical process by which energy is transferred nonradiatively from an excited molecular fluorophore (the donor) to another fluorophore (the acceptor) by means of intermolecular long-range dipole–dipole coupling. FRET can be detected by alteration of detectable fluorescent emission occurring when the transfer of excitation energy is allowed through an overlap in donor emission spectra and acceptor absorption spectra. The FRET effect occurs within the proximity of angstrom distances (10–100 Å). Thus, FRET can accurately measure physical interactions among proteins or among domains within a single protein molecule [11]. Several GFP variants are known to be suitable as FRET candidates (e.g., cyan and yellow). A slightly different use of FRET can be found in the enzyme substrate detection scheme. Catalytic reporter systems consist of a recombinant enzyme and an exogenous fluorogenic substrate. A reporter system based on an enzyme (e.g., β-lactamase) makes use of a substrate (e.g., cephalosporin) flanked by a pair of FRET donor and acceptor fluorophores. Recombinant β-lactamase acts to cleave the cephalosporin substrate and thus disrupts FRET between the two fluorophores. The cells that express the β-lactamase-driven gene(s) of interest can be sorted out by eliminating cells with FRET signals. A catalytic reporter offers highly sensitive probe scheme since it can be detected at a thousand times less concentration (~10^{-4} μM) than GFP that requires a concentration of 0.1–1 μM [12].

3. IMAGE ANALYSIS APPLICATIONS IN CANCER DIAGNOSTICS AND DRUG TARGET DEVELOPMENT

There are numerous scoring protocols for cancer diagnostics and drug development that demand accurate visual interpretation. To produce automated versions of these scoring guidelines, cellular imaging systems are required to offer the recognition of single cellular objects, the measurement of probes within the recognized object, the number of positively stained objects, and definition of cellular subpopulations and statistical evaluation [13]. To meet these requirements, the most critical step is the segmentation of objects from cellular images. Since individual cells touch or overlap each other in tissue or in culture, it is extremely difficult to recognize a whole cell as an object. Often, automated analysis can be performed only from the cells whose boundaries are well defined.

In treating advanced breast cancers, the patients with high level expression of HER2 protein can be treated by the monoclonal antibody therapy, Trastzumab. A guideline for scoring HER2 protein expression using immunohistochemistry (IHC) (HercepTest™, DAKO Corp, Carpinteria, CA) involves visual estimation of membrane immunostaining intensity (gray value) and the percentage of cells with completely intact membrane staining (%CM). If more than 10% of cells have medium to intense staining in completely intact membrane, the specimen is scored positive (*i.e.,*. overexpressed).

With the help of cellular imaging software called Hersight™ from Applied Imaging Corp (San Jose, CA), automated HER2 scores could be generated. In Fig. 1.a, immuno-staining patterns of cell membrane bound HER2 is shown in bright field micrograph. In Fig. 1.b, software detected positively stained cell membrane. Briefly, cell membrane was segmented by applying a "well" operation with a 5 by 5 structuring element on the intensity image [14]. Binary membrane mask was obtained after applying thresholding. The gray value was measured based on this mask. In Fig. 1.c, nuclear objects were detected by a blob detection algorithm [15]. Segmentation artifacts were removed according to their shape features such as roundness, ferret ratio, and area. In Fig. 1.d, nuclear objects with completely intact membrane were shown. A seed fill was performed with each labeled nucleus as seed and membrane mask as limit, so that an isolated nuclear region was created if a nucleus (or nuclei) was surrounded by completely intact membrane. A nucleus with completely intact membrane was found only if the corresponding

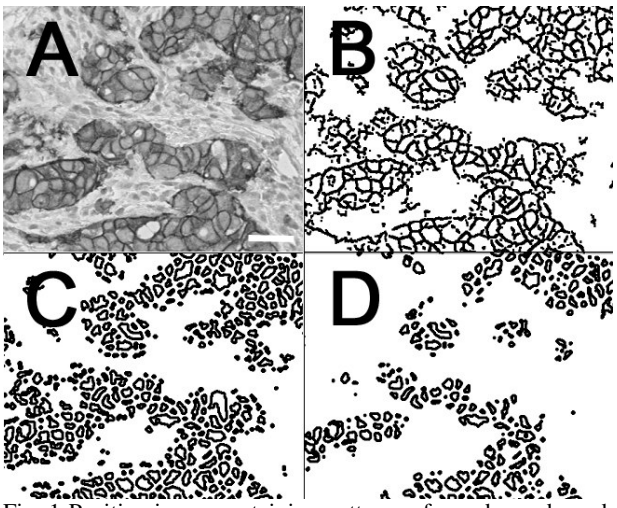

Fig. 1 Positive immuno-staining patterns of membrane bound HER2 protein (Bar: 20 μm) (A); Cell membrane (B); Nuclei (C); Nuclei with completely intact nuclei (D).

seed filled region contained a single nucleus. The %CM was calculated by 100 • the number of nuclei with completely intact membrane over the total number of nuclei. A training set consisted of randomly selected 25 negative and 25 positive images that were scored previously by experts. Positive gray values and %CM were set at 187 and 10% that separated the above two groups perfectly. Software classified 636 test images with 94.5% accuracy (109 true positives, 35 false positives, 492 true negatives, 0 false negatives). False positive images showed borderline gray values (from 171 to 187) that may be used to prompt expert's attention to confirm the suggested positivity.

Fluorescence *in situ* hybridization (FISH) detects disease-related abnormalities in tissue using fluorescently labeled DNA probes bound to genetic targets. FISH offers much higher sensitivity than standard cytogenetic methods, although FISH tends to be more expensive and labor-intensive. In finding overexpression of Her2/neu gene, FISH based analyses produced more accurate result than IHC based ones [16]. The guideline for scoring Her2/neu gene amplification using FISH (PathVysion™, Vysis, Downers Grove, IL) involves visual estimation of the ratio between the number of gene signals and reference chromosomal signals in a field of view. If the ratio is greater than 2.0, the specimen is scored positive (*i.e.,* amplified).

In an automated analysis, FISH signals in each fluorescent channel were digitized in multiple focus planes with the help of a motorized stage (z-axis) since signals are distributed in the 3D cell nucleus. A series of images was, then, transformed into a 2D image by projecting the brightest intensity values along the z-axis at each pixel location [15]. Thus, unreliable signal detection in each 2D plane and time consuming 3D processing were avoided without losing true rendition of 3D signal distribution. In Fig 2.a, a breast cancer specimen shows amplified Her2/neu gene signals and reference chromosomal signals. Cellular imaging software from Applied Imaging Corp (San Jose, CA), Neusight™, segmented each nucleus (in highlighted contour) reference chromosomal signals (in boxes) and amplified Her2/neu gene signals (in highlighted mask) (Fig 2.b and Fig. 2.c). Briefly, each nucleus was segmented by tophat

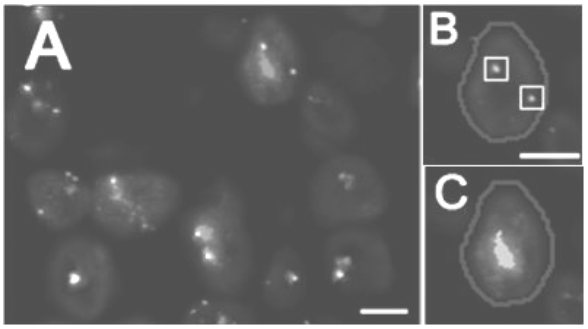

Fig. 2 Amplified Her2/neu gene signals by FISH in a breast cancer specimen (Bar: 5 μm) (A); Automatically detected two chromosome signals (Bar: 5 μm) (B); Amplified Her2/neu gene signals (C)

operation and artifacts were removed according to predetermined shape and size criteria. FISH signals within the nuclear mask were segmented by a similar method that used a smaller structuring element. Autofluorescent artifacts were removed if FISH signals at the same location were equally strong in multiple fluorescence channels. Gene signals were calculated by dividing the signal area with a predetermined average area of single gene signals. Isolated chromosomal signals were counted. The ratio of the gene signals and the chromosomal signals was obtained in each field of view. The ratio data were highly correlated to the manual ratio data generated by visual counting ($\rho = 0.93$, $n=31$, $p < 0.01$). The automated method accurately discriminated all Her2/neu amplified cases with the ratio > 2.0.

With the introduction of genomics age, the drug development paradigm has been changing significantly to take advantage of the power of genomics-based approaches. Traditional ways of drug development heavily relied on empirical selection of candidate compounds without previous knowledge on the gene(s) responsible for a disease process. In contrast, drug development in recent years begin with the process of identifying critical target genes of the disease via a genomics-assisted R&D pipeline and screen for and develop compounds that can specifically modulate the activity of the target gene. Therefore, in the future drug development it becomes increasingly important to accurately identify the molecular process whose alteration caused a disease and define the critical gene that should be targeted for therapeutic intervention.

The cellular imaging system can be a powerful high throughput tool for identifying drug targets in a sensitive and reliable manner [17]. Fig. 3.a shows the image of cells where a potential cancer gene has activated the GFP reporter system that was designed to monitor the aberrant activity of a carcinogenesis pathway. Briefly, an oncogenic candidate gene was introduced into cells. If the gene had oncogenic potential, the overexpression of the gene would activate a relevant transcription factor (TF) as predicted by a proven carcinogenesis process. Then, the activated TF would be able to bind to the TF binding sites in the recombinant DNAs (Fig. 3.b.) already introduced into cells. This binding turned on the expression of GFP reporter gene that was eventually detected by a fluorescence imaging system as in Fig. 3.a. The candidate cancer gene indeed

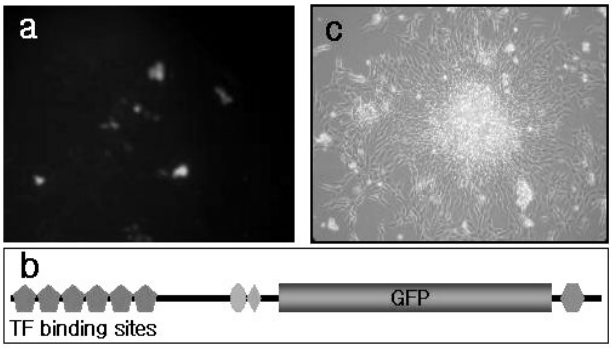

Fig. 3. Fluorescence microscopic image of cells that accumulated green fluorescent protein (GFP) after the introduction of a candidate drug target gene (A); Schematic diagram of the GFP reporter construct for a transcription factor (TF) whose activity was monitored in the cellular imaging assay (B); Multi-layered growth pattern shows tumorigenic activity of the candidate oncogene identified from the cellular imaging-based drug target assay (C).

demonstrated a strong oncogenic activity shown as multi-layered growth pattern when tested in a tissue culture system (Fig. 3.c).

The imaging-based drug target identification is considerably more sensitive than conventional biochemical assays since it can reveal the signal at a single cell level. The technology may well be applied as a tool for functional analysis of large number of disease-associated genes identified by gene expression profiling such as DNA chip analysis. Accordingly, the imaging-selected candidate genes need to be validated further by biochemical, genetic and clinicopathological means before they can be selected as the ultimate target for drug screening. The imaging-based target identification system may be adapted for a cell-based, high contents drug screening with appropriate modifications.

4. SUMMARY AND PERSPECTIVES

Advanced microscopy and probe technologies were briefly outlined and image analysis applications were introduced in the fields of molecular diagnostics and drug target development. In combination with vast amount of biological databases and bioinformatics tools, advanced cellular imaging meets the challenge of realizing molecular diagnostics and drug target development.

Although gross histologic changes can be efficiently recognized a human observer, quantitative description of such complex cellular events can be often beyond our capabilities. A substantial challenge to cellular imaging comes from the fact that an increasing amount of cellular parameters and molecular markers, as well as their interrelations have to be determined on a single-cell level and in a quantitative manner. Cellular imaging-based analysis equipped with a variety of computer vision tasks will continuously increase the accuracy and reliability of measuring cellular biomolecules detected by highly advanced probe technologies and microscopy.

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