Novel Biological Imaging Techniques Using Functional Nano-particles

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Abstract

Recent advances in nano-biotechnology let us have an expectation to revolutionize prevention, diagnosis and treatment of disease. Nano-materials for medical applications are expected to grasp pharmacokinetics and the toxicity for application to medical treatment on the aspect of safety of the nano-materials and nano-devices. We describe generation of CdSe nano-particles (Quantum Dots (QDs)) conjugated with monoclonal anti-HER2 antibody (Trastuzumab), for single molecular in vivo imaging of breast cancer cells. We established a high resolution *in vivo* 3D microscopic system for a novel imaging method at molecular level. The cancer cells expressing HER2 protein were visualized by the nano-particles in vivo at subcellular resolution, suggesting future utilization of the system in medical applications to improve drug delivery system to target the primary and metastatic tumors for made to order treatment. We also describe novel fluorescence measurement by fluorescence tomography based on acousto-optic modulation imaging for sentinel node navigation using fluorescent nano-particles for breast cancer surgery in experimental model, which have shown the potential to be an alternative to existing tracers in the detection of the sentinel node. We developed novel contrast media of silica coated silver iodide beads. This contrast medium has a unique property of long lasting contrast enhancement. It would be used for pre-operative marking of tumors for complete dissection. Future innovation in cancer imaging by nano-technology and novel measurement technology will provide great improvement, not only in clinical field, but also in basic medical science for the development of medicine.

1. Introduction

Molecular targeting anti-cancer therapeutics by tumor-specific antibodies has been of great interest in basic science such as oncology, pharmacology and nano-medicine. This approach will allow to increase therapeutic efficacy and to decrease systemic adverse events. Quantitative investigation of dynamics of such drug delivery *in vivo* is crucial to enable the development of more effective drug delivery systems. One of the best ways to perform this is to apply new technology on biophysics that the positions of proteins are detected quantitatively at single molecule level with nanometer resolution.

However, the specific delivery processes *in vivo* is not known at single particle level. Conventional imaging modalities such as computed tomography, magnetic resonance imaging, positron emission tomography and organic fluorescence or luminescence imaging have insufficient spatial and temporal resolution to analyze the pharmacokinetics of drugs at the single particle level *in vivo*.

To address the issue, real-time single particle tracking using quantum dots (Q-dots) has been applied to the study of drug delivery. Q-dots fluorescence nano-crystals, were expected to be as the good biomarker because of their intense brightness and stability, in contrast to existing organic dyes and GFP. In cultured cells, single particle tracking has yielded invaluable information on the function of purified proteins.

Recent work shows the antibody-conjugated Qdots have allowed real-time tracking of single receptor molecules on the surface of live cells. However, no real-time single particle tracking in live animals have been reported, and it is uncertain that single particle of Qdots could be observed or tracked in live animals. We analyzed the movement of single functional Qdots in the tumor of mice from a capillary vessel to cancer cells by a highly sensitive measurement system.

2. Materials and Methods

2.1. Single molecular imaging of an anticancer agent

We made conjugations of Qdot (Quantum Dot Corporation, Hayward, CA) and trastuzumab (Chugai

pharmaceutical Co., LTD., Tokyo, Japan) with a Qdot 800 Antibody Conjugation Kit (Quantum Dot Corporation, Hayward, CA) via poly ethylene glycol (M.W. 2000) and heterobifunctional cross-linker 4-(maleimidomethyl)-1-cyclohexanecarboxylic acid N-hydroxysuccinimide ester (SMCC).

The final concentration of Qdots and trastuzumab complexes (QT-complexes) was determined by measuring the conjugate absorbance at 550 nm and using an extinction coefficient of 1,700,000 $M^{-1}cm^{-1}$ at 550 nm.

The human breast cancer cell line KPL-4, which overexpresses HER2 and is sensitive to trastuzumab, was kindly supplied from Dr. J. Kurebayashi (Kawasaki Medical School, Kurashiki, Japan). KPL-4 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum. A suspension of KPL-4 was transplanted subcutaneously to the dorsal skin of female Balb/c nu/nu mice at 6-10 weeks of age (Charles River Japan, Yokohama, Japan). Mice bearing a tumor volume of 100-200 mm^3 were selected for experiments. All operations on animals were in accordance with the institutional animal use and care regulations. This study was approved by Animal Care and Use Committee of Tohoku University.

QT-complexes were injected into tail vein of mice. The mice were placed under anesthesia by the intraperitoneal injection of a ketamine and xylazine mixture at a dosage of 95 mg/kg and 5 mg/kg, respectively. The temperature of mice was maintained at 37 ^oC by a thermo-plate and objective lens heater. The modified DSFC method was used to fix the exposed mouse tumor on the stage of the microscope. Two sterilized polyvinyl chloride plates (0.5mm thickness) containing a window were mounted so as to fix the extended double layer of dorsal skin including the tumor site. Skin between chambers sutured around the window, and the tumor could be located in the center of the window and fixed without influence from the beating of the heart and breathing. The tumor was placed surface down on the neutral saline mounted cover slip on a viewing platform of an inverted microscope. The mouse was fixed to a metal plate on the stage designed to stabilize the chamber. Tumors can be visualized directly by means of this set-up.

The mice were sacrificed by CO_2 overdose, after imaging. The tumors were removed and divided for histological and immunohistochemical examination. In the histological Qdot uptake study, tumors were frozen and cryosectioned 6 microns thickness, fixed with acetone at 0^oC and examined with an imaging system. For immunohistological examination, tumors were fixed in 10% neutral-buffered formalin overnight and then transferred into ethanol before processing and paraffin embedding. Immunohistochemical analysis was performed on paraffin sections at 6 microns thickness using the HercepTest (Dako Cytomation, CA) to confirm HER2 expression.

As shown in Fig. 1, the optics system for 3D observation consisted primarily of an epi-fluorescent microscope (IX71, Olympus) with modifications, a Nipkow lens type confocal unit (CSU10, Yokogawa) and an electron multiplier type CCD camera (iXon 887, Andor). The confocal unit adopts multi-beam scanning using about a thousand beams that are simultaneously emitted through a pin-hole disk to facilitate high-speed scanning. And the EMCCD has advantage that offering unsurpassed sensitivity performance, and has been shown to yield markedly improved signal/noise ratio. The objective lens was moved by a piezo actuator with a feedback loop for stabilizing the position of the focus. A computer controlled the piezo actuator in synchronization with the image acquisitions in order that the objective lens remained within the exposure time of the CCD camera. An area of ~30 x 30 μm^2 was irradiated by a green laser (532 nm, Crystalaser).

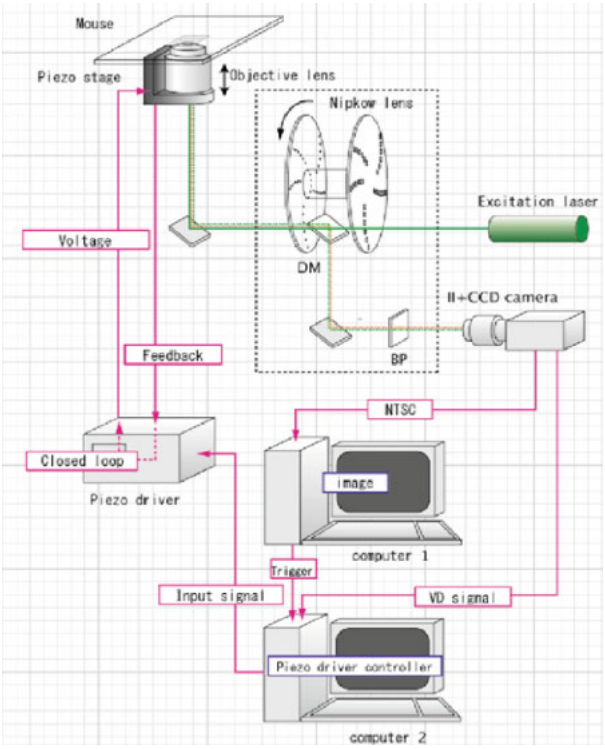

Fig. 1. The optics system for 3D observation

The xy-position of the fluorescent spot was calculated by fitting to a 2D-Gaussian curve. The single molecule could be identified by the fluorescence spot. And quantitative and qualitative information such as velocity, directionality and transport mode was obtained using time-resolved trajectories of particles. The resolution of the position was determined from the position of immobile QT-complexes in a chemically fixed tumor cell. The resolution of the x and y directions of images taken at an exposure time of 33 ms was 30 nm taking into consideration the standard deviation.

2.2. Fluorescence tomography based on acousto-optic modulation imaging

The experimental setup comprises a continuous wave diode pumped solid state laser system Verdi V-6; Coherent, Inc. as a light source, an ultrasound transducer, and a photomultiplier tube (PMT) as a detector (Fig. 2). The laser beam is reduced and collimated to 1 mm diameter and enters the water tank through a glass window. A focustype ultrasonic transducer (38 mm focal length, 3 mm focal diameter, V314-SU; Olympus-NDT) that is driven by a 1 MHz continuous sinusoidal wave is incorporated into the side wall of the water tank, where the ultrasound beam traverses the incident axis of the laser beam. Sound pressure in the sound-field focus region was 4.1X104 Pa in water.

The signal from the PMT is fed into a spectrum analyzer and the intensities of the resonant frequencies are detected using a narrow bandwidth (100 Hz). To obtain a two-dimensional tomographic image of fluorescence intensity through scanning of the ultrasound focus, a water tank equipped with a transducer is mounted on a two-axis translational stage and scanned in 500 μ m steps along the *X* axis (parallel to the incident laser beam) and the *Y* axis (parallel to the ultrasound beam).

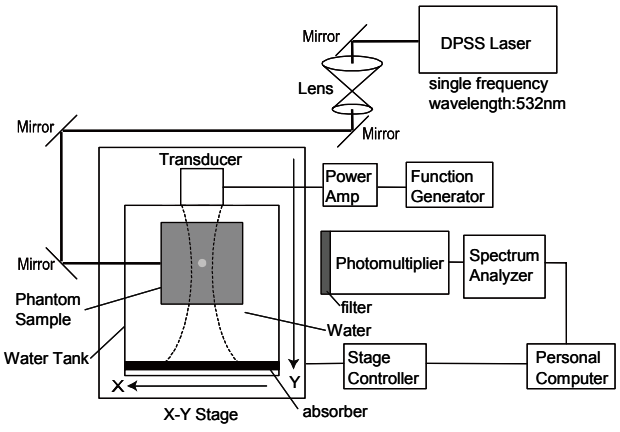

Fig. 2. Fluorescence tomography based on acousto-optic modulation imaging

It is controlled using a personal computer that is synchronized with a spectrum analyzer. A tissue phantom is suspended independently from the holder, which is located above the water tank; the phantom is immersed in the water without touching the water tank. The optical arrangement of the phantom and incident light remains unchanged during scanning of the ultrasound beam. The imaging experiments are conducted using a wavelength of 532 nm and an incident beam signal intensity of 60 mW.

The light modulation mechanism is inferred to be induced through density variation of the medium in the ultrasound field, which engenders changes of the refractive index and optical scattering coefficient. When fluorescent pigment is present in the sound field, the density variation of the medium engenders modulation of the photon density through deflection of light between two successive scattering events in the gradient of the refractive index, thereby causing fluorescence intensity modulation. Variation of the scattering coefficient also causes modulation of the fluorescence intensity.

We used gel tissue phantoms of isotropic light-scattering media made of 5% agar (Inagel; Ina Food Industry Co. Ltd., Nagano, Japan) that were prepared through dilution of Intralipid (Intralipid 10%; Fresenius Kabi AG, Germany), which is a clinically useful fat emulsion that is often used for tissue phantom studies to elucidate light propagation in scattering media. The final volume concentration of Intralipid in the phantom was 40 ml/ *l* in 5% agarose gel with the water-glycerin (20%) solution. The agarose gel was molded to 40X40X75 mm^3.

A fluorescent region in the phantom was formed by embedding a fluorescent material that contains fluorescent microspheres (530 nm absorption peak, 590 nm emission wavelength, fluoresbrite carboxylate microspheres, NYO; Polyscience, USA) molded with columnar agarose gel (5 mm long, 3 mm diameter).

The reduced scattering coefficient of the phantom was estimated as 0.61 mm^{-1} at the excitation wavelength.

2.3. Nano-sized silver iodide beads as X-ray contrast media

We used the silver-iodide I ultra-fine particles with silica coating in this experiment (Fig. 3). Silica-coating was performed to prevent acute and fatal reactions. We used several kinds of different size (20-100nm) of AgI beads. Silica coating beads were made by the Stober method.

We administered 58.6mg/kg AgI beads (28.3±5.9nm) into a rabbit by intravenous injection, and we took images by a X-CT to evaluate the enhancement of AgI beads to cancerous lesions.

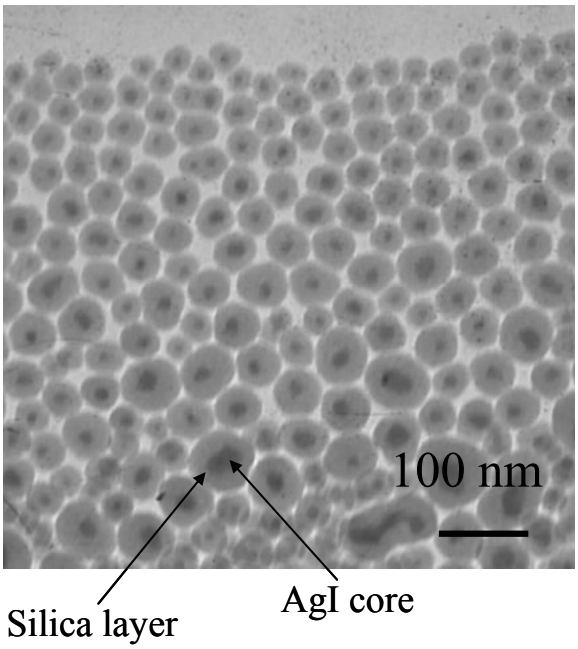

Fig. 3. TEM image of silica coated AgI beads

3. Results

3.1. Single molecular imaging of an anticancer agent

Two hours after the injection, many complexes had migrated into the tumor interstitial area close to the tumor vessels. Six hours after the injection, QT-complexes had bound to the KPL-4 cell membrane on which the HER2 protein is located. We also observed in pursuing the transport of QT-complexes from the peripheral region of the cell to the perinuclear region (Fig. 4).

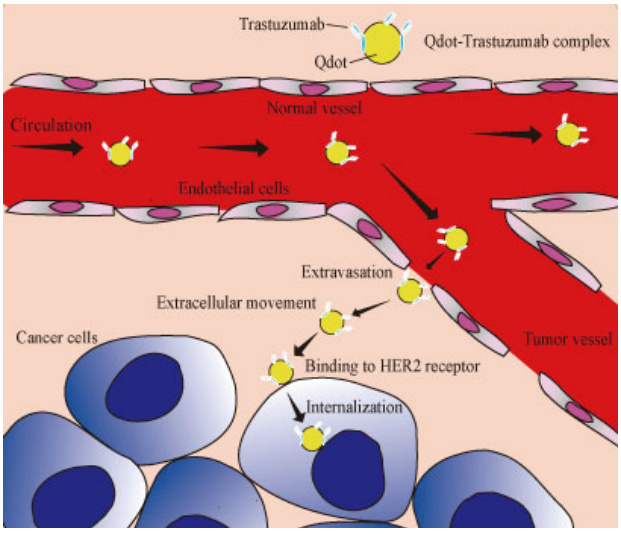

Fig. 4. Tranport of the QT-complex *in vivo*

3.2. Fluorescence tomography based on acousto-optic modulation imaging

We could observe two fluorescent regions in a phantom with an embedded 9 mm gap along the *X* axis. Both the image Fig. 5(a) and the *X*-axis profile Fig. 5(b) show the two well-separated fluorescence peaks, which correspond to the embedded positions of both regions, as shown in the photograph sectioned on the longitudinal plane of the phantom Fig. 5(c).

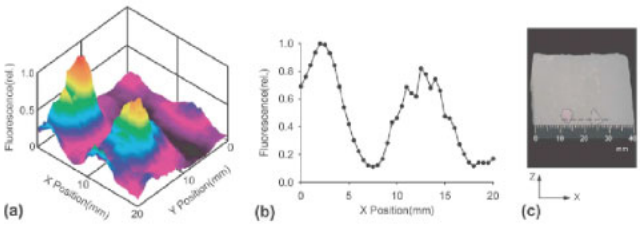

Fig. 5. Fluorescence intensity profile of the two fluorescence dye embedded in turbid media

3.3. Nano-sized silver iodide beads as X-ray contrast media

The liver and spleen were enhanced gradually for 60 minutes (Fig. 6). The enhancement was maintained for 24 hours. Then enhancement was gradually reduced for 7days. The enhancement was completely disappeared by 7 days.

4. Discussion

We successfully captured the specific delivery of single QT-complexes in tumor vessels to the peri-nuclear region of tumor cells in live mice after QT-complexes had been injected into the tail vein of mice. Six stages were detected (Fig. 4), 1) vessel circulation, 2) extravasation, 3) movement into the extracellular region, 4) binding to HER2 on the cell membrane, 5) movement from the cell membrane to the perinuclear region after endocytosis and 6) in the perinuclear region. The transport speed of QT-complexes in each process was highly variable, even in the vessel circulation. The movement of the complexes in each process was also found to be ”stop-and-go”, *i.e.*, the complex remaining within a highly restricted area and then moving suddenly. This indicates that the movement was promoted by a motive power and constrained by both the 3D-structure and protein-protein interactions. The motive power of the

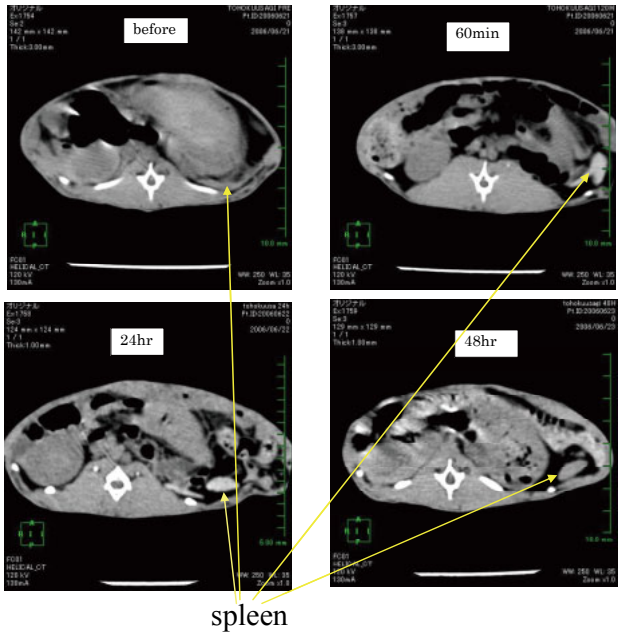

Fig. 7. Changes in enhancement of spleen by AgI beads

movements was produced by blood circulation (essential in processes 1 and 2), diffusion force driven by thermal energy (2, 3 and 4) and active transport by motor proteins (5). The cessation of movement is most likely induced by a structural barricade such as a matrix cage (2, 3 and 4) and/or specific interaction between proteins, *e.g.*, an antibody and HER2, motor proteins, and rail filaments such as actin filaments and microtubules.

The molecular mechanism underlying the movement and its cessation during delivery of nano-particles in animal models is the fundamental basis of drug delivery. There have been many different approaches to tumor-targeting "nanocarriers" including anti-cancer drugs, for passive targeting such as Myocet, Doxil and for active targeting such as MCC-465, anti-HER2 immunoliposome. There is still very little understanding of the biological behavior of nanocarriers, including such crucial features as their transport in the blood circulation, cellular recognition, translocation into the cytoplasm, and final fate in the target cell. These results suggest that the transport of nano-carriers would be quantitatively analyzable in the tumors of living animals by the present method. This approach thus should afford great potential new insight into particle behavior in complex biological environments. Such new insight in turn will allow rational improvements in particle design to increase the therapeutic index of the tumor targeting nanocarriers.

Nanocrystal semiconductor quantum dots conjugated with antibody may serve fundamentally as new controllable materials for medical purposes including cancer molecular imaging.

This new technology of fluorescence measurement serves possibility of nano-sized fluorophores as clinical diagnostic agents as well as markers for basic research. It may become as a key technology to overcome the limit of fluorescence measurement. New applications of fluorophores are expected as made to order surgery based on accurate detection of cancer.

The depth of targets is a serious problem in fluorescence measurement of living tissue. The local excitation illumination within tissue exponentially attenuates due to absorption and scattering from the surface to that depth. This problem of lack of transmission prevents us from detection in tissues deeper than 1 cm from the surface of the body at present [10]. We can detect the SN of small animals like rats, but may have difficulty in detection in larger animals because of the depth at which SNs. For example, lymph node in human is buried in fat and it locates deeper than 1 cm. Detection technique to find SN up to 2 cm depth is recommended. To solve this problem, we should select appropriate wavelength of fluorescence and fluorescent materials, and also develop imaging techniques. Hemoglobin absorbs light in the range of visible light below 650 nm, and water absorbs light above 1100 nm. But in the near infrared range between 650 and 1100 nm, the absorption of light in living tissue is minimum. This range is called the optical window. Besides collagen, NADH and FAD are substances that *in vivo* have the fluorescent wavelengths in the range of 400 to 500 nm. So, from this point of view, NIR range has the advantage for the fluorescence measurement. We are also investigating the application of semiconductor nano crystal that has extremely stronger fluorescent intensity than usual fluorescent beads to increase the detection ability and fluorescence tomography based on acousto-optic modulation imaging.

The AgI beads were coated with silica. This coating has a multifunctional effect. The silica coated particles have considerable stability and durability. Physically, the surrounding structure prevents adverse particle effects; for example, an allergic reaction to iodine. Silica coating can be performed for various types of particle. The thickness of the silica can be adjusted to modify the size of the particle. The size of nano-particles may play a important role to enhance cancerous lesion, for vessels in cancerous lesion has larger pores than normal vessels. The large pores of

tumor vessels arrow nano-particles in size less than 100nm to pass vessel walls. It can be used for the development of drug delivery system for cancer treatment and diagnosis.

Nano-biotechnology should be a great aid to improve the tailor made medicine by their hyper sensitive and super selective property for diagnoses. Advanced sensing technologies such as single molecule imaging technique and acousto-optic modulation imaging technique are also required to make the best use of the functional nano-materials for achievement of hyper sensitive and super selective imaging. These novel products of advanced technologies may realize revolution of medicine in near future.

Acknowledgement

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