Up-and-coming Complementary Imaging Technique in Translational Nanomedicine; MALDI-IMS, Confocal Microscopy and NIRF

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Matrix-assisted laser desorption/ionization - imaging mass spectrometry (MALDI-IMS) is a newly emerging technique in nanomedicine to evaluate the 3D nano-drug distribution within the experimental tissue. Time-dependent mapping provides detecting the penetration of metabolites among the cells by ion density image which can be obtained instantly up to 5 µm without requiring any specific labels (label-free technology) in order to monitor the distribution of drug. Imaging the location of novel metabolites can be discovered by the aid of a suitable photoactive MALDI matrix. As well as, the nanoparticles can be followed up precisely by three-dimensional confocal microscopy technique in wide time range. Both equilibrium phase materials (nanocrystals) and non-equilibrium phase materials (nanogels) can be visualised up to 20 µm to maintain monitoring particle interactions throughout cells in tissue by confocal microscopy. Real space in real time analysis of nanomedicines would be achieved by this powerful technique. As the final complementary imaging technique, NIRF (near-infrared fluorescence) provides *in vivo* monitoring of nanomedicines during the blood circulation of drugs. Especially in the field of anti-cancer imaging, this technique is very advantageous during progress of disease by versatile fluorescent probes, in near infrared region. NIR fluorophores maintain less scattering during deep photon (cm based) penetration into the tissue. Above mentioned complementary imaging techniques as an Up-and-Coming opportunity for translational nanomedicine (TNM) applications will provide time-effective approach for investigation of new pharmaceuticals.

Keywords: MALDI-IMS; Confocal Microscopy; NIRF; translational nanomedicine; complementary imaging

1. Nano-enabled imaging technology for personalized therapy

Nano-enabled devices are convenient for improving successful development of simultaneous imaging and therapy. Thereby, multifunctional nanomaterials have been expanding by interwining drug delivery, molecular imaging and clinical oncology. Since the molecular diversity through the body is a formidable challenge, bringing cellular phenotypes to light which will guide target-specific drug delivery is essential. After the era of generic medicine put forward *one-size-fits all* has been converted to personalized medicine by the aid of multimodal and complementary imaging for gaining anatomic, pharmacokinetic and pharmacodynamic information about drug metabolism [1, 2].

1.1 Early defined target/off-target nanodevices shorten the time to translate the technology

Today, amount of nanomaterials have tremendously increasing in both scientific and industrial fields. Range of drug efficiency, distribution and clearance of the candidate material influence both its target and off-target conditions concerning various types of cancer [3]. In reality, commercial potential of the candidate drug depends on safety in order to venture the risk to invest time and money. The key point is to be able to evaluate the candidate drug in early stage at the bench before moving on [4]. The most promising and newly arised microscopic approaches have been tested for the diversification of success translation to the clinic swiftly, such as IMS tissue biopsies [5]. Leveraging the technology from preclinical to clinical stage would be possible by three complementary imaging techniques; MALDI-IMS, Confocal Microscopy and NIRF over the bench side in earliest stage of drug development [5-12].

Considering the biodistribution of the candidate drug can be trailed in general optically in preclinical stages by the aid of consuetudinary florecent molecule. In fact, desired intention is to evaluate not only its action among the cells that it prefers; but also, measuring the response of cells that had been affected [11, 12]. Early defined off-target nanodevice shorten the time to translate the technology to the clinical stage. Even if, elegant nanodevice could be designed, the vast majority do not reach to the tumor tissue. The other crucial challange is where unbounded ones acumulate in another part of the body that can cause further complications. The most superior nanodevice, which would be only effective in tissue of interest, must be designated before clinical trials [3, 13]. Advanced microscopy techniques can shorten the time during early bench studies in order to translate nanodevice to clinical stage during therapy to patients [6, 12].

The potential pharmocokinetics behavior thereof reflection actual chemical insight of candidate material could essantially display the behavior of nanomaterials *in vivo*. Faster assesment of candidate material depends on imaging potential both *in vitro* and *in vivo* during early bench stages. *In vivo* application of traceable candidate nanodevice within the cell and throughout the whole body would give real clue to invest money. From investor pespective, translation can be able to carried out by reducing risk before moving technology to the clinic [3]. MALDI-IMS, Confocal Microscopy and NIRF would be attitude as a whole imaging technique as the *Up-And-Coming Complementary Imaging Technique* in early stages [5-10, 14, 15].

The understanding just before to go beyond the imaging by using MALDI was only possible by visualisation just as other microscopic techniques. Since early beginning, this technique has been developed via by the simple sample preparation, broad range observation of vast of molecules and continuous improvement of its handy software especially in medical applications. Today, MALDI-IMS become a widely used technique for structure-specific molecular imaging based on the mass differences in tissues, which has been impossible by classical microscopic techniques.

Complex metabolism of glycans, lipids and proteins can be simply analyzed. Likewise, biomarkers of pathological stages can be visualized such as molecular portrait during the progress of disease and drug metabolism. This technique has become an advantage through biopsy during histopathological evaluations; and, especially for cancer diagnosis and therapy even during surgical operations; such as, *iknife* [16, 17]. Although MALDI-IMS has many drawbacks, the technique is under improvement for clinical applications. These drawbacks are totally related with standardization and medical validation which includes the poor sensivity of measurement, low resolution, restrained analysis range and lack of result reproducibility [18].

On the other hand, the laser confocal microscope provides analyzing the intracellular regions of several cell components even under cellular trafic during the nanoparticle penetration progress. Technique is useful for intact, alive tissues. In fact, it can be also possible via labelling by using organic dye, crystal or SWCNT should be used into nanoparticle under ~200 μm in order to visualize its cell penetration [19].

Near-infrared fluorescence (NIRF) is a newly emerging *in vivo* imaging strategy which is used with the purpose of cancer detection and characterization, even during surgical operations. It has high dimensional resolution, high sensitivity and advantage of real-time applicability. It provides the detailed molecular information about targetted tumors by using special fluorescence-imaging agents which could be excited near-infrared wavelengths. By illuminating the cell surface, NIR light could penetrate beneath the tissue up to 3-4 cm with less scattering in order to obtain a 2D-image. NIRF would overcome the limitations or disadvantages of other imaging techniques as a complementary module. The superior advantage of NIR fluorescence would be very valuable for both preclinical and clinical imaging applications of cancer treatment [20-22].

1.2 Nanotheranostics: New Materials for Image-Guided Therapy

Theranostic has been represented by combining diagnose and theraphy in order to improve drug efficiency,which first named by Funkhouser and coworkers in 2002 [23]. Nanotheranostics term has been defined as nano-based materials that bears both imaging agent and therapeutics to carry out real time biodistribution of material for the dose modification; site specific therapeutic release to reduce off-target toxicity and suitable targeted therapy for personalized medicine[24]. Especially for the cancer, *intra- and inter-* heterogeneity of tumor designates the fate of therapy; so that, the obtained specimen from physical biopsy generally fails in phenotypic characterization. Clinicians indicate primary tumor cells that can represent totally different phenotypes from metastatic foci, insomuch that, diverse cancer phenotypes can come together within a given tumor. Besides, adaptive resistance of the tumor cells through the therapy is an undeniable fact. Molecular profiling of the body and multitargeting theraphy is not enough to overcome cancer cells; so that sometimes tumor could inevitably evolve and repeat itself. Therefore, real-time imaging and modified targeting are the main issues of the theranostic nanomedicine to recompense cancer cell resistance. Even if, actual imaging methods; such as, PET, CT and MRI can provide great resolution; new multimodal and complementary imaging techniques have been needed more and accordingly evolved and experimented for accurate pharmaceutical denouement[1]. Nanotheranostics should be in small range size, approximately prefarable 10-100 nm spherical, have high drug loading capacity, capable in targeting to the desired tissues with minimal off-target effect, high drug bioavailability, biocompatibility, and sophisticated imaging in order to monitor the theraphy. Optical, ultrasound, magnetic and nuclear imaging could be feasible by the aid of Synthetic Fluorophores, Semiconductor Fluorescent Crystals, Quantum Dots (QDs), Single-Walled Carbon Nanotubes (SWTNs), Special Dyes (indocyanine green, NIR region fluorescent cyanine forms, dialkylcarbocyanine fluorophores), for monitoring by different imaging techniques.Therefore, MALDI-IMS as the most promising label free technology that can be complementary alternative way for enlightening route of the therapy [2, 18, 25, 26].

2. MALDI-IMS Imaging Technique

Traditional imaging techniques just as histological staining, magnetic resonance imaging (MRI) establish cursory location of pictures of the anatomy and the physiological processes of the body; on the contrary, MALDI-IMS techniques lay bare molecular information in the biological samples. Moreover, spatial examined samples by using MALDI-IMS can be reanalyzed with other classical imaging techniques after matrix precisely removed without damaging the biological sample as label-free. Thereby, comparative results can be lapped in order to assess real structural and molecular view of sample that put into completely a new standpoint for imaging technology.

The oldest technique, SIMS (secondary ion mass spectrometry) which is firstly developed and introduced in 1962 by Castaing & Slodzian by examining desorption and ionization processes [27]. Then the technique was applied first to biological samples by Winograd and Todd [28, 29]. DESI-MS (desorption electrospray ionization-mass spectrometry) is the youngest technique developed by Cooks research group, which its importance -as the ambient mass spectrometry techniques- enable rapid analysis in original state of biological sample [30]. Even if, DESI-MS techniques served as recognizing tumor parts of the tissues in several cancer types by analyzing lipid constituents, the most notable feature is subtyping the grading of tumor cells by high cross-validation [31]. Most recent commercial project, *iknife technology* has been developed in order to gently seperate tumor cells from healthy tissue, which is established by MS analysis even during electrosurgical dissection in real time without any precursor histopathological analysis [16].

Even if, among the three major IMS approaches; SIMS, DESI and MALDI are complementary, MALDI-IMS has been widespread and soft approach since 1990s [17, 32, 33]. Actually, MALDI-IMS method is simple and useful and provide less complicated data. The analyzed sample is subjugated to the suitable matrix which includes of small organic molecules designated according to pulsed-laser beam absorption of the matrix-analyte crystals (Table 1). Molecules through the surface are incorporated into formed matrix crystals which properties (size, shape, etc.) are related to the solvent, incubation time and matrix concentration. Laser beam cause energy absorption that trigger explosive desorption of the matrix-analyte crystals that enable sample ionization [34, 35].

Table 1 Well-known Matrix Types and examples from applied tissues for MALDI-IMS

Abbreviation	Matrix Name	Applied Tissues	Reference
(CHCA)	α-cyano-4-hydroxycinnamic acid	human colon carcinoma cell line HCT116	[36]
(DHB)	2,5-dihydroxybenzoic	neuropeptides in pituitary tissue	[37]
(SA)	sinapinic acids	12-µm coronal brain sections	[38]
(9-AA)	9-aminoacridine	phospholipids and sulfatides of rat brains	[39]
(DNPH)	2,4-dinitrophenylhydrazine	paraffin-embedded samples	[40]

MALDI-IMS with TOF can provide lateral resolution up to 10 mm and high mass range (100,000 Da) for MS-MS characterization. When it is desired to analyze distribution of low molecular mass ; such as, small molecules, drugs etc., incorrectly applied matrix cause delocalization of the molecules [41]. Solving this problem can be possible by using optimized amount of suitable matrix by spraying onto the sample surface by rapid action during the experiment. The other prefarable technique is radiolabelling by using radioisotopes. This technique provides monitoring of the desired compound after radiolabelled with a stable radioisotope in the body. The major challange is mislabelling thereof the inability of drug discrimination in the organism from its metabolites. Moreover, labelled part of the drug may be moved away that radiated by an additinal signal just as a labelled real drug.

Contrarily, MALDI-IMS technique unambiguously designates drug in defined tissue from its metabolite.The future prospect of the developer is to display real pharmacokinetic and pharmacodynamic behaviour of drug in the targetted tissue [42-44]. Drug metabolism and its interaction of endogenous substances are other parameters to forecast the personalized therapy. Although, it seems to realize three promising MALDI imaging techniques, IMS ionization approach is more useful and widespread technique than DESI and SIMS. Today, MALDI imaging techniques have best resolution and sensitivity in order to monitor drug distribution and accumulation in the cells, tissues and even whole body in real time by both using best scanning speed in high quality and best simple methodology [45].

3. Confocal Microscopy Imaging Technique

The theranostic mechanism of penetration among cell membrane is the other speculative issue. Confocal microscopy cannot only display nanotheranostic penetration between outer/inner layers of lipid membrane; but also, monitor intracellular internalization by measuring the half-life of nanoparticles. Theranostics can pass across the cell membrane either by endocytosis or passive targeting. During this progress of adhesion of nanoparticle to the fluid cell membrane

by causing alteration on morphology of cell membrane and effects the penetration resulting off-target adhesion that outcomes toxicity. Besides, physical properties of theranostics including size, surface composition and charge bring about rate of adhesion ability; for instance, large theranostics above 500 nm in diameter, could distrupt the penetration in membrane [46]. Likewise, theranostics are sometimes enclosed by endocytic vesicles, which indirectly penetrate to the cytosol. On the contrary, direct internalization are intended [47].

In fact, this techniqe is not label-free; even if, sample should be in high density and monitoring of penetration can be possible up for at least 200 μm. On the other hand, fluorescent nanoparticle can be detected up to 10 nm [19]. Thereby, the laser confocal microscope has been already carried out even analyzing the intracellular regions of several cell components [48]. The laser scanning confocal microscope (LSCM) based on point by point illumination of high density biological tissue. Likewise, it can be provided by scattered light from the focal plane for imaging purposes. The laser or blue light is oriented by a dichroic mirror towards a pair of mirrors that provide scaning light in *x- and y-* axis. Then the light passed from microscope objective that excites the fluorescent molecule (crystal, dye or SWCNT) in nanoparticle which goes under penetration progress within the cell. The fluorescent light from biological tissue returns back among same rotue. The dichroic mirror across the pinhole is stated in the conjugate focal that provides rejection of all out-of-focus light obtained from the tissue. Finally, perceived light is measured by the detector impending to convert 2D images of sample slices to the 3D by the aid of computer reconstruction [49-51]. The well-known, other microscobic techniques based on damaging on tissue thereof are sectioning and fixation; however, (LSCM) provides measurements under intact and alive conditions of desired tissue [52]. Confocal laser scanning microscopy (LSCM) is versatile technique as the complementary to put forward images from cell or tissue samples by laser scanning on an optical environment. Obtained images are more featured in high resolution with depth selectivity than other traditional microscopic tehniques. The main ability of its optical sectioning images is regenerated point by point scaning up to 200 μm [9, 52].

This technique historically has been developed firstly by Marvin Minsky in the 1950s who had been visualized human nervous system by using the light source as the carbon arc lamp [53]. It was recognized that techique prevents multiple scattering and provides low contrast images [54]. Then, Confocal laser scanning microscopy (LSCM) was developed in 1978 by using laser to scan the three dimentional image of the biological object by the point by point technique just like designing of the scanning electron microscope for the three-dimensional analysis of cells and tissues by using fluorophore labelling technique [55].

Confocal microscopy is frequently the applied best method in order to monitor meanwhile fixed and slow dynamic cell structure. This technique cannot be eligible for fast dynamic cell scanning/imaging. Generally, mostly desired issue is not only to obtain three dimentional images of the cells but also to enlighten slow speed cellular traffic during the internalization of nanoparticles [56].

4. NIRF Imaging Technique

Early cancer detection and therapy with theranostics *in vivo* are on the spotlighted concept of nanomedicine. From this perspective, new technical developments in addition to microscopic imaging technologies have been broadened by adding new ability just as noninvasive detection of early cancer. Recent promising development in imaging technology plus nanotechnology are based on NIRF probes [57, 58]. By this technique, it can be carried out sophisticated imaging by the aid of bright, photostable and well-soluble NIR emitting fluorophores for *in vivo* optical imaging [59]. On the other hand, nanotheranostics have to become eligible for both targeting and imaging of the tumors via either active or passive targeting just as NIR nanoprobes [60, 61].

In fact, the newly NIRF nanoprobes have the roles future potential as the delivery vehicles for fluorescence imaging, photoacoustic imaging, and multimodal *in vivo* imaging of cancer. By using NIRF (650–900 nm) molecular materials as the probes which has high resolution, low autofluorescence when it is compared with other *in vivo* imaging techniques [62-64], NIRF imaging has relatively innocent owing to its nonionizing radiation. On the other hand, NIRF imaging agents are always utilized at much lower concentrations than other imaging agents; such as, MR, CT [57].

New investigations in nanomaterials establish tremendous development in their designs as the NIRF nanoprobes in order to monitor molecular imaging *in vivo* for diagnosis of tumors and assessment therapeutic effect; even if, their absorption, distribution, metabolism and excretion are highly variable. Thereby, their clinical investigations most of candidate probes have not completed, yet because of their redesign and optimization throughout the progress remain many years [65].

NIR nanoprobes can be categorised as NIR dye load nanoprobes, carbon nanotubes, QDs and gold-based nanostructures. Theranostic-based agents used as the NIR nanoprobes can be classified as organic materials (polymeric materials, liposomes, etc.) and inorganic materials (metals, SWCNT, nanorods, quantum dots (QDs), etc.) [66-68].

It is undeniable issue that NIRF imaging is the best technique ever up-to-date in order to monitor the progress of cancer *in vivo*. This technique could be used as a complementary imaging for the purpose *in vivo* multimodal cancer imaging which has been described as the combination of NIRF imaging with nuclear imaging techniques such as PET or single-photon emission CT, or MRI [57, 69, 70]. Although a few number of theranostic and nanoprobes have been approved, roles in nanomedicine will have been broadened in the near future [66, 71-73]. NIRF imaging can serve as

the complementary imaging in order to monitor cancer progress *in vivo* by real time when combined with other *in vitro* applied microscopic methods. This harmless and relatively noninvasive method herald of new technology to broaden *in vivo* imaging techniques.

Acknowledgements: Authors thanks to both TUBITAK and Coordination Unit of Scientific Research Projects of (BAP) in Abant Izzet Baysal University for their financial support. Also, we are grateful to DEVA HOLDING A.S. has being provided us anticancer active agents during our studies

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