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Molecular imaging of immune cells for adoptive immunotherapy (AIT)

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Abstract:
Molecular imaging, as defined by the Society of Nuclear Medicine, is "the visualization, characterization and measurement of biological processes at the molecular and cellular levels in humans and other living systems" [1]. Molecular imaging techniques typically include molecular magnetic resonance imaging (MRI), magnetic resonance spectroscopy (MRS), optical bioluminescence, optical fluorescence, targeted ultrasound, single-photon emission computed tomography (SPECT), and positron emission tomography (PET). Continued development and the wider availability of scanners dedicated to small animal imaging studies, can provide a similar in vivo imaging capability in mice, primates, and human, can enable smooth transfer of knowledge and molecular measurements between species, thereby facilitating clinical translation of novel imaging agents and techniques. This brief review will summarize the several methods for non-invasive cell tracking for adoptive immunotherapy of malignant tumours.

Key Word: *Molecular imaging*; *immune cells; immunotherapy; MRI; SPECT; PET*

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I. Introduction

Cell-based therapy shows excellent promise for the treatment of cancer. The capacity to non-invasively track the delivery of various therapeutic cells (e.g. T cells, dendritic cells and stem cells) to the tumour site, facilitating the subsequent differentiation/proliferation of these cells, would provide us with a greater knowledge of the mechanisms of cancer development and intervention [2]. At present, there are two ways to track a cell: direct (cells are labelled with certain tags, which can be detected directly with suitable imaging equipment) and indirect cell labelling (which typically uses a reporter gene approach) [3]. The methods for tracking different cell types (e.g. immune cells, stem cells, and cancer cells) in cancer are now well-established, and include fluorescence, bioluminescence, PET, SPECT and MRI. The initial purpose behind the development of the non-invasive tracking of immune and stem cells was to potentially apply it to cancer therapy. However, the tracking of cancer cells could further aggregate our understanding of cancer development and tumour metastasis [3]. Safety is a significant concern for future clinical applications. The ideal imaging modality for tracking therapeutic cells in cancer patients requires imaging tags to be non-toxic, biocompatible, and highly precise. Each imaging modality has its benefits and limitations; they are more complementary than competitive [4]. MRI, radionuclide-based imaging techniques, and reporter gene-based methods all fulfil particular niches, yet work together towards the same ultimate goal: personalized medicine for cancer patients. This review article will define and describe adoptive immune cell therapy (AIT) and outline the principles of molecular imaging in order to reveal different imaging modalities and labelling strategies. Finally, the article will discuss the benefits and drawbacks of AIT.

Key words: molecular imaging, adoptive immune cell therapy, molecular imaging probs.

The concept of AIT

Cancer treatment by adoptive immune cell therapy (AIT) is a form of immunotherapy that relies on the in vitro activation and/or development of immune cells [5]. In this method, immune cells (in particular, CD8+ T lymphocytes, NK T cells and dendritic cells) can potentially be harvested from a tumour-bearing patient. The cells are activated and/or extended in vitro in the presence of cytokines and other growth factors and later transferred back into the same patient to induce tumour regression. AIT (figure 1) moves the in vitro generation and activation of T-lymphocytes away from the immune-suppressive tumour microenvironment, thereby providing optimum conditions for potent anti-tumour activity [5].

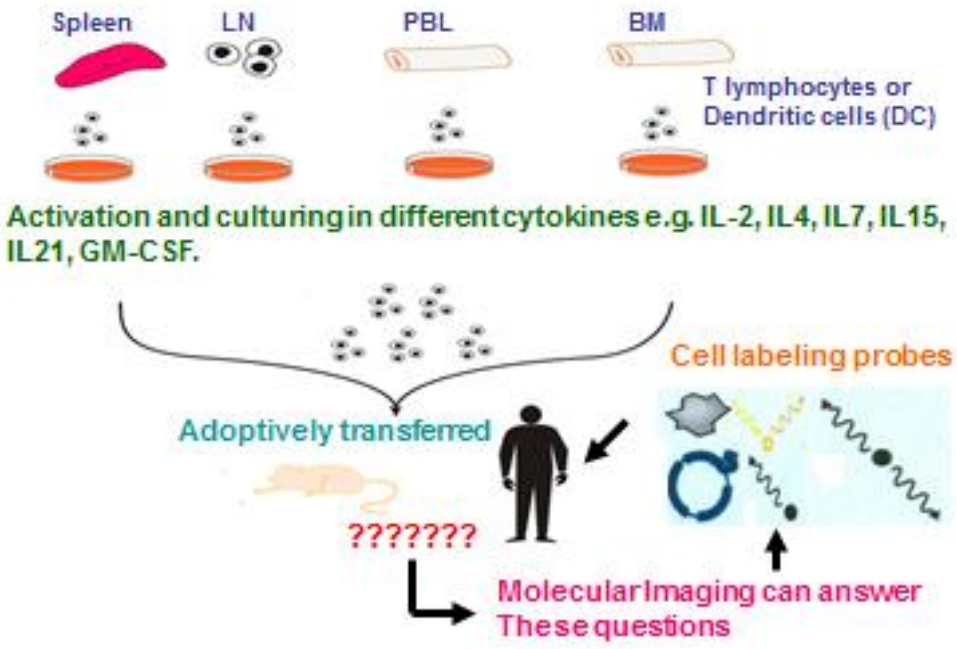

Fig.1 Visual illustration of AIT.

As per Figure 1 above, AIT follows the following 3-step process. First: isolation (from spleen, lymph nodes LN, peripheral blood vessels PBL, and bone marrow BM). Second: activation (in different cytokines: interleukins 2,4,7,15,21, and granulocyte-macrophage colony-stimulating factor (GM-CSF). Third: reinfusion of tumour-specific immune cells (T-lymphocytes or DCs) to patients with the same tumour. Relevant questions regarding this process include: Do they localize at the tumour site? Where and when do they localize? How long do they persist in vivo? Do they retain their proliferation and function? All of these questions can be answered by using molecular imaging.

Molecular Imaging as a guide for AIT

Several imaging modalities exist to track cells in animal models. However, only a few of these can be used in humans due to safety, technical and cost considerations. Figure 2 illustrates the common cell tracking modalities, using the most common imaging techniques and parameters and their application towards longitudinal use [5]. The imaging techniques relevant to clinical cell tracking include scintigraphy with gamma cameras, positron emission tomography (PET), SPECT and magnetic resonance imaging (MRI). Computed tomography (CT) is frequently used in conjunction with SPECT and PET to give anatomic context. In general, techniques that are dependent on radioactive isotopes are limited by the half-lives of the isotopes and are thus difficult to apply in longitudinal imaging [5].

However, an intriguing approach that might be a good compromise is to use an injectable label, for example, a radiolabeled antibody to track specific cells, i.e. in vivo labelling [6]. This can then be repeatedly injected for longitudinal cell tracking [7]. However, non-specific label uptake or slow clearance can confound data interpretation. Furthermore, techniques using radioactive isotopes or ionizing radiation can be restricted by exposure limits in humans. The absence of radiation is, therefore, one of the reasons why MRI is an attractive alternative. Other reasons include its extremely high resolution, tomographic ability and inherent soft-tissue contrast. On the downside, MRI can be several orders of magnitude less sensitive and less quantitative than techniques using radioactive isotopes. Similar to scintigraphy, MRI cells are labelled before they are transferred to the patient, although labels can be targeted in vivo [5], or cells can also be transduced to express reporter genes [8]. Optical imaging techniques using fluorescence or bioluminescence have proven invaluable in small animal models. These techniques are extremely fast and amenable to repeated sessions in the same animal. Existing markers, such as green fluorescent protein (GFP) or luciferase expression, can be exploited. In the case of bioluminescence imaging, a major advantage is the total absence of background, allowing for data acquisition in a relatively quantitative manner. However, the limited depth penetration of these wavelengths of light currently restricts the techniques to small animals for non-invasive imaging. Accordingly, optical agents have not been applied to in vivo cell tracking in humans [9].

Finally, the most widely-used imaging modality in the clinic does not exploit the visible light spectrum at all. Acoustic imaging, such as ultrasound imaging, is the most common structural imaging modality in the clinic. The technique gives real-time (4D) images with a limited field of view. Targeted labels have been developed for this technique [9], although human cell tracking has so far not been achieved. Due to resolution and penetration limits, ultrasound imaging is best-suited to gather structural data of superficial and echogenic structures and appears less applicable to cell therapy.

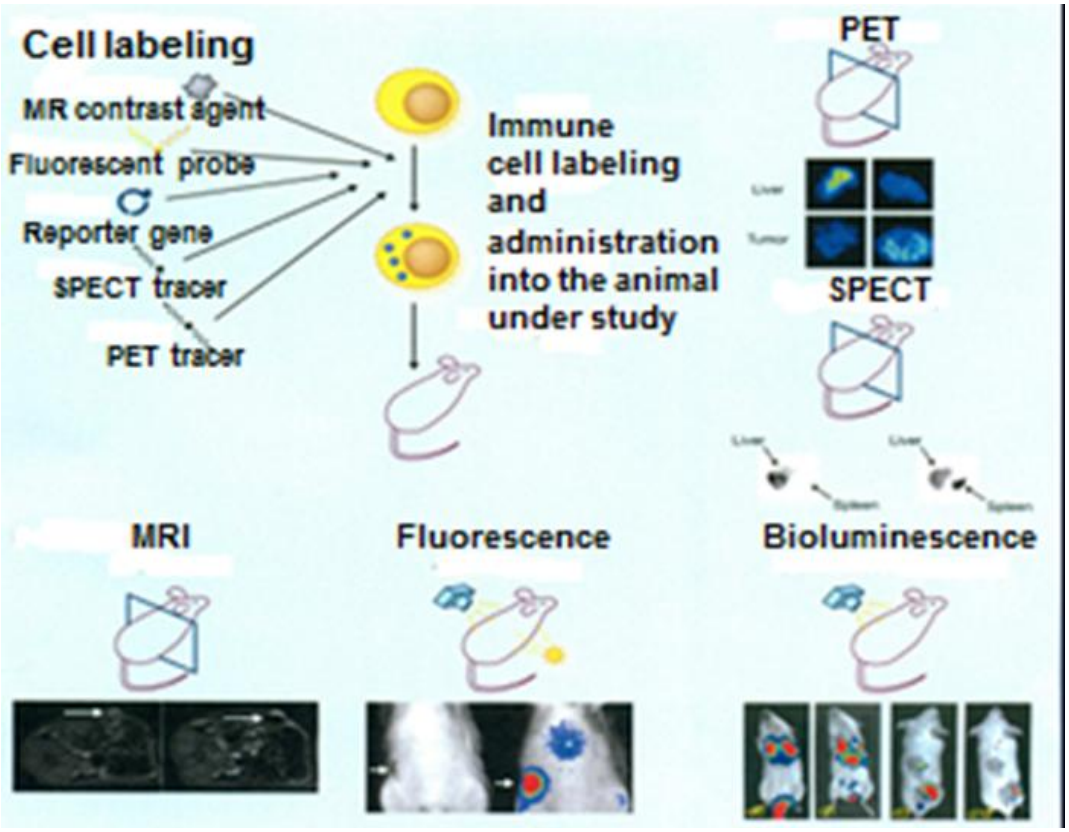

Fig.2 Imaging modalities for *in vivo* cell tracking.

As per figure 2 above, PET and SPECT techniques using radioactive isotopes may facilitate the longitudinal monitoring of cells if the labelling occurs in vivo. Optical imaging methods have not been applied in humans for cell tracking due to depth penetration limitations.19 F MRI has also been applied specifically to cell tracking in animals. However, these imaging techniques have been used in the clinic, and cell tracking has been carried out in preclinical models; therefore, they are marked as "potential" [10].

Cell labelling techniques

Cells must be suitably labelled to be detected via imaging to distinguish them from the surrounding cellular environment. In both preclinical and clinical situations, cells are typically labelled ex vivo before transfer to the host for cell tracking studies. Another option is in vivo labelling, for instance, via labelled antibodies specific to the transferred cells. Both techniques have pros and cons, which will be discussed in the following sections. A recent review lists some general requirements for using novel agents for clinical cell tracking [11], namely that these cell labels must be shown to be non-toxic to cells in culture or animal models. The labelled cells should be extensively characterized to determine any effect of labelling on cell functionality and so that the label synthesis can be done using compounds approved for human use.

Direct and indirect labelling strategies

In direct labelling, cells are harvested and labelled ex vivo with paramagnetic nanoparticles, fluorophores or radiotracers, thereby allowing them to be visualized by MRI, FLI and SPECT/PET, respectively. The great advantage of direct labelling is the simplicity of the labelling protocols. In general, these labels bind to specific intracellular targets and easily penetrate the cell membrane. This strategy has two drawbacks: the level of labelling depends on the cell's ability to retain the label (different cell populations display different phagocytic capacity, different membrane composition, etc.) and, more importantly, it can be

used only for the longitudinal monitoring of terminally differentiated cells such as dendritic cells and macrophages. This is because, in proliferating cells, the label may be lost or diluted as a result of cell death or cell cycle events (as shown in Figure. 3a). In indirect labelling, cells are transduced ex vivo with a vector carrying a reporter gene (luciferase, green fluorescent protein, Na/I symporter, transferrin receptor, etc.). The expression of the reporter protein generates a signal that makes it possible to track the cells directly in the body. Depending on the promoter or the regulatory element used to create the construct, this strategy is used to study a specific cell process or to determine a cell's viability. A constitutive promoter provides information about living cells and their homing and localization in the target organ. Instead, by using a tissue-specific promoter or specific regulatory sequences, it is possible to evaluate a specific activation or differentiation of the labelled cells. It is thus necessary for many of these reporter proteins to administer a substrate (e.g. luciferin for direct labelling strategy).

Luciferase, or a probe, involves radiotracers that bind to or can be internalized by the specific expressed receptor (e.g. Na/I symporter) in order to localize the labelled cells. This strategy is fundamental in tracking the migration and homing of proliferating cell populations and is useful in potentially evaluating their activation and division. Indeed, by inserting an exogenous reporter gene, it becomes possible to follow a specific cell progeny over time. Because the reporter protein will will only be permanently expressed in these cells, without signal dilution over time (as can be seen in Figure.3b), the signal is only lost when the cell dies.

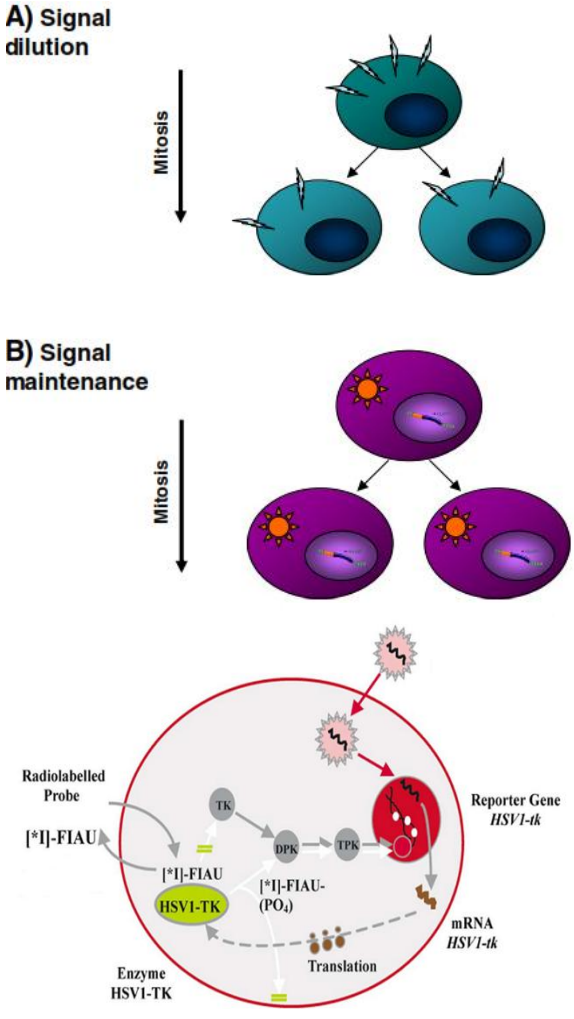

Fig. 3 Direct vs indirect cell-labelling strategies.

A. Direct labelling procedures are based on the imaging of cells labelled ex vivo with radiolabelled molecules, paramagnetic particles or fluorescent probes, which are then reinfused. This technique is useful for the in vivo imaging of terminally differentiated cells, such as dendritic cells and macrophages, while it does not allow for long-term monitoring of cell viability and proliferation in the body, because the label is diluted at each mitotic event.

B. Indirect imaging is based on transgenes, whose expression can be monitored in vivo using a transgene-specific probe. With this technique, a stable genetic modification of the cells is necessary to monitor their fate and the fate of their progeny in vivo over time. This approach is vital because it facilitates the imaging of proliferating cell populations during their migration, activation, division and hopefully, during their differentiation throughout the body.

Labelling probes

Many early reports have investigated the tracking of T lymphocytes in cancer for imaging that uses different labelling probes (see figure 4 below), including fluorescence, bioluminescence, MRI, SPECT and PET techniques [14-21]. Most of these early reports used fluorescence imaging techniques to track T cells. For in vivo applications, utilizing the near-infrared (NIR, 700-900 nm) window is advantageous since the absorbance spectra for all biomolecules reach minima in this region, providing a clear window for optical imaging [23]. The migration of T cells to tumours has been investigated by using NIR organic dyes as the fluorescent labels, such as IRDye800CW [24] and VivoTag 680 [25]. Both dyes bear an amine-reactive N-hydroxysuccinimide group for cell labelling, which was found to be biocompatible and suitable for monitoring cells at multiple resolutions in real-time in their native environments via NIR fluorescence imaging.

Modern fluorescence imaging techniques can trace the migration of cells in living animal models. Unfortunately, the outcomes of monitoring immune cells injected into the gastric cancer orthotropic model were unsatisfactory due to a low signal-to-noise ratio [4]. However, in recent years, the growth of a near-infrared fluorescence imaging technique has made it potentially better at tracing living cells in deep tissue [5]. Xiaohuidu et al. established an orthotopic gastric carcinoma nude mouse model and dynamically monitored cytokine-induced killer (CIK) cells. In this study, cytotoxic T lymphocytes (CTLs) were labelled with the near-infrared fluorescent dye, DiR (1,1'-dioctadecyl-3,3,3',3'-tetramethyl indotricarbocyanine iodide). Their results showed that labelling with DiR had no significant impact on the biological properties of CIK cells and CTLs, suggesting that DiR is fit for usage in living animal experiments. This finding is consistent with recent studies employing a similar lipophilic carbocyanine dye DiI [8,9]. Kalchenko et al. [6] successfully applied DiR to stain human leukaemia G2L cells, mouse lymphocytes and rat red blood cells for in vivo tracer experiments. Granot et al. [7] also utilised DiR to mark fibroblast cells, then observed a target ovarian cancer tumour some distance away from the injection site. The properties of near-infrared wavelengths make them ideal for imaging in deep tissue.

Although bioluminescence imaging (BLI) has been widely used for T cell trafficking in other diseases, such as inflammation [26-27], its use in monitoring T cell trafficking for tumours has not been well studied. Due to the central nervous system's presumed "immune privilege", it is commonly believed that T cells have difficulty reaching tumours located in the brain. In one study, the biodistribution and anti-tumour activity of adoptively transferred T cells specific for an endogenous tumour-associated antigen (gp100) was expressed by tumours implanted in the brain and was subsequently investigated [28]. BLI of luciferase expression in the antigen-specific T cells demonstrated the accumulation of transduced T cells in the bone marrow and the brain tumour, which suggested that peripheral tolerance to endogenous tumour-associated antigens can be overcome to treat tumours in the brain.

With exquisite soft tissue contrast, MRI is another widely used imaging modality for cell tracking in vivo [40]. In one early study, a highly derivatized cross-linked iron oxide (CLIO) nanoparticle was used to label CDLs for in vivo tracking of injected cells at near single-cell resolution with MRI [40]. In a melanoma model, MRI demonstrated the extensive three-dimensional spatial heterogeneity of antigen-specific T-cell recruitment to the tumours and temporal regulation of T-cell recruitment within the tumour. It was suggested that serial administration of CDLs homed towards different intratumoral locations, which may consequently offer a more effective treatment regimen than a single bolus administration. Nuclear imaging techniques (i.e. PET and SPECT) have much better clinical potential than optical imaging in that they have superb tissue penetration capability and are highly quantitative [9-29].

PET/SPECT imaging has been frequently used for T cell tracking. Radiolabeled T lymphocytes have been used in cancer patients for imaging using SPECT techniques [14-21]. Most of them used 111 In-oxine as the radiolabel approved by the Food and Drug Administration (FDA). A 64Cu-labeled antibody was used for animal studies to track transferred T cells (expressing the antigen recognizable by the antibody) with PET in living mice [30]. It was found that not all tumour-specific T cells were localized to the tumours; some also homed to the major lymphoid organs. Another report labelled T cells with 124I-iodo-2'-deoxyuridine (124I-IdU) in order to monitor their homing to tumours with PET imaging [31]. A significantly higher accumulation of 124I-IdU in the targeted tumours than the control tumours was observed. Another study compared the efficiency, stability, and toxicity of radiolabeling activated T lymphocytes with three different agents: 99mTc-hexamethyl propylene amine oxime (TcHMPAO),111In-oxine, and 18F-2-fluoro-2-deoxy-d-glucose (18F-FDG, the most widely used PET tracer in the clinic) [32]. It was found that the mean labelling efficiencies of 111In oxine and 18F-FDG were higher than that of 99mTc-HMPAO. Although none of the three agents induced any

significant alteration in cell viability or immune-phenotype, both Inoxine and 18F-FDG induced a loss of cytotoxic activity of lymphocytes against ovarian carcinoma cells.

Several groups have investigated the use of the herpes simplex virus type 1 thymidine kinase (HSV1-tk) gene and its mutants as a reporter gene for various biomedical applications, including cell tracking. In addition, a fusion of the HSV1-tk gene and other reporter genes, such as fluorescent proteins and/or bioluminescent enzymes, can enable the multimodality imaging of the transfected cells. They provided not only a convenient means of cross-validation, but also good translational potential. In some cases, the cells could be labelled with a PET or SPECT probe, 2'-fluoro-2'-deoxy-1-beta-D-arabinofuranosyl-5- Iiodouracil (124I-FIAU) and 2'-fluoro-2'-deoxy-1-beta-D-arabinofuranosyl-5- I-iodouracil(131I-FIAU) respectively [33], and then injected and tracked over time in live tumour-bearing mice. In most cases, the expression of the HSV1-tk gene or its mutants in the transfected T cells can be visualized by PET imaging after the injection of a reporter probe, such as 2'-18F-fluoro-2'-deoxy-1-beta-d-arabinofuranosyl-5-ethyluracil (18F-FEAU) [34,35] or 9[4-18F-fluoro-3-(hydroxymethyl)butyl]guanine (18F-FHBG) [36-40]. These cell tracking studies revealed important insights into cancer immunotherapy. For example, one study found that naive T cells did not localize to the tumour site, indicating that preimmunization was required [36]. Such an observation would have been challenging to reach if imaging were not used. Another study reported that the minimum detection threshold of T cells engineered to express the HSV1-sr39tk gene was approximately 7 × 105 T cells in the spleen and 1 × 104 T cells in the lymph nodes [37].

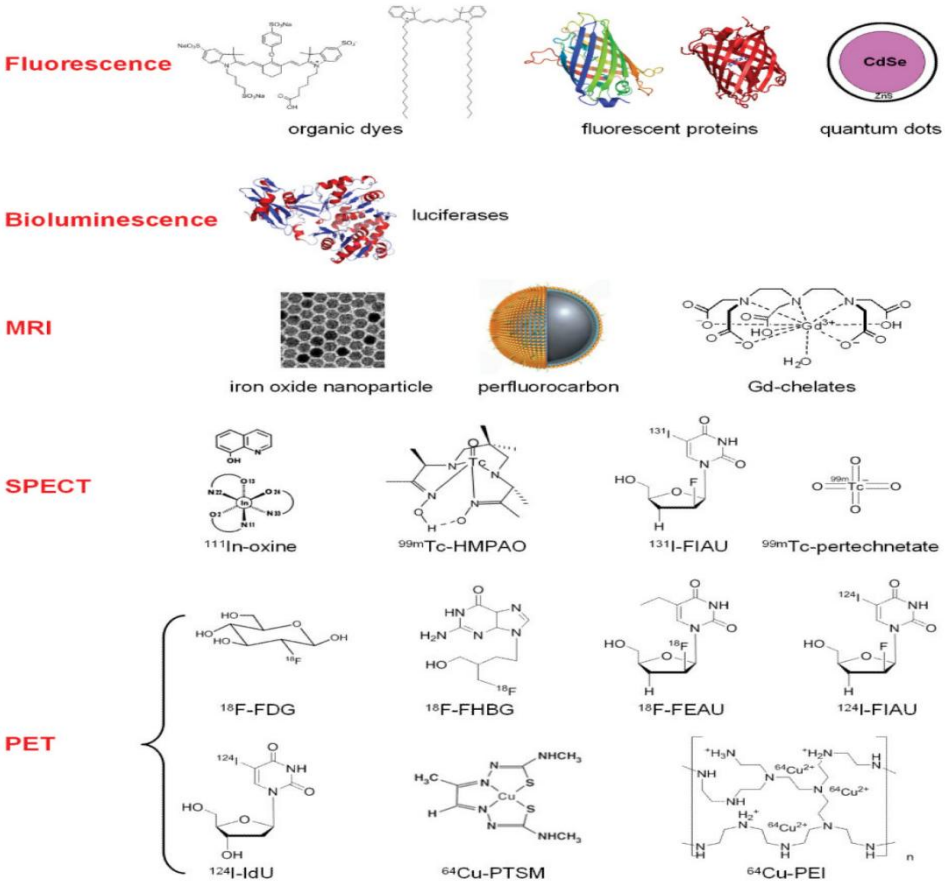

Fig.4 Common labelling probes for AIT

II. Conclusion

A wide variety of labels and imaging techniques have been explored for labelling and tracking cells in cancer and cancer therapy (see figure 4). Although the cell types can vary (e.g. immune cells, stem cells, and cancer cells), the labelling strategies are essentially the same. The direct labelling of cells with image tags is easier than indirect labelling in most cases, and the safety profiles of direct cell labelling techniques are generally quite good. However, the disadvantage of direct cell labelling is that the label itself is detected rather than the live cells of interest. With reporter gene techniques (i.e. indirect cell labelling), only live cells are detected; thus, they can provide greater insights into cell migration, differentiation, and proliferation in vivo.

Each imaging modality has its benefits and weaknesses in terms of sensitivity, tissue penetration, spatial resolution, and clinical potential (Table 1). Optical imaging is mainly applicable to preclinical studies where light penetration is less of an issue than with cancer patients. BLI cannot be used in human studies, while tracking labelled cells with MRI, SPECT, and PET may be performed in patients. A combination of various imaging modalities can provide complementary information. As a matter of fact, many reporter gene-based cell labelling studies have incorporated multiple reporter genes. For example, fluorescent genes (e.g. GFP and RFP) can facilitate cell sorting to isolate the cells of interest, BLI (with luciferases) can enable in vivo long term monitoring of cells in a quantitative manner in small animal models, and PET can allow for more clinically relevant, highly sensitive detection of the injected cells or the daughter cells.

With these tools in hand, scientists can investigate the various aspects of cancer development and cancer therapy in a manner that was previously considered impossible. Future development and validation of various cell labelling/tracking techniques will further strengthen the arsenal for cell-based imaging and therapy of cancer.

Table (1) A summary of comparisons between different imaging modalities

Modalities	Resolution	Sensitivity	Tissue presentation	Quantitative capability	Cost	Clinical trial
Fluorescence	medium	medium	poor	poor	$	low
Bioluminescence	low	high	poor	good	$$	none
MRI	high	low	good	poor	$$$	high
SPECT	medium	high	good	good	$$	high
PET	low	high	good	good	$$$	high

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