

A new tracer technology for therapeutic cell tracking

Good nuclear tools make for better techniques.

IVD Technologies
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In vitro neutron-beam detection of labeled in vivo probes could help physicians analyze organ function.

Colloidal fluorescein/europium is a multidetection nanomaterial that can be visualized by both fluorescence and electron microscopy, and can also be quantified by neutron interrogation technology (NIT).

In order to exploit recent advances in genomic knowledge to improve disease diagnosis and therapy, it is necessary to understand more fully the means of quantitatively probing for physiological function in vivo. An emerging technique, neutron interrogation technology (NIT), offers a novel approach to studying physiological processes. NIT introduces the use of a neutron beam to detect specifically labeled probes designed for diagnostic and therapeutic applications. This article discusses NIT and uses therapeutic cell tracking as an illustration of the power of this new detection technology as an in vitro diagnostic. One exciting aspect of NIT is its ability to be combined with a series of diagnostic imaging technologies to provide unique physiological information.

The Need for a New Detection Technology

The predominant IVD tracer technologies fall into two general classifications, radioactive and optical. Radioactive tracers offer many advantages, including high sensitivity, low background, high throughput, detectability in opaque tissues, and readily available measurement equipment. But the disadvantages of radioactivity as a label, mainly waste disposal and hazardous radiation, have resulted in a search for a replacement technology.

Optical labels, including fluorescence, have filled the need somewhat. These labels work well in most IVD applications, but not in cases where it is necessary first to introduce a functional probe into the body and then to quantify the probe in a biological sample. Since most biological samples, such as tissue or blood, are opaque or have high background activity, optical probes work poorly unless they are extracted from the sample prior to measurement.

NIT was developed to remedy this problem of quantifying labeled materials in biological samples. The principal application of NIT involves studying probes that have been administered to subjects in vivo and are then measured in isolated samples such as blood, organs, and other tissues.

NIT is a two-stage process. First, a labeled probe is synthesized and administered to the subject. Next, after that probe is extracted, instrumentation is used to measure it in isolated samples in vitro.

The first stage of NIT should be familiar to any laboratory scientist. Atoms of a metal (the tracer) are linked to a molecule or incorporated into a macromolecular structure such as an antibody, ligand, nanoparticle, or microsphere. The metals available to use as tracers are stable isotopes (not radioactive) and are biologically rare. Gold, lanthanides, and the like (see Figure 1a) are not found in normal biological samples. One or more of

these physiological probes is administered to the subject; each of them is able to have its own tracer. At some later time, biological samples of interest are taken from the subject and dried. Up to this point, there is no radioactivity. No further processing of the labeled sample is necessary for NIT analysis.

The second process stage is an assay of the sample with a neutron beam. Neutrons, a component of atomic nuclei, have a variety of properties that make them ideal for diagnostic and therapeutic applications in the life sciences. Chief among these is their high depth of penetration of biological tissues, which distinguishes neutrons from light photons. The generation of a neutron beam requires either a nuclear reactor, a radioactive neutron source, or a linear accelerator.

Historically, these technologies have not been readily accessible to the life scientist. This has limited the contribution of NIT to the field. In part through receiving grant support for new technology development from the National Institutes of Health Small Business Innovation Research (SBIR) program, BioPhysics Assay Laboratory Inc. is beginning to make NIT more accessible by providing an assay service to supply neutrons. Instrumentation that would allow NIT to be usable by any laboratory is now under development at BioPAL, again with some NIH grant support. It is expected that this will become commercially available in the near future.

For assaying the probe in the sample, the laboratory technician exposes the sample to a neutron beam. During the exposure, some of the neutrons combine with a small percentage of the atoms in the sample, including the metallic atoms of the probe, causing these atoms to become unstable, that is, radioactive. These unstable atoms begin to release energy in several forms, including alpha and beta particles, that can be used in therapeutic applications, as well as high-energy photons that are useful in diagnostic applications.

The high-energy photons emitted are quantified in an automated spectroscopic instrument that can quantify each distinct energy peak. Unlike the energy distributions associated with tritium and carbon 14 decay, the energy spectra of metallic probes are very sharp and well separated; they do not overlap (see Figure 1b). Data are reported in disintegrations per second. Once the measurements are made, the sample can either be disposed of in radioactive waste or archived for re-assay at a later date. Either way, owing to the short half-lives of the metals used as probes, the samples decay to background in a matter of a few weeks.

NIT has a number of properties that afford the life scientist great flexibility in planning and performing diagnostic assays.

First, unlike light photons, neutrons have no charge and are not readily attenuated within the sample. Therefore, NIT is matrix independent; measurements can be made in blood, lymph, brain, or even bone samples without the need for extraction prior to assay.

NIT can be used for multiple parallel assays. The NIT instrumentation developed for counting high-energy photons was designed to have the ability to read across a broad spectrum of photon energies. At least 14 different labels can be measured simultaneously in a single sample.

Prior to neutron irradiation, NIT is a non-radioactive tracking technology; the metallic probes are stable isotopes. Therefore, during the in vivo phase of the testing process,

the laboratory scientist does not have to deal with issues of radiation safety or radioactive waste.

The shelf life of a stable-isotope-labeled probe is potentially much longer than that of a radioactive label. Isotope half-life and radiolysis often limit the usefulness of any radioactively labeled material. By the time such material is employed, it may have a much lower specific activity and purity than it had when originally produced. Likewise with fluorescently labeled materials: issues involving quenching of the label, along with endogenous fluorescence, can limit the usefulness of any particular preparation.

NIT is a very robust technology in terms of sample handling. Once samples are taken, they can be stored indefinitely at room temperature and in the presence of ambient light. Because only a small portion of the label becomes radioactive during the NIT process, samples can be archived for years and then reactivated with neutrons at a later date in order to be remeasured. This is especially important for preclinical trials, when questions could arise during data analysis.

NIT has the property of variable sensitivity. Increasing the duration of exposure to the neutron beam, or the intensity of the neutron beam, increases the sensitivity of the assay proportionately.

Furthermore, the same samples can be measured at different sensitivity levels. When NIT users are not sure which level of sensitivity to use, many will run their samples first at the standard—that is, lowest—sensitivity. If the probes are undetectable, then the same samples can be rerun at a higher level of sensitivity. The tracers often become detectable. In many cases of experiments performed using insufficient quantities of radioactive or fluorescent labels, the detection methodology cannot be boosted to achieve detection. The whole process has to be redone—a very expensive proposition, especially in preclinical studies.

This array of attractive properties has led to NIT being used in a growing number of applications in life sciences research, including measurement of regional blood flow,¹⁻⁴ growth factor tracking,⁵ renal and liver function assaying,⁶⁻⁸ and measurement of the efficacy of compound-administration technology.⁹ An exciting new application of NIT is its use in in vivo tracking of therapeutic stem cells.

Stem cells are being studied for use in a variety of therapeutic targets. As a result, this therapeutic field has been growing rapidly. One major safety and efficacy question is where the therapeutic stem cells migrate once they have been administered in vivo. Recent data indicate that the vast majority of the administered cells may not stay at the target site but, instead, travel elsewhere in the body.¹⁰

The question of how many cells travel to each of the various organs of the body has to be answered. Cells that do not stay at the intended site of delivery may cause undesirable side effects as a result of their migration. In order to perform the measurements, a new diagnostic technology is needed. NIT, used in conjunction with nanomaterial technology, has been demonstrated to solve this problem.

To label cells successfully requires that several conditions be satisfied. Ideally, the label should be taken up and internalized so that it will not easily be released from the cell. Experimental protocol may require prelabeling the cells one day and then using the labeled cells some later day. Also, experiments may involve a number of days in vivo, so the labeled cells must not release their label during that period. Enough label should be delivered to each cell to establish a reasonable level of sensitivity. Detection of

approximately 1000 cells per sample is usually considered sufficient, although single-cell detection is the ultimate goal.

A colloidal material has been developed in which any of a number of possible lanthanide metals is embedded in a polymeric matrix. These colloids have particles ranging from 30 to 300 nm in diameter and are stable to freezing and autoclaving. Such materials contain thousands of atoms of stable isotope, thus maximizing sensitivity in a minimum of probe volume. By way of illustrating the amplification gained by using metal colloids as probes, it has been calculated that a single 20-nm probe will contain between 100 and 500 atoms of metal, while a single 300-nm probe will contain more than 10⁶ atoms of metal.

Treatment of Myocardial Infarction

Research into the administration of stem cells for a variety of therapeutic applications has been progressing rapidly. Applications include correcting damage from diabetes, spinal cord injury, and Parkinson's disease, but reversing injury from myocardial infarction (MI) is of particular interest.

A major problem in MI therapy is administering the cells efficiently with minimal deleterious effects on the research subject. The primary method of delivering cells to the area of damage in the heart is through the insertion of a cardiac catheter. This technique requires only a minor incision; the chest and heart remain intact. The whole procedure can be performed in a much shorter time than open-heart surgery. The therapeutic cells are injected via the catheter into the beating heart wall one or more times while being visualized in real time through a fluoroscope.

To develop this technology for safe use in humans, animal models are employed. Such models can help in optimizing catheter design, delivery protocols, and the type of therapeutic cell, as well as training surgeons in catheter operation. The capability to count the number of cells delivered specifically to the damaged portions of the heart (versus other tissues such as the lung, liver, or spleen) is crucial for optimization.

Radioactive and optical labeling technologies have been used previously. While radioactive labels can count the cells in a limited fashion, they tend to diffuse rapidly, can be toxic to therapeutic cells, have a short half-life, and result in large quantities of radioactive waste. Optical labels, such as fluorescent molecules, can locate cells; however, fluorescence is not a quantitative technology *in vivo* because of the opaque nature of tissue. Moreover, many optical labels also diffuse from the cell over a short period. Because of these limitations with traditional labeling technologies, researchers have begun to employ NIT using stable-isotope-labeled materials.

Cell labeling with NIT probes is performed in the following manner. The therapeutic cells to be tested are incubated in culture with a nanoparticulate probe containing a suitable metal. The incubation period generally lasts between 1 and 24 hours. The cells are then washed multiple times with excess culture medium in order to remove any particles not taken up into the cells. At this point the cells are ready to use. (They may be frozen and stored for later use.)

Because the nanoprobe is stable, it will not lose detection effectiveness the way radioactive labels, with their short half-life, and fluorescent labels, as a result of quenching, do. The nanoprobe is entrapped within cytoplasmic vesicles (see Figure 2). Therefore, it does not diffuse from the cell like radioactive and fluorescent tracers.

Following labeling, a standard curve is developed that graphs the number of cells against disintegrations per minute to yield the detection range (see Figure 3). In the experiment reported in that figure, the sensitivity of cell detection was approximately 1000 cells.

Nanoprobes have been used with several types of cell, including mesenchymal stem cells, myoblasts, bone marrow cells, and fibroblasts. All cell types to date have been found to take up the label, most likely through pinocytosis, and concentrate it in long-lived cytoplasmic vacuoles. Further, no major toxicity has been evident. Therefore, to quantify the biodistribution of newly developed cell-based therapies, NIT, in combination with nanocolloids, is proving to be an enabling technology that can provide needed information where conventional technologies have been unsatisfactory.

Multiple Detection Technologies

Metal nanoprobes have properties that allow them to be detected by several different parametric techniques, including NIT, magnetic resonance imaging (MRI), time-resolved fluorescence (TRF), and electron microscopy. The exploitation of these multiple physical properties within a single colloidal particle can provide information to the researcher that is uniquely useful in the development of cellular therapies.

MRI Contrast Agent and NIT. Imaging cells as they are being administered to the experimental model for cellular therapy can help to optimize delivery procedures. MRI can provide the most detailed real-time images during such a procedure. Investigators are using MRI contrast agents to label cells so that the tagged cells can be imaged against the background of tissue.

While iron-based colloids, which darken the image, have previously been used for cell labeling, most researchers prefer gadolinium-based materials because they brighten. Researchers have been forced to use iron for contrast because iron will work when in particulate form, thereby providing a means for cellular uptake. It had been thought that because they must have access to protons in water for use in MRI imaging, gadolinium particles would not provide the necessary contrast for MRI.

Since BioPAL had experience in the synthesis of lanthanide nanoparticles for NIT, and since gadolinium is a lanthanide, BioPAL developed a gadolinium particle that functions as a contrast-brightening agent in the T1 tissue-relaxation process. This new particulate material has potency equivalent to gadolinium diethylene-triamine-pentaacetate (Gd-DTPA), the principal available gadolinium-based contrast agent.

TRF and NIT. Light-microscopic imaging of the administered cells in histological sections may provide information about the location of the administered cells along with identification of the cells that reside nearby. A fluorescent label would help to image such cells. Some lanthanides have the physical property of time-resolved fluorescence. TRF materials are able to sustain fluorescence even longer after the stimulating light is turned off than the autofluorescence of the surrounding tissue lasts. Nanoprobes exhibiting TRF have been developed and are highly fluorescent

Electron Microscopy and NIT. Imaging via the electron microscope can provide ultrastructural information. The labels used in the nanoparticles are metals. As such, they are electron opaque and therefore are visible at an ultrastructural level through the electron microscope. The labeled cells can be imaged in order to study their ultrastructural environment and relationships. Highly subtle interactions between the therapeutic cells and their neighbors can be studied.

The NIH SBIR program has provided grant support for the development of the multidetection nanoparticle technology.

NIT and Cellular Therapy

NIT in conjunction with labeled nanoparticles constitutes a technology that allows for the precise quantification and imaging of administered cells in cellular therapy. Until now, the technology has been used only with research animal models of disease. Nevertheless, this has enabled researchers to make great progress in optimizing their cells and delivery technology. The modeling studies allow researchers to better understand the potential for side effects, as well, by enabling them to determine how many cells are going where in the body. At this point in the development of cell therapy for MI, it has been reported that approximately 90% of the delivered cells do not stay at the target site but move to other sites in the body.¹¹ Much more work needs to be done to maximize the retention rate and to better evaluate biodistribution and associated risk.

The ability to perform three types of imaging (MRI, fluorescence, and electron microscopy) with the same nanomaterials is just becoming available to medical researchers. For the first time, it is possible to both image and quantify in the same subject; therefore, a number can be associated with the visible probe on the image. This multidetection technology provides a foundation for creating improved therapies.

Conclusion

Diagnostic tests for bodily organ function commonly rely on measurement of the concentration of a naturally occurring substance followed by determination of whether the quantity of that substance within the subject is at an abnormal level. This article has described a novel technique in which the diagnostic test measures how an externally delivered labeled probe was handled when it was in the body.

NIT is a new diagnostic technology that can provide a unique ability to track probes quantitatively in vivo and use them to analyze physiological functions. Currently, it is used primarily by researchers in drug discovery and preclinical trials, but it has started to attract the notice of physicians. Clinical applications beginning to be developed include the measurement of renal organ function.⁷

It will take some time before NIT materials are used clinically. However, application of NIT in the life sciences is growing because this technology is uniquely suited to address important questions that older, conventional technologies cannot. NIT allows for multiplexing with other assays in order to broaden the range of information available to a physician or scientist. And because a large variety of probe labels have NIT potential, a battery of quantitative assays can be performed on a single sample. NIT is a prime example of the new directions that diagnostics is taking in the 21st century.