DOE Workshop Report

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Medical Sciences Division
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Preface

In the late 1960's, Sol Spiegelman developed a method, called nucleic acid hybridization, that quantitated viral rna during Q-beta bacteriophage replication. Nucleic acid hybridization was subsequently used to identify and quantify specific rna and dna sequences both "in solution" and when one component was fixed in solid phase. The later techniques have led to mrna and dna localization and semi-quantitation in fixed tissues and in chromosome spreads. The obvious next experimental breakthrough will be imaging mrna transcripts in real time. The development of generic methods to image specific gene expression will result in major advances in our understanding of developmental biology, cancer induction and pathogenesis, and in the clinical detection of inherited and acquired diseases.

The Department of Energy's Office of Biological and Environmental Research brought together experts in nucleic acid biochemistry, gene expression and medical imaging to address the possibility of imaging gene expression and to define the major technical and scientific hurdles impeding development of this methodology. This workshop represents the first step in the Department's intent of applying the fruits of the Human Genome Program to nuclear medicine.

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Executive Summary

The Medical Sciences Division of the Office of Biological and Environmental Research (ber), Office of Science, U.S. Department of Energy (doe) convened a workshop on "Imaging Gene Expression *In vivo*" on June 15–17, 1999, in La Jolla, California. The workshop was intended to advance the doe/Medical Sciences Division mission in developing new radiopharmaceutical molecular approaches by bringing genomic sciences and biotechnology advancements in partnership with nuclear medicine. A major goal of the workshop was to identify molecular processes associated with disease causation, and prospective ligands and instruments for quantitative measurement and rapid data analysis of these processes in experimental animals and humans.

There were twenty two participants including eighteen outside experts and four Medical Sciences Division staff members present at the workshop. The participants were given the charge to discuss the following topics in their presentation and summary sessions during the workshop:

- What are the major hurdles in imaging steady state mrna in cells and whole animals in real time?
- Is it likely that a major funding initiative in this area will accomplish the goal of imaging gene expression at the mrna level in a reasonable length of time?
- What are the practical alternative methods to image gene expression other than methods based on mrna hybridization reactions?
- What advances in targeting and radiochemistry will expedite efforts to image gene expression?

The workshop consisted of 14 presentations and two summary sessions covering three general areas: (1) new technologies for imaging gene expression in cells in tissue culture *in vitro*; (2) a reporter gene approach for imaging gene expression in living organisms *in vivo*; and (3) the antisense approach for imaging any gene transcript in any person *in vivo*. It covered a broad range of potential gene imaging and methodology issues, interspersed with extensive discussion of both a formal and an informal nature, herein summarized in the proceedings section.

Imaging gene function at the mrna level in real time will help identify biological errors contributing to disease. Imaging action at the function, protein, mrna, or gene level, is a proposition with an increasing order of challenge, complexity, merit and reward. Nuclear medicine utilizes radiotracer and radionuclide imaging techniques to make relatively simple measurements at the metabolic level. It can provide information on the functional consequences of perturbing the activity of a particular gene through specific hormonal or drug stimulation or blockade. Imaging at the gene level is most challenging because only 1-2 targets would normally be available per cell, except in cases

of gene duplication and target amplification from manifestation of certain pathologies. Also, it is not certain that the dna target sites will be any more accessible when a gene is being expressed than when it is not. In terms of raw numbers, the prospects of imaging mrna over genes is improved, because the mrna copy number is 10 to 1000 fold higher than the gene copy number in the expressing cells. Proteins can be recognized by a binding event or detectable catalytic activity. They have been the traditional targets of imaging. In both cases, the target amount for imaging is much larger at the protein level than at the gene or mrna level. This is especially true when catalytic activity can be harnessed to generate, accumulate, or fixate the agent or product that is generating the imageable signal. Reporter genes have been used to generate an imageable signal *in vivo*. This method does require delivery of the imageable reporter gene; and the approach may be best suited to follow the response to gene therapy wherein reporter genes are expressed in parallel with therapeutic genes. However, the reporter gene approach is not designed to fulfill the mission of imaging any gene in any person. Although indirectly, imaging any gene in real time is possible, provided the regulation of the gene of interest can be faithfully replicated in the regulation of a reporter gene delivered to the target site. Also the activity of many genes can be assessed very well at the level of protein or protein function with antibody probes or with specific labeled ligands, inhibitors, or substrates. Imaging at the level of function is already well established, though new radiotracers to expand the diversity of molecular targets, which can be imaged, are needed. In summary, the goal of imaging any gene in any person is believed to be a mission with profound health benefits to society. The advances in antisense drug discovery means those antisense radiopharmaceuticals or more suitable antisense agents through combinatorial chemistry techniques, can be designed to hybridize to target transcripts in a highly specific way. Molecular signal amplification methods are needed that work *in vivo* at the mrna level. New drug targeting technology and chemistry must be developed such that the various biological barriers can be effectively and safely surmounted *in vivo*. Finally, techniques devised for fluorescent molecular imaging could be brought together to function in conjunction with radionuclide imaging, for *in vitro* screening and data extrapolation to *in vivo* conditions.

Proceedings

The Targets for Imaging Gene Expression

As the sequencing of the genomes of several higher animals nears completion, the prospects for evaluating gene function and activity on a genome-wide basis becomes very tantalizing. Ideally, of course, it would be desirable to have a convenient general imaging method, one which could evaluate the activity of any endogenous gene of interest, at any site, and at any stage of development and physiological or environmental condition. Clearly this is a very tall order, and a challenge which is rightfully stimulating thinking on new approaches and methodologies for imaging. While the ultimate task presented above may need to be addressed partially or indirectly at first, imaging gene expression *in vivo* will certainly have an exciting and productive future.

Imaging at What Level: Gene, mRNA, Protein, or Function?

Remarkable chemical probes, minor groove binding polyamides, are being developed to recognize with high fidelity any arbitrary sequence in duplex dna (*Dervan*[1]). Some of these agents have been refined to the point that they are able to act in cells to alter the activity of specific genes and to discriminate between mutant genes. Imaging at this level—that of the gene—is not an attractive prospect, however, because only 1–2 targets would normally be available per cell. Also, it is not certain that the dna target sites will be any more accessible when a gene is being expressed than when it is not. Gene duplication, which is an important manifestation of certain pathologies, would increase the potential target size, although the extent of amplification in natural systems is still relatively low (i.e., rarely more than 30 fold).

Intensive work on the development of antisense agents *(Crooke)* has yielded a toolbox of oligonucleotide analogs, some of which have now been demonstrated to act as therapeutic agents by blocking gene expression. The target of these agents is mrna, and their aim is to extinguish gene expression at this level. In terms of raw numbers, the prospects of imaging mrna is improved over that of imaging the gene itself, but not by a great amount. The numbers of mrna molecules per cell is still rather low, so the target size for imaging remains very small. For example, although some highly expressed genes may produce more than 1000 mrna copies per cell, this is rare; more typical numbers are in the hundreds of copies per cell, and most in the tens. The pharmacokinetic behavior of those antisense agents optimized for therapy also appears to be poorly suited

[1] Contributions to the discussion by specific participants are indicated by their names. Please refer to the List of Participants at the end of this report for full names and institutional affiliations.

for *in vivo* imaging: large doses are needed, protein binding is extensive, and the mrna target itself is consumed as a consequence of the hybridization process. Clearly, further evaluations and new design efforts would be required to optimize antisense agents for an imaging rather than a therapy paradigm (*Hnatowich*).

Proteins, of course, have been the traditional targets of imaging: there are those that can be recognized by a binding event (proteins that are bound by labeled antibodies, receptors that bind labeled ligands, enzymes that bind inhibitors) (*Herschman, Katzenellenbogen)*, and those that are detectable by their catalytic activity (enzymes that turn over substrates) (*Herschman, Crystal, Tjuvajev, Fowler*). In both cases, the target size for imaging is much, much larger at the protein level than at the gene or mrna level. This is especially true when catalytic activity can be harnessed to generate, accumulate, or fixate the agent or product that is generating the imageable signal.

Target Size and Signal Strength: The Prospects for Signal Amplification *In vitro* vs. *In vivo*

Given the small size of imaging targets at the dna or rna level, one is prompted to consider methods for achieving the highest levels of signal amplification, so that imaging might be at least technically feasible. As challenging as this will certainly be for imaging, one can still be impressed by the astounding levels of signal amplification that can be achieved in cell-free systems, in which single molecules of rna can be visualized by fluorescence in a sequence-specific manner. *Ward* reviewed the rolling circle amplification approach for the detection of single nucleotide mutations in cultured cells and showed how this could be used in a combinatorial fluorescent imaging approach to study the presence of gene mutations in all 23 human chromosomes. Can such high levels of signal amplification be achieved *in vivo*?

Large levels of fluorescence signal amplification can be achieved in cells in culture (*Tsien*), and *in vivo* enzymatic activities in tissues can lead to accumulation of chemiluminescent (*Contag*) or radiolabeled substrates or products in a target tissue-specific manner (*Piwnica-Worms, Tjuvajev, Fowler, Katzenellenbogen, Herschman*). *Tsien* showed that cells transfected with the $-lactamase reporter gene could be subjected to fluorescent microscopy imaging of gene expression using specially designed fluorescent imaging molecules whereby a fluorophor was connected to a quencher via a $-lactam linker. This could lead to functional approaches to gene discovery whereby cultured cells are 'sprayed' with the $-lactamase reporter gene; differential gene expression could then be examined following stimulation of the cultured cells under a variety of conditions.

The metabolic imaging approaches are well known to laboratories using positron emission tomography (pet). In this approach, positron labeled small molecules are administered to animals or humans *in vivo* and the metabolic entrapment of the small molecule within the target organ allows for an indirect imaging of gene expression using standard external detection modalities such as pet or single photon emission computed tomography (spect).*Katzenellenbogen* and *Fowler* reviewed the approaches used for

imaging steroid hormone receptors or components of the dopaminergic system in brain, respectively. In such instances, the targets are receptors or enzymes, the products of endogenous genes, although not general, these targets provide an opportunity for the measurement of the activity of these specific genes (at the functional protein level).

As so far developed, the other methods rely on reporter genes to generate an imageable signal. The reporter gene approach to imaging gene expression is an outgrowth of the classical metabolic imaging approaches of remarkable scope and versatility. In principle, one can use this method to measure the expression of any particular gene by inserting the promoter of the gene of interest in a position where it will regulate an imageable reporter gene in the same manner as the endogenous gene is regulated. *Herschman* showed that positron labeled acycloguanosines such as ganciclovir or penciclovir could be labeled with fluorine-18 (^{18}F), a positron emitting isotope. Genetically engineered adenovirus carrying the Herpes simplex thymidine kinase reporter gene was injected into mice and this adenovirus selectively targets (>99%) the liver in mice; there was a selective sequestration of the acycloguanosines in the mice transfected with the thymidine kinase reporter gene. The alternative approach is to use the dopamine receptor and ^{18}F labeled spiperone. *Tjuvajev* discussed a similar reporter gene approach. In this approach, a retrovirus is used to carry the thymidine kinase reporter gene, and the reporter small molecule is fluoroiodouracil (fiau) labeled with iodine-124 (^{124}I), suitable for pet imaging. The use of positron emitting isotopes is particularly suited to the reporter gene methodology since most useful positron emitting isotopes such as carbon-11 and fluorine-18 can not only be prepared in high enough specific activities, but both carbon and fluorine can readily substitute for atoms in organic molecules.

This method does require, however, the delivery of an exogenous gene, the imageable reporter gene, to the tissue or site where the activity is to be monitored. During discussion a consensus emerged that the reporter gene approach is not designed to fulfill the mission of imaging any gene in any person. The reporter gene approach may be best suited to follow the response to gene therapy wherein reporter genes are expressed in parallel with therapeutic genes. *Crystal* reviewed his work with *in vivo* gene therapy in humans. The therapeutic gene consisted of vascular endothelial growth factor (vegf) and this transgene was carried to myocardial cells via an adenovirus vector following direct intramyocardial injection in humans for the treatment of coronary artery disease. It was found that the adenovirus transgene was only expressed for approximately 3 weeks secondary to the host defense immune system. Because of the immune response to adenovirus, there would be minimal gene expression following the non-invasive, intravenous injection of this virus in humans. Nonviral gene delivery systems were deemed highly inefficient because only a small amount of the therapeutic gene is actually found in the nucleus. In contrast, targeting the therapeutic gene to the nucleus was achieved with much higher efficiency using adenoviral or retroviral vectors.

The antisense approach to quantitating and imaging gene expression has been widely used for more than a decade with *in vitro* approaches such as Northern blotting or in situ hybridization wherein an antisense phosphodiester (po) oligodeoxynucleotide (odn) is

hybridized to mrna either isolated from organ extracts (Northern blotting) or mrna in fixed organ specimens (in situ hybridization). However, as reviewed by *Crooke*, the po-odn is highly unstable *in vivo* owing to degradation by both exonucleases and endonucleases widely distributed in the body. Phosphorothioate (ps)-odns are nuclease resistant. However, *Crooke* noted that 1-10 mm concentrations of ps-odn must be achieved in liver *in vivo* in order to achieve a true antisense effect, i.e., a pharmacologic effect arising from the formation of an rna/dna heteroduplex within the cytosol of the target cell. Protein binding is crucial for effective action of most oligonucleotide analogs. Even though the issue of the optimal protein binding remains to be defined, for any water-soluble analog, a failure to bind to plasma proteins leads to clearance at glomerular filtration rate. Also, protein binding plays a key role in the distribution process. *Crooke* also reported that extremely lipophilic oligonucleotide analogs, with the exception of those compounds that have a lipophilic modification at one end and are hydrophilic in other areas, generally were devoid of good biological activity.

The generation of a cellular concentration of 1–10 mm requires the administration of 10-100 mg/kg intravenously in mice. Conversely, the maximum doses of ps-odn given intravenously in humans is approximately 2 mg/kg, a dose that may be too low to generate a true antisense therapeutic effect in tissues *in vivo*. Owing to the sulfur atoms, ps-odns are highly reactive molecules that interact with a variety of cell membrane surface proteins. Because of this property, ps-odns has therapeutic effects via non-antisense mechanisms. For example, the beneficial effect of the ps-odns in cmv retinitis may be related to a non-antisense effect whereby the ps-odn inhibits viral binding to target cells. Although ps-odns can actually inhibit rnase h, it is believed the antisense effect of ps-odns is due to the activation of rnase h via the formation of an rna-dna heteroduplex. The binding of only single ps-odn molecule to a target rna is sufficient to activate rnase h, it is somewhat surprising that such high concentrations (1-10 mm) are required to generate an antisense effect in mouse liver *in vivo*. The explanation for this paradox is probably that less than 1% of the total hepatic ps-odn is found in the cytosol where the target transcript and rnase h molecules are located. Greater than 99% of the intracellular ps-odn in the liver is confined to subcellular organelles such as endosomes and pre-lysosomal structures.

There was discussion as to whether radiolabeled antisense molecules would generate a signal sufficient for measurement by standard external detection modalities given the fact that only 1-100 mrna molecules may exist in any target cell.

A successful noninvasive, direct detection of messenger rna rather than through protein detection is dependent on several factors: the specific activity of the radiopharmaceutical probe; the number of mrna copies for the specific target; the concentration of cells expressing the specific mrna; the instrumentation sensitivity for detection; the background or nonspecific accumulation of the radiopharmaceutical; the ability of the radiopharmaceutical to reach the intracellular target; the control of cleavage, and the potential for amplification of the radio-label. All of these factors have major importance for developing novel technologies necessary to image gene expression using antisense probes. Two questions were raised during and since the conference: are

there enough copies for the known specific activities to allow reliable quantitation in a reasonable time, and secondarily what will be the background?

The minimum activity per ml can be calculated based on the number of mrna copies (assume 10 per cell), the specific activity of the radiopharmaceutical (assume one million curies per mole), and the concentration of cells (assume 200 million per ml). Under these conditions, one can expect 7400 disintegrations per minute per ml of cells. But this translates to a modern 3d-instrument detection event rate of about 240 events per minute for each target ml. With the assumption of a target to background ratio of 2, a pet instrument could quantitatively evaluate this tissue activity with 7 mm resolution in ten minutes of data collection with an uncertainty of 20 percent, according to calculations by *Budinger*. The expected specific activity for short lived pet tracers such as ^{18}F is 1,000,000 Ci/mole; however, for a longer-lived radionuclide appropriate for antisense studies, such as ^{124}I, the specific activity can be less depending on the chemical synthesis. Because the sensitivity of single photon tomography instruments is at least 20 times less than that of pet, for 7 mm resolution, the expectations of signal to noise will change according to specific activity and other factors (listed above). But of most importance as agreed by *Budinger* and *Katzenellenbogen*, even if the copy number is much higher than a few per cell, the background activity is likely to overwhelm the image as the mrna copies will be saturated at a local concentration of only 0.003 microCurie. Recall that 1.0 mCi distributed uniformly throughout the body would be an average concentration of about 0.015 microCi/ml. In addition to lowering background by delaying the time of imaging there are methods of amplification, which might overcome this problem.

There are multiple modes of antisense binding which contribute to therapeutic effect. The stimulation of rnase h activity is but one of the mechanisms, and may not be the most important for imaging. *Crooke* mentioned that there are many rnase h independent mechanisms which do not require degradation of, or binding to an rna target for antisense activity. Targeting such mechanisms may be useful for imaging and evaluation of a loss of rna to increase specificity.

Although not widely discussed at the meeting, it was noted that the peptide nucleic acids (pna) may have better potential as antisense imaging molecules for studies in humans because these molecules only hybridize to target transcripts, but do not activate rnase h and thus would not lead to degradation of the target transcript. Conversely, the use of a ps-odn for imaging purposes could lead to activation of rnase h and an undesired degradation of the target transcript during the imaging procedure. Describing the Isis experience about the pna therapeutics, *Crooke* pointed out that the bound pnas show excellent high dissociation temperatures (Tms) and that many purine rich pnas have been prepared. However, despite the increase in affinity seen with pna in many systems, a commensurate increase in therapeutic efficacy has not been observed. This may be due to the poor ability of pna molecules to hybridize with the helices. Also, it has been observed from detailed pharmacokinetic, toxicologic, and pharmacologic comparisons in animals, that pna molecules display very poor pharmacokinetics. However, optimizing charge and hydrophilicity may improve the pharmacokinetic

behavior.

Remarkable methods have recently been developed to extract from the endothelial cells lining the blood vessels of tissues, proteins whose expression is specific for those tissues (*Schnitzer).* These methods could be used to identify novel targets that might have potential for tissue imaging, using radiolabeled antibodies or other agents that would bind specifically to these proteins or would utilize their catalytic activity, should they prove to be enzymes.

The Development of Contrast: Getting the Signal to the Target and Away from the Background

It is not uniformly appreciated that for imaging to be successful *in vivo*, one needs to provide three things: (1) an adequate quantity of a receptor, a protein target, or an amplifying system to generate a signal of sufficient magnitude, so that it can be detected by the imaging device, (2) means for delivering the labeling agent or substrate to the target site so that the signal can be generated or accumulated, and (3) a means for clearing away the excess of labeling agent or avoiding its activation or accumulation at non-target sites. The latter two points are pharmacokinetic issues that can be safely ignored in most cell-free and *in vitro* situations, but which prove to be major challenges *in vivo*. As a consequence, *in vivo* imaging agents need to be carefully optimized not only in their role of providing the signal, but also in terms of the facility with which they can be delivered to the target (this involves issues of distribution and tissue penetration that follows administration of the dose by intravenous injection), and in terms of the effectiveness with which they can be cleared from non-target sites (this involves issues of non-specific binding, metabolism, and excretion). For many small molecules, these kinetic features can usually be achieved by careful design. However, it is likely that both points 2 and 3 will prove to be significant difficulties with oligomeric agents that are directed at dna or mrna targets.

Traversing the cellular barriers is a well-recognized challenge, especially for macromolecules as discussed by *Wagner* and others. *Pardridge* reviewed the three barriers: (1) the capillary endothelial barrier of the target organ, (2) the plasma membrane of the target cell, and (3) the intracellular endosomal membrane of the target cell. In order to achieve a successful *in vivo* imaging of gene expression with antisense radiopharmaceuticals, the intravenously administered antisense radiopharmaceutical must traverse these 3 biological barriers *in vivo* so that the radiolabeled antisense radiopharmaceutical may be delivered to the cytosol of the target cell where the target transcript resides.

Schnitzer reviewed the vascular proteomics technology for tissue-specific targeting of caveolae to overcome both endothelial and epithelial barriers to drug and gene delivery *in vivo*. Data on monoclonal antibody transcytosis in the lung indicate that the caveolae transport pathway may prove quite useful in getting imaging agents to their intended cells in difficult situations: when the target tissue is on the other side of the usually quite

restrictive endothelial and epithelial barriers. It may also be a way to get agents into the target cells - possibly by creating bifunctional targeting agents.

Targeting is most difficult in the brain owing to the additional presence of the blood-brain barrier (bbb). *Pardridge* reviewed the chimeric peptide technology for drug targeting across the bbb *in vivo*. In this approach, peptidomimetic monoclonal antibodies undergo receptor-mediated transcytosis through the bbb *in vivo* are in the form of antibody/avidin fusion proteins, and transport biotinylated peptide or antisense radiopharmaceuticals. The peptidomimetic monoclonal antibody that targets a specific receptor on the bbb may also undergo receptor mediated endocytosis in the target brain cells, with the same receptor on the plasma membrane of those target cells. The limiting factor in the delivery of antisense radiopharmaceuticals to the cytosol of brain target cells is the present lack of a suitable and safe endosomal release system built.

Endogenous Genes vs. Delivered Reporter Genes

The issue of imaging the expression of any gene in any person at any time was debated keeping in mind the current state-of-the-art in technology. Imaging in real time, although indirectly, is possible provided the behavior of the reporter gene faithfully represents the regulation of the gene of interest and can be delivered to the target site. But, because gene delivery is required, this approach is limited to animals or to situations where such genes can be delivered to appropriate target sites in humans. Also, what is being measured is not the transcriptional output of the endogenous gene itself, but the activity of a reporter gene surrogate whose regulation has been engineered by recombinant methods to provide a faithful reflection of the transcription of the endogenous gene. While these are tall orders, they are not outside of the scope of currently available technologies. The technology to produce knockout and transgenic experimental animals can also be adapted to introduce imageable reporter genes to assess specific gene expression.

Also the activity of many genes can be assessed very well at the level of protein or protein function, with antibody probes or with specific ligands, inhibitors, or substrates, respectively. Imaging at the level of function is already well established, and is constantly being expanded in an enterprising and promising fashion. Often, as well, the functional consequence of perturbing the activity of a particular gene, through specific hormonal or drug stimulation or blockade, can be assessed effectively, albeit indirectly, by making relatively simple measurements at the metabolic level. Here, well established methods for measuring oxygen, carbohydrate, and fatty acid consumption, can be used.

Concluding Remarks

In summary, the goal of imaging any gene in any person is believed to be a mission with profound health benefits to society should the goal be realized. Currently the expression of endogenous genes in animals (including humans) cannot be imaged, at least not directly. Realization of this goal may appear to be a very tall order at present. However, depending on the circumstances and the technologies that can be brought to bear on the specific molecular imaging situation of a general interest, and given the astounding pace of biotechnology development, it may be highly challenging but not an unattainable goal. The success, however, must come through well though out planning, team efforts and future research endeavor: the advances in antisense drug discovery means that antisense radiopharmaceuticals through combinatorial chemistry techniques can be designed to hybridize to target transcripts in a highly specific way. However, the antisense and combinatorial molecular chemistry technologies available for chemotherapeutic drug development, must be fully exploited and optimized for imaging. Molecular signal amplification methods are not yet available that work *in vivo* at the mrna level, and technological advancement in this area is well desired. Equally important is the hurdle of drug targeting technology, which must be developed to such an extent that the various biological barriers (endothelial, cell membrane, and endosomal membrane) can be safely surmounted *in vivo*, using genetically engineered drug-targeting systems. Clever techniques for harnessing specific protein transport systems may assist in macromolecular delivery to the brain. Finally, there should be instrumentation techniques devised for fluorescent molecular imaging technologies to function in conjunction with radionuclide imaging for *in vitro* screening and data extrapolation to *in vivo* conditions. These are some of the critical medical science and technology challenges, which were identified at the workshop. These challenges must be addressed through responsible encouragement and commitment to and from the scientific community of chemists, biologists and clinicians working together as a team towards this very rewarding goal for the next millennium.

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