(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
1 December 2005 (01.12.2005)

PCT

(10) International Publication Number
WO 2005/113022 A1

(51) International Patent Classification[7]: A61K 49/06

(21) International Application Number:
PCT/GB2005/001983

(22) International Filing Date: 19 May 2005 (19.05.2005)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0411417.9 21 May 2004 (21.05.2004) GB

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declaration under Rule 4.17:
— *of inventorship (Rule 4.17(iv)) for US only*

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: SYSTEM FOR MONITORING GENE EXPRESSION BY MAGNETIC RESONANCE IMAGING USING TARGETED CONTRAST AGENTS

(57) Abstract: The invention relates to a method of monitoring gene expression in a system comprising introducing to said system an MRI contrast agent linked to a targetting agent capable of binding to a cell surface antigen; and obtaining an MRI image from said system. The invention further relates to a method of monitoring gene expression in a system comprising introducing to said system nucleic acid comprising a promoter of interest operably linked to a nucleic acid sequence encoding a cell surface antigen; incubating said system for a time sufficient to allow gene expression to take place; introducing to said system an MRI contrast agent linked to a targetting agent capable of binding to said cell surface antigen; and obtaining an MRI image from said system. The targetting agent is preferably an antibody. The invention also relates to the use of superantibodies in MRI, in particular when linked to a contrast agent.

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MAGIC METHOD

Field of the Invention

The invention relates to methods of magnetic resonance imaging (MRI). In particular, the present invention relates to a generic method for the imaging of gene expression, and more in particular to methods involving magnetic antigen gene imaging contrast (MAGIC).

Background to the Invention

Magnetic resonance imaging (MRI) is widely applied in medicine. In brief, sensitive nuclei can be induced to produce radio frequency signals by the application of a magnetic field. The resulting free induction decay signals can be resolved by mathematical processes such as Fourier transformation and give rise to images providing anatomical information (MRI) or a frequency spectrum giving biochemical information (magnetic resonance spectroscopy or MRS).

The opportunity to generate images of living tissues or organisms by a non-invasive and non-destructive method has made MRI an extremely widespread imaging technique. The images generated correspond to high resolution maps of the local distribution of water molecules. The contrast produced in the images is due to differences in content or properties of the water within the area examined. The usefulness and interpretation of the images generated is influenced by manipulation of the magnetic pulse sequences used or more commonly by the use of magnetic resonance contrast agents. Contrast agents usually comprise biologically inert paramagnetic entities such as iron or gadolinium. Their delivery and targeting is a key problem area in MRI.

Contrast agents may be targeted passively. This can be by local application or by injection or other means of introducing soluble, granulated or particulate contrast agents into an area for examination. However, problems with passive targeting include difficulty in providing specific application of the contrast agent, and include counter action by the hosts' natural

defence mechanisms such as phagocytosis removing the individual particles of contrast agent from the body.

Other methods of introducing contrast agents include active targeting. Two such techniques are found in the prior art in this area.

The first is a receptor based technique for targeting the delivery of the contrast agent. In this situation, a receptor is placed in the area of interest and a ligand-contrast agent conjugate is used to specifically deliver the contrast agent to the receptor via the ligand. The second form of targeting in the prior art is targeting via localised activation. In this approach, an inert substrate-blocked form of a contrast agent is produced and flooded into the system. A specific enzyme is then targeted to the area of interest such that when the inert contrast agent is brought into contact with the localised activating enzyme, the substrate block is removed and the contrast agent is activated. In this way, although the contrast agent is present throughout the system, it is only activated in the areas of interest which have been targeted.

Louie *et al* (2000 Nature Biotechnology Volume 18 pages 321) described the use of MRI in monitoring reports of gene expression in living animals. They used an MRI contrast agent in which the access of water to the first co-ordination sphere of the chelated paramagnetic iron was blocked with galactopyranose as the blocking group. The reporter gene which they used was β-galactosidase. Upon contact with β-galactosidase, the galactopyranose blocking group is cleaved thereby activating the contrast agent. Using this system, β-galactosidase expression was followed in *Xenopus* embryos.

Bell *et al* (2000 Gene Therapy Volume 7 pages 1259) described the use of 'smart' MR contrast agents in monitoring gene expression. These smart contrast agents are at first inert and are then conformationally altered to produce an active form by enzymatic action of a marker gene. One such smart agent described is β-galactosidase sensitive agent as mentioned above. Another contrast agent described as being sensitive to β-galactosidase is also features a caged gadolinium attached to a fluorescent dye for detection by light microscopy. Furthermore, the calcium sensitive contrast agent DOPTA-GD is also described. However, each of these contrast agents suffers from the difficulty of their delivery to intact cells or

tissues. One further contrast agent is described is a gadolinium compound conjugated to DNA. Again, this is routed through cell surface receptors and results in internalisation of the agent together with the carrier into the cell. As noted above, this approach is potentially hazardous due to the loading of these chemicals into the actual cells. Bell *et al* note on page 1261 that there is no known generic MR marker for endogenous gene expression.

Moore *et al* (2001 Radiology Volume 221 pages 244) describe the use of human transferrin receptor as a marker gene in MRI. They describe the transfection of rat gliosarcoma cells to produce expression of the transferrin receptor, followed by administration of monocrystalline iron oxide nanoparticles in order to facilitate the detection. Again, cell internalisation of the particles is reported. This problem is reinforced in their own conclusion section on page 244. Their paper follows on from preliminary work with the asialoglycoprotein receptor which is again an endocytotic receptor.

Bogdanov *et al* (1998 TibTech Volume 16 pages 5) describe the use of marker genes in MRI. Marker genes described either encode intracellular enzymes such as thymidine kinase or β-galactosidase. They also describe the use of cell surface receptors such as the transferrin receptor in MRI.

Lanza *et al* (2003 MEDICAMUNDI Volume 47 pages 34) discuss targeting of paramagnetic nanoparticles. They describe passive targeting and also mention active targeting using ligands and receptors. The disclosure focuses on the example of fibrin targeted nanoparticles, and the integrin receptor is also mentioned, again in the context of ligand directed particles. Nanoparticles can be problematic due to their large size, which can restrict the target cells which can be imaged. Nanoparticles can have problems in tissue penetration.

Thus, the prior use of MRI to image gene expression has involved the insertion of marker genes either encoding enzymes or cell surface receptors. A major disadvantage of the former method is that there is a prerequisite for intracellular entry of the substrate for catalysis and hence, generation of the MR visible product. This has been overcome to a degree by the use of cell surface receptors, e.g., engineered transferrin receptor or tyrosinase. However, both

these methods involve the intracellular entry of significant amounts of iron which may have unknown physiological and metabolic effects.

There are numerous problems with a receptor type system of targeting. Firstly, extra copies of the receptor may themselves be undesirable. There may be signaling issues both on the targeted cells or indeed their neighbours as a function of the extra receptors being present. Furthermore, receptors are frequently internalised upon ligand occupation. This internalisation may be undesirable. Moreover, flooding a subject with excess ligand may itself have undesirable consequences. The ligands may be active in their own right, they may cross react with naturally occurring receptors and potentially a new coupling chemistry may need to be designed for each individual ligand used. This system thus has numerous problems and is difficult to generalise across diverse applications.

Similar problems are present with the enzyme based targeting system. Introducing potentially large amounts of foreign enzyme activity into a subject may itself be a hazardous procedure. Naturally occurring substrates or their analogues may be turned over at unacceptable levels in the subject as a consequence. Furthermore, the amount of contrast agent which would have to be introduced is very large since the contrast agent itself is not targeted and relies on being present throughout the system only to be cleaved into the active form by the localised enzyme. Thus, it can be a very expensive process. Furthermore, contrast agent chemistry in order to provide substrate dependent inactivation/protection is itself complicated and labour intensive.

The present invention seeks to overcome problems associated with the prior art.

Summary of the Invention

Prior art techniques for imaging gene expression via MRI have focused on the expression of foreign enzyme activities or exogenous receptors in cells as markers. As explained above, there can be numerous problems associated with spurious effects from receptor expression, or problems in maintaining (or caused by) enzyme activity with marker enzymes. The present invention avoids such problems by advantageously using cell surface antigens as marker

genes for MRI. This is beneficial in being more selective, reducing toxicity and avoiding build up of contrast agent in cells.

In the methods of the present invention, a cell surface antigen is expressed in the area of interest. An antibody charged (eg. conjugated) with ordinary contrast agent can then be applied to the system. In this way, the contrast agent is targeted to the area and to the cells of interest, but advantageously avoiding the detrimental effects associated with mis-expression of ligand/receptor pairings. Furthermore, this method avoids the problems of excessive interalisation which can be associated with systems such as the transferrin receptor.

Thus the invention advantageously provides a method for analysing gene expression via MRI using relatively inert marker molecules, and using readily available and well understood targeting molecules to delivery the contrast agent. The numerous further benefits from using the method according to the present invention are explained in more detail below.

In a broad aspect, the invention relates to nuclear imaging such as radionuclear imaging. This area is understood to include both magnetic resonance imaging (MRI) and positron emission tomography (PET), since both techniques rely on similar principles of supply of contrast agent following electromagnetic radiation based image readout. In the case of MRI this is brought about by detection of emitted radiowaves from resonating nuclei, and in the case of PET this is brought about by detection of emitted radiation following radioactive decay. However, it will be apparent from the context of the invention that in principle the only difference between these read-outs is the choice of contrast agent used. Embodiments of the invention should be read with this in mind, ie. applications described in the context of MRI can be adapted to PET simply by substituting the MRI contrast agent with a PET contrast agent. Indeed, in one embodiment both MRI and PET contrast agents are targetted on a single carrier/targetting molecule so the resulting system can advantageously be imaged using either MRI or PET or both. Preferably the invention relates to MRI.

In a broad aspect, the invention relates to a method of monitoring gene expression in a system comprising introducing to said system an MRI contrast agent linked to a targetting agent capable of binding to a cell surface antigen; and obtaining an MRI image from said system.

In another aspect the invention relates to a method of producing a magnetic resonance image comprising the step of linking a magnetic resonance contrast agent to a cell surface antigen present on the structure of interest.

The system may be any system amenable to MRI such as cells, tissues or preferably a living organism.

In a broad aspect, the cell surface antigen which is detected by the present invention may be inherently expressed in or on the cells to be imaged. The techniques may be used to follow real-time gene expression. However, preferably the cell surface antigen detected is caused to be expressed by the operator. This expression may be brought about by any suitable means known in the art, such as by gene activation, by induction of expression using physical or chemical agents, or by techniques involving recombinant nucleic acid. Preferably expression is brought about using recombinant nucleic acid. This might be by supplying a promoter and/or enhancer element, or directing production of a transcription factor or associated apparatus, or may be by provision of a transgene expressing the cell surface antigen of interest. Preferably expression of the cell surface antigen whose expression is being monitored is brought about by use of recombinant nucleic acid, preferably comprising sequence encoding the cell surface antigen of interest and a promoter capable of directing its expression in the cells of interest operably linked thereto.

In one aspect the invention relates to a method of monitoring gene expression in a system comprising inducing expression of a cell surface antigen in said system, incubating said system for a time sufficient to allow gene expression to take place, introducing to said system a nuclear imaging contrast agent linked to a targetting agent capable of binding to said cell surface antigen, and obtaining a nuclear image from said system.

Preferably inducing expression of a cell surface antigen in said system comprises introducing to said system nucleic acid comprising a promoter of interest operably linked to a nucleic acid sequence encoding a cell surface antigen.

Preferably the nuclear imaging is magnetic resonance imaging (MRI).

Thus, in another aspect the present invention relates to a method of monitoring gene expression in a system comprising introducing to said system nucleic acid comprising a promoter of interest operably linked to a nucleic acid sequence encoding a cell surface antigen; incubating said system for a time sufficient to allow gene expression to take place; introducing to said system an MRI contrast agent linked to a targetting agent capable of binding to said cell surface antigen; and obtaining an MRI image from said system.

Incubating to allow gene expression need not imply that gene expression occurs. If the conditions are such that the gene is shut down at the time of incubation, then this may itself be the read-out from that particular assay, as will generally be the case in negative control treatments. This step is simply to permit the transcription/translation machinery the time to express the gene if that is what will happen in the particular circumstances of the system at that time.

The targetting agent must be capable of binding to the cell surface antigen used in order to localise the constrast agent to the cell surface antigen. This binding may be reversible or irreversible. The binding may be covalent or non-covalent or may be by hydrogen bonding, salt bridging or van-der-waals interactions or by mere association. The binding may be direct or indirect or may be facilitated by one or more cofactors or may be mediated by one or more cofactors such as a sandwich arrangement. Preferably the binding is direct. Preferably the binding is by electrostatic/hydrogen bonding means.

Preferably the targetting agent is an antibody.

Preferably the antibody is an $H2K^k$ -FITC or a MACSelect K^k microbead, preferably a MACSelect K^k microbead.

The MRI image may be obtained using any suitable MRI equipment capable of responding to the contrast agent used.

The contrast agent may be any suitable agent known in the field and is preferably one in a form as mentioned hereinbelow. Preferably the contrast agent is a paramagnetic iron particle. Preferably said paramagnetic iron particle is approximately 1um to 20nm in size, preferably less than 100nm, preferably 50nm, or even smaller. In general, smaller is better since in the context of the invention it offers advantages of tissue penetration and easier clearance. Preferably the paramagnetic iron particle comprises an iron oxide core and a dextran coating. Preferably said dextran coating is approximately 50nm thick, which means that the iron and dextran coating is approximately 50nm in diameter. Preferably the iron oxide core is approximately 4-6nm in diameter and the dextran coating makes up the balance of the diameter, the total diameter preferably being approximately 50nm.

Preferably the contrast agent comprises dendrimers which are chain-like polymers to which substances such as gadolinium can be attached. These are advantageous for their small size.

Preferably the cell surface antigen is a class I MHC antigen.

Preferably the cell-surface antigen is the $H2K^k$ antigen, preferably the truncated $H2K^k$ antigen. The truncated H2Kk antigen has the advantageous feature of not activating second messenger systems, and not becoming internalised but rather staying on the cell surface.

In another aspect, the invention relates to the use of superantibody linked to a magnetic resonance imaging contrast agent in the production of a magnetic resonance image.

In another aspect, the invention relates to the use of superantibodies for imaging gene expression by MRI.

In another aspect the invention relates to use of H2Kk for imaging gene expression by MRI.

Detailed Description of the Invention

Analysis of gene expression/MRI

The ability to non-invasively map gene expression *in vivo* has tremendous implications for molecular medicine and biotechnology, and crucial in translational medicine and gene therapy. Longitudinal real-time imaging of individual animals obviates the necessity of sacrifice and harvesting of tissue to analyze gene expression required for traditional biological methods, which are *in vitro* based.

Approaches to imaging gene expression *in vivo* can be categorised into those that are based on optical, bioluminescence, near-infrared fluorescence, nuclear, or magnetic resonance imaging techniques. Depth penetration limits the use of optical, bioluminescence and near-infrared fluorescence techniques whereas nuclear imaging is limited by inherently lower spatial resolution. Magnetic resonance imaging (MRI) has excellent spatial resolution and depth penetration although suffers from inherently low sensitivity compared to the other techniques. However, with increasing magnetic field strengths and amplification strategies this can largely be overcome. Furthermore, the use of contrast agents greatly improves MR image generation, analysis and interpretation.

We have developed a new generic method for *in vivo* MRI of gene expression based on magnetic-antigen-gene imaging contrast (MAGIC). This involves the introduction of a marker gene coding for a cell surface antigen. The antigen in turn binds an appropriate antibody in a typical antigen-antibody interaction. In the method of the invention, the antibody is linked to a contrast agent, preferably conjugated to paramagnetic iron particles. The paramagnetic iron particles range in size, typically 20nm – 1um. The effect of these paramagnetic iron particles is to induce T2 relaxation of the surrounding water protons leading to attenuation of the MRI signal intensity, thus generating contrast. The iron oxide particles conjugated to the antibody preferably consist of an iron oxide core with a dextran coating (~50nm).

We illustrate the application of MAGIC using a truncated form of the $H2K^k$ antigen, a class I MHC antigen occurring endogenously in some mouse stains. This method can be applied to any antigen of choice providing contrast agent can be linked to an antibody recognising it. Preferably paramagnetic iron particles conjugated to the antibody are available. A variety of such conjugated antibodies are readily available commercially.

The method of the present invention has the advantage that the contrast agent does not need to enter the cell to generate gene expression related MR contrast. The method has the further advantage of avoiding the increased likelihood of adverse effects from such entry.

MRI may be conducted using commercially available apparatus. The actual mechanics of image generation or sample handling are not at the core of the present invention. The present invention relates to the method of producing images by targetting of the contrast agents to cell surface antigens. The actual output of an image following this contrasting technique can be accomplished using standard MRI equipment known in the art and described in different embodiments for example in Bell and Taylor-Robinson (Gene Therapy 2000 volume 7 pp1259-1264).

Cell Surface Antigens

This term is used to describe any cell surface antigen. These are sometimes referred to as cell surface markers. The important aspect is that they are displayed on the extracellular surface of the cell once expressed. In this manner, they are available for interaction with the moiety bearing the contrast agent without said moiety having to enter the cell. Furthermore, advantageously said contrast agent itself does not enter the cell.

Examples of such cell surface antigens are known to the skilled reader.

One group of cell surface antigens is known as the cell determinant or 'CD' antigen. This refers to any of a number of cell surface markers expressed by leukocytes and used to distinguish cell lineages, developmental stages, and functional subsets; such markers can be identified by specific monoclonal antibodies and are numbered CD1, CD2, CD3, etc. Markers used to identify T lymphocyte subsets were formerly called T antigens.

Other antigens that may be used include truncated CD4 and truncated human low-affinity nerve growth factor receptor (LNGFR). Both these and the targetting agents such as those directed against truncated H2K antigen are available from Miltenyi Biotec Inc, Germany with

microbeads (iron-dextran particles) conjugated to the relevant antibody. An advantage of using LNGFR is that it can avoid immune reactions which can occasionally be triggered by H2Kk, and so LNGFR is especially suitable for use in immune competent systems. However, since LNGFR is expressed in the CNS, choice will need to be made to take account of this.

Preferably the cell surface antigen is a T antigen.

Preferably the cell surface antigen is a cell determinant.

Preferably the cell surface antigen is a cell surface marker characteristic of a particular cell lineage. Preferably the cell surface antigen is a cell surface marker characteristic of a particular leukocytic cell lineage.

Preferably the cell surface antigen is CD4, preferably truncated CD4, CD2, LNGFR, H2Kk, preferably truncated H2Kk, myc tag, biotin/avidin (in which case avidin beads and/or anti-biotin beads may be used as targetting reagent, preferably anti-biotin beads which advantageously don't bind free biotin and are available in cGMP-grade). Preferably the antigen is truncated CD4, truncated H2Kk, or LNGFR. Alternatively, antigens can be made to be expressed on the cell surface such as by fusion to a cell surface protein; antigens in this category include myc tag, HA tag, lacZ, GFP, biotin, avidin or his tag. Preferably the antigen is a *bona fide* cell surface antigen ie. one that is naturally expressed on the cell surface without the need for fusion.

For human application, a non-endogenous cell surface antigen is preferred, preferably one which does not provoke an immune reaction, preferably one which does not trigger second messenger systems downstream (including on antibody binding).

Preferably the cell surface antigen is a major histocompatibility (MHC) antigen.

One group of MHC cell surface antigens is the class I antigens, major histocompatibility antigens found on virtually every cell, human erythrocytes being the only notable exception;

they are found on molecules consisting of two noncovalently bound chains. One, a 44,000-dalton polymorphic glycoprotein partially embedded in the cell membrane, is determined by an MHC gene (HLA-A, -B, or -C in humans); the other, beta$_2$-microglobulin, a 12,000-dalton nonpolymorphic protein, is determined by a non-MHC gene. Class I antigens are the classic histocompatibility antigens recognized during graft rejection and are also the antigens involved in MHC restriction.

One group of MHC cell surface antigens is the class II antigens, major histocompatibility antigens found only on immunocompetent cells, primarily B lymphocytes and macrophages; they are found on molecules consisting of two noncovalently bound chains, the 34,000-dalton alpha chain and 29,000-dalton beta chain, both glycoproteins partially embedded in the cell membrane and both determined by MHC genes. The human HLA-D, -DR, -DQ, -DT, -MB, -MT, and -Te loci are all associated with antigenic determinants on class II antigen molecules.

One group of MHC cell surface antigens is the class III antigens, a term used to refer to nonhistocompatibility antigens mapping in the major histocompatibility complex, e.g., the complement components C2, C4, and factor B.

Preferably the cell surface antigen belongs to a sub-group of the major histocompatibility (MHC) antigens as described above.

Advantageously, existing available immunohistochemical data may be used to identify possible targets / antibody pairings.

Preferably the cell surface antigen is not a receptor.

Preferably the cell surface antigen is not a ligand.

Preferably the cell surface antigen is not an enzyme.

The cell surface antigen/nucleic acid may be delivered by any suitable means known in the art such as transfection, transduction, injection, microinjection, infection, superinfection or

other technique for introduction. Preferably the cell surface antigen is delivered using nucleic acid encoding same.

In another embodiment, the cell surface antigen may be delivered by inducing its expression from a naturally occurring sequence in the genome of the cells/system of interest by appropriate treatment of said cells/system.

Targetting Agent

The targetting agent may be any moiety capable of binding to the cell surface antigen, and capable of supporting association with a MRI contrast agent. In this context, 'capable of' is meant to indicate that the contrast agent may be directly associated with the targetting agent, or may be indirectly associated with the targetting agent, for example by a two-step system such as a primary antibody targetting agent and a secondary antibody coupled to the contrast agent which delivers the contrast agent by binding to the targetting agent. A two-part system such as this can advantageously allow amplification of the signal. Furthermore, a two-part system can advantageously allow smaller components to be used and thus facilitate extravascation. Thus, the targetting agent may be single part entity or a multi-part entity. Preferably the targetting agent is a multi-part entity.

Preferably the targetting agent is not a ligand to a receptor.

Preferably the targetting agent is a polypeptide.

Preferably the targetting agent is an antibody or antibody fragment.

Preferably the targetting agent is a monoclonal antibody or fragment.

Advantageously targetting/linking can be carried out using an antibody sandwich technique to amplify the signal. For example, a primary antibody is used to attach to the cell surface antigen. A secondary antibody which is directed against the primary antibody is then used to deliver the contrast agent. Preferably a plurality of secondary antibodies will bind to each

primary antibody, thereby delivering a greater amount of contrast agent than would be achieved using only a labelled primary antibody.

Preferably the targetting agent is a superantibody or superantibody fragment. Superantibody technology is available to the skilled person for example via Innexus Biotechnology Inc., Kentucky, USA/Immpheron Inc., Ohio. USA.

Linking

The contrast agent is linked to the targetting agent so that it can be carried to the site of interest by same. The linkage may be by any suitable technique known in the art. This link may be covalent or non-covalent. It may be by standard conjugation techniques, or by multi-part linkage such as biotin-streptavidin or similar components.

Tracking gene therapy vectors and expression

MAGIC can be used to image gene transfer and/or to track cells. Advantageously it can be used to image the tracking of gene therapy vectors *in vivo*. The gene therapy vector (eg adenovirus, liposomes) can be tagged by an antibody/targetting moiety that is conjugated to a contrast agent, or recognized by an antibody/targetting moiety that is then recognized by another antibody/targetting moiety that is conjugated to the contrast agent, ie. in effect, direct and indirect labelling.

One way of working the invention in this manner is to use an antibody conjugated to biotin that recognizes the outer surface of a gene therapy vector (such as viral or viral-like particles) and then using an anti-biotin moiety conjugated to a contrast agent.

To apply the invention to the imaging of gene therapy vector expression, care should be taken to incorporate DNA that leads to the expression of antigen(s) not endogenously expressed by the host cells, otherwise signal to noise ratios may compromise the exercise.

In this embodiment, the invention may advantageously be used in following transfection with the gene therapy agents. Suitable antigens for use in this embodiment include those which only arise as a result of expression from or induced by the gene therapy vectors.

Further aspects and applications

Advantageously MAGIC extends the use of magnetically labeled antibodies such that the cell surface markers can be used to image gene expression for any gene of interest in any cell of choice. In effect, the marker gene such as the preferred *t*H2K^k gene is an imaging gene marker (comparable to lacZ in immunohistochemistry) whose product is used to report on the expression of genes also under the same genetic control. Thus, the present invention also enables the imaging of gene transfer.

SPIO (small paramagnetic iron oxide nanoparticles)-conjugated antibodies or antibody fragments can be used in the methods of the invention.

Use of [biotinylated-antibody, streptavidin and biotinylated-SPIO] and [biotinylated antibody and streptavidin] systems enable amplification, such that sensitivity is increased. Further, the use of such moieties rather than antibody-SPIOs means that the molecular weight may be kept low, improving extravasation from the circulatory system to the cells in such embodiments. Thus, embodiments of the MAGIC protocol using biotinylated antibody and streptavidin-conjugated SPIOs advantageously increase the sensitivity as well as delivery and clearance of these moieties and hence, increase the applicability of this method to imaging transgene expression in biological systems.

Thus, in general terms, the invention advantageously provides an MRI/PET technique for imaging gene expression using cell surface antigens such as H2Kk whereas prior art techniques used beta-galactosidase gene expression with cleavable chromogenic substrates such as X-gal and visual spectrum photomicrography.

For PET applications, the elements and considerations are as for the MRI described applications, except that a radiometal is preferably the contrast agent for PET.

In one embodiment, both MRI and PET are catered for in a single reagent, for example (MRI): [bead-chelator-radiometal] :(PET) wherein the chelator is linked to the targetting agent (preferably an antibody) and the two sites for attachment to the chelator are occupied as shown, one by a bead (for MRI readout) and one by a radiometal or radiohalide (for PET readout).

Preferably the chelator is DOTA, for example
(MRI): [bead-DOTA-radiometal] (PET) or (MRI): [bead-DOTA-radiohalide]:(PET).

Preferably gadolinium is chelated for MRI, thereby avoiding use of beads:
(MRI): [[gadolinium-DOTA]-radiometal] (PET). In this embodiment, stronger chelators are preferred to avoid toxicity of gadolinium leaching out of the chelator.

For a PET embodiment it may be convenient to radiofluorinate the targetting reagent eg. antibody directly eg. *via* lysine residues or iodination.

Introduction of the nucleic acid may be by any suitable means, such as using ExGEN-500 which is a cationic polymer (non-viral, non-liposome) gene delivery agent comprising a linear 22kDa polyelthylene imine compound.

The techniques of the invention may be applied to bioluminescence.

The invention is now described by way of example, in which reference is made to the following figures:

Figure 1 shows typical MRI images of cell pellets 48h after transfection with pMACSKk or pMACS4.1 at (a) TE = 30ms and (b) TE = 200ms.
Figure 2 shows a 4.7T MRI image of mouse 2 after injection with MACSelect K^k Microbeads
Figure 3 shows a 4.7T MRI image of mouse 3 after injection with MACSelect K^k Microbeads
Figure 4 shows a 9.4T MRI image of mouse 3 after injection with MACSelect K^k Microbeads

Figure 5 shows a 9.4T MRI image of the dissected skin grafts from mouse 3 after injection with MACSelect K^k Microbeads.

Figure 6 shows a 4.7T MRI image of mouse 1 showing the lower signal intensity of the H2K graft *in situ*.

Example 1: MAGIC MRI in mouse system

We demonstrate the use of MAGIC for the imaging of gene expression by MRI.

This generic method has the advantage of being readily accessibile to all researchers whilst having the flexibility to be tailored to individual researchers needs. As explained above, the method can draw upon existing available immunohistochemical data to identify possible targets and antibodies.

Methods and Materials

Cell Culture, Transfection and Harvest

HeLa cells (an adherent human cervical carcinoma cell line) were obtained from Cancer Research UK and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with heat inactivated 10% foetal calf serum (FCS, Invitrogen, Paisley, UK) at 37°C in a humidified atmosphere of 10% CO_2.

Four 6-well tissue culture plates were plated with HeLa cells (1.6×10^5 cells/well), 36h prior to transient transfection. Two wells were transfected with 1μg pMACSKk or pMACS4.1 (Miltenyi Biotech Ltd, Bisley, UK) at a ratio of 3:1 (Fugene 6, Roche Diagnostics Ltd., Lewes, UK:DNA). At 44h after transfection, the cells were washed with phosphate-buffered saline (PBS) and detached with trypsin-versene (0.3ml). Following detachment and neutralisation with DMEM supplemented with 10% FCS (2ml), the cells were spun and then resuspended in PBS (100μl) for cell counting using the Trypan Blue Exclusion assay prior to cell labelling by microbead or FITC-conjugated antibodies. Aliquots (5μl) from each sample were pooled and fixed for 20mins with 2% paraformaldehyde (1ml PBS) at 4°C. After

fixation, the cells were washed with PBS (1ml) twice and then stored in PBS prior to FITC labelling (see below).

Hela cells were also plated onto coverslips (200 x 200mm) contained in a 6 well plate. After culturing for 36h, the each well was transfected with either pMACSKk or pMACS4.1 as detailed above. At 44h after transfection, the cells were washed with PBS (1ml) and then fixed with 2% paraformaldehyde for 20mins at 4°C. After fixation the cells were washed twice with PBS (1ml, 5mins per wash), and then stored at 4'C prior to immunohistochemical analysis (see below)

Antibodies

The antibodies used in this study were anti-mouse H-2K^k-FITC and MACSelect K^k microbeads obtained from Miltenyi Biotec Ltd (Bisley, UK). Microbeads have previously been sold for cell sorting applications such as FACS – their use in imaging is another aspect of the present invention.

Cell Labelling for in vitro MRI and electron microscopy

Cells (3-4 x 10^6) transfected with pMACSKk or pMACS4.1 were spun down and resuspended in PBS (100μl) and MACSelect K^k Microbeads (25μl) added. After 30 mins incubation at 4°C, all cells were washed three times with PBS (1ml). Samples for MRI were counted and the cell viability assessed using the Trypan Blue Exclusion assay. Cells (~3 - 4 x 10^6 were pelleted in 0.5ml tubes (900g for 2 mins) for MRI. Cells were transfected, harvested and analysed by MRI in triplicate.

Samples for electron microscopy (EM) were pelleted (900g for 2 mins) and fixed with 3% glutaraldehyde (cacodylate buffer, 1ml) for 6h at room temperature. After fixation, the cells were washed and stored in cacodylate buffer at 4°C prior to preparation for EM analysis. For EM analysis, the samples were treated with 1% osmium tetraoxide and dehydrated with

ascending grades of alcohol and embedded in epoxy-resin. Ultra thin sections of 60-80nm thickness were stained.

Cell Labelling for Flow Cytometry Analysis

For flow cytometry analysis (FACS), anti-$H2k^k$ FITC (8µl) was added to cell suspensions (~10^6, 32µl) transfected with pMACSKk or pMACS4.1. After 15mins incubation at 4°C, the cells were washed twice with PBS (0.5ml) prior to FACS analysis. All data was acquired on a FACScan flow cytometer (Beckton Dickinson, San Diego, USA). Acquisition parameters were optimised for detection of FITC fluorophore (excitation at 488nm with an argon laser and detection above 505 nm). Ten thousand events were counted for each cell suspension to confirm $H2k^k$ expression in the pMACSKk transfected cells and the absence of $H2k^k$ expression in the pMACS4.1 transfected cells.

Immunohistochemistry

For immunohistochemical analysis, HeLa cells transfected with pMACSKk or pMACS4.1 were incubated with 1:10 anti-$H2k^k$ FITC for 15mins at 4°C. After FITC-labelling, the cells were imaged on a microscope.

MRI Data Collection and Analysis

The pelleted cells were placed in a water bath prior to placement into a ^1H tuned volume coil and MRI performed on a 4.7T Varian Inova MR spectrometer (Varian Ltd., Palo Alto, USA). A spin echo sequence was employed to measure the T2 values of the cell pellets by varying the echo time, TE (6ms – 400ms) with repetition time, TR=3000, FOV 100x100, matrix size 256x128, 1 average and 1 slice (8mm thick). Values of T2 (mean ± standard deviation) were calculated for the triplicates by measuring the signal intensities of the cell pellets at various TE values using ImageJ (NIH). The values were then fitted into the equation: signal intensity = $M_0 exp^{(TE/T2)}$ where M_0 is a constant to obtain T2 values using Prism (Graphpad Software, San Diego, USA).

MRI data

The T2 values of cells transfected with pMACSKk or pMACS4.1 and subsequently incubated with anti-$H2k^k$ microbeads were 57.6 ± 17ms and 424 ± 38.7ms, respectively ($P<0.0001$). Figure 1 illustrates the increased T2 relaxation properties of the pMACSKk transfected cells such that even at a short TE value of 20ms, the cells have already significantly attenuated the MRI signal compared to the pMACS4.1 transfected cells. This confirms $H2k^k$ expression only in the former transfected cells.

Figure 1 shows typical MRI images of cell pellets 48h after transfection with pMACSKk or pMACS4.1 at (a) TE = 30ms and (b) TE = 200ms.

Flow Cytometry Analysis

By FACS analysis, of a population of HeLa cells transfected with pMACSKk $H2k^k$ expression was observed on the cell surface of cells. No expression of $H2k^k$ antigens was observed from the population of pMACS4.1 transfected cells.

Immunohistochemistry

Immunohistochemical staining confirmed the expression of $H2k^k$ antigens on the surface of cells transfected with pMACSKk. Also, the absence of $H2k^k$ expression on the surface of cells transfected with pMACS4.1 was also established.

Example 2: The Use of Super-Antibodies for Imaging Gene Expression and Cell Tracking *in vivo*

Overview

Assessing molecular processes *in vivo*, especially temporal and regional variations in gene expression, has become one of the main objectives of modern biological and clinical imaging.

Several imaging modalities, including MRI, PET and BIL, are being developed to achieve these aims with variable results. Here we present a novel approach to image gene expression in vivo using a combination of superantibodies conjugated to membrane-translocating-segments (MTS) tagged with MRI/PET/BIL or ultrasound based contrast agents. This novel approach will not only allow imaging temporal and regional variation in gene expression at cellular (*in vitro*), organ (*ex-vivo*) and whole-living organism level, but could also be utilised for cell tracking applications.

Superantibodies tagged with the appropiate MTS and contrast agent will cross the cell membrane and only retained in cells that express the required antigen, while in cells where these antigens are not expressed, the superantibodies will leave the cell. Thus in an image, cells that have retained the tagged superantibodies will be clearly differentiated from other cells, which in turn will represent differences in gene expression.

This generic method will be of great utility in the biological and clinical setting, using a variety of imaging modalities, including MRI, PET, BIL and US. The methodology also has the flexibility to be used on its own or in combination with other therapeutic approaches, including gene therapy, superantibodies based therapies and conventional pharmaceutical interventions.

Example 3: H2Kk and persistence of effect

A H2Kk expression construct is transfected into target cells.

Contrast agent conjugated to targetting agent is infused into the system 1 day later. MRI imaging is performed.

As a control, liver and spleen are imaged by MR over the next seven days. Specific signal persists for 1-7 days, whereas nonspecific signal is cleared in approximately 24hours.

Histology is performed as a non-MR control reading out increased iron levels.

Example 4: Demonstration of M.A.G.I.C. in a murine skin graft model

In vivo and ex vivo measurements are shown in this model. The model used to demonstrate M.A.G.I.C. in vivo consists of Balb/c nu/nu mice (H2D haplotype) to which, the left side flank was grafted with skin from the tail of CBA mice (H2K haplotype) and on the right side, tail skin from a C57BL/6 mice (H2B haplotype) was grafted. MACSelect K^k Microbeads (Miltenyi Biotec) accumulate/bind at the H2K graft following their intravenous injection Each mouse acts as its own control since both H2K and H2B grafts were on the same mouse.

Animals and Treatment: A Balb/c nu/nu mouse was grafted on day 1 with tail skin (~6mm x 6mm) from CBA and C57BL/6 mice on the left and right flanks, respectively. On day 7, MACSelect K^k Microbeads (Miltenyi Biotec, 100 ul) were injected via the tail vein. MRI was performed on a 4.7T Inova scanner (Varian Inc., USA). The experiment was repeated with two more mice, mouse 2 and 3. However, the plaster that was covering the graft following grafting was carefully taken off 19h prior to MRI. After, the grafts were dissected and fixed in neutral buffered formalin.

MRI scanning - In vivo: For MRI, the mouse was placed centrally in a volume coil, and an oil capsule and a syringe of water placed on the left (H2K) and right side (H2B) of the mouse, respectively. MRI was performed of the abdomen (transverse plane) using a spin-echo sequence, TR=1500ms, TE=25ms, 2mm slice thickness, 2 averages, 35x35mm field of view and 256x192 matrix size. The first mouse was scanned with and then without the plaster covering the graft. Lubricating jelly was also placed on the grafts to determine if it could aid image analysis. The two other mice were scanned similarly but having had the plaster taken off the graft and no lubricating jelly was applied. Similarly, MRI was performed at 9.4T (Inova scanner, Varian Inc., USA) on mouse 3.

MRI scanning - Ex vivo: Following MRI *in situ*, the grafts were carefully dissected and fixed with formalin. The grafts were individually scanned (transverse plane) in formalin at 9.4T on a Inova scanner (Varian Inc., USA) to obtain high resolution T2-weighted images (TR=4000ms, TE=40ms, 30x30mm field of view, 256x256 matrix size, 40 averages and 0.5mm slice thickness) and T2 measurements. For measurement of T2, the spin echo

sequence was used with varying TEs (6.5, 20, 40, 80 100ms), TR=2000ms, 25x25mm field of view, 256x192 matrix size, 2 averages and 1mm slice thickness. T2 maps were generated from the data and T2 values were obtained for the H2B and H2K grafts using ImageJ (NIH, USA).

Figure 6 shows typical MR images obtained from a mouse with H2K and H2B grafts following MACSelect K^k microbeads. The H2K graft (dark stripe) appears darker than the H2B graft, suggesting accumulation of the MACSelect K^k Microbeads in the former graft. This was subsequently confirmed *ex vivo*. The high signal intensity on the surface of the mouse arises from both subcutaneous fat and the lubricating jelly.

The experiment was repeated with two more mice but the plaster was removed 19h prior to scanning to enable the mice to clean up the grafts. MRI was performed as for the first mouse but without application of lubricating jelly. The H2K graft appears less intense than the H2B graft in mouse 2 (Fig. 2), suggesting accumulation of microbeads at the former graft. MRI scans of mouse 3 shows the high signal intensity layer is broken by an area of low signal intensity area where the H2K graft is (Fig. 3, mouse 3), and also suggesting the accumulation of microbeads at this graft. Furthermore, the high intensity signal from the subcutaneous layer under the H2K skin graft appears attenuated by the presence of microbeads. (The microbeads induce increased susceptibility in the surrounding areas resulting in attenuation of the signal in these areas). MRI was repeated at 9.4T for mouse 3 and again, the H2K graft appears less intense than the H2B graft (Fig. 3).

Fig. 4 shows high-resolution T2-weighted MRI images of the dissected skin grafts from mouse 3. The H2K graft appears to be of lower intensity than the H2B graft, further supporting accumulation of MACSelect K^k Microbeads in the former graft. This is consistent with the T2 measurements (Table 1). The smaller T2 values of the H2K compared to the H2B grafts arises from increased susceptibility arising from iron in the microbeads.

Table 1. **Percentage normalised T2 values of the grafts for mice 2 and 3.**
(percentage normalised value = $T2_{graft}/T2_{formalin}$ X 100%)

	H2K	H2B
Mouse 2	50%	63%
Mouse 3	42%	57%

In summary, these studies show the accumulation/binding of MACSelect K^k Microbeads at the H2K graft, showing imaging of gene expression by MAGIC according to the present invention. In this example, inducing gene expression is carried out by the grafting procedure. The nuclear imaging is magnetic resonance imaging.

Example 5: MAGIC and PET

The nuclear imaging-based technology to image gene expression and gene transfer *in vivo* has been described mostly in connection with MRI, but it can also be readily applied to other imaging methodologies, including PET. PET is a powerful imaging technique, with excellent *in vivo* sensitivity but intrinsically low resolution. To label the required antibodies/antibody fragments for PET-based MAGIC, existing antibody-radiometal conjugation techniques are employed. Preferably, the radiometal is connected to the antibodies via a chelator which can complex the radiometal and also possesses a functional group for attachment to the antibody. Such chelators include EDTA (ethylenediaminetetraacetic acid), DTPA (diethylenetetraaminepentaacetic acid) and DOTA (1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid. Of these, DTPA and DOTA have been shown to be the most versatile as bifunctional forms are available. The bifunctionality allows the possibility of conjugation to both PET and MRI probes.

A preferred chelator-antibody conjugation method is via the sulfo-NHS route, the reaction occurs in aqueous solution at 4°C and pH 7.5 and thus, maintains antibody immunoreactivity, as is well known in the art. The chelator-antibody conjugates can then be directly radiolabelled with the radiometal.

By applying the MAGIC technique to PET, the scope of the technique both in preclinical and clinical studies is advantageously extended. Thus, to take advantage of the increased sensitivity offered by a PET-based MAGIC and to overcome its inherent low-sensitivity,

existing MRI-PET co-registration techniques are preferably used. This allows the sensitivity afforded by PET with the resolution available through MRI. Indeed, conjugation of both PET and MRI probes to the appropriate antibodies allows the application of the MAGIC method to be carried out simultaneously.

Example 6: In vivo MAGIC

Overview

To demonstrate MAGIC *in vivo*, nude mice are transfected with either pMACSKK.II or pMACS4.1 and then given MACSelect K^k microbead 24h after infection, the maximum expression of the antigens being expected to be 24-48h post transfection.

MACSelect K^k microbeads accumulate in many of the tissues of the pMACSKK.II transfected animals including the spleen and liver. They also accumulate in the pMACS4.1 transfected animals and also especially in the spleen and liver but non-specifically and are cleared much sooner than that in the pMACSKK.II transfected animals.

The presence of MACSelect K^k microbeads leads to attenuation of the MR signal in that area and also, a shortening of the T2 values of the protons in that area. The MR data are preferably correlated with histological data. The spleen and liver are dissected out and formalin fixed for Perl's staining which stains iron brown.

Animal Treatment: Balb/c nu/nu mice (n=8, 10 weeks old, Harlan UK) are used. Each mouse receives an injection of DNA-complexes (200ul) via the tail vein. Four mice receive complexes made with pMACSKK.II (Miltenyi Biotec) and four receive complexes made with pMACS4.1 (Miltenyi Biotec). A day later, the mice are scanned by MRI prior to an injection of MACSelect K^k microbead (150ul, Miltenyi Biotec, Bisley, UK) via the tail vein. The mice are then scanned at 24, 48 and 72h post-microbead dose. After the last MRI scan, the mice are killed and the spleen and liver dissected out and fixed in formalin for staining for iron with Perl's stain.

Formulation of DNA complexes: Gene transfer is performed *in vivo* using ExGen 500, polyethyleneimine, a non-viral, non-liposomal gene delivery reagent, capable to transfecting *in vivo* and *in vitro*. For each mouse, 50ug of either pMACSKK.II (Miltenyi Biotec) or pMACS4.1 (Miltenyi biotec) are added to a 5% glucose solution (to a total volume of 100ul), vortex-mixed gently and spun down briefly. Similarly, 22.5ul of ExGen 500 (Fermentas, UK) is added to a solution of 5% glucose (to a total volume of 100ul), vortex-mixed gently and spun down briefly. The diluted ExGen 500 is then added to the diluted DNA, vortex mixed immediately and spun down briefly. The mix is incubated for 10mins prior to injection into animals.

MRI: MRI is performed on an Inova 9.4T MR scanner (Varian Inc., USA). Anaesthesia is induced and maintained in mice during scanning using a 1-5% isoflurane-oxygen mix. The mice are individually placed within a home built MR quadrature coil so as to be able to image both spleen and liver. MR is performed using a FLASH sequence, TR=50ms, TE=1.8ms, 128 x 128 matrix size, 30mm x 30mm field of view and 6 transients. A spin-echo sequences is also employed with varying TE to measure T2 values (TR=1200ms, TE=12 and 24ms, 256 x 128 matrix size, 30 x 30 mm field of view and 1 transient).

Microbeads or similar can be detected by MRI in the liver and sometimes the spleen following injection as their iron content leads to signal attenuation in these organs. Our previous studies have indicated that non-specific accumulation of microbeads persists for a day post-injection in the liver and is minimal in the spleen, as indicated by signal attenuation in the MRI image and measurement of T2 values (T2 values are shortened by the presence of iron). Also, such studies have indicated that the specific accumulation of microbeads can persist for at least a week in both the spleen and the liver.

In this system, a decrease in the MRI signal intensity and shortening of T2 values in the liver and possibly the spleen following MACSelect K^k microbead injection due to gene transfection and expression of the truncated $H2K^k$ in these organs. (ExGen 500 has been shown to transfect both the liver and spleen in the mouse *in vivo* using 15 N/P equivalents of DNA to ExGen 500 (Bragonzi et al., 1999, Gene Therapy, 6, 1995-2004) with maximum expression 24-48h post-injection of the DNA complexes.)

The signal attenuation persists for at least a day in the mice transfected with the pMACSKK.II plasmid but the signal intensity returns back to baseline levels in mice transfected with the pMACS4.1 plasmid, a day post-microbead injection. Similarly, T2 values in the liver and spleen would be shorter in the pMACSKK.II plasmid treated animals compared to pMACS4.1 plasmid treated animals at greater than a day post-microbead injection. Such data evidences the expression and non-expression of $H2K^k$ in the pMACSKK.II and pMACS4.1 treated animals.

The MR data are correlated with the histological data. Iron content in the spleen and liver are stained brown by Perl's staining. A greater degree of staining in the pMACSKK.II transfected animals arising from increasing amounts of MACSelect K^k microbeads present arising from specific interactions between the microbeads and the H2Kk antigen.

Thus, the methods of the present invention are demonstrated *in vivo*.

CLAIMS

1. A method of monitoring gene expression in a system comprising
   (a) inducing expression of a cell surface antigen in said system
   (b) incubating said system for a time sufficient to allow gene expression to take place;
   (c) introducing to said system a nuclear imaging contrast agent linked to a targetting agent capable of binding to said cell surface antigen; and
   (d) obtaining a nuclear image from said system.

2. A method of monitoring gene expression in a system according to claim 1 wherein step (a) comprises introducing to said system nucleic acid comprising a promoter of interest operably linked to a nucleic acid sequence encoding a cell surface antigen.

3. A method according to claim 1 or claim 2 wherein the nuclear imaging is magnetic resonance imaging (MRI).

4. A method according to any of claims 1 to 3 wherein the contrast agent is a paramagnetic iron particle.

5. A method according to claim 4 wherein said paramagnetic iron particle is approximately 20nm to 1um in size.

6. A method according to claim 4 wherein the paramagnetic iron particle comprises an iron oxide core and a dextran coating.

7. A method according to claim 6 wherein the said iron and dextran coating is approximately 50nm in diameter.

8. A method according to any preceding claim wherein the targetting agent is an antibody.

9. A method according to any preceding claim wherein the cell-surface antigen is the $H2K^k$ antigen.

10. A claim according to any preceding claim wherein the cell surface antigen is a class I MHC antigen.

11. A method according to claim 8 wherein the antibody is a MACSelect K^k microbead.

12. A method of producing a magnetic resonance image comprising the step of linking a magnetic resonance contrast agent to a cell surface antigen present on the structure of interest.

13. Use of H2Kk for imaging gene expression by MRI.

14. Use of superantibody linked to a magnetic resonance imaging contrast agent in the production of a magnetic resonance image.

Figure 1

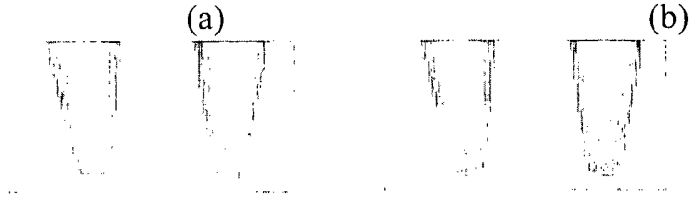


Figure 2

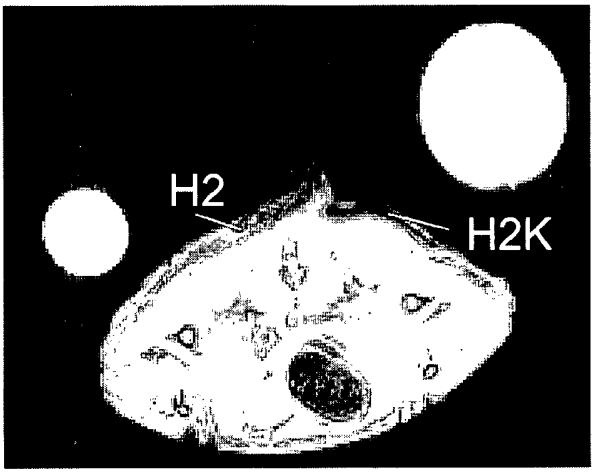


Figure 3

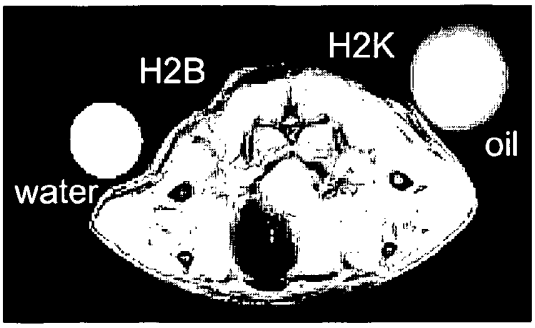


Figure 4

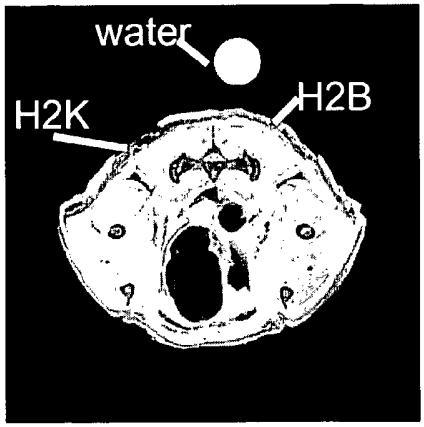


Figure 5

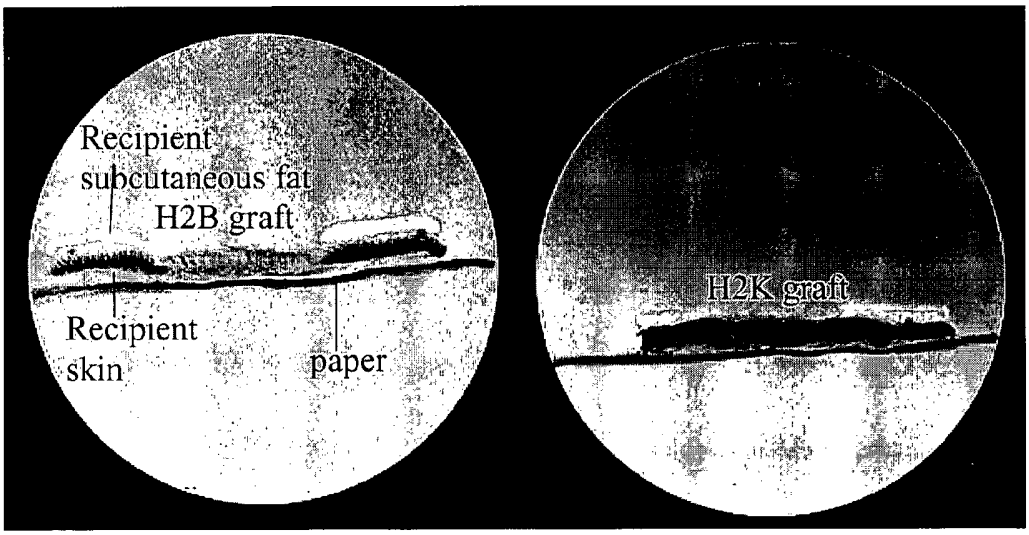
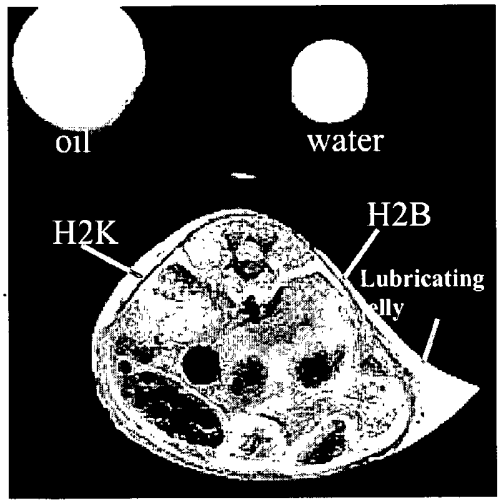


Figure 6



INTERNATIONAL SEARCH REPORT

Intern Application No
PCT/GB2005/001983

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K49/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, MEDLINE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ARTEMOV DMITRI: "Molecular magnetic resonance imaging with targeted contrast agents." JOURNAL OF CELLULAR BIOCHEMISTRY, vol. 90, no. 3, 15 October 2003 (2003-10-15), pages 518-524, XP002348192 ISSN: 0730-2312	1-8, 10-12,14
Y		9,13

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 October 2005	03/11/2005

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Wimmer, G

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Form PCT/ISA/210 (second sheet) (January 2004)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ARTEMOV DMITRI ET AL: "MR molecular imaging of the Her-2/neu receptor in breast cancer cells using targeted iron oxide nanoparticles." MAGNETIC RESONANCE IN MEDICINE, vol. 49, no. 3, March 2003 (2003-03), pages 403-408, XP002348193 ISSN: 0740-3194 the whole document	1-8, 10-12,14
Y		9,13
X	REMSEN LAURA G ET AL: "MR of carcinoma-specific monoclonal antibody conjugated to monocrystalline iron oxide nanoparticles: The potential for noninvasive diagnosis" AJNR, vol. 17, no. 3, 1996, pages 411-418, XP002348194 ISSN: 0195-6108 the whole document	1-8, 10-12,14
Y		9,13
X	SUWA TATSUSHI ET AL: "Magnetic resonance imaging of esophageal squamous cell carcinoma using magnetite particles coated with anti-epidermal growth factor receptor antibody" INTERNATIONAL JOURNAL OF CANCER, vol. 75, no. 4, 9 February 1998 (1998-02-09), pages 626-634, XP002348195 ISSN: 0020-7136 the whole document	1-8, 10-12,14
Y		9,13
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Y		9,13
X	WO 01/56582 A (HADASIT MEDICAL RESEARCH SERVICES AND DEVELOPMENT; HOCHBERG, ABRAHAM;) 9 August 2001 (2001-08-09)	1-8,12, 14
Y	page 26 - page 27	9,13

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4

INTERNATIONAL SEARCH REPORT

Inter Application No
PCT/GB2005/001983

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
Y	WEI Q ET AL: "Selection of genetically modified chicken blastodermal cells by magnetic-activated cell sorting" POULTRY SCIENCE, CHAMPAIGN, IL, US, vol. 80, no. 12, December 2001 (2001-12), pages 1671-1678, XP002975511 ISSN: 0032-5791 the whole document	9,13

INTERNATIONAL SEARCH REPORT

Information on patent family members

Interna Application No
PCT/GB2005/001983

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0156582 A	09-08-2001	AU 3047101 A	14-08-2001