Luminescent Imaging Technology as an Opportunity to *Reduce and Refine* Animal Experiments: Light at the End of the Tunnel?

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Summary

In vivo luminescent imaging technology has been introduced in experimental life science research several years ago and has rapidly gained wide acceptance. By making use of this technology substantially more information can be gained from animal experiments than was previously possible. The concept of the 3Rs describes the aim to Refine, Reduce and Replace animal models in research. The goal of the present paper is to systematically investigate the impact of luminescent imaging on the 3Rs. In particular, three examples of applications are explained in detail so as to be accessible to the reader unfamiliar with the procedure. The examples are subsequently analysed for and categorised according to their concrete effect on animal welfare as defined by the 3Rs.

Keywords: luminescent imaging, 3R concept, in vivo, mice, radiography, micro-CT

1 Luminescent and fluorescent imaging

A host of novel imaging technologies has entered the field of animal research over the last decade. Imaging methods applicable to small animals include radiotracer-based modalities, e.g. single photon emission computed tomography (SPECT) and positron emission tomography (PET), X-ray-based modalities, e.g. computed tomography (CT), optical technologies, e.g. fluorescence and bioluminescence, as well as μ-ultrasound and high-field magnetic resonance imaging (MRI) (Koo et al., 2006). Many imaging methods can also be used in patients and have, in fact, become established clinical routine. Optical imaging has emerged in recent years as a whole new field of imaging that is widely used in the field of life science research in animals (Sadikot and Blackwell, 2005; Luker and Luker, 2008). In contrast to other technologies, optical imaging is relatively inexpensive and does not require a comprehensive operative environment with highly trained experts. Nevertheless, it is a powerful tool that makes it possible to assess morphological structures or functional, metabolic or molecular events *in vivo* (Luker and Luker, 2008).

The advantages and drawbacks of either approach (fluorescent or bioluminescence imaging) have been expertly reviewed and discussed elsewhere (Troy et al., 2004). Both methods, however, share the limitation of visible light being scattered and absorbed in biological tissue, leading to an imprecise localisation of the photon interaction, especially in deep tissues (these limitations depend on the wavelength of the light source or dye, among other factors). Hence, optical imaging is not readily applicable to diagnostic imaging in patients, in which most organs of interest would be out of reach. Nonetheless, a number of clinical applications have been established (e.g. fluorescence retinal angiography) or may become available for diseases in relatively superficial locations, such as the diagnosis of breast cancer, for which fluorescent imagers are being clinically tested (Frangioni, 2008). In contrast, the emergence of fluorescence and luminescence imaging has had an immediate impact on animal experiments in life science research and drug discovery.

Indeed, within a few years, optical imaging devices have become commonplace in academic and industrial institutions alike. Such methods have been successfully used for the investigation of development, physiology, pathophysiology and

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pharmacological research in as diverse fields as, among others, immunology, infectious diseases, oncology, metabolic and cardiovascular disorders (Contag et al., 1998; Contag and Bachmann, 2002; Sadikot and Blackwell, 2005; Luker and Luker, 2008). Luminescent imaging, in particular, stands out by its unique advantages: (1) an extremely high sensitivity due to exceedingly low background signal, (2) the relative ease of use and the possibility to simultaneously image up to five mice and more, depending on the equipment, (3) no need for radioactivity and X rays, and (4) the moderate costs. However, luminescent imaging has a number of limitations, including its restriction to intracellular targets (Wehrman et al., 2006), and the fact that factors such as anaesthesia might alter luminescent signal intensity (Effects of anesthesia on host and tumor physiology: impact on bioluminescence measurements when compared to conscious mice. Reported by Schnell et al. at the 100th Annual Meeting of the American Association for Cancer Research 2009). Overall, the advantages of luminescent *in vivo* imaging appear to make it more widely used overall than other modalities, including fluorescent imaging, although the latter might become increasingly used in the future with the development of improved devices and fluorochromes that might reduce background fluorescence. Although the present review focuses on luminescent imaging for the reasons mentioned above, the conclusions derived also apply in principle to fluorescent imaging.

The 3Rs

The 3R concept was proposed and developed by Russel and Burch 1959, and published in "The Principles of Humane Experimental Technique: A concept to *Refine, Reduce and Replace* animals in experiments". It is based on the premise that excellent scientific work goes hand in hand with humane treatment of laboratory animals (Flecknell, 2002; Rusche, 2003; Leist et al., 2008). The 3R concept became gradually accepted, for example in the declaration of Bologna in 1999 (http://3r-training.tierversuch.ch/en/module_3r/3r-bologna_declaration/the_3rs.html) and presently forms the implicit basis of the Animal Welfare Acts of most European countries. In European jurisdiction, animal experiments are classed according to their aims. Hence, any researcher planning animal experiments is required (1) to demonstrate that no alternative method other than animal experiments is available, (2) to justify the number of animals used, and (3) to ensure that suffering and pain are kept to a minimum and are "ethically acceptable". The 3Rs have been reviewed and commented on elsewhere (Flecknell, 2002; Rusche, 2003; Leist et al., 2008), and can be briefly summarised as follows. Replacement includes the use of non-animal methods such as cell cultures, human volunteers or computer modelling to achieve the defined scientific goal. Refinement includes the use of methods that alleviate or minimise pain, suffering or distress, to avoid fear and generally to improve animal welfare for those animals that cannot be replaced. Reduction means obtaining the best quality and most precise information with the smallest possible number of animals. Experiments that are well-designed and well-conducted deliver reliable results and eliminate the need for repetition of the same tests. This also includes the use of methods that enable researchers to obtain more information from the same number of animals.

The aim of this paper is to investigate the potential of luminescent imaging to Reduce, Refine and Replace animal experiments. Three concrete examples of applications that have already been proven to be useful in life science research will be analysed and categorised according to their impact on the 3Rs: Replacement, Reduction and Refinement of animal experiments in biomedical research. As an introduction, the most important technologies and applications in the field of luminescent imaging are reviewed.

2 Principles, technical requirements and protocol

Optical imaging is based on the ability of visible light to travel through biological tissue. This property can be harnessed in two ways: either by excitation using an external light source (fluorescence imaging) or by active emission of light from a luciferase reporter enzyme within living animals (luminescence imaging). Various organisms such as bacteria, plankton, insects and fish have the natural ability to produce luminescence (Lapota, 1984; Meighen, 1993; Mensinger, 1997). By inserting the gene encoding for luciferase (*luc*) into, for example, bacteria, mammalian cells or fertilised eggs, luminescent cells or animals can be engineered. The *luc* gene from the North-American firefly *Photinus pyralis* is the most widely used bioluminescence reporter gene in life science research. The basic principle for creating luminescent light is a luciferase-catalysed reaction of molecular oxygen with luciferin (Fig. 1 A-B).

Systems for bioluminescent *in vivo* imaging that include a light-tight cabinet, a charged-coupled-device (CCD) camera as well as data processing and storage software are commercially available from several manufacturers (Fig. 1 C). Several authors have used the IVIS®-100 and IVIS®-Spectrum systems (Xenogen-Caliper Life Sciences, Hopkinton, MA), but a similar system (NightOWL II LB 983) is available from Berthold Technologies (Bad Wildbad, Germany) (Jenkins et al., 2003; Kutschka et al., 2006).

As an example, the IVIS®-100 consists of the following components:

- A light-tight imaging chamber
- A heated stage (to avoid hypothermia of the anaesthetised animals), which is limited and sized to hold 5 mice or 3 rats. A full gas anaesthesia system with an induction chamber (e.g. isoflurane) is included.
- A CCD camera (2048x2048 pixels), cryogenically cooled to -90°C by a closed-cycle refrigeration unit to minimise electronic background and maximise sensitivity
- The system is operated via a computer using the Living Image® 3.0 software. The software also serves to store the data and to display and analyse the images.

A typical protocol consists of mice expressing firefly luciferase (Fluc) from a cloned construct, either as transgenic animals following gene transfer or after implantation of cells engineered to express Fluc. The substrate D-luciferin is injected intraperitoneally, is distributed in the systemic circulation and is locally cleaved by Fluc in a reaction requiring, among others, ATP, O_2 and Mg^{2+} as cofactors to produce light. The unsur-

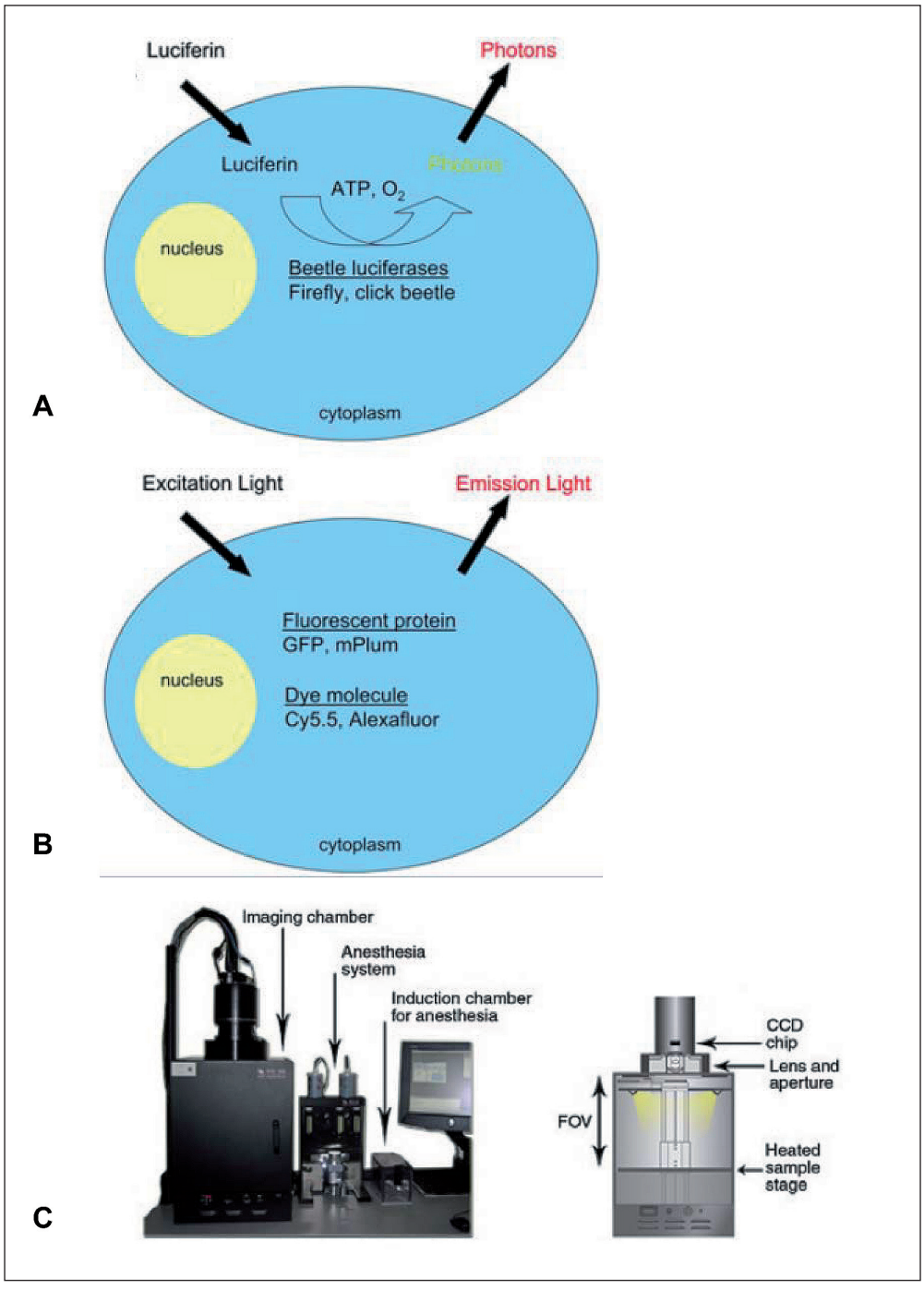

Fig 1: Luminescent imaging
Schematic representation of bioluminescent and fluorescent imaging. (A) Firefly luciferase expressed in engineered cells catalyses the reaction of luciferin in the presence of oxygen and ATP production to produce light photons. (B) Fluorescent imaging requires light to excite the reporter molecule (e.g. a fluorescent protein), resulting in emission of light with a defined emission spectrum that is characteristic for each reporter protein or dye. (C) Example of an *in vivo* imaging system: IVIS100 (Xenogen). Mice are anesthetised in the induction chamber and are kept under anaesthesia throughout the imaging session. The bioluminescent signal is detected by a cooled CCD camera. See Section 2 for further details.
Reproduced with permission from: (A, B) Luker et al., *J. Nucl. Med.* 2008, 49(1):1-4;
(C) Franke-Fayard B, et al., *Nat. Protoc.* 2006, 1(1): 476-85.

passed sensitivity of luminescent imaging is due to the absence of Fluc expression in mammalian tissues in conjunction with the almost complete absence of endogenous luminescence in living animals. The latter difference is obviously in contrast to fluorescent imaging, which always generates a fluorescent background signal, thus substantially reducing sensitivity. Indeed, luminescent imaging has been reported to detect as few as 10 cells engineered to express Fluc implanted into live wild type mice. Up to 5 mice or 3 rats are simultaneously anesthetised (e.g. using isoflurane), injected intraperitoneally with D-luciferin and placed on the heating stage of the imaging chamber. A series of images is then continuously acquired by the CCD camera and related to the imaging software. Depending on the strength of the luminescent signal, exposure time ranges between one second and several minutes. In a typical setting, serial images are acquired until peak signal intensity is reached (between 10 and 20 min after injection of the luciferin substrate). The luminescent signal of each image is integrated, corrected (e.g. flat field correction) and graphically displayed merged with a photographic image of the mouse to allow localisation of the lumi-

nescent signal. Quantifications are typically performed off-line after completion of the imaging session. Because the procedure of intraperitoneal injection requires minimal technical expertise and is little time consuming as many as 5 mice can easily be imaged simultaneously. In contrast, protocols requiring intravenous injections (e.g. fluorescent substrates or other forms of luciferase) require higher technical expertise and training and cause considerable delays, making it difficult if not impossible to image more than one mouse at a time. In a typical setting (e.g. implantation of cells expressing Fluc), the signal obtained can be several thousand-fold over background (Troy et al., 2004). Obviously, an important advantage over an X-ray or radionuclide-based approach is that luminescent imaging requires no costly facility, particular safety measures or operator training, and the imager can be used on the bench top of an ordinary laboratory. Finally, the costs are moderate: the imaging device costs between 150,000 and 200,000 $, requires only minimal maintenance and the substrate costs less than a dollar per mouse and imaging time point.

Importantly, with respect to the welfare of laboratory animals, these technologies cause only minimal distress and are performed under short-term anaesthesia. They typically encompass an intraperitoneal injection of the substrate, followed by the imaging session itself (typically around 20 min). The commonly used substrates are non-toxic and the entire procedure is well-tolerated, even if repeated on a daily basis over extended periods of time.

3 Concrete examples of applications and their impact on the 3Rs

3.1 Example from own studies: Luminescent imaging in cardiac cell therapy

The implantation of stem or progenitor cells into the injured heart holds promise as a treatment for patients with myocardial infarction. Reports from experimental investigations and early clinical trials suggest that cell therapy might benefit patients without other treatment options. However, the mechanisms by which these cells might contribute to improvement of cardiac function have not yet been clarified. Furthermore, the optimal cell type and method of delivery need to be determined. These

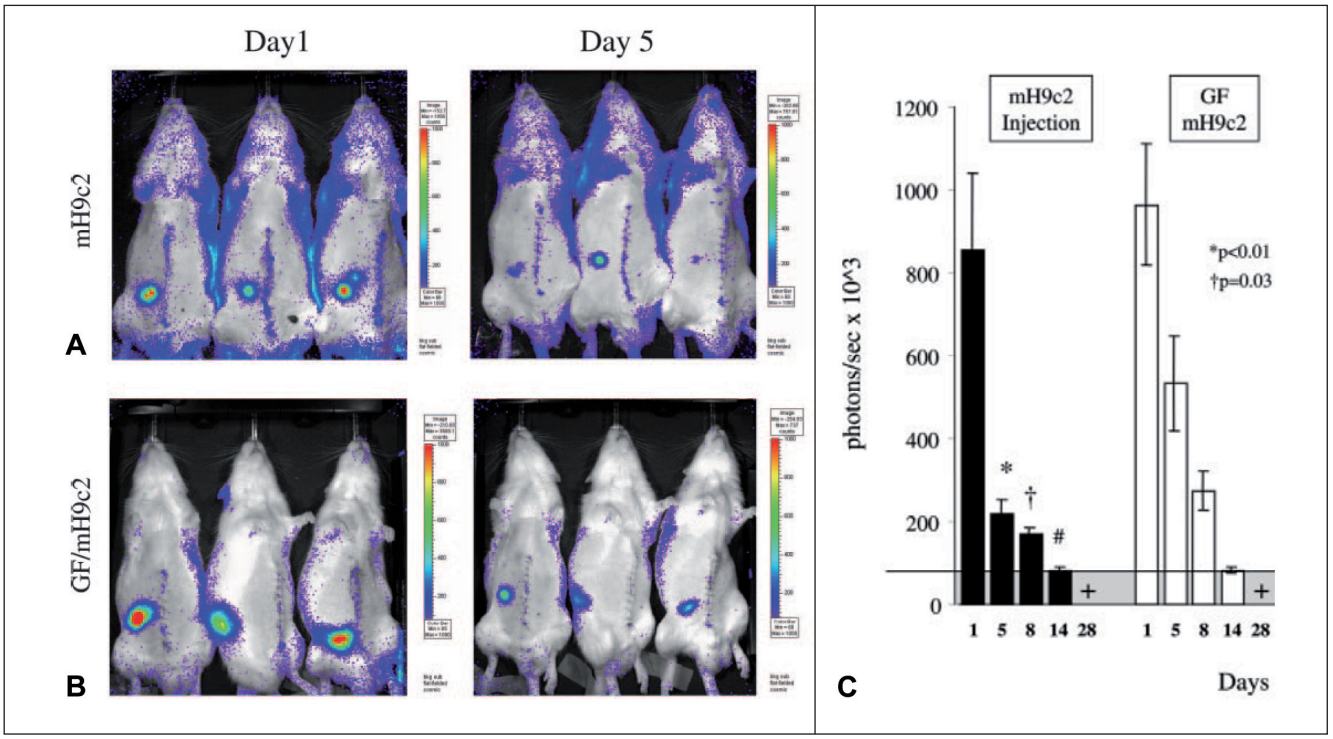

Fig. 2: Cardiac cell therapy
Optical bioluminescence imaging at different time points after cardiac cell grafting in a heterotopic heart transplant model. (A + B) representative luminescent images; (A) cells injected directly into the heart without gel foam support; (B) the same amount of cells transplanted into the heart within a gel foam support matrix. (C) Quantification shows up to 3-fold higher luminescence in hearts implanted with cells with the gel foam support matrix (white bars) compared to cells injected without the matrix (black bars); the difference, however, is only apparent at early time points up to 8 days after implantation, the signal declining to background after 14 days. **3R relevance:** assessing cell survival using conventional methods (immunofluorescence microscopy) would have required sacrifice of an entire group of animals at every predefined time point, which would have increased the number of animals used 5-fold. Furthermore, the number of mice needed per group is reduced by the fact that individual animals are followed over time, reducing variability. Reproduced with permission: Kutschka et al., *Circulation* 2006, 114 (Suppl. 1): 167-173.

questions will need to be answered in order to develop this promising approach into an effective therapy.

We have applied luminescent imaging to study the fate of cells injected into the infarcted heart in rats (Kutschka et al., 2006) (Fig. 2). Specifically, we tested the hypothesis, that cell survival is improved if the cells are transplanted within a collagen support matrix, compared with simple injection of a cell suspension. Subsequently, we tested whether increased survival of the transplanted cells would lead to improved cardiac function. Hearts were obtained from Sprague-Dawley rats. The explanted hearts were locally damaged *ex vivo* by cryoinjury, and H9C2 foetal cardiac myoblast cells were implanted into the infarcted area of the heart (in two groups: either with or without a collagen support matrix). The treated hearts were then transplanted into the abdominal cavity of a second, recipient rat (Kutschka et al., 2006). Through this experimental design, only minor surgery was required, thus obviating the need for a more traumatising thoracotomy. The primary readout was cardiac function, assessed non-invasively by echocardiography at different time points and invasively before the scheduled end of the experiment (28 days after surgery).

The main finding was that implantation of the H9C2 cells using a collagen-based support matrix did indeed significantly improve cardiac function after 28 days. Based on this observation, the expectation was to find increased cell survival following implantation within the collagen-based support. In order to investigate cell survival, hearts were harvested, processed and analysed by fluorescent microscopy. Surprisingly, however, it turned out that hardly any transplanted cell was alive by that late time point in either group. Hence, improved cardiac function was not explained by differences in cell survival at the time point of sacrifice, i.e. 28 days after surgery. In the absence of luminescent *in vivo* imaging, the results would have been puzzling, and all but uninterpretable.

Fortunately, luminescent imaging had been included in the study design from the start: the H9C2 cells had been genetically engineered to express Fluc prior to implantation, and luminescent imaging had been performed serially over time throughout the experiment. The analysis of the luminescent images made it possible to precisely follow the course of cell survival over time and to correlate the relative number of cells having survived at different time points with cardiac function (Fig. 2). As a matter of fact, the use of a collagen matrix was found to significantly increase cell survival, but only at *early* time points (up to 5 days). Cells thereafter gradually disappeared, independent of whether they had been implanted with the collagen matrix or without. Hence, surprisingly, cardiac function 28 days after surgery did not correlate with cell survival at this late time point. These findings clearly refute the hypothesis that contractile function of the implanted cells increases overall cardiac function in this model. By contrast, the finding of enhanced cell survival 5 days after implantation indicates a role for the cells in inducing angiogenesis and/or structurally stabilising the freshly infarcted myocardium (scar bridging). Hence, the inclusion of luminescent imaging in the experimental design revealed an unexpected link between the time course of cell survival and myocardial function in the setting of cardiac cell therapy.

Impact on the 3Rs

In total, more than 100 animal experiments were avoided by including luminescent imaging in the study described here, based on the following data and calculations: two groups, five time points, 2 animals/experiment, n=9/group (higher n/group required because of interindividual variability) (= Reduction).

Indeed, conventional techniques would have required sacrifice of the animals, section of the hearts, and staining for a marker expressed by the transplanted cells (e.g. green fluorescent protein (GFP)). In the experiments outlined above, the inclusion of luminescent imaging substantially improved the quality of results generated, as the results would have been difficult, if not impossible, to interpret without this additional information (= Reduction). Importantly, this additional valuable information was obtained without requiring any additional animal experiment (not even a pilot experiment) (= Reduction). The number of animals per group was also reduced compared with conventional techniques, because the ability to follow the fate of transplanted cells within an individual animal eliminates inter-animal variability, and because the time course of cell survival was followed in the same individual animals that were analysed for cardiac function at day 28, thus further reducing variability (= Reduction). Wild type animals were used, obviating the need to generate and breed transgenic animals. Only limited additional strain was imposed on the laboratory animals (four to five imaging sessions under short-term anaesthesia).

3.2 Example from own studies: Luminescent imaging in combination with other imaging modalities in cancer research

Cancer is one of the main causes of morbidity and mortality. The design of new treatments requires a thorough understanding of the time course and characteristics of tumour metastasis into remote tissues such as the bone. Furthermore, the efficacy of new pharmacological compounds needs to be demonstrated in experimental models before moving on to clinical trials.

We combined luminescent imaging with histomorphometry, radiography and micro-computed tomography (μCT) for the simultaneous monitoring of tumour burden and osteolytic lesions in a breast cancer bone metastasis model (Käkonen and Mundy, 2003; Strube et al., 2009). The aim of this study was to determine possible correlations between methods assessing tumour burden (luminescent imaging *in vivo* and histomorphometry postmortem) and morphological changes of bone (radiography and μCT). In this model, 1 x 10^5 MDA-MB-231(SA)/luciferase breast cancer cells were inoculated into the left cardiac ventricle of female athymic nude mice. The cell line was generated by stable transfection of the parental MDA-MB-231(SA) cell line (a gift from Professor T. Guise, University of Virginia, VA, USA) with a pRev CMV_Luc2 vector. Mice were anesthetised with 5% xylazine / 10% ketamine, and 1 x 10^5 cells in 100 μl PBS were injected intracardially. The mice developed osteolytic lesions within 3 weeks after injection of the cells. The tumour burden and tumour cell localisation (metastasis) were determined using bioluminescence imaging (NightOWL *in vivo* Imager, Berthold Technologies) and histological examination

(hematoxylin and eosin (H&E) staining). Tumour induced destruction of the bone was assessed using radiography (Faxitron X-Ray, Wheeling, USA) and micro-computed tomography (Tomoscope, VAMP GmbH, Erlangen, Germany). All measurements were performed at the end of the study (at day 22).

Luminescent imaging revealed the extent of luciferase-labelled tumour cell dissemination throughout the body of the animal, indicating the presence of metastasised tumour cells in the region of hind limbs, forelimbs, spine and skull (Fig. 3A). Tumour burden could be quantified by luminescence signal in different parts of the body in individual animals over time. Finally, histological examination provided a more detailed evaluation of structural changes in bone, revealing that the tumours were primarily located within the bone (Fig. 3B, animal 6). There was a strong correlation between *in vivo* luminescent imaging signal intensity and tumour area determined post mortem by histomorphometry in individual animals (Fig. 3A-C).

Similarly, a good correlation was observed between X-ray and μCT measurements, methods that were employed to monitor osteolysis. Radiography displayed a two-dimensional view of the hind limbs, whereas μCT measurements more accurately reflected morphological changes in bone in a three-dimensional manner. However, the localisation of osteolytic lesions correlated precisely between both methods (Fig. 3D). In addition, there was

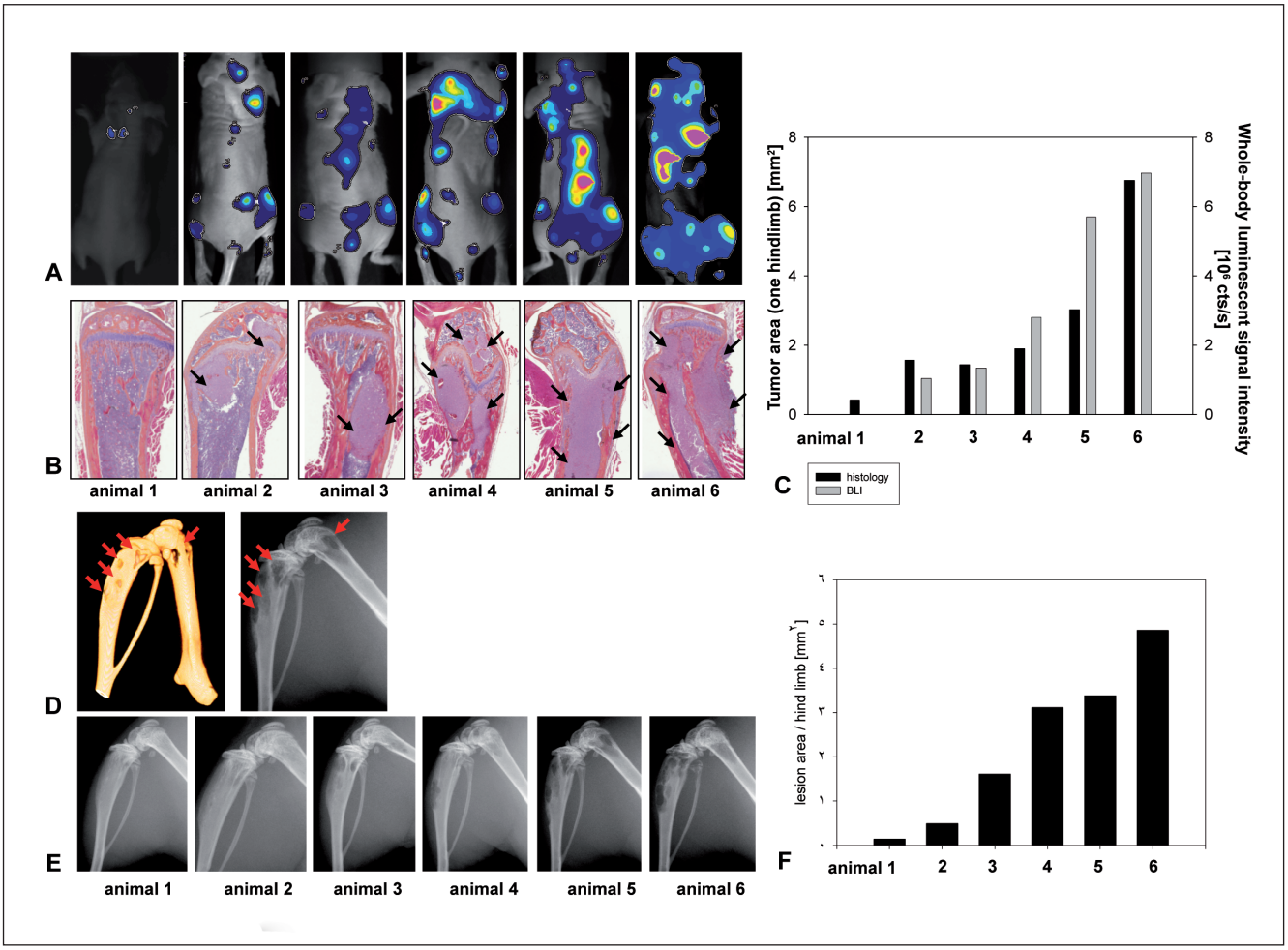

Fig. 3: Cancer research

Luminescent signal intensity and tumour area concomitantly increased in mice inoculated intracardially with MDA-MB-231(SA)/luc breast cancer cells. (A) Bioluminescence images and (B) H&E stained bone sections indicating tumour burden. (C) Quantification of bioluminescence signal intensity and tumour area measured in bone sections show excellent correlation. (D) Representative radiographs and μCT images of a hind limb indicating correlation of both methods (arrows indicate osteolytic lesions). (E) Radiographs of hind limbs and (F) corresponding lesion area.

3R relevance: the study shows an excellent correlation between the *in vivo* quantification of tumour burden in different parts of the body using luminescent imaging and histomorphometric quantification. Additional information is gained through μCT and radiography. Based on these data, future experiments in this model can be performed using substantially fewer animals by relying on luminescent imaging as the main outcome parameter. By imaging individual mice serially, interindividual variability is avoided, making it possible to further reduce the number of mice needed per group.

a strong correlation between the localisation of tumour cells and intensity of luminescent signal, and the localisation and severity of osteolysis as detected by radiography (Fig. 3E-F). These observations are in line with the hypothesis that bone destruction is the result of an increase in osteoclast activation induced by breast cancer cells (Käkonen and Mundy, 2003).

In conclusion, a strong correlation was observed between methods assessing tumour burden (luminescent imaging, histomorphometry) and technologies monitoring morphological changes in bone, such as radiography and μCT. Additionally, *in vivo* multimodal imaging, such as the combination of luminescent imaging and μCT, allows a simultaneous registration of tumour cell localisation and morphological changes in bones. The estimation of luminescent signal intensity, tumour area, lesion area and bone volume allows not only qualitative, but also quantitative analysis of osteolytic changes and tumour burden in bones.

Impact on the 3Rs

The present example describes a thorough characterisation of a model of tumour metastasis. Based on these data, subsequent experiments can be performed using dramatically fewer animals. The number of animals required in future experiments is estimated to be between 25 and 75% of the number that would have been needed otherwise (= Reduction).

In a bone metastasis model, tumour size cannot be assessed *in vivo* using conventional methods such as the caliper. Hence, it is necessary to sacrifice the animals at given time points to assess tumour size at necropsy. As a consequence, substantially more animals need to be used for 2 reasons: (1) since assessment of tumour size is terminal, a group of mice needs to be sacrificed at every time point of interest; (2) whereas repeated measurements in individual animals minimises variability (each animal serves as its own control), a higher number of mice per group is required using conventional techniques (= Reduction). Bioluminescent imaging (BLI) allows for longitudinal monitoring of tumour cell localisation and for the detection of whole-body tumour burden, which would be very difficult using conventional methods requiring for example the histological analysis of all tissues and bones. Thus, luminescent imaging provides substantially more information from the same number of animals than conventional methods (= Reduction and Refinement). Furthermore, because tumour burden can typically be quantified at earlier stages, due to the high sensitivity of luminescent imaging technology, a shorter overall time course of the experiment is possible, thus minimising the strain on the animals (= Refinement). The combination of these technologies has already been shown to be helpful in determining the efficacy of pharmacological compounds in a therapeutic setting (Strube et al., 2009). The evaluation of compounds is especially important, because bone metastasis is an area of high medical need. Thus, animal models are crucial to better understand the biology of bone metastasis and develop therapy options. In addition, a complete replacement of bone metastasis experiments is not possible due to the complex mechanisms involved, including manifold interactions between for example the bone microenvironment, osteoclasts, osteoblasts, tumour cells, immune cells, stroma and vasculature

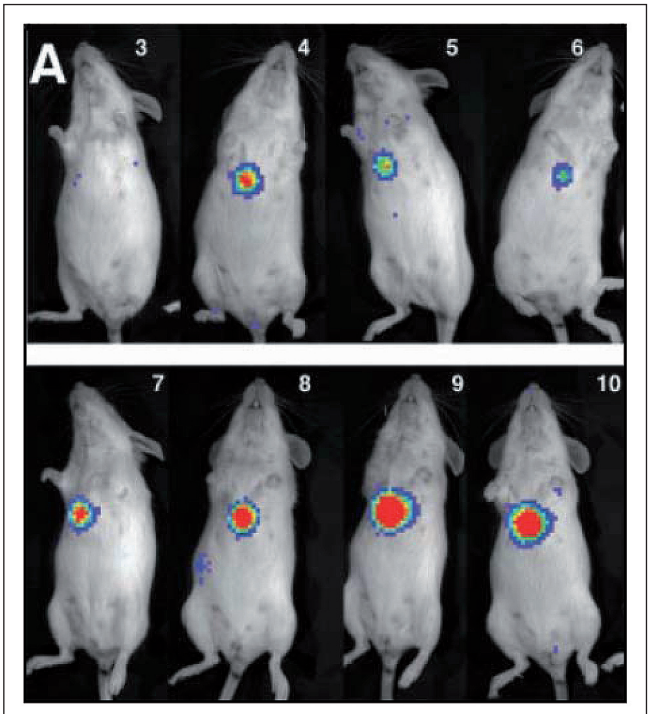

Fig. 4: Bacteriology
Luminescent imaging of the replication of *Listeria monocytogenes* in the gall bladder of mice. (A) A luminescent *hly* deletion of *L. monocytogenes* 10403S was inoculated intravenously into 6-week-old BALB/c mice. The mice were then imaged on the indicated days. One animal displaying the characteristic signal of gall bladder growth is shown. 3R relevance: luminescent imaging allowed the discovery that orally or intravenously inoculated L. monocytogenes localise to the gall bladder and replicate extracellularly. Such a finding would have been all but impossible using conventional techniques, which would have required sacrificing groups of animals at predefined time points, followed by whole body sectioning and staining to identify the area of residence of the bacteria. Hence, luminescent imaging in this application is a considerable refinement over existing technologies and allows reducing the number of animals used. Reproduced with permission: Hardy et al., *Science* 2004, 303: 851-853.

(Käkönen and Mundy, 2003). To date there are no cell and tissue culture models or computer simulations available for studying the processes in a whole living system (Festing, 2008).

3.3 Bacteriology: Listeriosis

Bioluminescent imaging has been applied by Jonathan Hardy and co-workers to the investigation of bacterial infections in an elegant series of experiments (Hardy et al., 2004). *Listeria monocytogenes* are the cause of Listeriosis, a form of food poisoning that can lead to severe illness or death, especially in immune-compromised people, or to foetal death or malformation in pregnant women, causing approximately 500 deaths per year in the US. Surprisingly much remains unknown about the

process of infection and colonisation of Listeriosis in man. It is assumed that *Listeria monocytogenes* has adapted itself to several organs within the human organism. The aim of this study was to investigate the time course and pattern of infection, colonisation and replication of *Listeria monocytogenes* after infection in mice. Using bioluminescent imaging, a previously unknown chronic carrier state was discovered in the gall bladder, suggesting a potential source of food contamination. These findings could have important implications for the prevention of Listeriosis.

Susceptible inbred BALB/c-mice are a well characterised animal model of infectious disease and immunity. In order to follow the fate of the bacteria after inoculation, a strain of *Listeria monocytogenes* was first made luminescent by chromosomal integration of a lux-kan transposon cassette (Hardy et al., 2004). Using bioluminescence imaging, the pattern and strength of luminescence after oral or intravenous inoculation with the luminescent bacteria could be followed in individual, living mice over time. Unexpectedly, a robust luminescent signal was found localised in the lower thoracic region, even though the animals appeared healthy (Fig. 4). The luminescence appeared 2 days after inoculation, and increased in intensity over time, suggesting active replication. The localisation of the bacteria was found to be in the gall bladder by post-mortem section, facilitated, again, by luminescence imaging using a technique called "image-guided dissection". Thus, localisation and extracellular replication of *Listeria monocytogenes* in the gall bladder was described in living animals for the first time by capitalising on the unique strengths of bioluminescence imaging. These important findings suggested the potential for a chronic carrier state as a novel source of contamination with Listeriosis, a major advance in our understanding of its infectiology.

Impact on the 3Rs

In the absence of luminescent imaging, a dramatically higher number of animal experiments would have been required to enable gaining the findings described here.

This example clearly demonstrates the scientific potential of luminescent imaging in the study of infectious diseases. Importantly, relatively few animal experiments were used. Indeed, without luminescent imaging, the discovery *per se* of the presence and multiplication of *Listeria monocytogenes* in the gall bladder would have required infection of numerous mice, followed by sacrificing them at defined time points, dissection of each entire mouse and analysis of all organs for the presence of *Listeria monocytogenes* (= Reduction). Using conventional techniques, such an undertaking would have posed a substantial strain on resources and, as a matter of fact, had not been successful earlier. Furthermore, the number of mice required to perform such a study using conventional techniques would have been dramatically higher: not knowing into which organ and at which time point to look, it would obviously have been necessary to perform many more animal experiments (= Reduction). Finally, after having identified the gall bladder as a reservoir of listeria, to characterise the time course of progression would have required, again, many additional animal experiments using conventional techniques: time points of interest would have been assumed, and a large group of mice would have been inoculated and sacrificed at predefined time points. Instead, few mice sufficed for a full characterisation of the time course of events and correlation with morbidity (= Reduction). Finally, the experiments were performed in wild type BALB/c mice, obviating the need to generate and breed transgenic mice.

4 Conclusion

Luminescent imaging technology has the potential to substantially contribute to the 3Rs, especially to the reduction of animal numbers used per experiment, and refinement of the experimental design. This is illustrated here by three concrete examples of successful applications to investigations in the field of oncology, cardiology and bacteriology described in this review. Arguably, reduction of animal experiments is the most important consequence of luminescent imaging with respect to animal welfare.

Reduction is achieved by reducing variability: as animals are followed over time, each animal serves as its own control: inter-individual variability is eliminated and higher statistical power is obtained, allowing the number of animals per group (n) to be reduced. Further reductions in the number of laboratory animals used can be achieved by performing functional and imaging studies in the same animal, thus reducing inter-individual variability. In principle, the potential to reduce the number of animals used is highest in experiments in which inherent inter-individual variability is largest. Furthermore, the quality of data obtained from an animal experiment can be enhanced, sometimes decisively, by performing imaging studies.

Refinement can be achieved by making it possible to define earlier, less stressful time points and milder degrees of disease. It also means that pathophysiological mechanisms and pathways can be elucidated in ways that would be either impossible or associated with additional stress to the animals if luminescent imaging was not available. In many instances, animal experiments are the only feasible method to answer complex biological questions and are not likely to be replaceable by other methods in the near future. Hence, it is an important goal to adapt and refine existing methods in order to minimise strain on the laboratory animals; luminescent imaging has already been proven to improve animal welfare by contributing to this important goal.

Replacement: it lies within the nature of this subject that replacement of animal experiments is not possible.

In conclusion, luminescent imaging contributes to animal welfare by reducing and refining *in vivo* experiments. The concrete examples in the present analysis show how this can be achieved. As optical imaging technology progresses, further advances are likely that will result in further improvements of animal experiments.

References

Contag, C. H. and Bachmann, M. H. (2002). Advances in in vivo bioluminescence imaging of gene expression. *Ann. Rev. Biomed. Eng. 4*, 235-260.

Contag, P. R., Olomu, I. N., Stevenson, D. K. and Contag, C. H. (1998). Bioluminescent indicators in living mammals. *Nat. Med. 4*, 245-7.

Festing, S. (2008). On the necessity for animal experimentation. *Bioessays 30*, 94-95; author reply 96-97.

Flecknell, P. (2002). Replacement, reduction and refinement. *ALTEX 19*, 73-78.

Frangioni, J. V. (2008). New technologies for human cancer imaging. *J. Clin. Oncol. 26*, 4012-4021.

Hardy, J., Francis, K. P., DeBoer, M. et al. (2004). Extracellular replication of Listeria monocytogenes in the murine gall bladder. *Science 303*, 851-853.

Jenkins, D. E., Oei, Y., Hornig, Y. S. et al. (2003). Bioluminescent imaging (BLI) to improve and refine traditional murine models of tumor growth and metastasis. *Clin. Exp. Metastasis 20*, 733-744.

Käkonen, S. M. and Mundy, G. R. (2003). Mechanisms of osteolytic bone metastases in breast carcinoma. *Cancer 97(3 Suppl),* 834-839.

Koo, V., Hamilton, P. W. and Williamson, K. (2006). Non-invasive in vivo imaging in small animal research. *Cell. Oncol. 28*, 127-139.

Kutschka, I., Chen, I. Y., Kofidis, T. et al. (2006). Collagen matrices enhance survival of transplanted cardiomyoblasts and contribute to functional improvement of ischemic rat hearts. *Circulation 114*, *1 Suppl.,* 167-173.

Lapota, D. L. J. (1984). Observations of bioluminescence in marine plankton from the Sea of Cortez. *J. of Experimental Marine Biology and Ecology 7*, 209-239.

Leist, M., Kadereit, S. and Schildknecht, S. (2008). Food for thought... on the real success of 3R approaches. *ALTEX 25*, 17-32.

Luker, G. D. and Luker, K. E. (2008). Optical imaging: current applications and future directions. *J. Nucl. Med. 49*, 1-4.

Meighen, E. A. (1993). Bacterial bioluminescence: organization, regulation, and application of the lux genes. *Faseb J. 7*, 1016-1022.

Mensinger, A. C. J. (1997). Luminescent properties of fishes from nearshore California basins. *J. of Experimental Marine Biology and Ecology 210*, 75-90.

Rusche, B. (2003). The 3Rs and animal welfare - conflict or the way forward? *ALTEX 20 (Suppl. 1)*, 63-76.

Sadikot, R. T. and Blackwell, T. S. (2005). Bioluminescence imaging. *Proc. Am. Thorac. Soc. 2*, 537-40, 511-512.

Strube, A., Hoffmann, J. et al. (2009). Sagopilone inhibits breast cancer bone metastasis and bone destruction due to simultaneous inhibition of both tumor growth and bone resorption. *Clin. Cancer Res. 15*, 3751-3759.

Troy, T., Jekic-McMullen, D., Sambucetti, L. et al. (2004). Quantitative comparison of the sensitivity of detection of fluorescent and bioluminescent reporters in animal models. *Mol. Imaging 3*, 9-23.

Wehrman, T. S., von Degenfeld, G., Krutzig, P. O. et al. (2006). Luminescent imaging of beta-galactosidase activity in living subjects using sequential reporter-enzyme luminescence. *Nat. Methods 3*, 295-301.

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