	Inventaris Wob-verzoek W17-09								
		wordt verstrekt				**weigeringsgronden**			
nr.	**document**	**reeds openbaar**	**niet**	**geheel**	**deels**	**10.1.c**	**10.2.e**	**10.2.g**	**11.1**
	NTS2017897								
1	Aanvraagformulier				x		x	x	
2	Projectvoorstel				x		x	x	
3	Niet-technische samenvatting	x							
4	Bijlage beschrijving dierproeven 1			x					
5	Bijlage beschrijving dierproeven 2			x					
6	Bijlage beschrijving dierproeven 3				x		x	x	
7	DEC-advies				x		x	x	
8	Ontvangstbevestiging				x		x	x	
9	Verzoek aanvulling aanvraag				x		x	x	
10	Reactie aanvulling aanvraag				x		x	x	
11	Advies CCD		x						x
12	Beschikking en vergunning				x		x	x	

AVD 103002017897

1.

0 6 MAART 2017

Aanvraag
Projectvergunning Dierproeven
Administratieve gegevens

- U bent van plan om één of meerdere dierproeven uit te voeren.
- Met dit formulier vraagt u een vergunning aan voor het project dat u wilt uitvoeren. Of u geeft aan wat u in het vergunde project wilt wijzigen.
- Meer informatie over de voorwaarden vindt u op de website www.zbo-ccd.nl of in de toelichting op de website.
- Of bel met 0900-2800028 (10 ct/min).

1 Gegevens aanvrager

1.1 Heeft u een deelnemernummer van de NVWA?
Neem voor meer informatie over het verkrijgen van een deelnemernummer contact op met de NVWA.

☑ Ja > *Vul uw deelnemernummer in* 10300
☐ Nee > *U kunt geen aanvraag doen*

1.2 Vul de gegevens in van de instellingsvergunninghouder die de projectvergunning aanvraagt.

Naam instelling of organisatie	Stichting Katholieke Universiteit Nijmegen
Naam van de portefeuillehouder of diens gemachtigde	Instantie voor dierenwelzijn
KvK-nummer	4 1 0 5 5 6 2 9

1.3 Vul de gegevens van het postadres in.
Alle correspondentie van de CCD gaat naar de portefeuillehouder of diens gemachtigde en de verantwoordelijke onderzoeker.

Straat en huisnummer	Geert Grooteplein 29
Postbus	9101, [redacted]
Postcode en plaats	6500HB Nijmegen
IBAN	NL90ABNA0231209983
Tenaamstelling van het rekeningnummer	UMC St Radboud

1.4 Vul de gegevens in van de verantwoordelijke onderzoeker.

(Titel) Naam en voorletters	☐ Dhr. ☑ Mw. [redacted]
Functie	[redacted]
Afdeling	[redacted]
Telefoonnummer	[redacted]
E-mailadres	[redacted]

1.5 *(Optioneel)* Vul hier de gegevens in van de plaatsvervangende verantwoordelijke onderzoeker.

(Titel) Naam en voorletters	☐ Dhr. ☑ Mw. [redacted]
Functie	[redacted]
Afdeling	[redacted]
Telefoonnummer	[redacted]
E-mailadres	[redacted]

1.6 *(Optioneel)* Vul hier de gegevens in van de persoon die er verantwoordelijk voor is dat de uitvoering van het project in overeenstemming is met de projectvergunning.

(Titel) Naam en voorletters	[redacted] ☐ Dhr. ☐ Mw.
Functie	Instantievoor Dierenwelzijn
Afdeling	[redacted]
Telefoonnummer	[redacted]
E-mailadres	[redacted]

1.7 Is er voor deze projectaanvraag een gemachtigde?

☑ Ja > *Stuur dan het ingevulde formulier Melding Machtiging mee met deze aanvraag*

☐ Nee

2 Over uw aanvraag

2.1 Wat voor aanvraag doet u?

☒ Nieuwe aanvraag > Ga verder met vraag 3

☐ Wijziging op (verleende) vergunning die negatieve gevolgen kan hebben voor het dierenwelzijn

Vul uw vergunde projectnummer in en ga verder met vraag 2.2

☐ Melding op (verleende) vergunning die geen negatieve gevolgen kan hebben voor het dierenwelzijn

Vul uw vergunde projectnummer in en ga verder met vraag 2.3

2.2 Is dit een *wijziging* voor een project of dierproef waar al een vergunning voor verleend is?

☐ Ja > Beantwoord dan in het projectplan en de niet-technische samenvatting alleen de vragen waarop de wijziging betrekking heeft en onderteken het aanvraagformulier

☐ Nee > Ga verder met vraag 3

2.3 Is dit een *melding* voor een project of dierproef waar al een vergunning voor is verleend?

☐ Nee > Ga verder met vraag 3

☐ Ja > Geef hier onder een toelichting en ga verder met vraag 6

3 Over uw project

3.1 Wat is de geplande start- en einddatum van het project? Startdatum	02-04-2017
Einddatum	01-04-2022
3.2 Wat is de titel van het project?	Nanoparticle imaging agents for cancer therapy
3.3 Wat is de titel van de niet-technische samenvatting?	Toepassing van nanodeeltjes voor kankertherapie.

3.4 Wat is de naam van de Dierexperimentencommissie (DEC) aan wie de instellingsvergunninghouder doorgaans haar projecten ter toetsing voorlegt?

Naam DEC	RU DEC
Postadres	Postbus 9101, 6500 HB Nijmegen [redacted]
E-mailadres	[redacted]

4 Betaalgegevens

4.1 Om welk type aanvraag gaat het?

☑ Nieuwe aanvraag Projectvergunning	€ 1.541,00	Lege
☐ Wijziging	€	Lege

4.2 Op welke wijze wilt u dit bedrag aan de CCD voldoen.
Bij een eenmalige incasso geeft u toestemming aan de CCD om eenmalig het bij 4.1 genoemde bedrag af te schrijven van het bij 1.2 opgegeven rekeningnummer.

- ☐ Via een eenmalige incasso
- ☑ Na ontvangst van de factuur

5 Checklist bijlagen

5.1 Welke bijlagen stuurt u mee?

Verplicht

- ☒ Projectvoorstel
- ■ Niet-technische samenvatting

Overige bijlagen, indien van toepassing

- ☐ Melding Machtiging
- ☒ DEC-advies en factuurinformatie

6 Ondertekening

6.1 Print het formulier uit, onderteken het en stuur het inclusief bijlagen via de beveiligde e-mailverbinding naar de CCD of per post naar:

Centrale Commissie
Dierproeven
Postbus 20401
2500 EK Den Haag

Ondertekening door de instellingsvergunninghouder of gemachtigde (zie 1.6). De ondergetekende verklaart:

- dat het projectvoorstel is afgestemd met de Instantie voor Dierenwelzijn.
- dat de personen die verantwoordelijk zijn voor de opzet van het project en de dierproef, de personen die de dieren verzorgen en/of doden en de personen die de dierproeven verrichten voldoen aan de wettelijke eisen gesteld aan deskundigheid en bekwaamheid.
- dat de dieren worden gehuisvest en verzorgd op een wijze die voldoet aan de eisen die zijn opgenomen in bijlage III van richtlijn 2010/63/EU, behalve in het voorkomende geval de in onderdeel F van de bijlage bij het bij de aanvraag gevoegde projectvoorstel gemotiveerde uitzonderingen.
- dat door het ondertekenen van dit formulier de verplichting wordt aangegaan de leges te betalen voor de behandeling van de aanvraag.
- dat het formulier volledig en naar waarheid is ingevuld.

Naam	[redacted]
Functie	Instantie voor dierenwelzijn
Plaats	Nijmegen
Datum	0 2 - 0 3 [redacted]
Handtekening	[redacted]

2.

Form
Project proposal

- This form should be used to write the project propsal of animal procedures.
- The appendix 'description animal procedures' is an appendix to this form. For each type of animal procedure, a separate appendix 'description animal procedures' should be enclosed
- For more information on the project proposal, see our website(www.zbo-ccd.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	10300
1.2	Provide the name of the licenced establishment.	Stichting Katholieke Universiteit Nijmegen
1.3	Provide the title of the project.	Nanoparticle imaging agents for cancer therapy

2 Categories

2.1 Please tick each of the following boxes that applies to your project.

[X] Basic Research
[X] Translational or applied research
[] Regulatory use of routine production
[] Research into environmental protection in the interest of human or animal health or welfare dier
[] Research aimed at preserving the species subjected to procedures

[] Higher education or training
[] Forensic enquiries
[] Maintenance of colonies of genetically altered animals not used in other animal procedures

3 General description of the project

3.1 Background

Describe the project (motivation, background and context) with respect to the categories selected in 2.

- For legally required animal procedures, indicate which statutory or regulatory requirements apply (with respect to the intended use and market authorisation).
- For routine production, describe what will be produced and for which uses.
- For higher education or training, explain why this project is part of the educational program and describe the learning targets.

Cancer therapy
Cancer is one of the main killers of humanity today. It is now increasingly accepted that cancer therapy must be tailored to the individual and the type of cancer, due to the huge amount of heterogeneity involved. Hence, there is now a lot of interest in personalised medicine. This requires the use of noninvasive imaging techniques, in order to monitor and optimise the therapy as needed (for example, to adapt the dosage). In particular, more advanced therapeutics, such as cell therapies, require the use of imaging in order to monitor their localisation and functionality in vivo.

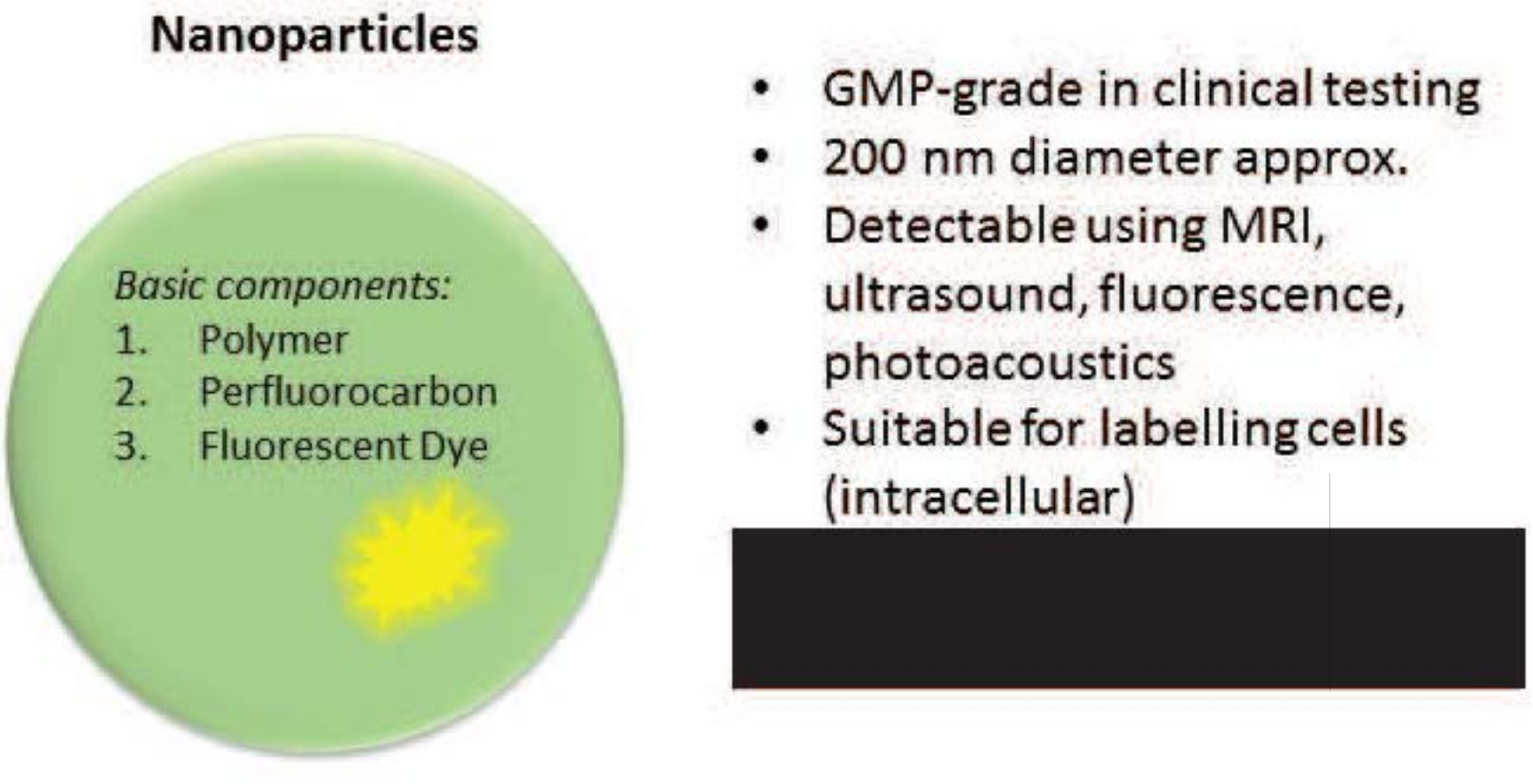
In this project, we plan on further exploring the use of imaging nanoparticles to cancer therapy., focusing on the imaging of cell therapy and the enhancement of tumor ablation therapies. The nanoparticles described here are currently in clinical testing as well.

Nanoparticles in cancer therapy
Polymeric particles have received a lot of attention over the past decades for various applications in nanomedicine, such as drug delivery or multimodal imaging [1][2][3][4][5]. These particles can be loaded, for example, with an imaging agents for imaging applications or with drugs or tumor antigens for therapy, or both. Our department has been working on developing polymeric nanoparticles for both clinical and preclinical use. These nanoparticles are being designed to be used for both the improvement of imaging (polymeric nanoparticles with imaging agents) [6] and therapy [7][8].

We are also producing these particles at GMP grade for an ongoing clinical trial at the Department of Tumor Immunology, thus the nanoparticles are fully expected to be safe for in vivo applications.

The nanoparticles (approx. 200 nm diameter) consist of an FDA-approved polymer, poly-lactic-co-glycolic acid (PLGA), entrapping a perfluorocarbon and a fluorescent dye. In some cases, the nanoparticle surface may be functionalised with a targeting antibody. This closely mimics the clinical nanoparticles we make, which consist of PLGA and a perfluorocarbon (either perfluoro-15-crown-5-ether or perfluorooctyl bromide) and a fluorescent

dye (either indocyanine or fluorescein). *All* the nanoparticles used in this entire DEC application will be PLGA-entrapped perfluorocarbon particles as described above. These nanoparticles are inherently suitable as imaging agents for multimodal imaging, particularly with MRI, fluorescence, ultrasound and photoacoustics. Additionally, they seem to be inherently suitable for ablative therapies (based on preliminary data). See figure below for a summary.



The nanoparticles are detectable using several imaging modalities: Magnetic Resonance Imaging (due to the perfluorocarbon); fluorescence (due to the fluorescent dye); ultrasound and photoacoustics (due to the structure of the nanoparticles).

Thus, through this project, we would like to further develop the nanoparticles for use in cancer therapy, both in imaging therapeutic cells and their ability to enhance tumour ablation, as detailed below.

1. Imaging of cellular therapies

Cellular therapy is the term which describes the transfer of cells to a patient to treat a disease or condition; for example, immune cells can be used to stimulate the immune system against tumors or inflammations [9] . For successful cellular therapy, it is essential that transferred cells are monitored post-transplant noninvasively, longitudinally, and quantitatively, such as by using *in vivo* imaging techniques. Cell tracking consists of following specific cells *in vivo,* this is often in term of their localization, fate, functionality or differentiation, which gives the ability to acquire specific information, for example regarding the number of cells in a region of interest (2). Such information is vital for the optimization and development of cellular therapies.

Although there are close to 30,000 clinical trials involving some form of cell therapy, there are none that have become clear successes. One of the main reasons for this is that it is nearly impossible to monitor cells in vivo noninvasively. For example, how do you know if the therapeutic cells have reached the correct location? Are they functional there? How long can they survive?

In order to answer such questions, imaging is essential, because it allows a noninvasive means to monitor cells in vivo. In fact, regulatory bodies such as the FDA and EMEA are strongly suggest that imaging be included in cell therapy trials (http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/CellularandGeneTherapy/ucm376136.htm#IV). There are currently no approved agents for clinical cell tracking, and thus this is an urgent problem. We think these nanoparticles would be strong contenders due to their clinical applicability and suitability for imaging with several imaging modalities.

These nanoparticles are currently in clinical testing for dendritic cell (DC) therapy within our department, but much remains unknown about them in vivo, particularly, relating to the fate of the nanoparticles after death of the therapeutic cells. We would like to answer questions regarding the processing and clearance of the nanoparticles and their biodistribution in vivo through this DEC. For that we would like to inject free nanoparticles and DCs labelled with our nanoparticles and follow them in vivo using imaging techniques, like MRI, photoacoustics and fluorescence imaging. We chose DCs as these cells are essential for cancer therapy and we have already shown that these cells can readily take up the PLGA-PFC nanoparticles.

Technological advances in imaging technologies have produced a variety of approaches for cell tracking and assessing the effects of cells on host tissues in small animals, including: MRI (1H, 19F), nuclear imaging (i.e., SPECT and PET), ultrasound, fluorescence, bioluminescence and photoacoustics (also known as MSOT). However, each modality has some limitations as shown in Table 1. To enable the widest possible reach with the platform and circumvent limitations of individual modalities, here, we will focus on developing probes suitable for MRI, ultrasound and MSOT imaging. Such agents are completely missing currently. As illustrated in the table below, MRI, ultrasound and MSOT imaging represent a powerful combination for non-invasive and safe monitoring of cellular therapies.

Table 1. Each imaging modality has pros and cons; *using a multimodal imaging approach, with the nanoparticles described here, overcomes weaknesses and maximises on strengths.*

Imaging technique	Clinical use	PROs	CONs	Relevance to cell tracking
Magnetic resonance imaging (MRI): Standard (^{1}H)	Established	Non-ionising, high resolution	Expensive, relatively long duration, patient specific issues (implants, movement)	Handful of clinical studies a decade ago, but not pursued further
MRI: ^{19}F	Emerging	Non-ionising, quantitative, not limited by half-life decay	Expensive, long durations	One published clinical study thus far (2014); handful more in progress. Technology is limited by availability of cell labels
Nuclear imaging (PET, SPECT)	Established	Extremely sensitive	Ionising, high cost, invasive, limited by half-life decay, repeated administration for longitudinal imaging	Whole body imaging; may not be suitable for e.g. stem cell due to radiotracers and possible DNA damage
Ultrasound	Established (anatomic)	Cheap, non-ionising, non-invasive, quick bedside scans	Low resolution, operator dependent	No clinical cell tracking work due to absence of suitable labels
Fluorescence	Histological or intra-operative	Low cost, safe, quantitative	Low penetration depth, photo bleaching	Validation of in vivo results in biopsies
MSOT	Emerging (iThera scanners only available)	Non-ionising, non-invasive, low cost	Low imaging depth or poor resolution	Not yet applied to cell tracking due to absence of suitable labels

Here, we will be demonstrating and optimising the use of our imaging nanoparticles (polymer-entrapped perfluorocarbon particles) for the imaging of dendritic cells in mice. We are certain that these data, together with our clinical data, will greatly aid in advancing our understanding of cellular therapies in vivo.

[redacted]

Finally, the last part of this project involves studying the biodistribution and in vivo fate of the nanoparticles. This is essential for their in vivo use, both for cell tracking and tumour ablation.

Hypothesis

We hypothetise:

1) The PLGA nanoparticles will allow imaging of cells for cancer therapy in vivo using imaging (such as MRI, fluorescence, ultrasound, photoacoustics).

[redacted]

More information about us or the work we do can be found at http://www.tumor-immunology.com/

Previous relevant research to this project
The nanoparticles have been an integral part of research from our department for the last several years, being involved in at least two other DECs involving imaging. Furthermore, the nanoparticles are now in clinical testing for cell tracking. These GMP-grade nanoparticles are identical to those that will be used in this project, except for differences in the choice of fluorescent dye. The only modifications will be the addition of a targeting antibody or a drug in some parts of this project. Thus, overall, we are confident that the nanoparticles are not toxic and are suitable for multimodal imaging. We have previousl shown that the particles are suitable for MRI and fluorescence imaging [3], and now we would like to extend this to ultrasound and photoacoustic imaging [patent pending]. Such imaging is feasible due to the unique facilities at RUMC, namely the PRIME, where all the imaging scanners and animal housing are in the same building.

[redacted]

are injected directly into a lymph node. Thus, no free particles are injected the label is never injected intraveneously. This is excellent for a first trial. However, we need to move towards in vivo or in site labelling, where the particles are injected intraveneously and are then taken up by specific cells.
Thus, experiments on biodistribution and also the ability of the particles to reach tumours need to be carried out, as described in this proposal. Furthermore, here, we will also test the efficacy of these particles for tumour ablation, based on some promising preliminary in vitro data (figure in section 3.2). We have no in vivo data on this aspect of the particles, although it would be a very exciting and relevant clinical application. Such experiments also require more knowledge of the biodistribution.
Hence, all these experiments are crucial to take us to the next stage of in vivo labelling, thus skipping laborious ex vivo cell culture and labelling steps, and tumour ablation.

The project is funded by an ERC Starting Grant and an ERC Proof of Concept Grant, both focusing on the developing the nanoparticles for therapeutic applications.

References:

[1] R. N. Mariano, D. Alberti, J. C. Cutrin, S. Geninatti Crich, and S. Aime, "Design of PLGA based nanoparticles for imaging guided applications.," *Mol. Pharm.*, vol. 11, no. 11, pp. 4100–6, Nov. 2014.
[2] G. Strohbehn, D. Coman, L. Han, R. R. T. Ragheb, T. M. Fahmy, A. J. Huttner, F. Hyder, J. M. Piepmeier, W. M. Saltzman, and J. Zhou, "Imaging the delivery of brain-penetrating PLGA nanoparticles in the brain using magnetic resonance.," *J. Neurooncol.*, vol. 121, no. 3, pp. 441–9, Feb. 2015.
[3] M. Srinivas, L. J. Cruz, F. Bonetto, A. Heerschap, C. G. Figdor, and I. J. M. de Vries, "Customizable, multi-functional fluorocarbon nanoparticles for quantitative in vivo imaging using 19F MRI and optical imaging.," *Biomaterials*, vol. 31, no. 27, pp. 7070–7, Sep. 2010.
[4] M. Figueiredo and R. Esenaliev, "PLGA Nanoparticles for Ultrasound-Mediated Gene Delivery to Solid Tumors.," *J. Drug Deliv.*, vol. 2012, p. 767839, Jan. 2012.

[5] Y.-R. Lee, Y.-H. Lee, S.-A. Im, K. Kim, and C.-K. Lee, "Formulation and Characterization of Antigen-loaded PLGA Nanoparticles for Efficient Cross-priming of the Antigen.," *Immune Netw.*, vol. 11, no. 3, pp. 163–8, Jun. 2011.
[6] M. Srinivas, A. Heerschap, E. T. Ahrens, C. G. Figdor, and I. J. M. de Vries, "(19)F MRI for quantitative in vivo cell tracking.," *Trends Biotechnol.*, vol. 28, no. 7, pp. 363–70, Jul. 2010.
[7] I. J. M. de V. Srinivas, Mangala, Jurjen Tel, Gerty Schreibelt, Fernando Bonetto, Luis-Javier Cruz, Houshang Amiri, Arend Heerschap, Carl G. Figdor, "PLGA-encapsulated perfluorocarbon nanoparticles for simultaneous visualization of distinct cell populations by 19F MRI," *Nanomedicine*, 2015. .
[8] L. J. Cruz, P. J. Tacken, I. S. Zeelenberg, M. Srinivas, F. Bonetto, B. Weigelin, C. Eich, I. J. De Vries, and C. G. Figdor, "Tracking Targeted Bimodal Nanovaccines : Immune Responses and Routing in Cells , Tissue , and Whole Organism," *Mol. Pharm.*, 2014.
[9] A. Al Faraj, N. Luciani, J. Kolosnjaj-Tabi, E. Mattar, O. Clement, C. Wilhelm, and F. Gazeau, "Real-time high-resolution magnetic resonance tracking of macrophage subpopulations in a murine inflammation model: a pilot study with a commercially available cryogenic probe.," *Contrast Media Mol. Imaging*, vol. 8, no. 2, pp. 193–203, Jan. .

[redacted]

3.2 Purpose

Describe the project's main objective and explain why this objective is achievable.

- If the project is focussed on one or more research objectives, which research questions should be addressed during this project?
- If the main objective is not a research objective, which specific need(s) does this project respond to?

The main goal of this project is to optimize and apply imaging nanoparticles to cancer therapy, through application in cell therapy and tumor ablation.

The project consists of three parts.
1. In vivo tracking of therapeutic cells pre-labelled with nanoparticles (Experiment group 1)
2. Analyzing the fate of nanoparticles after in vivo administration (Experiment group 2)

[redacted]

All Experiment groups will use the same nanoparticles.

The nanoparticles used here are currently in clinical use for imaging therapeutic dendritic cells (DCs) in melanoma patients. Here, we will optimize the nanoparticles in a preclinical model for cell imaging, and also further explore the nanoparticles in vivo, particularly in terms of their biodistribution and their direct therapeutic effect in cancer.
The nanoparticles has been previously tested for the 19F MRI cell tracking, [1] thus it is know that there is a possibility to track the nanoparticles and labelled cells in vivo.

[redacted]

The research projects range from fundamental to translational. Our group consists of people with both biological and chemical backgrounds, people with animal handling experience, and with an imaging expertise, and stays in close collaboration with fellow scientists of other backgrounds. That gives a strong technical and intellectual input on the various stages of our project. We also have suitable equipment available to carry all the necessary experiments.

[1] M. Srinivas, L. J. Cruz, F. Bonetto, A. Heerschap, C. G. Figdor, and I. J. M. de Vries, "Customizable, multi-functional fluorocarbon nanoparticles for quantitative in vivo imaging using 19F MRI and optical imaging.," *Biomaterials*, vol. 31, no. 27, pp. 7070–7, Sep. 2010.

3.3 Relevance

What is the scientific and/or social relevance of the objectives described above?

Scientific relevance

The nanoparticles are themselves imaging agents for multimodal imaging (MRI, fluorescence, ultrasound and photoacoustics). Furthermore, the fact that they are currently in clinical use makes them highly exciting. However, their current use has only been partially explored for cell imaging. Here, we will further characterise the particles for tumour ablation. This is a completely new application for such nanoparticles, and is thus very exciting.

The particles and their use for imaging and tumor ablation are currently the subject of 3 patents (pending), as well as several publications. They are also the main subject of various highly competitive grants, including an NWO Veni, and ERC Starting grant and ERC Proof of Concept, and an STW grant. Other than these granted proposals, several more are in process.

Novelty

The project is novel is several ways:

- The use of PLGA-entrapped nanoparticles for imaging with MRI is expected, but their signal in ultrasound and photoacoustics is not. Thus, demonstration of their in vivo utility for relevant applications such as cell tracking, is very exciting.

[redacted]

Finally, because we are already able to use the nanoparticles in humans, we are convinced that these agents have very high potential for cancer therapy, and would like to fully explore and exploit them for this purpose.

Societal relevance

There is a clear and urgent need to optimise cancer therapy. These nanoparticles are very likely to be able to contribute to improving cellular therapeutics, and potentially also noninvasive tumor ablation. The relevance of this research is clear. In fact, we are also looking to further this work commercially through a spin-off (this idea won the Dutch Life Sciences Venture Challenge, http://www.lifesciencesatwork.nl/conquest-winner-of-the-venture-challenge-spring-2015/), as are convinced of the possible impact of these nanoparticles to personalised medicine.
Furthermore, the dual-pronged approach taken here, both cell therapy [redacted], is a very power combination. In future projects, we can look into further enhancing the work through, for e.g., combining drug delivery with imaging [redacted]
Lastly, because the CCMO has already approved the nanoparticles used in this project for a clinical trial involving the imaging of dendritic cell therapy in melanoma patients, the work is certainly translatable, at least for the part involving cell therapy.
Overall, given that cancer is already a leading cause of mordibity and mortality worldwide, with the numbers expected to worsen (WHO statistics: http://www.who.int/mediacentre/factsheets/fs297/en/), the societal relevance of this project, and the societal benefit if the work is successful, is immense.

3.4 Research Strategy

3.4.1 Provide an overview of the overall design of the project (strategy).

The project consists of three parts. All parts of the project use the same nanoparticles, which are suitable for imaging with fluorescence, MRI, ultrasound and photoacoustics; and [redacted]

1. In vivo tracking of cells pre-labelled with polymeric nanoparticles containing an imaging agent (Experiment group 1)

The nanoparticles will be added to mouse DCs and incubated before the in vivo administration (the cells take up the nanoparticles). Pre-labelled DCs will be then tracked in vivo using following imaging modalities: ultrasound, photoacoustics, MRI, fluorescence to see whether cell reach a place of interest, e.g lymph nodes or a localised inflammation site. Tracking DCs in vivo will give us a better understanding of cells fate in vivo, regarding their localization and functionality.
These experiments closely mimic the ongoing clinical trial where human DCs are labeled with the same nanoparticles and injected in melanoma patients. We aim to corroborate and expand on the clinical data obtained, to resolve unanswered questions such as what happens to the label after cell death, which cannot be answered through the human trial.

2. Analyzing the biodistribution and clearance of the nanoparticles in vivo (Experiment group 2)

In order to study the fate of nanoparticles after in vivo administration, the nanoparticles will be directly administered into the mouse via foot pad, intravenous or intranodal injections. In a situation, where these types of injection will be insufficient (nanoparticles do not leave the injection site or inability to inject sufficient amount of nanoparticles) we will try further other injection types. The nanoparticles will be either targeted or non-targeted, by functionalising their surface with antibodies or other agents such as radioligands, in order to monitor their biodistribution and clearance in vivo. These data is essential for imaging and tumor ablation applications.
Again, these data will be vital to corroborate our human data as the in vivo fate of the nanoparticles cannot be studied in humans.

[redacted]

3.4.2 Provide a basic outline of the different components of the project and the type(s) of animal procedures that will be performed.

The three parts of this project aim at giving us answers on how we can optimize cancer treatment, through cell therapy and [redacted] Over the course of this project we will use different models and types of assays, which are listed below.

Types of models
Over the course of this project we will use different inducible tumor models: B16 melanoma, 9464D neuroblastoma and EL4 thymoma. Our department and our collaborators have experience with these models, thus the induction characteristics of these models are known (injection concentration, growth characteristics). For our purposes, tumor cells will be injected subcutaneously. For the imaging part of the project, where we will track the cells labelled with nanoparticles, we will also use inducible inflammation models (ear inflammation).

Numbers of models
To answer our questions, we will start our experiments with B16 melanoma model. However, tumors are very heterogeneous, with respect to growth and environment, thus very often, the proof of concepts is required to be confirmed in additional tumor models. That is why the number of different types of tumor models we will use is at most three. Not all experiments will be repeated in the second model though. Each results will be evaluated and the decision on which readout assay will need to be repeated in an alternative model will be made. This decision will be based on whether we see sufficient amount of the imaging agent accumulation within the tumor.

Types of experiments

To determine the effects of the various treatments the following assays will be used. Mice will be monitored at specific time points, with the minimal number needed for statistical significance. Collecting data by techniques such as flow cytometry is necessarily terminal, and increases the numbers of mice required, but no alternatives are available.

1) In vivo tracking of cells pre-labelled with polymeric nanoparticles containing an imaging agent
The nanoparticles containing an imaging agent will be added to mouse DCs and incubated before the in vivo administration. Pre-labelled DCs will be then tracked in vivo using various imaging modalities. We will also use the inflammation model (ear inflammation) to check whether DCs reach the inflammation site.
-donor mice for mouse DCs
-Following pre-labelled DCs in vivo using different imaging modalities (MRI, fluorescent imaging, ultrasound and photoacostics, at most 5 times within a two week period, and they will be done in a continuous session where the mouse will remain under anesthesia, duration of max. 2h per session per mouse) (All mice that will be used for below assays will be also used here)
-Influx of DCs and related immune cells into the lymphoid organs will be monitored by imaging, and the results validated by established techniques such as by flow cytometry (FACS) and histology
Go/no go decisions will depend on the quality and reproducibility of the data. When the quality appeared good and significance is reached, the results can be trusted and further variants/fine-tuning is not necessary. In this case, we need to be able to reproducibly detect the imaging agent or labeled cells in the relevant region (lymph node or region of inflammation) using in vivo imaging (MRI/fluorescence/ultrasound/photoacoustics), and be able to validate the data using ex vivo analyses, such as histology.

2) Analyzing the fate of nanoparticles after in vivo administration
Here, we will examine the fate of the nanoparticles in vivo after intravenous administration. The same nanoparticles as in the parts 1 and part 3 will be used, except we will also look at the effect of adding a targeting agent to the nanoparticle surface on its biodistribution. The targeting will be either against cell-specific surface markers on DCs or on tumor cells. The nanoparticles will typically be injected just before or after the tumor inoculation, and biodistribution will be monitored through imaging and validated through histology, as in the previous part of this project.

For each tumor cell line we will run a pilot experiment. The purpose of the pilot experiment is to establish the right set up for the experiment and to see what would be the best imaging times needed per each mouse, as this is not known. We believe that carrying out a pilot will help us reduce the overall numbers of mice needed.
-Transfer of nanoparticles encapsulating imaging agents (targeted and non-targeted) and following them in vivo will be monitored using imaging techniques at most 5 times within a two week period, they will be done in a continuous session where the mouse will remain under anesthesia, duration of max. 2h per session per mouse. Each mouse can be imaged with all of these techniques, so that lowers the amount of mice needed per experiment
-In vivo biodistribution of imaging agents will be determined by flow cytometry and histology on excised organs and tissues. The uptake will be studied overtime, thus there is no possibility to combine this and above group

Go/no go decisions will depend on the quality and reproducibility of the results. Here, we need reproducible biodistribution data in both in vivo (imaging) and ex vivo (e.g. histological) analyses. In this project we would like to detect and show the distribtion of our nanoparticles, in particular we would like to see if they will accumulate in the tumor. The goal of the experiment 2 is to see whether the nanoparticles will reach the tumor site, while in experiment 3 we would like to study the influence of the presence of nanoparticles [redacted]

[redacted]

Total number of animals in the project

Taken together, the total number of animals in the projects will be then 395 mice.

3.4.3 Describe the coherence between the different components and the different steps of the project. If applicable, describe the milestones and selection points

[redacted]

The particles were originally developed for the imaging of cell therapeutics, and are now also in clinical testing for this application. In this project, we will use the same nanoparticles to study (1) cell therapy, specifically of dendritic cells; (2) biodistribution; and (3) enhancement of tumor ablation. The only modifications to the nanoparticles will be the addition of a targeting antibody in biodistribution studies, and the inclusion of drugs to further enhance tumor ablation.

The studies are all closely linked, and not only because they all use the same nanoparticles (PLGA-entrapped perfluorocarbon particles), but also because all the mouse models aim at improving cancer therapy, whether in dendritic cell therapy or tumor ablation; and tumor ablation therapy itself requires the use of imaging.

3.4.4 List the different types of animal procedures. Use a different appendix 'description animal procedures' for each type of animal procedure.

Serial number	Type of animal procedure
1	In vivo tracking of cells pre-labelled with polymeric nanoparticles containing an imaging agent
2	Analyzing the fate of nanoparticles after in vivo administration. The nanoparticles will be non-targeted or targeted to various cells
3	[redacted]

4.

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website www.zbo-ccd.nl.
- Or contact us by phone. (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	10300	
1.2	Provide the name of the licenced establishment.	Stichting Katholieke Universiteit Nijmegen	
1.3	List the different types of animal procedures. Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.	Serial number 1	Type of animal procedure In vivo tracking of cells pre-labelled with polymeric nanoparticles containing an imaging agent

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

In this part we will use the nanoparticles to label immune cells and track the pre-labelled cells through in vivo imaging in order to study their localization, viability, and functionality. These are therapeutic dendritic cells (DCs) as used in cancer therapy. To validate the in vivo imaging data, we will need to carry out standard tests such as histology and flow cytometry.

For that, we will collect cells from donor mice and use those for the labeling with nanoparticles. Pre-labelled cells will be then directly administered into the mouse via foot pad, intravenous or intranodal injections. This will be done under anesthesia. The procedure mimics that used in an ongoing clinical trial using the same nanoparticles, except that the human subjects are not anasthesized during injection of the nanoparticle-labeled dendritic cells.

In some experiments, local inflammation will be induced using an injected agent. This is necessary in order to study the effects of inflammation on cell therapeutics.

Readout parameters in this experimental part will be:
- In vivo cell tracking of immune cells pre-labelled with nanoparticles for imaging. Pre-labelled cells will be tracked in vivo using different imaging modalities (MRI, fluorescent imaging, and photoacoustics). The mice will be imaged at most 5 times within a two week period. Imaging will be done in a continuous session where the mouse will remain under anesthesia, duration of max. 2h per session per mouse. Each mouse can be imaged with all of these techniques, so that lowers the amount of mice needed per experiment. What is more, all these imaging modalities are available within the same laboratory, so logistically doing imaging with several modalities is feasible.
- Mice will be monitored at specific time points, starting with the time of injection.
- To look at the influx of DCs and related immune cells into the lymphoid organs or to the inflammation site mice will be sacrificed to collect the organs and inflamed tissue. Those organs/tissue will be then tested with flow cytometry (FACS) viability assay, and histology. A mouse will be sacrificed at specific time point and organs/tissues will be collected. This will be done to study the influx of cells over time.

Collecting data by techniques such as flow cytometry is necessarily terminal, and increases the numbers of mice required, but no alternatives are available. However, the use of such established techniques is necessary to validate the in vivo imaging data obtained.
Since the nanoparticles are approved for clinical testing, we are confident that they will not prove toxic to the mice. Furthermore, the use of in vivo imaging allows use to reuse each mouse for more than one imaging session, although the sessions will be kept short (2 hours) and sufficiently spaced out (at least 24 hours, and preferably longer) and limited to a maximum of 5 per mouse. This is to reduce any side effects due to anasthesia (isoflurane). Some mice will need to be sacrificed at earlier time points, for validation of the imaging data through histology. Finally, because we are studying the immune system and its response to cancer therapy, we will be able to use only female mice to avoid sex-related differences in their immune systems.

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

Procedures cell tracking
Mouse immune cells will be extracted from donor mice and incubated together with the nanoparticles.
These pre-labelled cells will be then injected via foot pad, intravenous or intranodal injections and followed using multimodal imaging (MRI, fluorescence and photoacoustics) in a limited number of sessions (see Experimental approach).
Ear inflammation will be induced in a group of mice to study the migration of pre-labelled cells to the inflammation site.

In all, we do not expect to follow cells for 2 weeks.
For example, a possible protocol is: Isolation of dendritic cells from donor mice, and then in vitro labeling of these cells. The labeled cells are then injected intravenously in a mouse with ear inflammation (day 0). The mouse is then imaged in vivo on days 1, 3, 7 and 14 using MRI, fluorescence and photoacoustics without waking up during the imaging session. Some mice in the group are sacrificed after imaging at these time points for histological analyses to validate the in vivo imaging data.

Procedures readout assays
Imaging techniques will be used to track the injected cells. The imaging session will include taking an image immediately before injection of cells, and several images after the injection. We envisage that a maximum of five imaging sessions, with a maximum of 1 session per day, over the course of a maximum of 2 weeks will be enough to record changes.
At specific time points post injection (matching the imaging schedule outlined in the example above), some animals will be sacrificed via cervical dislocation. Then organs will be harvested from the mice, after which these tissues are processed further in the lab for analysis via the mentioned platforms (FACS, histology, viability assay). This is to validate the in vivo imaging data.

Procedures to induce localised inflammation
Localised inflammation will be induced through injection of agents such as LPS, possibly mixed in an adjuvant such as Freund's adjuvant. The will be induced either in the ear or in the upper leg of the mouse (only one site per mouse).

Anesthetics
The imaging and cell tracking sessions will involve time isoflurane inhalation. Each animal will stay under anesthesia for max. 2 h per session, max. 5 times over the course of 2 weeks. It is necessary to anasthesise the mice during imaging, to minimise motion. No pain or other discomfort is expected.

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

The number of mice needed to be able to reach statistical significance will be calculated for each individual experiment using a power analysis. For this we will use the validated software program G-power 3.0.8. The type of statistical test that will be performed is dependent on the nature of the data generated. For instance, comparison of more than two groups, a kaplan meier curve, or a correlation plot all require different statistics. The power that will be used in these tests will be 80%, as this is the lowest number that still gives reliable outcomes, while keeping the numbers of mice

low. Alpha values will be kept at 0.05, while variances needed for these analyses will be estimated based on former experiments or data from literature. If needed (more groups are compared at same time) a bonferroni correction will be taken into account.
Former studies helped us in our choice in which tumor model to invest and in which not.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Species
For our in vivo experiments we will use wild type mice. Studying the in vivo behavior nanoparticles encapsulating an imaging agents is not possible in 'lower' species. In literature, mice are commonly used to study the in vivo behavior of various polymeric particles. Additionally, the immune system of mice is recognized as sufficiently comparable to the human immune system (when primates are excluded). From an immunological point of view, almost all relevant components that are present in men are present in mice, too.

Origin
We will obtain our mice from registered EU breeding companies, or from our own breeding facility.

Estimated numbers
The total number of animals for these experiments is 150 mice.

- 50 mice to be donors for mouse DCs
- 100 mice for following pre-labelled DCs in vivo and to study the influx of DCs into the lymphoid organs and inflammation sites

Mouse number estimation:
-Donor mice for mouse DCs: The cells cannot be cultured ex vivo, thus cells must be acquired from donors for each experiment. Some cells will also be required to optimise labeling procedures, optimise imaging procedures and study any effects of labeling on the cells. Therefore, an estimate is 15 mice for ex vivo analyses and optimisations, an 35 to provide donor cells for other mice for in vivo imaging = 50 mice.
-In vivo imaging: 2 groups of mice (one inflammation model, non-treated control), (up to 4 routes of injection, e.g. intraveneous, subcutaneous, intramuscular or footpad) with 2.5 time points and 4 animals per group= 100 mice.
(2.5 time points calculated as an average, as most mice will not be sacrificed at an imaging time point due to the noninvasive nature of the procedure; however, at each time point some mice will need to be sacrificed to validate the in vivo data using established techniques such as histology)

Life stages
Our experiments will preferably be done in mice younger than 6 months. However, depending on the specific subquestion, sometimes older mice can be valuable too. This will ensure every mouse is well-used. Unless there is a specific explainable need for it, mice will not be allowed to grow older than one year.

Sex
Only female mice will be used as the donor and recipient cells must be a good match. We chose to use only female mice because (1) it is not possible to transfer cells between male and female mice due to the possibility of immune reactions, and (2) strong sex differences in immune reactions are known to occur. [1] Therefore, we chose to use only female mice.
[1] Kovats, S. Estrogen receptors regulate innate immune cells and signaling pathways. *Cell. Immunol.* 294, 63–69 (2015).

Species	Origin	Maximum number of animals	Life stage
wild type mice	commercial supplier/own in house breedings	150	all ages (except for embryonic)

C. Re-use

Will the animals be re-used?

[X] No, continue with question D.
[] Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

[] No
[] Yes > Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Replacement.
A significant part of our research is being done with in vitro cultures using cell-lines and tissue-like phantoms. However, it is necessary to use animals for further experiments as the in vivo behavior of nanoparticles may differ from in vitro set up. At the moment, it is not possible to mimic this sufficiently in vitro, or with computer models (so without animals).

Reduction.
Our experiments are designed with the minimal number of mice needed to answer the questions in our studies. The minimal group size needed to reach statistical significance will in the majority of cases be calculated using power analyses. In cases where this is not possible we base ourselves on the number of cells needed per experiment, divided by the number of cells that can be obtained per mouse. We strive to make optimal use of the material from each mouse by combining experiments wherever possible.

Refinement.

All procedures with the animals will be performed by experienced researchers/caretakers to keep the discomfort for the animals as low as possible. The procedures and models we use in this proposal are described in literature to give reliable data. In these models there are currently no methods available to reach further refinement. When opportunities with respect to refinement arise during the course of the project we strive to implement these wherever possible.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

In addition to the items mentioned under 'refinement' the extra precautions that are taken to reduce pain/stress or other discomfort are:
-Mice will first be made acquainted with the handling by the researcher, before they enter the actual experiment.
-Animals from one group will be housed together as much as possible. Randomization will be done as early in the experiment as possible, and re-randomization will be prevented if the experimental setup allows this. This will prevent unnecessary disturbance of social structures.
-Frequent checking of the animals before and during the experiment will prevent unnecessary discomfort.
- Each mouse can be imaged with all of the imaging techniques within one imaging session, which means the mouse will be anesthetized only once per each imaging session

Repetition and Duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

not applicable

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

[X] No
[] Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

[X] No > Continue with question H.
[] Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

[] No > Continue with question I.
[X] Yes > Will anaesthesia, analgesiaor other pain relieving methods be used?

[] No > Justify why pain relieving methods will not be used.

[X] Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

Injection of nanoparticle-loaded cells may cause some discomfort. The injections of pre-labelled cells will be followed by imaging sessions, thus the use of anesthesia (isoflurane) is needed to keep the animal still during imaging. The animals may experience some discomfort while recovering from anesthesia. The mice will be imaged at most 5 times within a two week period. Imaging will be done in a continuous session where the mouse will remain under anesthesia, duration of max. 2h per session per mouse. Each mouse can be imaged with all of these techniques,which means the mouse will be anesthetized only once per each imaging session. This can bring the times each mouse will experience a discomfort to a minimum. During long sessions (max. 2 hours) each animal will be closely monitored to prevent any unnecessary discomfort. To prevent sudden drops in mouse body temperature, we will use special heating platforms (when possible). In a situation when we see too low heart beat rate or too low temperature, we will immediately stop the imaging session and help the mouse to recover from anesthesia by warming it up.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals welfare may be expected?

Anesthetics:
Imaging with various imaging modalities will be done under general anesthesia using isoflurane.

Analgesics:
The level of pain expected from the injections or imaging sessions will not need the use of analgesics .

Experiment-related forms of discomfort:
-Injection of nanoparticles and cells or therapies
- injection of agents for localised inflammation
-anesthesia

Explain why these effects may emerge.

The adverse effects may result from:
-Injection of nanoparticles and cells or therapies
-inducing localised inflammation
-anesthesia

Indicate which measures will be adopted to prevent occurrence or minimise severity.

Each mouse can be imaged with all of the techniques we are going to use,which means the mouse will be anesthetized only once per each imaging session. This can bring the times each mouse will experience a discomfort to a minimum. During long sessions (max. 2 hours) each animal will be closely monitored to prevent any unnecessary discomfort. To prevent sudden drops in mouse body temperature, we will use special heating platforms (when possible). In a situation when we see too low heart beat rate or too low temperature, we will immediately stop the imaging session and help the mouse to recover from anesthesia by warming it up.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

[] No > Continue with question K.
[X] Yes > Describe the criteria that will be used to identify the humane endpoints.

The humane endpoints as present in the code of practice 'dierproeven in het kankeronderzoek' will be applied. Next to this, the following test specific humane endpoints will be used.

1. There is a significant drop in general condition or cachexia occurs. Loss of weigth of more than 15% in 2 days

Next to these the general humane endpoints will be applied:
1. The animal experiences more than little, additional, discomfort as a result of conditions not related to the experiment (e.g. injuries/wounds/infections)
2. The animal experiences more discomfort than justified for the purpose of the experiment and weighed by the DEC
3. (reliable and applicable)results cannot be achieved because of conditions not related with the experiment
4. The objective of the experiment has been reached

Indicate the likely incidence.

Drop in general condition or cachexia <2%
Significant ulceration is occurring 5-7%

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned (non-recovery, mild, moderate, severe).

Mice not receiving injections 'mild'
Mice receiving injections of pre-labelled cells 'mild'
Mice under (long) anesthesia 'moderate'
Imaging sessions 'mild'
Induction of inflammation 'mild'

The cumulative discomfort for all the animals in this study has been set **from 'mild' (80% mice) and moderate (20% mice).**

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

[] No > Continue with Section 3: 'Signatures'.
[X] Yes > Explain why it is necessary to kill the animals during or after the procedures.

The animals will be sacrificed when there is a need to collect the tissue for analysis or the end is reached. The animals will also be sacrificed when humane endpoints are reached.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

[] No > Descripe the method of killing that will be used and provide justifications for this choice.

[X] Yes

5.

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website www.zbo-ccd.nl.
- Or contact us by phone. (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	10300	
1.2	Provide the name of the licenced establishment.	Stichting Katholieke Universiteit Nijmegen	
1.3	List the different types of animal procedures. Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.	Serial number 2	Type of animal procedure Analyzing the fate of nanoparticles after in vivo administration. The nanoparticles will be non-targeted or targeted to various cells

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

In this part we will study the biodistribution of the nanoparticles in vivo. This is essential for both cell tracking and ablation enhancement experiments. The same nanoparticles (developed for imaging) will be used, as in the rest of the project. The only modification will be functionalisation of the nanoparticle surface, for example through the addition of a targeting antibody. Targeted or non-targeted nanoparticles will be directly administered into the mouse via foot pad, intravenous or intranodal injections.
In this part of the project we will use 3 tumor models: B16 melanoma, 9464D neuroblastoma and EL4 thymoma. The tumor cells will be injected subcutaneously.

Readout parameters in this experimental part will be:
- In vivo imaging is the main readout parameter here. Nanoparticles will be tracked in vivo using different imaging modalities (MRI, fluorescence, ultrasound, photoacoustics). The mice will be imaged at most 5 times within a two week period at specific time points. Imaging will be done in a continuous session where the mouse will remain under anesthesia, duration of max. 2h per session per mouse. Each mouse can be imaged with all of these techniques, so that lowers the amount of mice needed per experiment. What is more, all these imaging modalities are available within the same laboratory, so logistically doing imaging with several modalities is feasible.
- The in vivo results will be validated by conventional ex vivo analyses. To look at the in vivo uptake of nanoparticles, relevant organs/tissue will be excised then tested with flow cytometry and histology ex vivo. A mouse will be sacrificed at a specific time point and organs/tissues will be collected, after in vivo imaging. This is essential to validate the in vivo imaging data.
Collecting data by techniques such as histology is necessarily terminal, and increases the numbers of mice required, but no alternatives are available.

For example, a typical protocol would be: A group of mice receives an intraveneous injection of nanoparticles. The mice are then imaged at day 0, day 1, day 4 and day 8 using in vivo MRI and fluorescence imaging. Some mice from the group are sacrificed after imaging at days 1 and 4 for histological analyses of relevant tissues (such as the liver) for validation of the in vivo imaging data.

Since the nanoparticles are approved for clinical testing, we are confident that they will not prove toxic to the mice. Furthermore, the use of in vivo imaging allows use to reuse each mouse for more than one imaging session, although the sessions will be kept short (2 hours) and sufficiently spaced out (at least 24 hours, and preferably longer) and limited to a maximum of 5 per mouse. This is to reduce any side effects due to anasthesia (isoflurane). Some mice will need to be sacrificed at earlier time points, for validation of the imaging data through histology. Finally, because we are studying the immune system and its response to cancer therapy, we will be able to use only female mice to avoid sex-related differences in their immune systems.

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

Procedures nanoparticle transfer

Nanoparticles (PLGA-entrapped perfluorocarbon) will be injected via foot pad, intravenous or intranodal injections followed using multimodal imaging (MRI, ultrasound and photoacoustic imaging using high frequency scanner). The route of injection will be finalized based on the functionality of the injected cells (ie can the injected cells reach lymph nodes, and can these can detected via in vivo imaging).
Some mice will receive only particles, while other will receive cells labeled with particles. The free particles serve as important controls, so we can see if the cells are actively migrating.
Further, the mice will be imaged at most 5 times within a 14-day period. Imaging will be done in a continuous session where the mouse will remain under anesthesia, duration of max. 2h per session per mouse. Each mouse can be imaged with all of these techniques, so that lowers the amount of mice needed per experiment. What is more, all these imaging modalities are available within the same laboratory, so logistically doing imaging with several modalities is feasible.

The exact imaging schedule will be determined experimentally, although within a 14-day limit. For example, a mouse is injected with dendritic cells labeled with the nanoparticles in the foot pad, and then imaged on days 1, 3, 9 and 14 post-injection using MRI, fluorescence and ultrasound imaging. Because all the imaging scanners are located close to each other, the mouse can be imaged using all these modalities without waking up from anasthesia.
Here, we will also use tumor models in order to look whether the nanoparticles accumulate within the tumor. We will use 3 tumor models: B16 melanoma, 9464D neuroblastoma and EL4 thymoma. The tumor cells will be injected subcutaneously.

Procedures readout assays
Imaging techniques will be used to trace the injected nanoparticles, primarily through in vivo images. However, the in vivo data will need to be validated using an established technique, namely histology. Thurs, at specific time points (such as days 1, 3, 9 and 14 post injection), animals will be sacrificed via cervical dislocation. Then organs (such as liver, spleen, lymph nodes and tumour) will be harvested from the mice, after which these tissues will be processed further in the lab for analysis via the mentioned platforms (FACS, histology, viability assay).

Anesthetics
The imaging and cell tracking sessions will involve isoflurane inhalation. Anasthesia is necessary to keep the mice immobilised during imaging. Mice will be monitored for breathing and/or heart rate, and temperature when anasthetics are used.

Some animals may need chlorophyll-free feed (readily available from standard suppliers) in order to reduce background from chlorophyll in the gut in the fluorescence images.

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

The number of mice needed to be able to reach statistical significance will be calculated for each individual experiment using a power analysis. For this we will use the validated software program G-power 3.0.8. The type of statistical test that will be performed is dependent on the nature of the data generated. For instance, comparison of more than two groups, a kaplan meier curve, or a correlation plot all require different statistics. The power that will be used in these tests will be 80%, as this is the lowest number that still gives reliable outcomes, while keeping the numbers of mice low. Alpha values will be kept at 0.05, while variances needed for these analyses will be estimated based on former experiments or data from literature. If needed (more groups are compared at same time) a bonferroni correction will be taken into account.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Species

For our in vivo experiments we will use mice. Studying the in vivo behavior nanoparticles encapsulating an imaging agents is not possible in 'lower' species. In literature, mice are commonly used to study the in vivo behavior of various polymeric particles. Additionally, the immune system of mice is recognized as sufficiently comparable to the human immune system (when primates are excluded). From an immunological point of view, almost all relevant components that are present in men are present in mice, too. In this part, we will use wild type mice.

Origin

We will obtain our mice from registered EU breeding companies, or from our own breeding facility.

Estimated numbers

The total number of animals in these experiments is 125 mice.

- 25 mice for pilot experiment
- 50 mice to study in vivo uptake of imaging agents
- 50 mice for in vivo tracking of nanoparticles and cells

Mouse number estimation:

-pilot experiment to set up tumour models and establish optimal time points for imaging = 25 mice

-in vivo biodistribution requires 2 types of particles (targeted and non-targeted), 4 groups of mice (3 tumor models and non-treated controls), 3 time points = 24 = 25 mice (rounded up); furthermore some targeted agents will be tested only in a few mice to determine their efficacy before moving on to a full experiment with several time points as in the previous case, thus this would be (4 agents, 3 time points, 2 mice per time point)= 24 mice= rounded up to 25; in total = 50 mice

-Especially promising nanoparticles from the previous experiments, will be further validated using standard histology or flow cytometry, which requires more mice per time points; therefore 3 groups of mice (2 targeted agents and non-targeted control), 3 time points with 5 mice per time point = 45 mice, rounded up to 50 mice

(rounding up is necessary, as some mice may not develop tumours)

Life stages

Our experiments will preferably be done in mice younger than 6 months. However, depending on the specific subquestion, sometimes older mice can be valuable too. This will ensure every mouse is well-used. Unless there is a specific explainable need for it, mice will not be allowed to grow older than one year.

Sex

Only female mice will be used to avoid differences in results due to gender-related differences in the immune system. We chose to use only female mice because (1) it is not possible to transfer cells between male and female mice due to the possibility of immune reactions, and (2) strong sex differences in immune reactions are known to occur. [1] Therefore, we chose to use only female mice.

[1] Kovats, S. Estrogen receptors regulate innate immune cells and signaling pathways. *Cell. Immunol.* 294, 63–69 (2015).

Species	Origin	Maximum number of animals	Life stage
wild type	commercial supplier/own in house breedings	125	all ages (except for embryonic)

C. Re-use

Will the animals be re-used?

[X] No, continue with question D.
[] Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

[] No
[] Yes > Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Replacement.
A significant part of our research is being done with in vitro cultures using cell-lines and tissue or tissue-like phantoms. However, it is necessary to use animals for further experiments as the in vivo behavior of nanoparticles may differ from in vitro set up. At the moment, it is not possible to mimic this sufficiently in vitro, or with computer models (so without animals), or in 'lower' species.

Reduction.
Our experiments are designed with the minimal number of mice needed to answer the questions in our studies. The minimal group size needed to reach statistical significance will in the majority of cases be calculated using power analyses. In cases where this is not possible we base ourselves on the number of cells needed per experiment, divided by the number of cells that can be obtained per mouse. We strive to make optimal use of the material from each mouse by combining experiments wherever possible.

Refinement.

All procedures with the animals will be performed by experienced researchers/caretakers to keep the discomfort for the animals as low as possible. The procedures and models we use in this proposal are described in literature to give reliable data. In these models there are currently no methods available to reach further refinement. When opportunities with respect to refinement arise during the course of the project we strive to implement these wherever possible.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

In addition to the items mentioned under 'refinement' the extra precautions that are taken to reduce pain/stress or other discomfort are:
-Mice will first be made acquainted with the handling by the researcher, before they enter the actual experiment.
-Animals from one group will be housed together as much as possible. Randomization will be done as early in the experiment as possible, and re-randomization will be prevented if the experimental setup allows this. This will prevent unnecessary disturbance of social structures.
-Frequent checking of the animals before and during the experiment will prevent unnecessary discomfort.
- Each mouse can be imaged with all of the imaging techniques within one imaging session, which means the mouse will be anesthetized only once per each imaging session

Repetition and Duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

not applicable

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

[X] No
[] Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

[X] No > Continue with question H.
[] Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

[] No > Continue with question I.
[X] Yes > Will anaesthesia, analgesiaor other pain relieving methods be used?

[] No > Justify why pain relieving methods will not be used.

[X] Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

Injection of nanoparticle-loaded cells may cause some discomfort. The injections of pre-labelled cells will be followed by imaging sessions, thus the use of anesthesia is needed (mainly to prevent motion during the imaging, not due to pain). The animals may experience some discomfort while recovering from anesthesia. The mice will be imaged at most 5 times within a two week period. Imaging will be done in a continuous session where the mouse will remain under anesthesia, duration of max. 2h per session per mouse. Each mouse can be imaged with all of these techniques,which means the mouse will be anesthetized only once per each imaging session. This can bring the times each mouse will experience a discomfort to a minimum. During long sessions (max. 2 hours) each animal will be closely monitored to prevent any unnecessary discomfort. To prevent sudden

drops in mouse body temperature, we will use special heating platforms (when possible). In a situation when we see too low heart beat rate or too low temperature, we will immediately stop the imaging session and help the mouse to recover from anesthesia by warming it up.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals welfare may be expected?

Anesthetics:
Injection of the pre-labelled cell suspension and imaging with various imaging modalities will be done under general anesthesia using isoflurane.

Analgesics:
The level of pain expected from the injections or imaging sessions will not need the use of analgesics . We will try to not use analgesics during the tumor growth as it may interfere with the final result of the experiments.

Experiment-related forms of discomfort:
-Injection of nanoparticles and cells or therapies
-injection of tumor cells
-tumor growth
-anesthesia

Explain why these effects may emerge.

The adverse effects may result from:
-Injection of nanoparticles and cells or therapies
-injection of tumor cells
-tumor growth
-anesthesia

Indicate which measures will be adopted to prevent occurrence or minimise severity.

Each mouse can be imaged with all of the techniques we are going to use,which means the mouse will be anesthetized only once per each imaging session. This can bring the times each mouse will experience a discomfort to a minimum. During long sessions (max. 2 hours) each animal will be closely monitored to prevent any unnecessary discomfort. To prevent sudden drops in mouse body temperature, we will use special heating

platforms (when possible). In a situation when we see too low heart beat rate or too low temperature, we will immediately stop the imaging session and help the mouse to recover from anesthesia by warming it up.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

[] No > Continue with question K.
[X] Yes > Describe the criteria that will be used to identify the humane endpoints.

The humane endpoints as present in the code of practice 'dierproeven in het kankeronderzoek' will be applied. Next to this, the following test specific humane endpoints will be used.

1. There is a significant drop in general condition or cachexia occurs. Loss of weigth of more than 15% in 2 days
2. End of the experiment

Next to these the general humane endpoints will be applied:
1. The animal experiences more than little, additional, discomfort as a result of conditions not related to the experiment (e.g. injuries/wounds/infections)
2. The animal experiences more discomfort than justified for the purpose of the experiment and weighed by the DEC
3. (reliable and applicable)results cannot be achieved because of conditions not related with the experiment
4. The objective of the experiment has been reached

We need the tumours to be large enough for the EPR effect to occur, and also for carrying out HIFU ablation. After that, we need to observe the treated mice (in relation to non-treated controls), to see how tumour growth is affected by the treatment. This is probably quite a slow process, thus the mice will need to be monitored for relatively long periods after treatment. Therefore, we currently cited the humane endpoint in this manner.

Indicate the likely incidence.

Drop in general condition or cachexia <2%
The total tumor mass reaches 2cm3: 50% (depending on the experimental setup up to 100% is possible)
Significant ulceration is occurring 5-7%

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned (non-recovery, mild, moderate, severe).

Mice not receiving injections 'mild'
Mice receiving injections of pre-labelled cells 'mild'
Mice under anesthesia 'mild'
Imaging sessions 'mild' to 'moderate'
Induction of tumors 'mild'
Tumor growth 'moderate'

The cumulative discomfort for all animals will be 'mild' (50% mice), except those who will undergo longer imaging sessions and tumor growth. For those the cumulative discomfort will be 'moderate' (50% mice).

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

[] No > Continue with Section 3: 'Signatures'.
[X] Yes > Explain why it is necessary to kill the animals during or after the procedures.

The animals will be sacrificed when there is a need to collect the tissue for analysis or the end is reached. The animals will also be sacrificed when humane endpoints are reached.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

[] No > Descripe the method of killing that will be used and provide justifications for this choice.

[X] Yes

6.

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website www.zbo-ccd.nl.
- Or contact us by phone. (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	10300	
1.2	Provide the name of the licenced establishment.	Stichting Katholieke Universiteit Nijmegen	
1.3	List the different types of animal procedures. Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.	Serial number 3	Type of animal procedure Improvement of tumor ablation therapies via the use of nanoparticles encapsulating an imaging agent and drugs

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

[redacted]

- Influx of DCs and related immune cells into tumor will be determined by flow cytometry (FACS), viability assay, and histology.

[redacted]

Since the nanoparticles are approved for clinical testing, we are confident that they will not prove toxic to the mice. Furthermore, the use of in vivo imaging allows use to reuse each mouse for more than one imaging session, although the sessions will be kept short (2 hours) and sufficiently spaced out (at least 24 hours, and preferably longer) and limited to a maximum of 5 per mouse. This is to reduce any side effects due to anasthesia (isoflurane). Some mice will need to be sacrificed at earlier time points, for validation of the imaging data through histology. Finally, because we are studying the immune system and its response to cancer therapy, we will be able to use only female mice to avoid sex-related differences in their immune systems.

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

Procedures tumor models
For the experiments in this part we will use tumor bearing mice. Tumor cells will be injected subcutaneously. In the typical model tumors will become palpable after approximately 10 days. From that day on, tumors will be checked frequently (2-3 times/week), and their growth will be measured using calipers, to ensure that humane endpoints will be detected in time.

Procedures treatments
First, nanoparticles will be injected intraveneously in tumour-bearing mice. In case this does not result in sufficient concentration of nanoparticles in the tumour, intra-tumoural injections may be necessary. Once nanoparticles are present in a tumor, in situ tumor treatments will be done using ablation methods [redacted] Each animal will remain under anesthesia for at most 2 hours, during which it will be closely monitored. Breathing frequency will be monitored and kept constant between 30 and 60 per minute. Body temperature will be measured with a rectal thermometer and maintained using a heated air flow device. [redacted]

Procedures readout assays
Directly after the ablation treatment, the animals will undergo an imaging session. The lesions size will be measured using imaging techniques (MRI,

fluorescence and photoacoustic imaging) in order to establish whether the formation of lesion was enhanced by the presence of imaging agents. During the imaging session animals will remain under anesthesia using isoflurane.
Animals will be divided into groups depending on the experimental part.
To establish the influence of nanoparticles on the lesion size animals will be split into two groups where group 1 will be sacrificed right after the treatment and imaging session and organs will be harvested for FACS and histology; and group 2 will be kept for 48 hours. After 48 hours animals in group 2 will undergo an imaging session (MRI, fluorescence and photoacoustic imaging) with the use of isoflurane inhalation anesthesia. Each animal in this group will be imaged and the time of the session is expected to last max.2 hrours. After imaging animals in group 2 will be sacrificed for tissues harvesting. These tissues will be further processed in the lab for analysis via the mentioned platforms.
[redacted] and its influence on tumor development, mice will be imaged directly after the treatments (MRI, fluorescence and photoacoustic imaging) with the use of isoflurane inhalation anesthesia. Next, the animals will be closely monitored over the course of 4 weeks, when tumors will be measured with calipers. For this measurement animals will not have to anesthetized. During those 4 weeks animals will be imaged using ultrasound and photoacoustic imaging at most 4 times, once a week.

Imaging sessions are necessary to measure the lesion size, tumor growth, and to detect any signal from nanoparticles encapsulating an imaging agent.

Anesthetics
In these experiments the exact growth location of the tumor needs to be secured, and therefore all tumor inoculations will be done under anesthesia. Same holds true for the in situ tumor treatments. This will involve one time isoflurane inhalation.

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

The number of mice needed to be able to reach statistical significance will be calculated for each individual experiment using a power analysis. For this we will use the validated software program G-power 3.0.8. The type of statistical test that will be performed is dependent on the nature of the data generated. For instance, comparison of more than two groups, a kaplan meier curve, or a correlation plot all require different statistics. The power that will be used in these tests will be 80%, as this is the lowest number that still gives reliable outcomes, while keeping the numbers of mice low. Alpha values will be kept at 0.05, while variances needed for these analyses will be estimated based on former experiments or data from literature. If needed (more groups are compared at same time) a bonferroni correction will be taken into account.
In special cases that have a strong pilot-character, and for which a-priori variances are difficult to estimate, the minimum number of mice that still allows simple forms of statistics will be used per group.
Former studies helped us in our choice in which tumor model to invest and in which not.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Species

For our in vivo experiments we will use mice. Studying the in vivo behavior and role of nanoparticles encapsulating an imaging agents is not possible in 'lower' species. In literature, mice are commonly used to study the in vivo behavior of various polymeric particles and ablation therapies. Additionally, the immune system of mice is recognized as sufficiently comparable to the human immune system (when primates are excluded). From an immunological point of view, almost all relevant components that are present in men are present in mice, too. In this part, we will mainly use wild type mice.
We will obtain our mice from registered EU breeding companies, or from our own breeding facility.

Estimated numbers

The number of animals needed for these experiments is 120 mice.

- 90 mice to study the influx of DCs and related immune cells into tumor
- 30 mice to study the release of the therapeutics from nanoparticles and their effect on tumor cells

Mouse number estimation:
-tumour-bearing mice [redacted] 6 groups of mice (3 tumor models with one group each that receive treatment and one group that do not receive treatment to act as controls), with 3 intensities of ablation treatment and 5 mice per group = 90 mice -a selected tumour-bearing model from the previous experiments will receive particles that also contain an antitumoral therapeutic agent; thus 3 groups of mice (no particles, particles without therapeutics, particles with therapeutics) and 10 mice per group (a large number is needed to allow sufficient mice for procedures such as histology and flow cytometric analyses of tumor growth)= 30 mice

Life stages
Our experiments will preferably be done in mice younger than 6 months. However, depending on the specific subquestion, sometimes older mice can be valuable too. This will ensure every mouse from our breeding is well-used. Unless there is a specific explainable need for it, mice will not be allowed to grow older than one year.

Sex
Only female mice will be used, as the immune system can differ between male and female mice. We chose to use only female mice because (1) it is not possible to transfer cells between male and female mice due to the possibility of immune reactions, and (2) strong sex differences in immune reactions are known to occur. [1] Therefore, we chose to use only female mice.
[1] Kovats, S. Estrogen receptors regulate innate immune cells and signaling pathways. *Cell. Immunol.* 294, 63–69 (2015).

Species	Origin	Maximum number of animals	Life stage
wild type	commercial supplier/own in house breedings	120	all ages (except for embryonic)

C. Re-use

Will the animals be re-used?

[X] No, continue with question D.
[] Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

[] No
[] Yes > Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Replacement.
A significant part of our research is being done with in vitro cultures using cell-lines and tissue or tissue-like phantoms. However, it is necessary to use animals for further experiments as the in vivo behavior of nanoparticles and their influence on tumor ablation may differ from in vitro set up. At the moment, it is not possible to mimic this sufficiently in vitro, or with computer models (so without animals), or in 'lower' species.

Reduction.
Our experiments are designed with the minimal number of mice needed to answer the questions in our studies. The minimal group size needed to reach statistical significance will in the majority of cases be calculated using power analyses. In cases where this is not possible we base ourselves on the number of cells needed per experiment, divided by the number of cells that can be obtained per mouse. We strive to make optimal use of the material from each mouse by combining experiments wherever possible.

Refinement.
All procedures with the animals will be performed by experienced researchers/caretakers to keep the discomfort for the animals as low as possible. The procedures and models we use in this proposal are described in literature to give reliable data. In these models there are currently no methods available to reach further refinement. When opportunities with respect to refinement arise during the course of the project we strive to implement these wherever possible.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

In addition to the items mentioned under 'refinement' the extra precautions that are taken to reduce pain/stress or other discomfort are:
-Mice will first be made acquainted with the handling by the researcher, before they enter the actual experiment.
-Animals from one group will be housed together as much as possible. Randomization will be done as early in the experiment as possible, and re-randomization will be prevented if the experimental setup allows this. This will prevent unnecessary disturbance of social structures.
-Frequent checking of the animals before and during the experiment will prevent unnecessary discomfort

-Each mouse can be imaged with all of the imaging techniques within one imaging session, which means the mouse will be anesthetized only once for the ablation treatment and post ablation imaging session
-Tumor growth will be closely monitored to avoid overgrowth and to monitor any effects on the animals themselves
-Close monitoring of animals, by measuring body temperature and heart rate during the experiment

Repetition and Duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

not applicable

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

[X] No
[] Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

[X] No > Continue with question H.
[] Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

[] No > Continue with question I.
[X] Yes > Will anaesthesia, analgesiaor other pain relieving methods be used?

[] No > Justify why pain relieving methods will not be used.

[X] Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

During the tumor growth and when tumor reaches the size needed for treatment (around 7 mm) it can be expected that there will be mild forms of discomfort associated with the growth of the tumor. Giving continuous pain relief is unnecessary with this level of pain, and moreover, this will significantly influence the immune system. Therefore, in general for this experimental part analgesics will not be used.
Exceptions are the injection of nanoparticles, tumor cells and the tumor treatments. Not all injections of tumor cells will need to be done under general anesthesia, but in some specific cases where precise subcutaneous localization is required, the use of anesthetics is vital.
We will monitor the mice closely for any adverse reactions to the ablation treatment, such as burns. However, these are extremely unlikely, as we will keep the energy intensity low to mimic that used in the clinic.
During long treatment and imaging sessions (max. 2 hours) each animal will be closely monitored to prevent any unnecessary discomfort. Breathing frequency will be monitored and kept constant between 30 and 60 per minute. Body temperature will be measured with a rectal thermometer and maintained using a heated air flow device. During the imaging sessions to prevent sudden drops in mouse body temperature, we will use special heating platforms (when possible). After the treatment animals will be recovered from anesthesia and frequently checked to monitor any adverse post-treatments effects, such as slower movement and signs of severe discomfort.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals welfare may be expected?

Anesthetics:
As the tumors need to be positioned at a very exact spot for in situ tumor ablation, injection of the nanoparticles or tumor cell suspension will be done under general anesthesia using isoflurane.
[redacted] imaging will also be done under isoflurane inhalation to make sure an animal keeps still. The imaging (with the use of MRI, fluorescence and photoacoustic imaging) will be done directly after [redacted] and at most 2 more time over the course of a week. Each of this imaging modalities can used within one imaging session, which means the animals will have to be anesthetized only once per session. The imaging session will last at most 2 hours.

Analgesics:
It can be expected that there will be mild forms of discomfort associated with the growth of the tumor. Giving continuous pain relief is unnecessary with this level of pain, and moreover, this will significantly influence the immune system. With respect to the in situ tumor ablation treatments we have tried various anesthetics/analgesics in the past. In combination with the ablation the animals however appeared to cool down significantly and even died more often. Moreover, these compounds are known to influence the immune significantly, what further can influence the results of our studies. The current use of isoflurane leads to no increased mortality. The post-operative stress/pain is reduced to normal levels within one hour after treatment.

Experiment-related forms of discomfort:
-Growth of subcutaneous tumors.
-Discomfort as experienced [redacted]
-Injection of nanoparticles and cells or therapies
-injection of tumor cells
-anesthesia

Explain why these effects may emerge.

Adverse effects may result from:
-Injection of nanoparticles and cells or therapies
-injection of tumor cells
-tumor growth
-anesthesia (isoflurane)
[redacted]

Indicate which measures will be adopted to prevent occurrence or minimise severity.

Each mouse can be imaged with all of the techniques we are going to use,which means the mouse will be anesthetized only once per each imaging session. This can bring the times each mouse will experience a discomfort to a minimum. During long sessions (max. 2 hours) each animal will be closely monitored to prevent any unnecessary discomfort. To prevent sudden drops in mouse body temperature, we will use special heating platforms (when possible). In a situation when we see too low heart beat rate or too low temperature, we will immediately stop the imaging session and help the mouse to recover from anesthesia by warming it up.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

[] No > Continue with question K.
[X] Yes > Describe the criteria that will be used to identify the humane endpoints.

The humane endpoints as present in the code of practice 'dierproeven in het kankeronderzoek' will be applied. Next to this, the following test specific humane endpoints will be used.

1. There is a significant drop in general condition or cachexia occurs. Loss of weigth of more than 15% in 2 days
2. The total tumor mass exceeds 2cm3.
3. Significant ulceration is occurring. Not all wounds/necrotic patches on the tumor are ulcerations. A photo guide for the most often used tumor models present at the CDL will be used to distinguish between the various situations

Next to these the general humane endpoints will be applied:
1. The animal experiences more than little, additional, discomfort as a result of conditions not related to the experiment (e.g. injuries/wounds/infections)
2. The animal experiences more discomfort than justified for the purpose of the experiment and weighed by the DEC
3. (reliable and applicable)results cannot be achieved because of conditions not related with the experiment
4. The objective of the experiment has been reached

Indicate the likely incidence.

Drop in general condition or cachexia <2%
The total tumor mass reaches 2cm3: 50% (depending on the experimental setup up to 100% is possible)
Significant ulceration is occurring 5-7%

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned (non-recovery, mild, moderate, severe).

Mice receiving injections of pre-labelled cells or nanoparticles 'mild'
Mice under anesthesia 'mild'
Imaging sessions 'mild'
Induction of tumors 'mild'
Tumor growth 'moderate'
Tumor treatment 'moderate'

The cumulative discomfort for animals will be 'mild' (30% mice) , except those who will undergo longer imaging sessions or in which tumor growth will be extended. For those the cumulative discomfort will be 'moderate' (70% mice).

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

[] No > Continue with Section 3: 'Signatures'.
[X] Yes > Explain why it is necessary to kill the animals during or after the procedures.

The animals will be sacrificed when there is a need to collect the tissue for analysis or the end is reached. The animals will also be sacrificed when humane endpoints are reached.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

[] No > Descripe the method of killing that will be used and provide justifications for this choice.

[X] Yes

7.

DEC-advies

A. Algemene gegevens over de procedure

1. Aanvraagnummer: 2016-0045
2. Titel van het project: Nanoparticle imaging agents for cancer therapy
3. Titel van de NTS: Toepassing van nanodeeltjes voor kankertherapie.

4. Type aanvraag:
   ☒ nieuwe aanvraag projectvergunning
   ☐ wijziging van vergunning met nummer

5. Contactgegevens DEC:
   - naam DEC: RUDEC
   - telefoonnummer contactpersoon: [redacted] bereikbaar op maandag, dinsdag, en donderdag van 9:00 tot 15:00 uur
   - e-mailadres contactpersoon: [redacted]

6. Adviestraject (data dd-mm-jjjj):
   ☒ ontvangen door DEC: 22-12-2016
   ☐ aanvraag compleet
   ☒ in vergadering besproken: 10-01-2017 en 07-02-2017
   ☐ anderszins behandeld
   ☒ termijnonderbreking(en) van 17-01-2017 tot 24-01-2017 en van 13-02-2017 tot 17-02-2017
   ☐ besluit van CCD tot verlenging van de totale adviestermijn met maximaal 15 werkdagen
   ☒ aanpassing aanvraag: 24-01-2017 en 17-02-2017
   ☒ advies aan CCD: 02-03-2017

7. De inhoud van dit project is afgestemd met de IvD en deze heeft geen bezwaren tegen de uitvoering van het project binnen deze instelling.

8. Eventueel horen van aanvrager: n.v.t.

9. Correspondentie met de aanvrager
   - Datum: 17-01-2017
   - Datum antwoorden: 24-01-2017

   - Gestelde vragen en antwoorden:

     Project Proposal:
     -3.1: De nanodeeltjes worden al geproduceerd en gebruikt in een klinische trial. De commissie neemt aan dat hier proefdieronderzoek aan vooraf is gegaan. Wat is er al bekend over deze nanodeeltjes en hoe verhoudt het onderzoek in dit project zich tot de klinische trial? Wat is de meerwaarde van dit proefdieronderzoek?
     Antwoord: The nanoparticles are in a clinical trial where therapeutic cells (dendritic cells) are labelled with the nanoparticles ex vivo, and the labelled cells are injected directly into a lymph node. Thus, no free particles are injected the label is never injected intraveneously. This is excellent for a first trial. However, we need to move towards in vivo or in site labelling, where the particles are injected intraveneously and are then taken up by specific cells.
     Thus, experiments on biodistribution and also the ability of the particles to reach tumours need to be carried out, as described in this proposal.

Furthermore, here, we will also test the efficacy of these particles for tumour ablation, based on some promising preliminary in vitro data (figure in section 3.2). We have no in vivo data on this aspect of the particles, although it would be a very exciting and relevant clinical application. Such experiments also require more knowledge of the biodistribution.

-3.2: De haalbaarheid is nog niet voldoende toegelicht met referenties naar eigen werk.
Antwoord: The nanoparticles have been previously tested for the 19F MRI cell tracking,[1] thus it is know that there is a possibility to track the nanoparticles and labelled cells in vivo.

[redacted]

-3.4.1: Zijn er nog geen data over de biodistributie van deze nanodeeltjes? In experiment groep 2 is sprake van targeted nanodeeltjes. Zijn deze targeted voor tumoren of voor ontsteking? Waarom is het interessant om de biodistributie van nanoparticles te onderzoeken bij een lokale inflammatie? (zie ook de vraag over DAP1, A1)
Antwoord: We are interested in moving forward from ex vivo labelling of therapeutic cells, as in the current clinical trial, to in vivo labelling, where the label is specifically taken up by the target cells. This would allow us to skip laborious ex vivo cell culture steps, and would also greatly reduce cost and risk to the patient. For such experiments, we need data on the in vivo biodistribution after intravenous administration, and also when the nanoparticles are equipped with a targeting agent.
Finally, it is known that some tumours and regions of inflammation show an enhanced permeability and retention (EPR) effect,2 where the poorly formed vasculature is "leaky" and allows accumulation of certain agents at the tumour or site of inflammation. Since our nanoparticles are small enough for this, we would like to study the biodistribution of the nanoparticles under these conditions. If successful, this would provide a technique to image tumours or localise inflammation, both of which would be very useful in the clinic.

-3.4.2: Zijn de no go momenten goed gedefinieerd? Wat gebeurt er als de biodistributie ongunstig blijkt, of als er geen effect is op tumorgroei? De onderzoekers hebben hier de berekening van de aantallen dieren toegevoegd. De commissie verzoekt hen deze hier weg te laten en toe te voegen aan de desbetreffende DAPs. Het lijkt alsof de dieren voor de pilot tweemaal worden geteld.
Antwoord: In this project we would like to detect and show the distribution of our nanoparticles, in particular we would like to see if they will accumulate in the tumor. We are not interested in studying whether the presence of nanoparticles affect tumor growth. Regarding the animal number calculations, those have been removed from PP and are now described in DAP.

Description of Animal Procedures:
De uitwerking van het doel van het onderzoek in de DAPS lijkt wat onzorgvuldig. De onderzoekers worden verzocht dit beter uit te werken. Het is voor de commissie lastig te volgen waarom men deze experimenten wil doen met deze aantallen dieren.

*DAP1
-A1: Waarom willen de onderzoekers het effect van ontsteking onderzoeken? Dit is onvoldoende duidelijk uit het project proposal.
Antwoord: The answer to this question is presented in section 3.4.1 of PP.

-A: Klopt het dat er geen tumorgroei zal plaatsvinden bij de dieren in dit onderdeel van de aanvraag? Zo ja, dan graag alle verwijzingen naar tumorgroei verwijderen. Zo nee, dan graag tumorgroei toevoegen op de plaatsen waar dit vermeld dient te worden.
Antwoord: The references to tumor growth have been removed from this section.

-B: Waarom is het belangrijk om vrouwelijke dieren te gebruiken voor dit onderzoek? De commissie meent dat de gegeven reden (bij A1, wordt hier gevraagd) ook gebruikt kan worden om het gebruik van mannelijke dieren te rechtvaardigen.
Antwoord: We chose to use only female mice because (1) it is not possible to transfer cells between male and female mice due to the possibility of immune reactions, and (2) strong sex differences in immune reactions are known to occur.[3] Therefore, we chose to use only female mice.

-K: Het ongerief veroorzaakt door de plaatselijke ontsteking ontbreekt.
Antwoord: It's been corrected to mild.

-K: Het ongerief van langdurige narcose en het bijkomen daaruit is matig.
Antwoord: It's been corrected.

-K: Het cumulatief ongerief is nog niet beschreven. (geldt ook voor andere DAPs). Indien dit niet voor alle dieren hetzelfde is, graag aangeven welke mate van cumulatief ongerief voor welk deel van de dieren geldt.
Antwoord: The cumulative discomfort has been set to mild or moderate, depending on the DAP and assays.

*DAP2
-A1: Welke tumormodellen worden gebruikt, en hoe worden deze aangebracht? De beschrijving daarvan ontbreekt hier en bij A2.
Antwoord: We will use 3 tumor models: B16 melanoma, 9464D neuroblastoma and EL4 thymoma. The tumor cells will be injected subcutaneously.

-A2: Volgens het PP 3.4.2 wordt in dit onderdeel de biodistributie van de nanodeeltjes onderzocht, terwijl hier en bij B en H2 ook sprake is van cellen met nanodeeltjes die worden geïnjecteerd en gevolgd. Graag in overeenstemming brengen met elkaar.
Antwoord: Some mice will receive only particles, while other will receive cells labeled with particles. The free particles serve as important controls, so we can see if the cells are actively migrating.

-J:. Waarom is het voor dit onderzoek nodig de tumoren zo groot te laten worden dat 50 tot 100% van de dieren hierdoor een humaan eindpunt zal bereiken?

[redacted]

-K: het ongerief van tumorgroei is nog niet ingeschat.
Antwoord: It was corrected to moderate.

*DAP3

[redacted]

[redacted]

-K: Het ongerief van tumorgroei is matig wanneer de tumor max 2 cm^3 kan worden.
Antwoord: It's been corrected.

Niet-technische samenvatting:
-3.1 Dit project draait niet om de ontwikkeling van nanodeeltjes, maar om uitbreiding van de toepasbaarheid van bestaande nanodeeltjes.
-3.1 In dit onderzoek zal [redacted] worden. Dit komt onvoldoende tot uiting in de NTS.
-De onderzoekers worden verzocht te checken of de beantwoording van bovenstaande vragen over het Project Proposal en de DAPs ook leidt tot aanpassingen in de NTS.
Antwoord:
Above points have been addressed and the changes were applied in the NTS.

References

1. *Srinivas, M. et al. Customizable, multi-functional fluorocarbon nanoparticles for quantitative in vivo imaging using 19F MRI and optical imaging. Biomaterials 31, 7070–7 (2010).*
2. *Hill, T. K. & Mohs, A. M. Image-guided tumor surgery: Will there be a role for fluorescent nanoparticles? Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology (2015). doi:10.1002/wnan.1381*
3. *Kovats, S. Estrogen receptors regulate innate immune cells and signaling pathways. Cell. Immunol. 294, 63–69 (2015).*

- Datum: 13-02-2017
- Datum antwoorden: 17-02-2017

Project Proposal:
-3.4.2: De onderzoekers antwoorden dat zij niet geïnteresseerd zijn in het antitumor effect van de nanodeeltjes. Dat is volgens de DEC in tegenspraak met het feit dat in DAP3 onderzocht wordt hoe tumorablatie therapie effectiever kan worden gemaakt met behulp van de nanodeeltjes die een "imaging agent" en geneesmiddelen bevatten.
Antwoord: In this project we would like to detect and show the distribution of our nanoparticles, in particular we would like to see if they will accumulate in the tumor. The goal of the experiment 2 is to see whether the nanoparticles will reach the tumor site, while in experiment 3 we would like to study the influence of the presence of nanoparticles on tumor ablation.

Description of Animal Procedures:
*DAP1
-A: nog niet alle verwijzingen naar tumorgroei zijn verwijderd uit deze DAP (zie onderdeel I).
Antwoord: The references to tumor growth have been removed from the DAP1 (I).

-K: percentages dieren met licht of matig ongerief ontbreken (geldt voor alle DAPs).
Antwoord: The percentage of animals experiencing discomfort have been added in part K of all DAPs.

Niet-technische samenvatting:
-De onderzoekers worden verzocht te checken of de beantwoording van bovenstaande vragen over het Project Proposal en de DAPs ook leidt tot aanpassingen in de NTS.
Antwoord: The NTS has been checked and adjusted if needed.

- De antwoorden hebben geleid tot aanpassing van de aanvraag. De beantwoording van de vragen van de commissie is niet altijd even zorgvuldig. De commissie is echter van mening dat zij desondanks een ethische afweging kan maken.

10. Eventuele adviezen door experts (niet lid van de DEC): n.v.t.

B. Beoordeling (adviesvraag en behandeling)

1. Het project is vergunningplichtig (dierproeven in de zin der wet).
2. De aanvraag betreft een nieuwe aanvraag.
3. De DEC is competent om hierover te adviseren.
4. Er is geen betrokkenheid van DEC-leden bij deze projectaanvraag, waardoor onafhankelijkheid en onpartijdigheid zijn gewaarborgd.

C. Beoordeling (inhoud)

1. Deze aanvraag heeft een concrete doelstelling en kan getypeerd worden als een project. De opzet komt het best overeen met voorbeeld 4B uit de handreiking 'Invulling definitie project' van de CCD. Het is helder welke handelingen individuele dieren zullen ondergaan. Hierdoor is ook duidelijk welk ongerief individuele dieren zullen ondergaan. De aanvrager heeft, zowel binnen de doelstellingen en bijlagen dierproeven, als tussen de doelstellingen, beschreven op basis van welke criteria zij zal besluiten het project wel of niet te continueren. De DEC is er daardoor van overtuigd dat de aanvrager gedurende het project op zorgvuldige wijze besluiten zal nemen over de voortgang van het project en er niet onnodig dieren gebruikt zullen worden.

2. Voor zover de DEC weet is er geen "tegenstrijdige" wetgeving die het uitvoeren van de experimenten in de weg zou kunnen staan.

3. De in de aanvraag aangekruiste doelcategorieën zijn in overeenstemming met de hoofddoelstelling.

   Belangen en waarden

4. Het directe doel van het project is het ontwikkelen en verbeteren van nanodeeltjes voor het in

vivo imagen van cellulaire therapie en voor effectievere ablatietherapie (eventueel in combinatie met medicijnafgifte) in muismodellen voor kanker. Het uiteindelijke doel is het verbeteren van celtherapieën voor kanker (doordat de therapeutische cellen in beeld gebracht kunnen worden en de therapie kan worden bijgestuurd) en van tumor ablatie. In eerste instantie zal de biodistributie van (targeted) nanodeeltjes en DCs met nanodeeltjes worden onderzocht. Later worden ook effecten op tumor ablatie en de lokale afgifte van geneesmiddelen onderzocht. Celtherapie voor kanker wordt al in de kliniek toegepast, maar heeft nog niet altijd het verwachte effect. Onduidelijk is nog of de toegediende cellen daadwerkelijk aankomen op de goede locatie, of ze daar functioneren en hoe lang ze effectief blijven. Wanneer deze cellen in het lichaam in beeld gebracht kunnen worden (hetzij na in vitro labeling, hetzij na in vivo labeling) kan dit aanknopingspunten bieden voor het verbeteren van de therapie. Uit voorafgaande in vitro experimenten [redacted] er nanodeeltjes in het tumorweefsel aanwezig zijn. De aanvragers zullen de aanwezigheid van (targeted) nanodeeltjes in tumorweefsel vaststellen en onderzoeken of dit leidt tot effectievere vernietiging van tumorweefsel. De DEC is daarom van mening dat er binnen dit project een directe relatie is tussen het doel van deze projectaanvraag en het uiteindelijke doel.

5. De belangrijkste belanghebbenden in deze projectaanvraag zijn de proefdieren, de onderzoekers en de doelgroep/patiënten.
   Voor de proefdieren geldt dat hun welzijn en integriteit worden aangetast. De dieren zullen beperkt worden in hun natuurlijke gedrag en gedurende de proeven zullen de dieren stress ondervinden en pijn ondergaan. Uiteindelijk zullen ze in het kader van het onderzoek gedood worden. De dieren hebben er belang bij hiervan gevrijwaard te blijven.
   Voor de onderzoekers geldt dat het publiceren van belangrijke nieuwe wetenschappelijke inzichten resulteert in een goede wetenschappelijke reputatie, hetgeen vaak de sleutel is voor het verkrijgen van nieuwe onderzoeksmiddelen en mogelijkheden. Dit kan door de onderzoeker zelf van belang geacht worden, maar dient naar de mening van de DEC geen rol te spelen in de ethische afweging over de toelaatbaarheid van het gebruik van proefdieren. Het gaat uiteindelijk om de vraag of dit onderzoek belangrijke maatschappelijke en wetenschappelijke doelen dient (gezondheid, kennis).
   Voor patiënten is dit onderzoek van belang, omdat het kan bijdragen aan een verbetering van hun kwaliteit van leven. Gerichte behandeling op basis van mechanistisch inzicht kan bijdragen aan een betere diagnostiek en behandeling met minder bijwerkingen. Dit kan er toe leiden dat de patiënt weer gezond wordt, dan wel een betere kwaliteit van leven heeft. Kunnen beschikken over adequate behandelingen voor ernstige ziekten, zoals kanker, is van groot belang voor de samenleving.

6. De aanvrager geeft niet aan nadelige effecten op het milieu te verwachten. De DEC ziet geen aanleiding om aan te nemen dat die toch op kunnen treden.

Proefopzet en haalbaarheid

7. De kennis en kunde van de onderzoeksgroep en andere betrokkenen bij de dierproeven zijn voldoende gewaarborgd. De commissie is overtuigd van de kwaliteit van het werk van de aanvrager, zoals blijkt uit de in de aanvraag vermelde publicaties van deze onderzoeksgroep. De aanvragers beschikken over voldoende kennis en kunde om te kunnen voldoen aan alle zorgvuldigheidseisen omtrent het verrichten van dierproeven.

8. De doelstellingen van het project zijn realistisch en de voorgestelde experimentele opzet en uitkomstparameters sluiten hier logisch bij aan. Bovendien heeft deze groep veel ervaring in dit onderzoeksveld en met de voorgestelde dierproeven. De DEC is dan ook van mening dat het

project goed is opgezet, en dat deze strategie en experimentele aanpak kunnen leiden tot het behalen van de doelstelling binnen het kader van het project.

Welzijn dieren

9. Er is geen sprake van de volgende bijzonderheden op het gebied van categorieën van dieren, omstandigheden of behandeling van de dieren:
   ☐ Bedreigde diersoort(en) (10e lid 4)
   ☐ Niet-menselijke primaten (10e)
   ☐ Dieren in/uit het wild (10f)
   ☐ Niet gefokt voor dierproeven (11, bijlage I richtlijn)
   ☐ Zwerfdieren (10h)
   ☐ Hergebruik (1e lid 2)
   ☐ Locatie: buiten instelling vergunninghouder (10g)
   ☐ Geen toepassing verdoving/pijnbestrijding (13)
   ☐ Dodingsmethode niet volgens bijlage IV richtlijn (13c lid 3)

10. De huisvesting en verzorging van de dieren zijn conform de eisen in bijlage III van richtlijn 2010/63/EU.

11. Het cumulatieve ongerief als gevolg van de dierproeven is realistisch ingeschat en geclassificeerd. Het ongerief wordt hoofdzakelijk veroorzaakt door de herhaalde anesthesie die nodig is voor het imagen van de dieren en door de onderhuids groeiende tumoren waarvan de groei gedurende langere tijd wordt gevolgd. De commissie is van mening dat het cumulatief ongerief voor de dieren hierdoor kan oplopen tot maximaal matig ongerief voor de dieren die een tumor dragen en/of herhaaldelijk en langdurig onder anesthesie gaan. Voor de overige dieren is de ongeriefcategorie licht.

12. De integriteit van dieren wordt in beperkte mate aangetast door de onderhuids groeiende tumoren en doordat een aantal muizen geschoren wordt om de fluorescentie van cellen in het lichaam te kunnen meten. Beide hebben invloed op hun uiterlijk en gedrag.

13. De criteria voor humane eindpunten zijn voldoende specifiek gedefinieerd en toegesneden op het experiment. Het percentage dieren dat naar verwachting een humaan eindpunt zal bereiken is ingeschat op basis van eerdere resultaten met deze onderhuids groeiende tumoren. De commissie is het eens met deze inschatting en de gehanteerde humane eindpunten. Die zijn in overeenstemming met wat in het kankeronderzoek gebruikelijk is.

3V's

14. De aanvrager heeft voldoende aannemelijk heeft gemaakt dat er geen geschikte vervangingsalternatieven zijn. De onderdelen van het project die in vitro bestudeerd kunnen worden (bijvoorbeeld met fantomen) zijn al uitgevoerd. Voor het beantwoorden van de resterende onderzoeksvragen zijn dierproeven noodzakelijk. Er vindt een humane trial plaats met DCs die gelabeld zijn met nanopartikels, maar een aantal onderzoeksvragen (zoals de histologische bevestiging van de biodistributie van de cellen en de biodistributie van losse nanopartikels) kan alleen bij proefdieren worden onderzocht.

15. Het maximale aantal te gebruiken dieren is realistisch ingeschat en is proportioneel ten opzichte

van de gekozen onderzoeksopzet en de looptijd. De onderzoekers hanteren een goede strategie om ervoor te zorgen dat er met het kleinst mogelijke aantal dieren wordt gewerkt waarmee nog een wetenschappelijk betrouwbaar resultaat kan worden verkregen. Waar mogelijk worden experimenten gecombineerd om optimaal gebruik te kunnen maken van het beschikbare materiaal. Door de stapsgewijze aanpak waarin de resultaten uit eerdere proeven worden gebruikt voor het design van vervolgexperimenten wordt onnodig gebruik van proefdieren voorkomen.

16. Het project is in overeenstemming met de vereiste van de verfijning van dierproeven. Muizen zijn de minst complexe vertebraten met een immuunsysteem dat vergelijkbaar is met dat van mensen. De muizen worden met meerdere technieken onder anesthesie geimaged zonder dat ze tussentijds bijkomen, zodat ze niet elke keer last hebben van het ontwaken uit anesthesie. De effectiviteit van de therapie wordt onderzocht met onderhuidse tumormodellen die minder ongerief voor de dieren veroorzaken dan orthotoop groeiende tumoren. De DEC is ervan overtuigd dat de beschreven proefopzet de meest verfijnde is en dat de dierproeven zo humaan mogelijk worden uitgevoerd.

17. Het betreft geen wettelijk vereist onderzoek.

    Dieren in voorraad gedood en bestemming dieren na afloop proef

18. De aanvrager zal in het project alleen vrouwelijke dieren gebruiken. Hiervoor is de volgende onderbouwing gegeven: Er worden alleen vrouwelijke muizen gebruikt omdat er grote verschillen in immuunreacties kunnen optreden tussen mannelijke en vrouwelijke dieren. Donormuizen voor de isolatie van DCs dienen van hetzelfde geslacht te zijn als de ontvangende muizen. De DEC is van mening dat de aanvrager in voldoende mate wetenschappelijk heeft onderbouwd dat het om de doelstellingen met zo min mogelijk dieren te bereiken noodzakelijk is om de proeven met alleen vrouwelijke dieren uit te voeren.

19. De dieren zullen in het kader van het project gedood worden. Dit is noodzakelijk om verschillende weefsels te kunnen onderzoeken voor het beantwoorden van bepaalde onderzoeksvragen. De gebruikte dodingsmethode staat vermeld in bijlage IV van richtlijn 2010/63/EU.

20. Er worden in deze projectaanvraag geen landbouwhuisdieren, honden, katten of niet-humane primaten gebruikt (en dus ook niet gedood om niet-wetenschappelijke redenen).

    NTS

21. De niet-technische samenvatting is een evenwichtige weergave van het project en begrijpelijk geformuleerd.

D. Ethische afweging

1. Rechtvaardigt het belang van de doelstelling van het project het ongerief dat de dieren wordt aangedaan, en is aan alle zorgvuldigheidseisen (3V's) voldaan?

2. Er vindt een lichte of matige aantasting van welzijn en integriteit van de proefdieren plaats (beschreven in C9 tot C20). De doelstellingen kunnen niet zonder dieren behaald worden. De onderzoekers doen al het mogelijke om het lijden van de dieren en het aantal dieren te

beperken.
Voor patiënten is dit onderzoek van belang, omdat het kan bijdragen aan een verbetering van hun gezondheid en kwaliteit van leven. De DEC kent daar veel gewicht aan toe om de volgende redenen. Celtherapie voor kanker is een veelbelovende vorm van immuuntherapie. Een aantal kankerpatiënten wordt al behandeld met deze therapie, maar de behandeling is niet altijd voldoende effectief. Tumor ablatie is een weinig invasieve behandeling waarmee tumoren (gedeeltelijk) vernietigd kunnen worden, maar die in de praktijk nog weinig ingezet wordt vanwege een beperkte effectiviteit . De resultaten van dit project zullen bijdragen aan de ontwikkeling van effectievere celtherapie en effectievere tumor ablatie. Het is aannemelijk dat de doelstellingen op termijn behaald zullen worden. De commissie acht het ontwikkelen van effectievere celtherapie en tumor ablatie van substantieel belang.

3. De DEC is overtuigd van het belang van de doelstellingen: ontwikkelen en verbeteren van nanodeeltjes voor het *in vivo* imagen van cellulaire therapie en voor effectievere ablatietherapie (eventueel in combinatie met medicijnafgifte) in muismodellen voor kanker. Het uiteindelijke doel daarvan is het verbeteren van celtherapieën voor kanker (doordat de therapeutische cellen in beeld gebracht kunnen worden) en van tumor ablatie. De DEC is van mening dat de belangen van de patiënten voldoende zwaar wegen om het schaden van de belangen van de proefdieren (om gevrijwaard te blijven van een aantasting van hun welzijn en integriteit) te rechtvaardigen. De commissie is overtuigd van de kwaliteit van het werk van de aanvrager. De DEC is van mening dat het project goed is opgezet, en dat de gekozen strategie en experimentele aanpak kunnen leiden tot het behalen van de doelstelling binnen het kader van het project. De aanvrager heeft voldoende aannemelijk gemaakt dat er geen geschikte vervangingsalternatieven zijn, dat het doel niet met minder dieren behaald kan worden, dat de gebruikte aanpak de meest verfijnde is en dat zij zal kunnen voorkomen dat mens, dier en het milieu onbedoelde negatieve effecten ondervinden als gevolg van de dierproeven.
De DEC is van oordeel dat het hier boven geschetste belang de onvermijdelijke nadelige gevolgen van dit onderzoek voor de dieren, in de vorm van angst, pijn of stress, rechtvaardigt. Aan de eis dat het belang van de experimenten op dient te wegen tegen het ongerief dat de dieren wordt berokkend, is voldaan.

E. Advies

1. Advies aan de CCD
   ☒ De DEC adviseert de vergunning te verlenen

   ☐ De DEC adviseert de vergunning te verlenen onder de volgende voorwaarden
   - Op grond van het wettelijk vereiste dient de projectleider bij beëindiging van het project een beoordeling achteraf aan te leveren die is afgestemd met de IvD.
   - Voor de uitvoering van dit project is tevens ministeriële ontheffing vereist
   - Overige door de DEC aan de uitvoering verbonden voorwaarden, te weten...

   ☐ De DEC adviseert de vergunning niet te verlenen vanwege:
   - De vaststelling dat het project niet vergunningplichtig is om de volgende redenen:…
   - De volgende doorslaggevende ethische bezwaren:…
   - De volgende tekortkomingen in de aanvraag:…

2. Het uitgebrachte advies is gebaseerd op consensus.

3. Er zijn geen knelpunten of dilemma's geconstateerd – zowel binnen als buiten de context van het project - die de verantwoordelijkheid en competentie van de DEC overstijgen.

8.

Centrale Commissie Dierproeven

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Onze referentie
Aanvraagnummer
AVD103002017897

Bijlagen
2

Datum 2 maart 2017
Betreft Ontvangstbevestiging aanvraag projectvergunning Dierproeven

Geachte [redacted]

Wij hebben uw aanvraag voor een projectvergunning dierproeven ontvangen op 2 maart 2017. Het gaat om uw project "Nanoparticle imaging agents for cancer therapy". Het aanvraagnummer dat wij aan deze aanvraag hebben toegekend is AVD103002017897. Gebruik dit nummer wanneer u contact met de CCD opneemt.

Wacht met de uitvoering van uw project
Als wij nog informatie van u nodig hebben dan ontvangt u daarover bericht. Uw aanvraag is in ieder geval niet compleet als de leges niet zijn bijgeschreven op de rekening van de CCD. U ontvangt binnen veertig werkdagen een beslissing op uw aanvraag. Als wij nog informatie van u nodig hebben, wordt deze termijn opgeschort. In geval van een complexe aanvraag kan deze termijn met maximaal vijftien werkdagen verlengd worden. U krijgt bericht als de beslisperiode van uw aanvraag vanwege complexiteit wordt verlengd. Als u goedkeuring krijgt op uw aanvraag, kunt u daarna beginnen met het project.

Factuur
Bijgaand treft u de factuur aan voor de betaling van de leges. Wij verzoeken u de leges zo spoedig mogelijk te voldoen, zodat we uw aanvraag in behandeling kunnen nemen. Is uw betaling niet binnen dertig dagen ontvangen, dan kan uw aanvraag buiten behandeling worden gesteld. Dit betekent dat uw aanvraag niet beoordeeld wordt en u uw project niet mag starten.

Meer informatie
Heeft u vragen, kijk dan op www.centralecommissiedierproeven.nl. Of neem telefonisch contact met ons op: 0900 28 000 28 (10 ct/minuut).

Datum:
2 maart 2017
Aanvraagnummer:
AVD103002017897

Met vriendelijke groet,

Centrale Commissie Dierproeven

Deze brief is automatisch aangemaakt en daarom niet ondertekend.

Bijlagen:
- Gegevens aanvraagformulier
- Factuur

Datum:
2 maart 2017
Aanvraagnummer:
AVD103002017897

Gegevens aanvrager

Uw gegevens

Deelnemersnummer NVWA:	10300
Naam instelling of organisatie:	Stichting Katholieke Universiteit Nijmegen
Naam portefeuillehouder of diens gemachtigde:	[redacted]
KvK-nummer:	41055629
Straat en huisnummer:	Geert Grooteplein 29
Postbus:	9101
Postcode en plaats:	6500 HB NIJMEGEN
IBAN:	NL90ABNA0231209983
Tenaamstelling van het rekeningnummer:	UMC St Radboud

Gegevens verantwoordelijke onderzoeker

Naam:	[redacted]
Functie:	[redacted]
Afdeling:	[redacted]
Telefoonnummer:	[redacted]
E-mailadres:	[redacted]

Datum:
2 maart 2017
Aanvraagnummer:
AVD103002017897

Gegevens plaatsvervangende verantwoordelijke onderzoeker

Naam:	[redacted]
Functie:	[redacted]
Afdeling:	[redacted]
Telefoonnummer:	[redacted]
E-mailadres:	[redacted]

Gegevens verantwoordelijke uitvoering proces

Naam:	[redacted]
Functie:	Instantievoor Dierenwelzijn
Afdeling:	[redacted]
Telefoonnummer:	[redacted]
E-mailadres:	[redacted]

Over uw aanvraag

Wat voor aanvraag doet u?	[x] Nieuwe aanvraag [ ] Wijziging op een (verleende) vergunning die negatieve gevolgen kan hebben voor het dierenwelzijn [ ] Melding op (verleende) vergunning die geen negatieve gevolgen kan hebben voor het dierenwelzijn

Over uw project

Geplande startdatum:	2 april 2017
Geplande einddatum:	1 april 2022
Titel project:	Nanoparticle imaging agents for cancer therapy
Titel niet-technische samenvatting:	Toepassing van nanodeeltjes voor kankertherapie
Naam DEC:	RU DEC
Postadres DEC:	Postbus 9101, 6500 HB Nijmegen [redacted]
E-mailadres DEC:	[redacted]

Betaalgegevens

De leges bedragen:	€ 1.541,-
De leges voldoet u:	na ontvangst van de factuur

Checklist bijlagen

Verplichte bijlagen:	[x] Projectvoorstel [x] Beschrijving Dierproeven [x] Niet-technische samenvatting
Overige bijlagen:	[x] DEC-advies

Ondertekening

Naam:	[redacted]
Functie:	Instantie voor dierenwelzijn
Plaats:	Nijmegen
Datum:	2 maart 2017

Centrale Commissie Dierproeven

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Onze referentie
Aanvraagnummer
AVD103002017897
Bijlagen
2

Datum 2 maart 2017
Betreft Factuur aanvraag projectvergunning Dierproeven

Factuur
Factuurdatum: 2 maart 2017
Vervaldatum: 1 april 2017
Factuurnummer: 170897
Ordernummer: Kostenplaats en kostensoort: [redacted]
projectnummer: 2016-0045 Verantwoordelijk onderzoeker: [redacted]

Omschrijving	**Bedrag**
Betaling leges projectvergunning dierproeven Betreft aanvraag AVD103002017897	€ 1.541,00

Wij verzoeken u het totaalbedrag vóór de gestelde vervaldatum over te maken op rekening NL29INGB 070.500.1512 onder vermelding van het factuurnummer en aanvraagnummer, ten name van Centrale Commissie Dierproeven, Postbus 93144, 2509 AC te 's Gravenhage.

9.

Van: [redacted]
Verzonden: vrijdag 31 maart 2017 11:06
Aan: [redacted]
CC: [redacted]
Onderwerp: AVD1030002017897: Aanvullende informatie

Geachte Instantie voor Dierenwelzijn,

Op 2 maart 2017 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project 'Nanoparticle imaging agents for cancer therapy' met aanvraagnummer AVD103002017897. Wij hebben nog een vraag over uw aanvraag. In deze e-mail leest u wat wij nog nodig hebben en wanneer u een beslissing kunt verwachten.

- U geeft aan alleen vrouwelijke dieren te gebruiken. Hoewel we er begrip voor hebben dat u het geslacht van donoren en acceptordieren wilt matchen, is de gebruikte argumentatie voor het gebruiken
van alleen vrouwelijke dieren in het project eerder als niet voldoende beschouwd. Kunt u aangeven waarom het niet mogelijk is om, met behoud van de mogelijkheid donoren- en acceptordieren te matchen, toch beide geslachten te gebruiken. U wordt verzocht aan te geven of er voor de te gebruiken modellen en uitleesparameters bekend is of er geslacht specifieke verschillen zijn.

Opsturen binnen veertien dagen
U heeft veertien dagen de tijd om de ontbrekende informatie aan te leveren. De CCD zou uw aanvraag echter graag in de eerstvolgende CCD vergadering bespreken. Wij vragen u daarom de informatie uiterlijk donderdag 6 april 2017 aan te leveren. U kunt dit aanleveren via NetFTP.

Wanneer een beslissing
De behandeling van uw aanvraag wordt opgeschort tot het moment dat wij de aanvullende informatie hebben ontvangen. Als u goedkeuring krijgt op uw aanvraag, kunt u daarna beginnen met het project.

Meer informatie
Heeft u vragen, kijk dan op www.centralecommissiedierproeven.nl. Of neem telefonisch contact met ons op: 0900 28 000 28 (10 ct/minuut).

Met vriendelijke groet,

[redacted]

Centrale Commissie Dierproeven
www.centralecommissiedierproeven.nl

Postbus 20401 | 2500 EK | Den Haag .....
... T: 0900 2800028
E: info@zbo-ccd.nl (Let op: nieuw e-mail adres)

AVD103002017897-(2016-0045-05-04-2017)

Our response is :

We thank the CCD for looking at our proposal and raising relevant questions. We were asked about why we chose to use only female mice in our experiments.

The main reason that we opted for only female mice is that we are studying inflammation and cancer, and these conditions are known to exhibit sexual dimorphisms, both in humans and in mice [see references 1-6 below]. This is a problem for us, as we are studying inflammation and related processes in our experiments. Importantly, our previous data (from the last 10 years almost) is entirely in female mice, and we are building on that work in this project.

Thus, including male mice would increase variability in our data, as well as increase complexity in planning the experiments, in terms of matching donors and recipients. This would lead to an overall increase in the numbers of mice required, and therefore we opted to use only female mice.

References:
1. Systemic Inflammation and sexual dimorphism: more than meets the eye. Crit Care Med 2007.
2. Sexual dimorphism in cancer. Nat Rev. Cancer 2016.
3. Sexual dimorphisms in leukocyte trafficking in a mouse peritonitis model. J. Leukol Biol. 2015.
4. Secual dimorphisms of adrenal steroids, sec hormones and immunological biomarkers and possible risk factors for developing rheumatoid arthrritis. Int. J. Endocrinol. 2015.
5. Sexual dimorphisms in the immune system of catechol-O-methyltransferase knockout mice. Immunology 2012.
6. Discovery of sexual dimorphisms in metabolic and genetic biomarkers. PLoS Genet 2011.

[redacted]

Radboud University Medical Center (RadboudUMC)

[redacted]

12.

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

Stichting Katholieke Universiteit Nijmegen
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Onze referentie
Aanvraagnummer
AVD103002017897
Bijlagen
1

Datum 20 april 2017
Betreft Beslissing aanvraag projectvergunning Dierproeven

Geachte [redacted]

Op 2 maart 2017 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "Nanoparticle imaging agents for cancer therapy" met aanvraagnummer AVD103002017897. Wij hebben uw aanvraag beoordeeld.

Op 05 april 2017 heeft u uw aanvraag gewijzigd. Op 31 maart 2017 hebben wij u gevraagd het gebruik van alleen vrouwelijke dieren te onderbouwen. Wij kunnen ons vinden in uw toelichting.

Beslissing
Wij keuren uw aanvraag goed op grond van artikel 10a van de Wet op de Dierproeven (hierna: de wet). Hierbij gelden de voorwaarden zoals genoemd in de vergunning.
Met het oog op artikel 10a, lid 1, zijn er algemene voorwaarden gesteld.

U kunt met uw project "Nanoparticle imaging agents for cancer therapy" starten. De vergunning wordt afgegeven van 20 april 2017 tot en met 1 april 2022.

Overige wettelijke bepalingen blijven van kracht.

Procedure
Bij uw aanvraag heeft u een advies van de Dierexperimentencommissie RU DEC gevoegd. Dit advies is opgesteld op 2 maart 2017. Bij de beoordeling van uw aanvraag is dit advies betrokken overeenkomstig artikel 10a, lid 3 van de wet. Wij kunnen ons vinden in de inhoud van het advies van de Dierexperimentencommissie. Dit advies nemen wij over; inclusief de daaraan ten grondslag liggende motivering.

Er worden aanvullende algemene voorwaarden gesteld.
Het DEC-advies en de in de bijlage opgenomen beschrijving van de artikelen van de wet- en regelgeving zijn de grondslag van dit besluit.

Bezwaar
Als u het niet eens bent met deze beslissing, kunt u binnen zes weken na verzending van deze brief schriftelijk een bezwaarschrift indienen.
Een bezwaarschrift kunt u sturen naar Centrale Commissie Dierproeven, afdeling Juridische Zaken, postbus 20401, 2500 EK Den Haag.

Bij het indienen van een bezwaarschrift vragen we u in ieder geval de datum van de beslissing waartegen u bezwaar maakt en het aanvraagnummer te vermelden. U vindt deze nummers in de rechter kantlijn in deze brief.

Bezwaar schorst niet de werking van het besluit waar u het niet mee eens bent. Dat betekent dat dat besluit wel in werking treedt en geldig is. U kunt tijdens deze procedure een voorlopige voorziening vragen bij de Voorzieningenrechter van de rechtbank in de woonplaats van de aanvrager. U moet dan wel kunnen aantonen dat er sprake is van een spoedeisend belang.

Voor de behandeling van een voorlopige voorziening is griffierecht verschuldigd. Op http://www.rechtspraak.nl/Organisatie/Rechtbanken/Pages/default.aspx kunt u zien onder welke rechtbank de vestigingsplaats van de aanvrager valt.

Meer informatie
Heeft u vragen, kijk dan op www.centralecommissiedierproeven.nl. Of neem telefonisch contact met ons op: 0900 28 000 28 (10 ct/minuut).

Centrale Commissie Dierproeven
namens deze:

[redacted]

Bijlagen:
- Vergunning
  Hiervan deel uitmakend:
  - DEC-advies
  - Weergave wet- en regelgeving

Centrale Commissie Dierproeven

Projectvergunning

gelet op artikel 10a van de Wet op de Dierproeven

Verleent de Centrale Commissie Dierproeven aan

Naam:	Stichting Katholieke Universiteit Nijmegen
Adres:	Postbus 9101
Postcode en plaats:	6500 HB NIJMEGEN
Deelnemersnummer:	10300

deze projectvergunning voor het tijdvak 20 april 2017 tot en met 1 april 2022, voor het project "Nanoparticle imaging agents for cancer therapy" met aanvraagnummer AVD103002017897, volgens advies van Dierexperimentencommissie RU DEC. Hierbij is afgeweken van het DEC-advies. Er worden aanvullende voorwaarde(n) gesteld. Zie samenvatting
De functie van de verantwoordelijk onderzoeker is [redacted] Voor de uitvoering van het project is Instantievoor Dierenwelzijn verantwoordelijk.
De aanvraag omvat de volgende bescheiden:

1 een aanvraagformulier projectvergunning dierproeven, ontvangen op 2 maart 2017
2 de bij het aanvraagformulier behorende bijlagen:
a Projectvoorstel, zoals ontvangen per digitale indiening op 2 maart 2017;
b Niet-technische Samenvatting van het project, zoals ontvangen per digitale indiening op 2 maart 2017;
c Advies van dierexperimentencommissie d.d. 2 maart 2017, ontvangen op 2 maart 2017.
d De aanvullingen op uw aanvraag, ontvangen op 05 april 2017

Aanvraagnummer:
AVD103002017897

Naam proef	Diersoort/ Stam	Aantal dieren	Ernst	Opmerkingen
3.4.4.1. In vivo tracking of cells pre-labelled with polymeric nanoparticles containing an imaging agent				
	Muizen (Mus musculus) /	150	20% Matig 80% Licht	
3.4.4.2. Analyzing the fate of nanoparticles after in vivo administration. The nanoparticles will be non-targeted or targeted to various cells.				
	Muizen (Mus musculus) /	125	50% Matig 50% Licht	
[redacted]				
	Muizen (Mus musculus) /	120	70% Matig 30% Licht	

Voorwaarden

Op grond van artikel 10a1 lid 2 van de Wet op de dierproeven zijn aan een projectvergunning voorwaarden te stellen

De vergunning wordt verleend onder de voorwaarde dat go/no go momenten worden afgestemd met de IvD.

In artikel 10, lid 1 sub a van de wet, wordt bepaald dat het verboden is een dierproef te verrichten voor een doel dat, naar de algemeen kenbare, onder deskundigen heersende opvatting, ook kan worden bereikt anders dan door middel van een dierproef, of door middel van een dierproef waarbij minder dieren kunnen

worden gebruikt of minder ongerief wordt berokkend dan bij de in het geding zijnde proef het geval is. Nieuwe onderzoeken naar alternatieven kunnen tot gevolg hebben dat inzichten en/of omstandigheden van het aangevraagde project in de vergunningsperiode wijzigen, gedurende de looptijd van deze vergunning. Indien bovenstaande zich voordoet dient aanvrager dit in afstemming met de IvD te melden bij de CCD. De CCD kan in een dergelijke situatie aan de vergunning nieuwe voorwaarden verbinden en gestelde voorwaarde wijzigen of intrekken.

Centrale Commissie Dierproeven

Aanvraagnummer:
AVD103002017897

Weergave wet- en regelgeving

Dit project en wijzigingen
Volgens artikel 10c van de Wet op de Dierproeven (hierna de wet) is het verboden om andere dierproeven uit te voeren dan waar de vergunning voor is verleend. De dierproeven mogen slechts worden verricht in het kader van een project, volgens artikel 10g. Uit artikel 10b volgt dat de dierproeven zijn ingedeeld in de categorieën terminaal, licht, matig of ernstig. Als er wijzigingen in een dierproef plaatsvinden, moeten deze gemeld worden aan de Centrale Commissie Dierproeven. Hebben de wijzigingen negatieve gevolgen voor het dierenwelzijn, dan moet volgens artikel 10a5 de wijziging eerst voorgelegd worden en mag deze pas doorgevoerd worden na goedkeuren door de Centrale Commissie Dierproeven.
Artikel 10b schrijft voor dat het verboden is een dierproef te verrichten die leidt tot ernstige mate van pijn, lijden, angst of blijvende schade die waarschijnlijk langdurig zal zijn en niet kan worden verzacht, tenzij hiervoor door de Minister een ontheffing is verleend.

Verzorging
De fokker, leverancier en gebruiker moeten volgens artikel 13f van de wet over voldoende personeel beschikken en ervoor zorgen dat de dieren behoorlijk worden verzorgd, behandeld en gehuisvest. Er moeten ook personen zijn die toezicht houden op het welzijn en de verzorging van de dieren in de inrichting, personeel dat met de dieren omgaat moet toegang hebben tot informatie over de in de inrichting gehuisveste soorten en personeel moet voldoende geschoold en bekwaam zijn. Ook moeten er personen zijn die een eind kunnen maken aan onnodige pijn, lijden, angst of blijvende schade die tijdens een dierproef bij een dier wordt veroorzaakt. Daarnaast zijn er personen die zorgen dat een project volgens deze vergunning wordt uitgevoerd en als dat niet mogelijk is zorgen dat er passende maatregelen worden getroffen.
In artikel 9 staat dat de persoon die het project en de dierproef opzet deskundig en bekwaam moet zijn. In artikel 8 van het Dierproevenbesluit 2014 staat dat personen die dierproeven verrichten, de dieren verzorgen of de dieren doden, hiervoor een opleiding moeten hebben afgerond.
Voordat een dierproef die onderdeel uitmaakt van dit project start, moet volgens artikel 10a3 van de wet de uitvoering afgestemd worden met de instantie voor dierenwelzijn.

Pijnbestrijding en verdoving
In artikel 13 van de wet staat dat een dierproef onder algehele of plaatselijke verdoving wordt uitgevoerd tenzij dat niet mogelijk is, dan wel bij het verrichten van een dierproef worden pijnstillers toegediend of andere goede methoden gebruikt die de pijn, het lijden, de angst of de blijvende schade bij het dier tot een minimum beperken. Een dierproef die bij het dier gepaard gaat met zwaar letsel dat hevige pijn kan veroorzaken, wordt niet zonder verdoving uitgevoerd. Hierbij wordt afgewogen of het toedienen van verdoving voor het dier traumatischer is dan de dierproef zelf en het toedienen van verdoving onverenigbaar is met het doel van de dierproef. Bij een dier wordt geen stof toegediend waardoor het dier niet meer of slechts in verminderde mate in staat is pijn te tonen, wanneer het dier niet tegelijkertijd voldoende verdoving of pijnstilling krijgt toegediend, tenzij wetenschappelijk gemotiveerd. Dieren die pijn

kunnen lijden als de verdoving eenmaal is uitgewerkt, moeten preventief en postoperatief behandeld worden met pijnstillers of andere geschikte pijnbestrijdingsmethoden, mits die verenigbaar zijn met het doel van de dierproef. Zodra het doel van de dierproef is bereikt, moeten passende maatregelen worden genomen om het lijden van het dier tot een minimum te beperken.

Einde van een dierproef
Artikel 13a van de wet bepaalt dat een dierproef is afgelopen wanneer voor die dierproef geen verdere waarnemingen hoeven te worden verricht of, voor wat betreft nieuwe genetisch gemodificeerde dierenlijnen, wanneer bij de nakomelingen niet evenveel of meer, pijn, lijden, angst, of blijvende schade wordt waargenomen of verwacht dan bij het inbrengen van een naald. Er wordt dan door een dierenarts of een andere ter zake deskundige beslist of het dier in leven zal worden gehouden. Een dier wordt gedood als aannemelijk is dat het een matige of ernstige vorm van pijn, lijden, angst of blijven schade zal blijven ondervinden. Als een dier in leven wordt gehouden, krijgt het de verzorging en huisvesting die past bij zijn gezondheidstoestand.
Volgens artikel 13b moet de dood als eindpunt van een dierproef zoveel mogelijk worden vermeden en vervangen door in een vroege fase vaststelbare, humane eindpunten. Als de dood als eindpunt onvermijdelijk is, moeten er zo weinig mogelijk dieren sterven en het lijden zo veel mogelijk beperkt blijven.
Uit artikel 13d volgt dat het doden van dieren door een deskundig persoon moet worden gedaan, wat zo min mogelijk pijn, lijden en angst met zich meebrengt. De methode om te doden is vastgesteld in de Europese richtlijn artikel 6.
In artikel 13c is vastgesteld dat proefdieren geadopteerd kunnen worden, teruggeplaatst in hun habitat of in een geschikt dierhouderijsysteem, als de gezondheidstoestand van het dier het toelaat, er geen gevaar is voor volksgezondheid, diergezondheid of milieu en er passende maatregelen zijn genomen om het welzijn van het dier te waarborgen.
De Minister heeft vrijstelling ontheffing verleend volgens artikel 13c, die de afwijkende methode van doden op basis van wetenschappelijke motivering ten minste even humaan acht als de in de richtlijn opgenomen passende methoden.