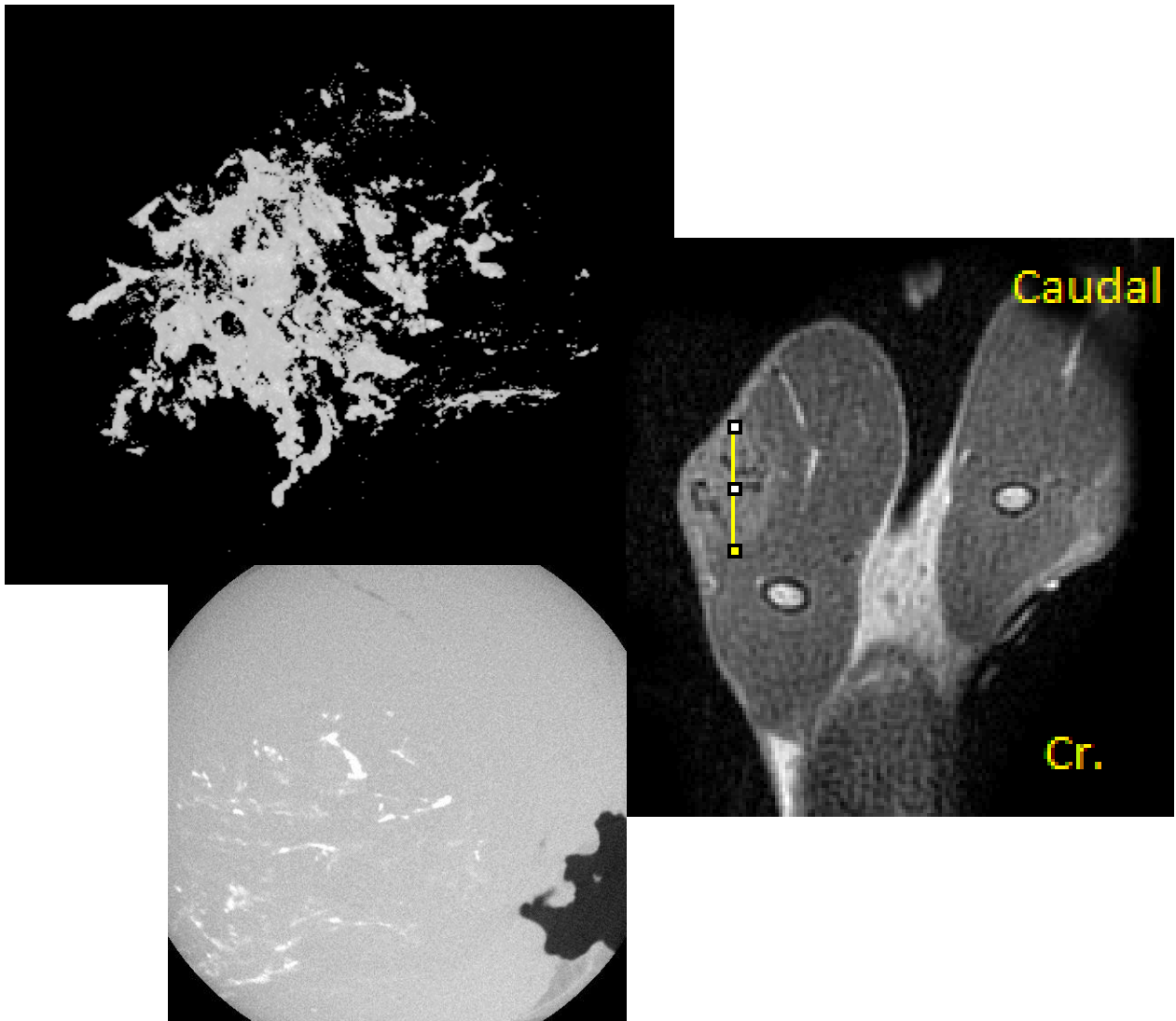# The biodistribution of holmium loaded microspheres in intratumoural injections

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Index

General introduction

The University Clinic for Companion Animals and the University Medical Centre in Utrecht have been performing experimental studies with radioactive microspheres to treat (mostly) inoperable tumours. The technique, called microbrachytherapy, can be applied to different types of tumour such as squamous cell carcinoma and fibrosarcoma. The radioactive microspheres are polylactic acids which contain the radioactive isotope holmium-166 (^{166}Ho). These are called ^{166}Ho-PLLA-MS (abbreviation Ho-MS). The treatment of the tumour with these particles may lead to the decrease or death of tumour tissue. This will improve the quality of life and/or may increase the lifespan of the patients.(1,5)

The distribution and effectiveness of holmium-166 loaded microspheres in the tumour tissue is important to investigate. The treated tumours may vary in type, size and consistence and thus the treatment may differ in outcome.(2,6) In a histological analysis of treated tumours with holmium-166, the conclusion was drawn that a qualitative analysis can be done with two-dimensional microscope images, but this was insufficient for the quantitative analysis (2). The use of different image modalities like MRI and (micro)CT can provide a better overview of different structures inside the object of investigation and the microspheres.

In this study, the MRI is used to give an anatomical overview of the tumour tissue and (micro)CT for the imaging of the holmium-166 loaded microspheres on a very detailed level. Combining these different techniques in a spatial manner could provide a complete 3D map of the tumour. However, the spatial alignment of these techniques proves to be very difficult due to the following:

I.   The orientation of the tissue is quickly lost during fixation because of morphological changes and excision of the tissue. This results in a change of tumour appearance. In vivo MRI images show an in situ tumour. The CT and ex vivo MRI show the fixated tumour for imaging. It is therefore difficult to compare these images. (3,4)

II. The MRI and CT images consist of several slices of certain thickness in a predefined angle. Comparing histological coupes and these slices demands slicing these in exactly the same direction and angle (termed the region of interest, ROI). This method has not been adequately described before in studies. (3,4)

III. Landmarks (anatomically or physically applied) are necessary to keep record of the original orientation in vivo. However, these landmarks are used to registrate the complete organ and not solely the tumour. There is no model marker designed for these kind of studies. (3,4)

In this study, the feasibility of using different image modalities to compare and illustrate the tumour and the biodistribution of holmium microspheres are evaluated.

Therefore, the research question is:

Which image modalities help map the biodistribution of holmium loaded microspheres in intratumoural injections?

In this study, the methods of using markers are explored before using imaging to answer the research question.

Substudy 1: Markers

The experiments in substudy 1 were carried out to find a method of marking tumour tissue so the orientation would not be lost (after fixation) in MRI, CT and histology. The tissues were put into an external fixation substrate, such as agar, paraffin and Falcon tubes. In the conducted literature search, no markers were mentioned which were used in this manner. The chosen markers which would be fixated externally into the tissue are needles, air columns, contrast agent and catheters. The needles and contrast were reasoned to create contrast in the surrounding tissue and become lighter on the CT images. The air columns and catheters would become darker on the CT images because of the air and therefore become visible. The internal markers used were sutures, because these would probably also provide some contrast and are frequently used as markers in larger tissues and organs for orientation.

Experiment 1   Paraffin

Goal
The goal of this experiment is to understand the method of fixation (to hold in place) of a piece of meat in paraffin. Paraffin is a common product used in histology to fixate tissues and is a hard substance. In this block of paraffin containing the meat, markers will be placed. These markers and the contrast solution will be tested under a CT to investigate their effectiveness in being shown on the scans. Also, the ease of cutting through paraffin will be tested.

Materials
o Paraffin	Pathology
o Template for paraffin icecubeholder	Supermarket
o 10 pieces of streaky bacon	Supermarket
o 3 Falcon bottles	UMC
o 4% formalin	UMC
o Needles with different gauges (16,20,22,23,26 and 27G)	UKG
o Really small catheters (16, 20 and 22G)	UMC
o Suture material Ethilon 2-0	UKG
o Contrast fluids	UMC

Methods
All tissues will remain in the formalin for preservation. The streaky bacon was cut into pieces of about 1 cm^3. Two Falcon tubes were filled with 40 ml formalin 4% and four pieces of tissue were inserted into each tube (image 1). In one Falcon tube, 22ml formalin 4% was mixed with 1.98 grams I_2 and 4.1 grams KI, this would result in a solution of 10% I_2KI in 4% formalin. Two tissue pieces were placed in this tube. All three tubes were kept overnight. From the preliminary tests, it became evident that the use of the icecubeholder as a template was very effective. Shown in images 2,3 and 4 are the different ways of pouring paraffin and placing the tissues. In template 1, the bacon was put in the template and filled with paraffin. In template 2, the paraffin was first poured into the template, solidified, then the bacon was placed and the paraffin was poured completely into the template. In template 3, the template was filled with paraffin and the tissue was pushed into the warm paraffin. The templates were cooled off in a bowl filled with water with a temperature around 15 degrees Celsius. The first method described had the disadvantage that it did not cover the tissue with paraffin on the bottom side and is therefore not used for the further experiments. The last method described had the disadvantage that the tissue has to be pushed through the hardening paraffin and therefore may cause (pressure) damage to the tissue. Because of the disadvantages of the afore mentioned procedures, the method used in template 2 was used in further experiments.

Image 1: The workplace with the three Falcon tubes with streaky bacon.

Test 1: Cut

On block #4 and #7 some cutting experiments were performed.
Block #4 was cut in half and block #7 was cut into three pieces with a microtome. It was shown that cutting parts closer to each other than 0.5 cm would break the paraffin and thus endanger the tissues. If done carefully, the slices are practically vertical and the tissue surface is visible and held in place. The manner of pouring the paraffin or how to place the tissue should be investigated further, because there are some artefacts. (some air/formalin between the different paraffin layers)

Images 2 and 3: The different methods of pouring paraffin into the template
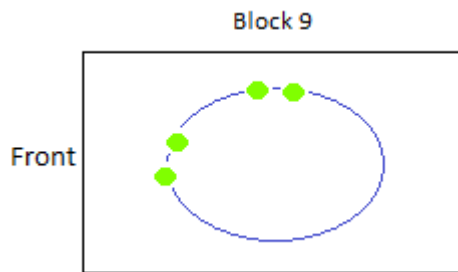

Block #4:

Block #7:

Test 2: Markers

In separate paraffin blocks, the different markers will be tested in this manner.

Block #9	Block #5	Block #3 and #5	Block #6	Block #3
Paraffin	Paraffin	Paraffin	Paraffin	Paraffin
Complete coverage	Complete coverage	Complete coverage	Complete coverage	Complete coverage
Sutures ethilon 2-0	Air column with catheter	Air column created by needle	Air columns filled with contrast 1	Needles
Tissue present	Tissue present	Tissue present	Tissue present	Tissue present
No contrast	No contrast	No contrast	Contrast 1	No contrast

Block #9


The view is from above in the figure. Sutures were placed with ethylon 4-0 (seen from the sticker) with one at the underside, two at the left side and one at the front. The sutures were easy to be placed.

Block #5

The catheters were inserted fairly easy, but the paraffin has to be warm. If the paraffin is not warm, the needles bent (especially the thin ones) and the paraffin crumbled off (especially the thick ones). It was not investigated what happened by insertion into cold paraffin. The radiodense catheters were placed into the block. The description is as seen from the sticker. The 16Gs (black) were placed caudal-cranial, the 20G (pink) and 22G (blue) were placed dorsal-ventral at the cranial side. At the caudal side, three holes were made dorsal-ventral. One hole was made with a 16G needle, the second hole was made by use of a 20 G needle and the third hole was made with a 26G needle. These are the air columns.
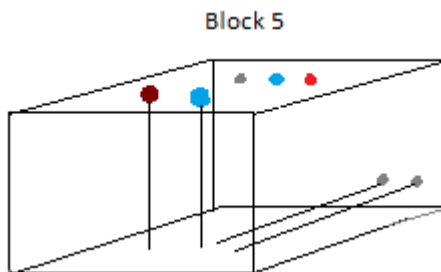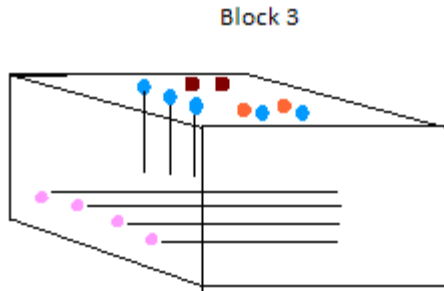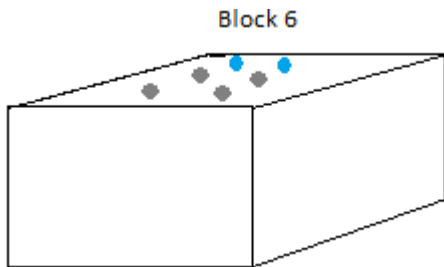
Block #3

Sterican needles (stainless, chromium-nickel steel) were placed in this block. Three 20G needles were placed dorsal-ventral (as seen from the sticker) and four 27G needles were placed left to right at different distances from each other. Furthermore, air columns were made. On the cranial side, two air columns were made with a 20G needle far cranial and two columns made with a 23G needle. On the caudal side, two 22G holes were made.

Needlegauges:
20 G
22 G
23 G
27 G

Block #6

In this block, columns were made filled with contrast 1. We placed cranially four holes made with a 16G needle and more caudally two holes with 20G. The columns were filled with the contrast fairly difficult, with pouring a drop onto the block and inserting the needle. The drop would get into the hole. The holes were softly coated with paraffin, so the contrast would not get out of the hole. A few days later, it was visible that the contrast fluid had migrated to the lower layer of first put in paraffin.
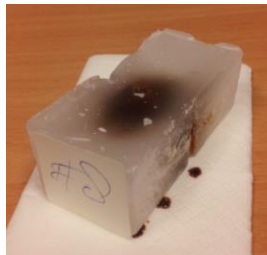

Needlegauges:
16 G
20 G

Test 3: Contrast agent in tissue

The contrast agent was prepared as described earlier and put in a Falcon tube with bacon. A bacon piece fixated with contrast agent was put in a paraffin block. No markers were placed.

| Block #8 |
| --- |
| Paraffin |
| Complete coverage |
| No markers |
| Tissue present |
| Contrast 1 |
| Contrast model |

A few days later, it is seen that the contrast agent is seeping through the paraffin. Or the density of paraffin is not great enough, there were tiny holes or the paraffin is being dissolved.

Experiment 2   Agar

Goal

The goal of this experiment is to understand the method of fixation (to hold in place) of a piece of meat in agar. Agar was used as a mold for directing needles in previous experiments and was thus shown to be effective in past tests for strength and useable under MRI scans. In this block of agar containing the tissues, markers will be placed. These markers will be tested under a CT to investigate their effectiveness in being shown on the scans. Moreover, a contrast solution will be tested to see if this becomes visible on CT. Also, the ease of cutting through agar will be tested.

Materials

o	Agar	UMC
o	Template for paraffin icecubeholder	Supermarket
o	Streaky bacon	Supermarket
o	4% formalin	UMC
o	Needles with different gauges (16,20,22,23,26 and 27G)	UKG
o	Really small catheters (16, 20 and 22G)	UMC
o	Suture material Ethilon 2-0	UKG
o	Contrast fluids	UMC
o	Paper towels	UMC
o	Cutting set	own property

Methods

All tissues will remain in the formalin for preservation. The streaky bacon was cut into pieces of about 2,5 x 1,5 x 1,5 cm. 9 pieces of tissue were inserted into a box filled with formalin 4%. In one Falcon tube, 22ml formalin 4% was mixed with 1.98 grams I_2 and 4.1 grams KI, this would result in a solution of 10% I_2KI in 4% formalin. One tissue piece was placed in this tube. All samples were kept overnight.

20.04 grams of agar MP was dissolved in 500mL distilled water. 10.5 mg MnCl was added. The suspension was put in the microwave to heat the substance and to facilitate the dissolvement of the agar. Then the suspension was placed on a heater with thermometer and a magnet to stir the suspension. The temperature was monitored and the suspension was kept in liquid state in this manner. After a few minutes, the suspension was placed in the vibrating bath to eliminate the bubbles. After pouring the agar into the icecubeholder at a temperature between 50 to 60 degrees Celsius, the agar was placed in the fridge 10 minutes to cool down.

Test 1: Cut

Block # 15	Block # 16
Agar	Agar
Complete coverage	Complete coverage
No markers	No markers
Tissue present	Tissue present
No contrast	No contrast
Cutting model	Cutting model

Cutting into the agar is fairly easy, but it is harder to cut more than once into one block. To cut once is easy when the tissue is surrounded with enough agar. Cutting in a distance of 0.5 cm, the agar may break and/or the tissue falls out of the agar. This is comparable to paraffin. But it is better to handle than paraffin.

Test 2: Markers

| Block # 10	Block # 11
Agar	Agar
Complete coverage	Complete coverage
Sutures, ethilon 2-0	Air columns with catheters
Tissue present	Tissue present
No contrast	No contrast
Suture model	Catheter model

Block # 12	Block # 13
Agar	Agar
Complete coverage	Complete coverage
Air columns made by needles	Needles
Tissue present	Tissue present
No contrast	No contrast
Air columns model	Needle model

Blok 10

Sutures

Blok 11

Blok 12

Needlegauges:
20 G
23 G

Blok 13
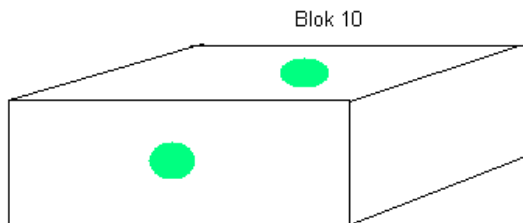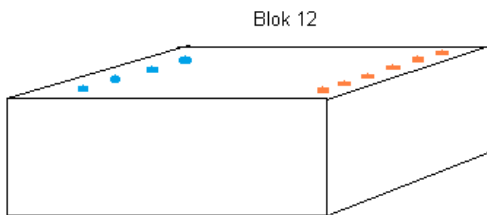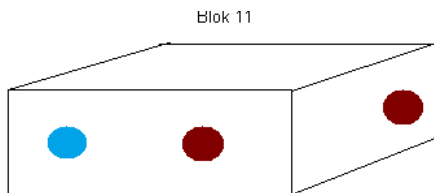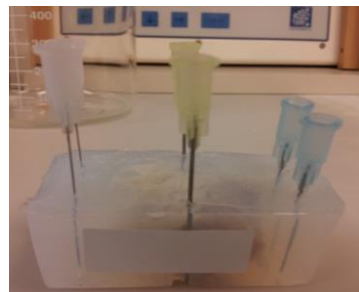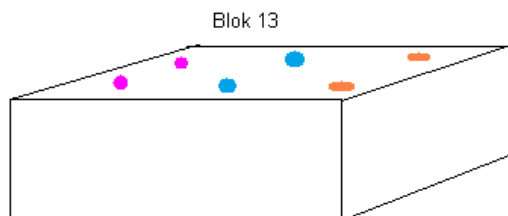
Needlegauges:
20 G
23 G
27 G

Putting in the needles was easy when the agar was warm or cold, this did not make a difference. The direction of the needles was easy to lead. The sutures were placed in the tissue before these were engulfed in agar. Placing sutures is fairly easy. The catheters were placed in the agar block, this was also easy to do when the agar was still warm.

Test 3: Contrast agent in tissue

The contrast agent was prepared as described earlier and put in a Falcon tube with bacon. A bacon piece fixated with contrast agent was put in an agar block. No markers were placed. The contrast agent does leak out of the agar, so the agar does not keep the contrast agent contained within the tissue. This makes agar as a substance unuseable with contrast.

Block # 14
Agar
Complete coverage
No markers
Tissue present
Contrast 1
Contrast model

Experiment 3   Falcon tubes

Goal

The goal of this experiment is to understand the method of fixation (to hold in place) of a piece of meat in a Falcon tube filled with formalin. In the tissues, markers will be placed. These markers will be tested under a CT to investigate their effectiveness in being shown on the scans. Moreover, a contrast solution in the Falcon tube will be tested to see if this becomes visible on CT.

Materials

- Streaky bacon   Supermarket
- 3 Falcon bottles   UMC
- 4% formalin   UMC
- Needles with 26G and 27G   UKG
- 22G catheters   UMC
- Suture material monocryl 4-0   UKG
- Paper towels   UMC
- Cutting set   own property

Methods

All tissues will remain in formalin, they were kept overnight in 4% formalin. The streaky bacon was cut into pieces of about 2,5 x 1,5 x 1,5 cm. The tissues were fitted with their own markers. The Falcon tubes were then filled with 40 ml formalin 4%.

Test 1: Markers

Tube 0	Tube 1	Tube 2	Tube 3
In formalin	In formalin	In formalin	In formalin
-	Sutures, monocryl 4-0	Air column with catheter	Needles
Tissue present	Tissue present	Tissue present	Tissue present
Contrast 1	No contrast	No contrast	No contrast
Contrast model	Suture model	Catheter model	Needle model

The markers were placed in the tissue outside the tubes and then placed in the formalin filled tube.

Tube 1

Tube 3

Tube 1　　　Tube 2　　　Tube 3

Sutures
Needlegauges:
20 G
26 G
27 G
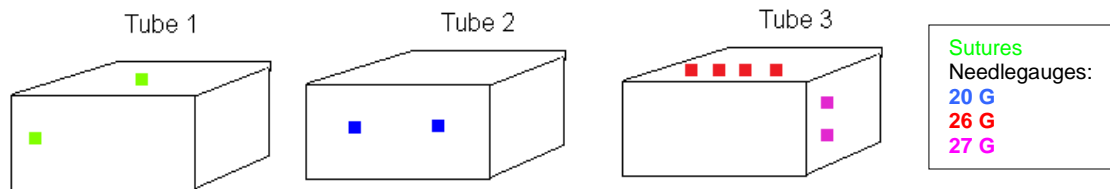
Experiment 4　　　CT imaging

Goal

This experiment was performed to determine the visibility of the different orientation markers for (micro)CT and the usefulness/visibility of the different embedding methods and to determine the usefulness of the contrast agent I_2KI. The blocks made in the experiments 1-3 were scanned.

Materials

- A large box to organize the different blocks, including plastic to cover the box
- Paraffin blocks of previous experiments (# 3, 4, 5, 6, 8 and 9)
- Agar blocks of previous experiments　　(# 10 - 16)
- Falcon tubes with loose tissue　　　　　(# 0,1,2,3)

Methods

All the different paraffin and agar blocks with the falcon tubes were organised in a box before heading off to the CT scanner. A Tena lady was used to cover the CT table before placing the box on it. There should be enough space between the elements to be able to differentiate the items in the scan. The scanner characteristics were a tesla 16 scanner. (*mAs 30, slice thickness 1 mm, 110 kV, a total of 385 pictures of the whole box*)

Groundplan

Analysis of the CT scans

Cutting models

Block #15	*Block # 4*	*Block # 16*
Cutting model	Cutting model	Cutting model
Agar	Paraffin	Agar
Complete coverage	Complete coverage	Complete coverage
No markers	No markers	No markers
Tissue present	Tissue present	Tissue present
No contrast	No contrast	No contrast
9.25 visible	9.36 visible	9.419 visible
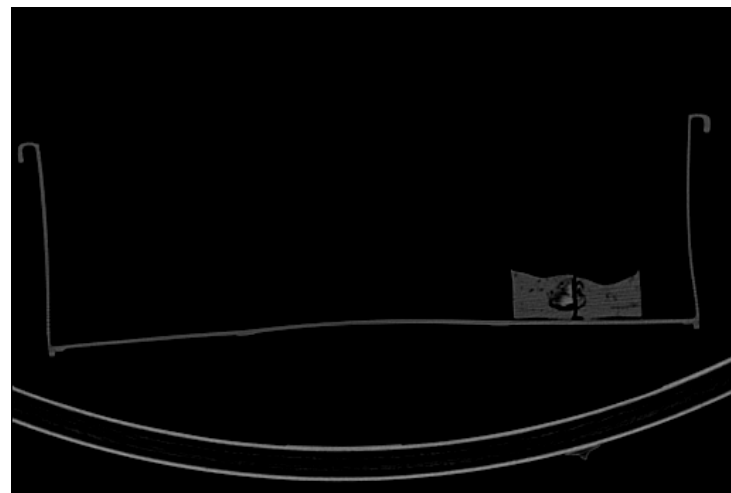
Block #4 (slide 9.385)
The tissue is surrounded with air and the tissue pieces were not placed
exactly against each other so the placement is oblique.

Air columns model

Block #12	*Block #3*	*Block #5*
Air columns model	Air columns model	Air columns model
Agar	Paraffin	Paraffin
Complete coverage	Complete coverage	Complete coverage
Air columns made by needles	Air columns made by needles	Air columns made by needles
Tissue present	Tissue present	Tissue present
No contrast	No contrast	No contrast
9.22 visible	9.248 visible	9.447 visible

Block #12 (slide 9.99)
Five air columns are visible as black columns.
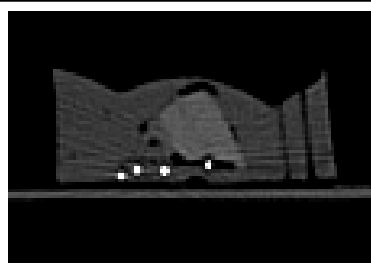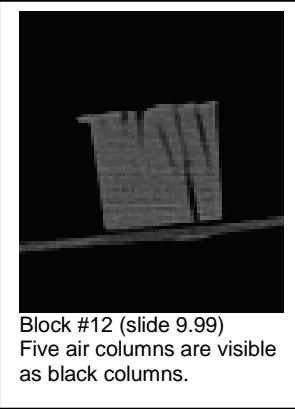
Block #3 (slide 9.269)
The 20G air columns are visible on the far right.
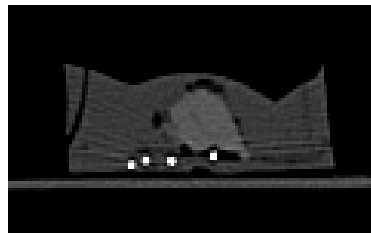
Block #3 (slide 9.271)
Most left 22 G air column is visible
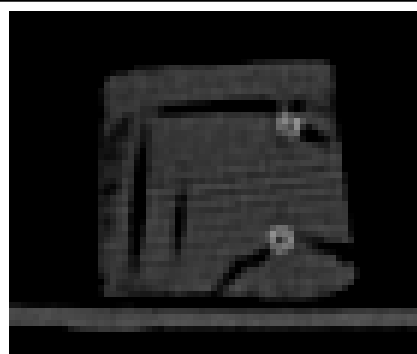
Block #5 (slide 9.447)
Two air columns are visible (left 16G and right 20G). The 27G air column is invisible.

Needle models

Tube 3	Block #3	Block #13
Needle model	Needle model	Needle model
In formalin	Paraffin	Agar
-	Complete coverage	Complete coverage
Needles	Needles	Needles
Tissue present	Tissue present	Tissue present
No contrast	No contrast	No contrast
9.134 visible	9.248 visible	9.188 visible
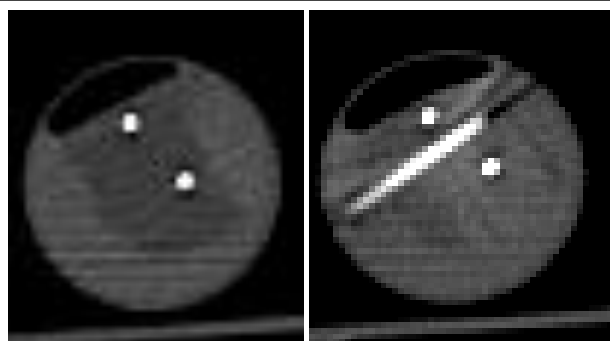
Tube 3
(slide 9.231, left) The 27G needles go into the tissue. These needles are very white. There seems to be diagonal stripes through the needles and surrounding tissue and some black around the needles, presumably air.
(slide 9.236, right) The 26G needle are visible.
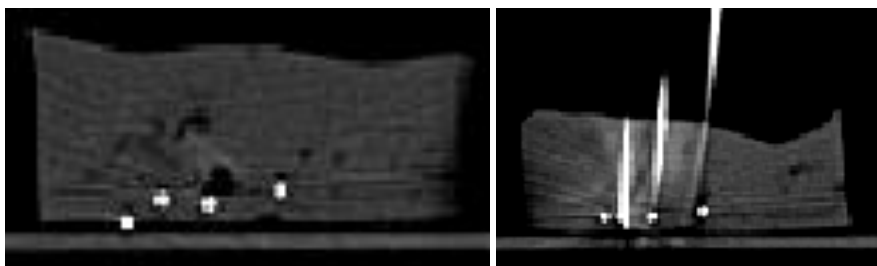
Block #3
(slide 9.256) The four needles (27G) are visible as very white, small points horizontally.
(slide 9.277) The three needles (20G) are visible vertically. These give diagonal dispersion.
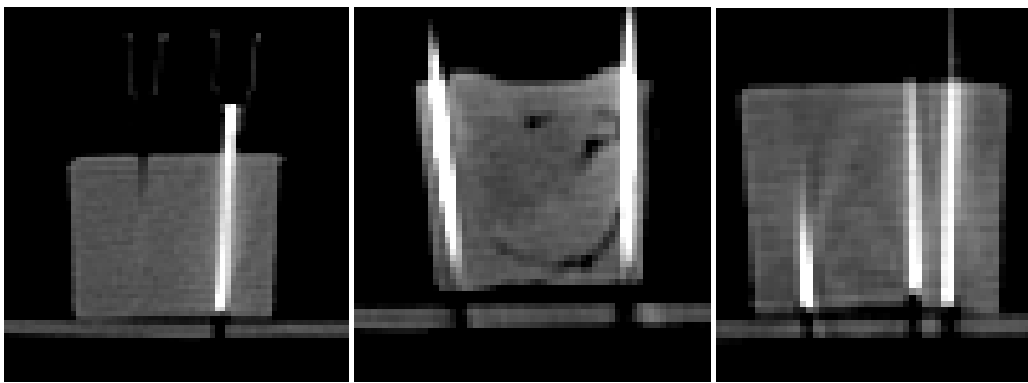
Block #13
(slide 9.142, left) The 23G needle is visible in white and produces a black artefact beneath the needlepoint
(slide 9.174, middle) 20G needles are visible
(slide 9.205, right) 27G needles are visible

Catheter models

Block # 11	Tube 2	Block #5
Catheter model	Catheter model	Catheter model
Agar	In formalin	Paraffin
Complete coverage	-	Complete coverage
Air columns with catheter	Air column with catheter	Air column with catheter
Tissue present	Tissue present	Tissue present
No contrast	No contrast	No contrast
9.26 visible	9.18 visible	9.412 visible
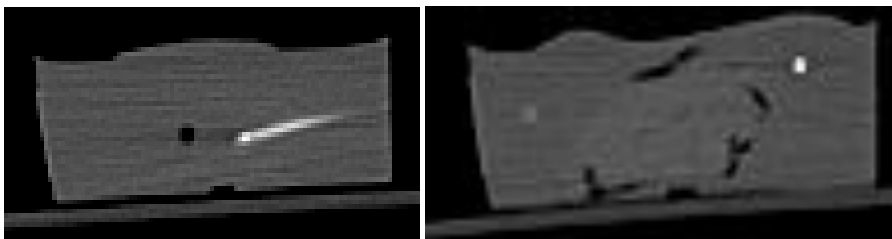
Block #11
(slide 9.41, left) 22G catheter visible
(slide 9.82, right) left 20G visible and right 22G visible
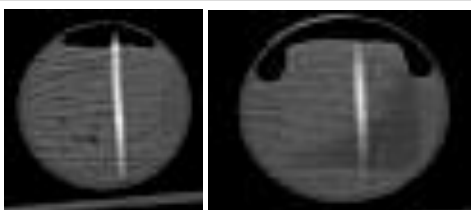
Tube 2
(slide 9.218, left) 20G catheter visible
(slide 9.246, right) 20 G catheter visible
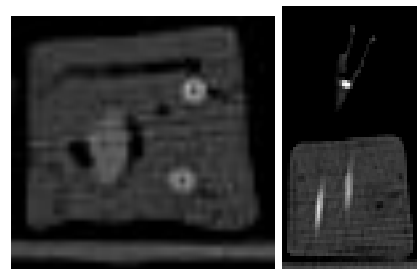
Block #5
(slide 9.464) 16G catheters visible horizontally
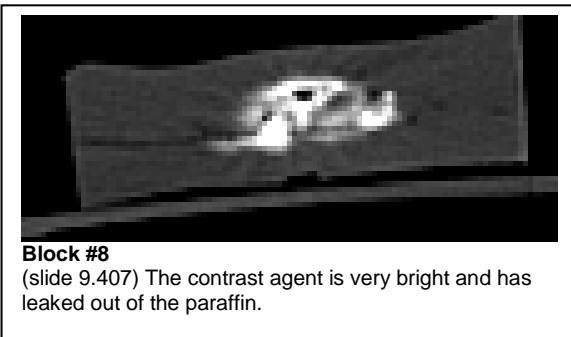(slide 9.517) 20G and 22G are visible vertically

Suture models

Block #9	Tube 1	Block # 10
Suture model	Suture model	Suture model
Paraffin	In formalin	Agar
Complete coverage	-	Complete coverage
Sutures, ethilon 2-0	Sutures, monocryl 4-0	Sutures, monocryl 4-0
Tissue present	Tissue present	Tissue present
No contrast	No contrast	No contrast
9.458 visible	9.180 visible	9.129 visible

Block #9
(slide 9.468) The tissue is visible but the sutures are not.
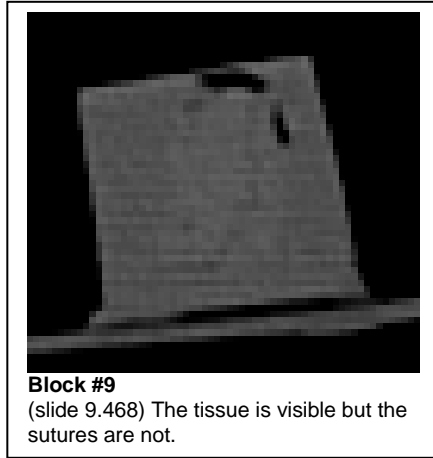
Contrast models

Block #8	Block #6	Tube 0	Block #14
Contrast model	Contrast model	Contrast model	Contrast model
Paraffin	Paraffin	In air	Agar
Complete coverage	Complete coverage	-	Complete coverage
No markers	Air columns filled with contrast 1	No markers	No markers
Tissue present	Tissue present	Tissue present	Tissue present
Contrast 1	Contrast 1	Contrast 1	Contrast 1
9.398 visible	9.472 visible	9.200 visible	9.27 visible

Block #8
(slide 9.407) The contrast agent is very bright and has leaked out of the paraffin.

Tube 0
(slide 9.212) The tissue is very white with some grey patches.

Block #6
(slide 9.483)white spots visible, these look more like droplets than columns. The columns were probably filled with more air than contrast agents. There is also a solemn column with air without any agent.

Block #14
(slide 9.40) the tissue is very bright

Discussion

General
The icecubeholder is very handy, but it is too small for the tumours to fixate them. It measures maximally 2x2 centimetres.

Paraffin
Paraffin is very easy to prepare and easy to obtain at the pathology department. It becomes hard without cooling within an hour. It fixates the tissue and stays stiff for weeks. Moreover, it does not decompose and the formalin and contrast cannot leak out of the paraffin, unless there are some holes. However, it is difficult to cut pieces within 0.5 cm range of each other, because the paraffin breaks. It is also not transparent and the tissue is surrounded by air because the tissue shrinks in the paraffin, due to dehydration after some time. In the paraffin bubbles are visible.

Agar
It is very easy to prepare the agar and it becomes hard in the refrigerator after approximately 15 minutes. The tissue does not shrink significantly because the agar has enough moisture to sustain the tissue and the agar is transparent. Also the tissue stays well fixated within the agar and cutting the tissue is very easy, even in short distances. The addition of manganese(II)chloride to enhance the contrast in CT and MRI is very handy. Disadvantages of agar are that the substance decomposes and the formalin and contrast leak out of the agar after some time. The tissue is also surrounded by a thin layer of air during preparation. The agar has no bubbles due to the fabrication process.

Falcon tubes
The Falcon tubes can be filled with formalin and thus no other material is required to fixate the tissue. In the tubes, free movement of the tissues is possible but restricted. This way the orientation of the tissue can be lost, therefore markers are needed in/on the tissue. There is a limit to the range of markers in the tube, like catheters and needles. They cannot be inserted in every direction and they cannot stick out the tissue too far. Contrast agent cannot be used as a marker, but can be used as a contrast for the outer layer.

Cutting
Cutting with a microtome or a scalpel makes no differences. Agar is easier to cut through than paraffin and the tissue stays better inside the agar than in the paraffin during cutting, which is probably due to the ease of cutting through agar. Paraffin is hard to cut through, which causes the tissue to sometimes 'jump' out (tissue is easier to cut through). Moreover the paraffin is very breakable, which causes the paraffin to crumble off the tissue. The cutting edges are not visible on the CT if they are placed directly next to each other.

Sutures
Sutures are very easy to place in the tissue, there are no apparent differences between ethilon or monocryl. Another advantage is that the sutures can be placed right after resection of the tissue. The sutures used are not visible on the CT because these are not radio dense enough. It is difficult to cut along the sutures if the orientation is unknown. Trying this it is most likely that the suture itself gets cut. Moreover, it is relatively difficult to make a scale due to the small surface of the material. There is radio dense suture material.

Air columns
The air columns are easy made with needles and are easier made in agar than in paraffin. It is important to prevent the column from being clogged with (other) material. All outer diameters (16, 20, 22 and 23G) are visible and their differences too. Air is also visible on a MR scanner and it is easy to cut through. However, to the naked eye it is difficult to find the air column within the block and thus to cut along.

Needles
Needles are very easy to use, but according to the material of the embedding and the thickness of the needle, the needles can slightly bend in the embedding material. The embedding material determines the ease of determining the direction of the needle. The needles (20,23 and 27G) are all clearly visible in the CT and by eye and are very white and sometimes even create dispersion. Iron needles seem to be too radio dense, which causes artefacts under the needle tip. Iron needles also create artefacts on MR images. Cutting through a needle will be difficult due to the density. With smaller needle gauges, the dispersion will be less but it would be even more difficult to put the needle straight in.

Catheters
The catheters are easy to put in the tissue because of the needles, but the direction is not easy to control according to the material and needle gauge. The catheters are clearly visible in the CT. In parallel direction, they show white lines, in perpendicular direction round circles are seen. No clear dispersion was seen. The catheters of the UMC (16G and 20G) are more visible than the catheter of the UKG (22G). The catheters are easy to cut through and are not influenced by the presence of tissue. It may be difficult to cut along a catheter, because you may cut through the catheter. The radio dense catheters are more radio dense than the paraffin and agar.

Contrast
The use of contrast really affects the imagery in CT, because the substance becomes so bright that all other information is lost. It is however clearly visible. The agent is also difficult to use because it cannot be contained by agar, it leaks fairly easy through the material. Putting contrast into self-made columns is very hard because of the suspected capillary action. Agar with this contrast is not useable, because it would make the Ho-MS less visible on the CT and is too radio dense.

Overall conclusion
The conclusion of these experiments was that all these tissue embedding/surrounding materials provided too little contrast to the tissue, so these cannot be used as markers. The agar appeared more useful than paraffin because it was much easier to cut through, good fixating properties and transparent. Radio dense catheters pointed out to be the most suitable markers, because of the visibility on CT, by eye, the minimal dispersion and it is easy to use.

Because of the little contrast between tissue and the used surrounding material, we decided that the best "surrounding material" for the tissues would be air. Therefore we designed a platform with air holes as a kind of grid and covered the tissues with cling film to keep the tissue in place. None of the tested markers was ultimately used, because the grid has holes in it which can be used as markers themselves. Below is an image of the grid.

Substudy 2: in vivo MRI

Introduction

In vivo MRI scans were made of the three tumours before, during and after the injections of the Ho-MS. Only the last scans were used to see the full effect and extent of the holmium. Bone becomes white on a t2-weighted MRI scan, tissue becomes more grey and black indicates air. MRI scanners use strong magnetic fields and radiowaves to visualise the different parts of the body. The holmium is seen as a black cloud. It is therefore easy to recognize the Ho-MS and the MRI is useful as a diagnostic image. The whiter substance on top of the rabbit is the agar mould used to direct and stabilise the injections. To process the MRI images, the programme ImageJ was used.

Preparation of the tumours

The tumours are native of previous experiments in which Vx2 tumours were grown on the thighs of two New Zealand White Rabbits subcutaneously in the period of May- July 2013. When the tumours had reached a size large enough to treat, they were injected with holmium-165-PLLA-microspheres. The first rabbit (306870) developed two tumours, one on each thigh. The tumour on the right thigh, tumour 1, measured 30 x 25 x 10 mm prior to fixation and 25 x 58 x 25 mm after fixation. It was one solid mass and injected once.

The tumour on the left thigh, tumour 2, consisted of two parts, one small part, which measured 4 x 4 x 4 mm (before fixation), and a larger part, which measured 20 x 20 x 8 mm before fixation and 27 x 21 x 20 mm after fixation. This tumour was injected twice.

The second rabbit (306816) developed only one tumour, tumour 3, on the left thigh which measured 40 x 40 x 20 mm after fixation.

During microsphere injection the tumours were scanned using a MR scanner. After the microsphere injection, the rabbits were euthanised. After euthanasia the tumours were resected, marked with sutures and put in the formalin for preservation and fixation.

Protocol
1. In vivo MRI (1.5 Tesla) imaging of the tumours.
2. Euthanise the rabbit.
3. Excise the VX2 tumour (1,5 cm width and 1 cm deep margins) injected with non-radioactive 166-HoMs.
4. Place sutures on the dorsal and cranial end of the excised tissue for orientation.
5. Create a landmark for slice location based on the in vivo orientation of the MRI slices (in the transverse plain).
6. Fixate in 4% formaldehyde.
7. When fixated wash excess formaldehyde from the tissue using sterile demineralised water.
8. Use 2 % (w/w), 20 mg/l MnCl3 cooled down to 39 degrees to fixate position of the tissue for further ex vivo imaging.
9. Perform ex vivo 7 Tesla and Microct imaging

DEC
The DEC number is DEC nr: 2011.III.08.080 (approved until September 2014). The responsible researcher is Dr. J.F.W. Nijsen (art.9; UMCU), the executive researchers are
B.S. van Leeuwen (art. 9; DGK) and Dr. S.A. van Nimwegen (art. 9; DGK) and the bio technician is A. Barten (art. 12; UMCU). The UU-FVM cornerstone is AVM.

Scans of Tumour 1

Image 1
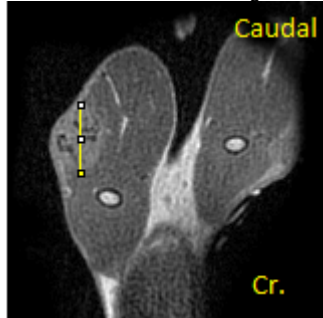PRS20130611_VX2_WIP_3D_UTE_koosh_1.1.mm_cor_CLEAR_47_1x1.hdr
Images after needle withdrawal (PRS20130611_VX2, 1 depot)
128.00x128.00 pixels (160x160); 32-bit; 16 MB
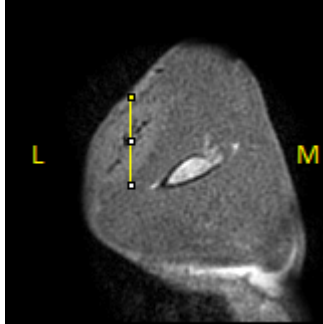
Coronal view
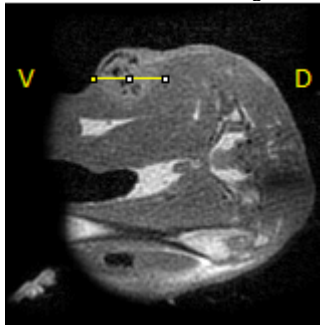**Tumor 2, slice 40/160, XZ 80, YZ 80**
Lateromedial maximal length: 24 mm
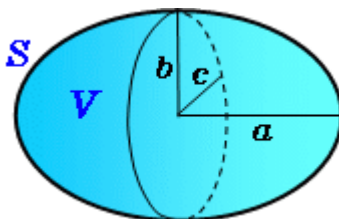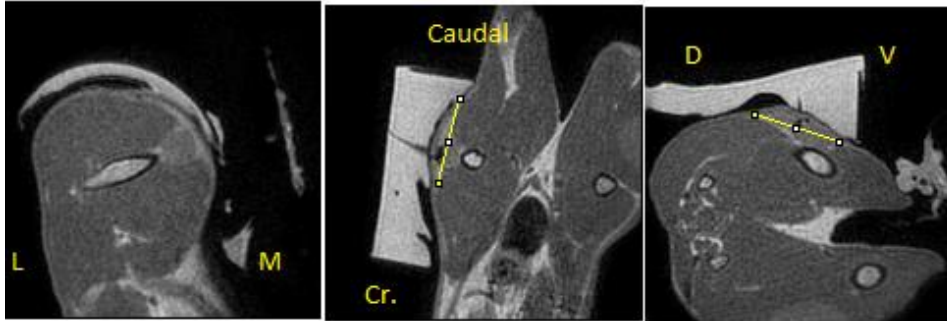

Sagittal view
Tumor 2, slice 37/160, XZ 80, YZ 66
Craniocaudal maximal length: 27,20 mm

Transverse view
Tumor 2, slice 39/160, XZ 68, YZ 66

From the scans of tumour 1, the volume of the tumour was determined.
4/3 π x (27.20mm/2 · 28,80mm/2 · 24mm/2) = 9819.3821mm³ = 9.8194 cm³

Volume of an ellipsoid: 4/3 π x (a·b·c)

A = craniocaudaal
B = dorsoventraal
C = lateromediaal

Image 2
PRS20130611_VX2_WIP_3D_UTE_koosh_1.1.mm_cor_CLEAR_47_1x1.hdr
Images after needle withdrawal (PRS20130611_VX2, 1 depot)
128.00x128.00 pixels (160x160); 32-bit; 16 MB
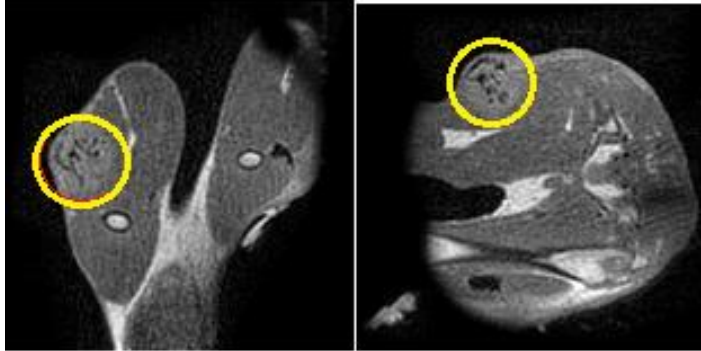
Tumour 1: Depot
The depot is located within the tumour, well distributed but in plaques. The entry with the needle cannot be seen. This can be seen clearly in image 2.

Scans of Tumour 2

Image 3
PRS20130611_VX2_WIP_3D_UTE_koosh_1.1.mm_cor_CLEAR_15_1x1.hdr
Images after needle withdrawal
128.00x128.00 pixels (160x160); 32-bit; 16 MB

Coronal view   Sagittal view   Transverse view
Tumour 2, first depot, slice 68/160, XZ 85, YZ 78
The yellow line within the black area is the measuring tape.
Craniocaudal maximal length: 36,35 mm
Dorsoventral maximal: 39,23 mm
Lateromedial length: 10,76 mm

Image 4
PRS20130611_VX2_WIP_3D_UTE_koosh_1.1mm_cor_CLEAR_30_1x1.hdr
Images after needle withdrawal
128.00x128.00 pixels (160x160); 32-bit; 16 MB
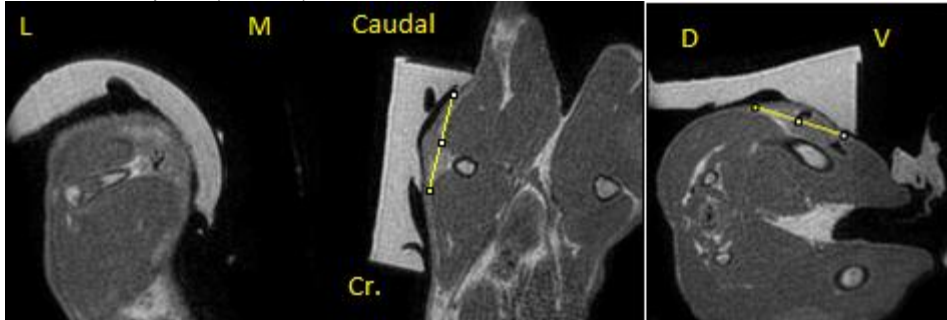
Coronal view   Sagittal view   Transverse view
Tumour 2, second depot, slice 60/160, XZ 78, YZ 74
Craniocaudal maximal length: 39,35 mm
Dorsoventral maximal: 34,29mm
Lateromedial length: 12,79 mm

Tumour 2: Volume

From the first depot scans of tumour 2 (image 3) , the volume of the tumour was determined.
4/3 π x (36,35mm/2 · 39,23mm/2 · 10,76mm/2) = 8034.03311 mm^3 = 8.03403311 cm^3.
The volume obtained from the second depot scans (image 3) was:
4/3 π x (39,35mm/2 · 34,29mm/2 · 12,79/mm2) = 9036.10749 mm^3 = 9.03610749 cm^3
The scans do not differentiate between the sizes of the two lobes of the tumour, so the measurements on the MRI scans are the full tumour size.
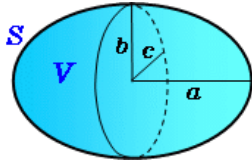
Volume of an ellipsoid: 4/3 π x (a·b·c)

A = craniocaudaal
B = dorsoventraal
C = lateromediaal

Image 5
PRS20130611_VX2_WIP_3D_UTE_koosh_1.1.mm
_cor_CLEAR_15_1x1.hdr
Images after needle withdrawal
128.00x128.00 pixels (160x160); 32-bit; 16 MB

Coronal view
Tumour 2, First depot, slice 58/160, YZ 80, XZ 78

Transverse view
Tumour 2, First depot, slice 65/160, YZ 80, XZ 80

Sagittal view
Tumour 2, First depot, slice 59/160, YZ 76, XZ 79

Image 6
PRS20130611_VX2_WIP_3D_UTE_koosh_1.1mm-
_cor_CLEAR_30_1x1.hdr
Images after needle withdrawal
128.00x128.00 pixels (160x160); 32-bit; 16 MB

Coronal view
Tumour 2, second depot, slice 74/160, XZ 74 , YZ 102

Transverse view
Tumour 2, second depot, slice 80/160 , XZ 74 , YZ 74

Sagittal view
Tumour 2, second depot, slice 76/160 , XZ 80 ,YZ 104

Tumour 2: Depots

First depot, image 5

The depot is located within the tumour, well distributed but more lateral and extends to the femur bone and within the tumour margins. The place of insertion is very evident, there has not been a lot of distribution, it remains clustered.

Second depot, image 6

The second depot is located more ventral, medial and caudal to the tumour, next to the femur bone. The injection of the second depot did not enter the tumour but ended up in the muscle due to needle instability. It does consist of two halves. This is clearly evident in the scans.

Scans of Tumour 3

Image 7
PRS20130722b_VX2_WIP_3D_UTE_koosh_1.1.mm_cor_CLEAR_38_1x1.hdr
Images after second needle withdrawal
127.90x127.90 pixels (224x224); 32-bit; 16 MB

Coronal view   Sagittal view   Transverse view
Tumor 3 slice 51/224, XZ 81 ,YZ 134
Craniocaudal maximal length: 13.70 mm
Dorsoventral maximal length: 14,27 mm
Lateromedial maximal length: 10,14 mm
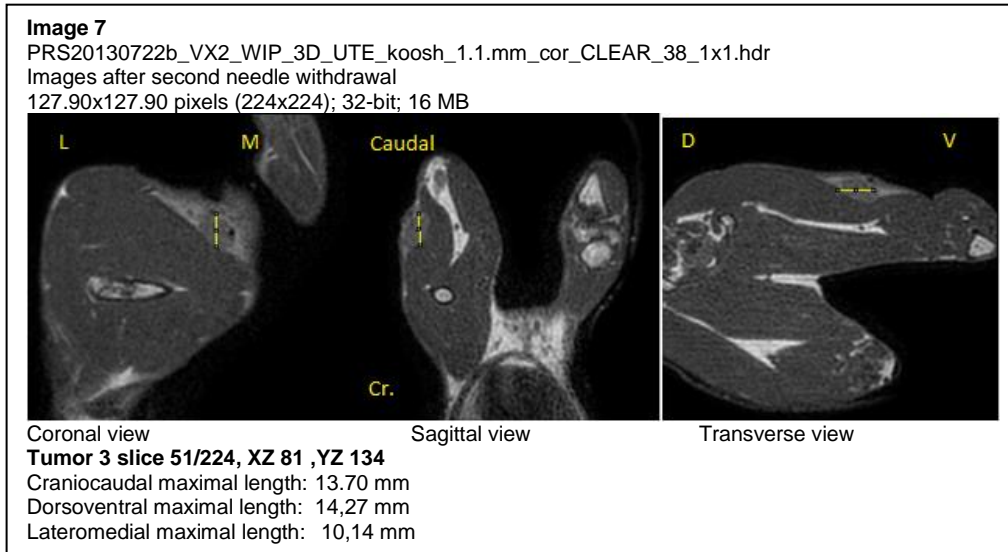
Tumour 3: Volume

From the scans of tumour 3, seen in image 7, the volume of the tumour was determined.
$4/3 \pi \times (13,70mm/2 \cdot 14,27mm/2 \cdot 9,14mm/2) = 1038.3601$ mm^3 = 1.038 cm^3.
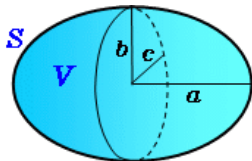
Volume of an ellipsoid: $4/3 \pi \times (a \cdot b \cdot c)$

A = craniocaudaal
B = dorsoventraal
C = lateromediaal

Tumour 3: Depots

As shown in image 8, the three depots have been inserted between the tumour and the skin with a part inserted into the lateroventral part of the tumour. It seems to have a roundish appearance.

Image 8
PRS20130722b_VX2_WIP_3D_UTE_koosh_1.1.mm_cor_CLEAR_38_1x1.hdr
Images after second needle withdrawal
127.90x127.90 pixels (224x224); 32-bit; 16 MB

Coronal view   Sagittal view   Transverse view
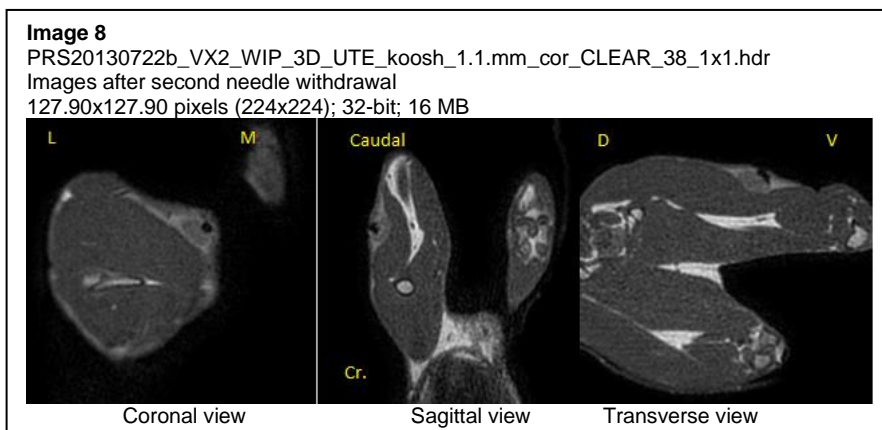
Substudy 3: MicroCT

Introduction

MicroCT

The microCT used is a Quantum FX microCT used by Erasmus in Rotterdam. The maximal scanning length is 200 mm. De diameter of the gantry is 193mm and 65 mm. The conclusive resolution is about 10 um versus 40 um in normal CTs. The scanning time is 17 seconds, in high resolution it takes two minutes.

Fixation of the tumours

Tumour 1, fixated to the grid.   Tumour 2, fixated to the grid.   Tumour 3, fixated to the grid.
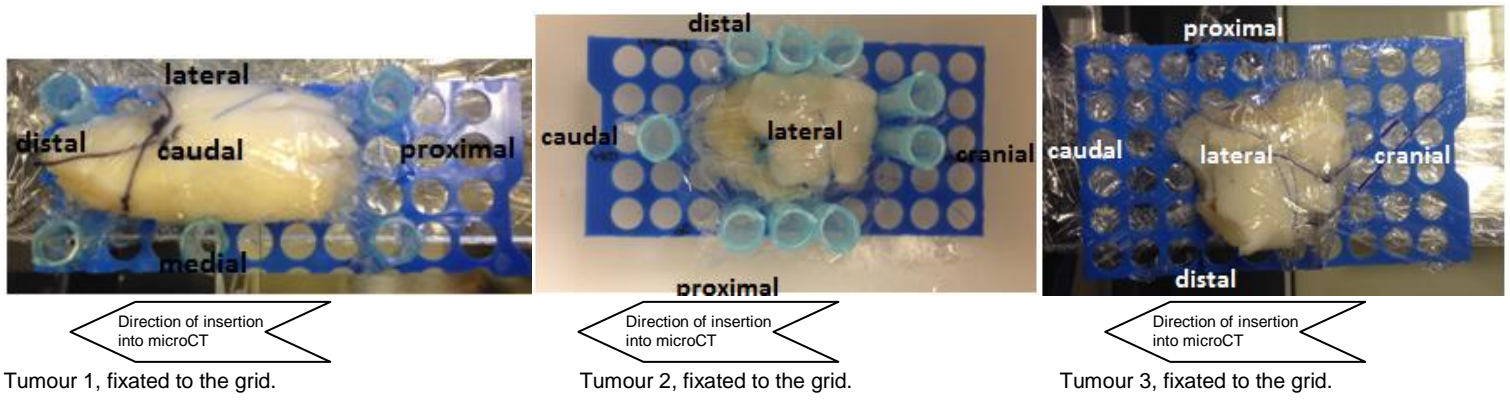
The tumours were put on a grid with round holes (pipette tip card) after evaluation of substudy 1. The grid was cut into proper dimensions so the grid would be able to fit into the microCT scanner. The tumour was attached to the grid by two to four sutures depending on the tumour size and structure. Hereafter the tumour and grid were covered with (stripes of) cling film. The cling film was attached to the grid in different ways to examine the most useable way of attachment. Pipette tips were cut on both sides to create smaller tips to fit into the microCT scanner. The pipettes were able to fixate the cling film and tumour. The tumours were still surrounded by tissue.

Tumour one was covered with stripes of cling film. The cling film was attached to the grid using the pipette tips. The pipette tips were pushed through the plastics. Tumour two was covered with a whole piece of cling film. The cling film was attached to the grid using the pipette tips. For this tissue one piece of cling film was used, because the tumour exists of 2 parts that have to be fixated towards each other. From a preliminary test, it has been proven to be crucial that the grid is not covered on the bottom part. It otherwise lets air sit between the layers and the formalin does not properly leak out of the construction. Tumour three was covered with stripes of cling film that were knotted under the grid. After fixation of the tumours to the grid the tumours were put back into the formalin.

Transport of the tumours

The tumours were transported from the UMCU to the Erasmus University Rotterdam in a plastic bucket surrounded by two trash bags in a cardboard box. In Rotterdam, the bucket was withdrawn from its packaging in a flow cabinet (because of the formalin) and the tumours were put in an empty plastic box. This box was taken to the scan room. In the scan room the tumours were scanned individually. Putting the whole setting including grid, tumor and fixation material on a scan table for rats. For the follow-up, another method of transportation should be investigated further.

Scans of Tumour 1

An overview scan A was made to determine the settings. *(settings: 90kV, 80µA, FOV 60 mm, std 17 sec).* On this scan it became clear that the MS (microspheres) injection was inserted into the center of the tumour. A more precise scan B was made (*settings: 90kV, 160µA, FOV 60mm, 4,5min).* The tumour setting was clearly visible. A nice cloud of MS clusters can be seen throughout most of the tissue, although in this large overview the contrast between tissue and MS clusters is not great (tissue is relatively white).

Hereafter the tumour was scanned in scan C. *(settings 90kV, 160µA, FOV 30 mm and technique fine 3 min.)* On this scan it became clear that we had enlarged the right area and we could zoom into it further. The MS seems to lay in rows behind and on top of each other. Scan D was made to zoom into the area with the cloud of MS. *(Scan settings: 90 kV, 160µA, FOV 20mm, time 4,5min.)* Small and large MS clusters can be visualised, clearly showing an interstitial pattern. The MS seems to be ordered in rows throughout the whole field of view.
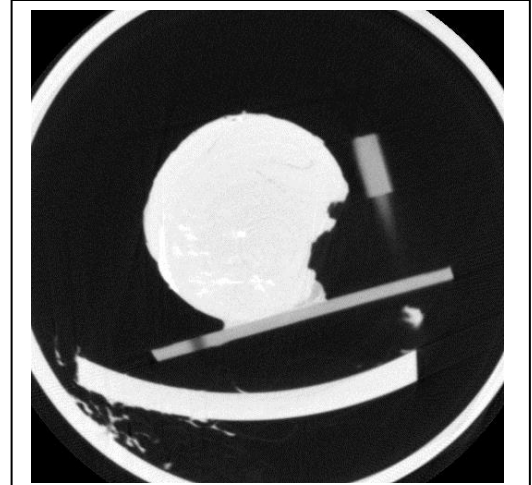
Tumour 1. Scan B. FOV 60 mm, scan time 4,5 minutes. Showing the setting, the tumour and a heterogeneous distribution of MS.
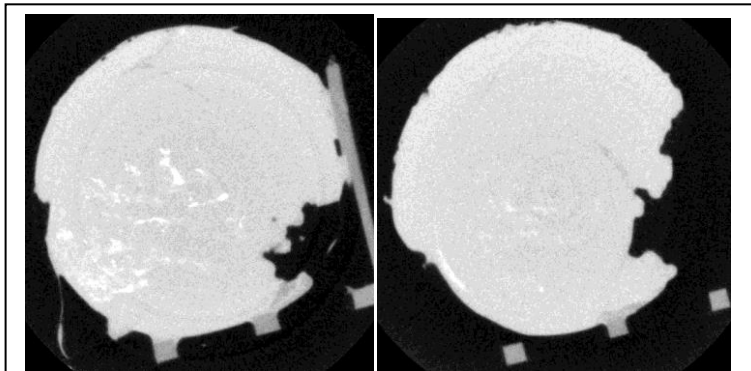
Tumour 1. Scan C. FOV 30mm scan time 3 min.
MS clusters clearly visible in a big cloud.
MS seems to lay ordered behind each other.
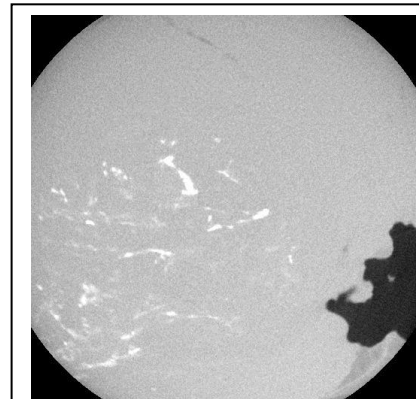
Tumour 1. Scan D. FOV 20mm, scan time 4,5min.
Small and large MS clusters can be visualised.

Scan E was made. *(settings: 90kV, 160µA, FOV 10mm, scan technique fine 3 min)*
This scan provided images with more noise than the images with the larger FOVs, and also some ring artifacts were visible as well. Finally, a sca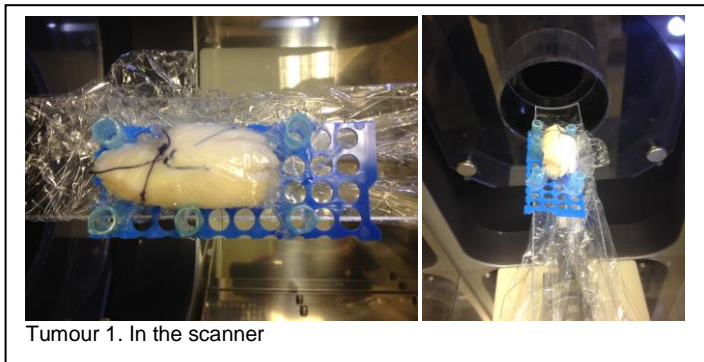n F with a field of view of 5 mm was made. (*settings: 90kV, 160µA, FOV 5mm, 3 min*). Again, a lot of noise was seen on the images, this made it impossible to localise individual microspheres.
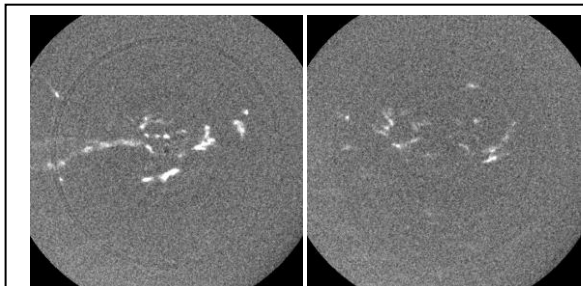
Tumour 1. Scan E. FOV 10mm, scan time 3 min.
MS clusters are visible.
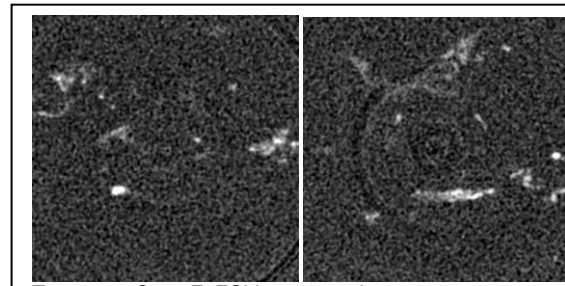
Tumour 1. Scan F. FOV 5mm, 3 min.
MS, noise and ring artifacts visible.

Tumour 1. In the scanner

On the 3D animations of the tumours the afore mentioned problem of tissue brightness is most prominent in the 3D reconstruction of the FOV 60mm images. This problem can partly be solved by increasing the threshold value. With an extreme elevation of the threshold value the Ho-MS cluster can be visualised, although it is not clear anymore if all the MS are being visualised now (see image A).

By slowly increasing the threshold value first, the outside of the setting can be seen and later the most radio dense parts of the setting can be seen. The plastic grid can roughly be seen until threshold 195 (image B), afterwards something of the grid can still be seen for a while, but after threshold 195 parts of the grid start to disappear. The most appropriate threshold for the visualisation of the MS should be investigated. 3D reconstructions made of images with a smaller FOV show MS clusters earlier, at lower thresholds, then those of images of larger FOVs. This could already be predicted because the contrast between tissue and MS is larger on the images with a small field of view than on those with a large FOV.

A remarkable item is that the purple sutures (most probably ethicon, PDS 2) are very clearly visible in the 3D image (image B), when the threshold is increased, although the blue sutures polypropylene are not visible in the 3D image.
As can be seen on the first scans (images A and B) ,the edges of the tissue and the setting can be visualised. On the scans hereafter only a part of the setting can be seen, this means that the orientation becomes more difficult.
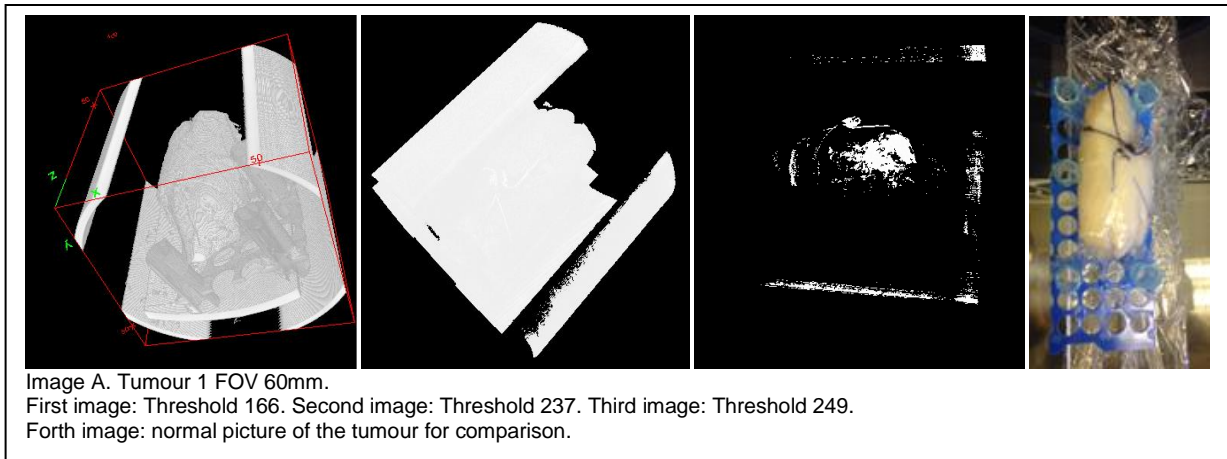
Image A. Tumour 1 FOV 60mm.
First image: Threshold 166. Second image: Threshold 237. Third image: Threshold 249.
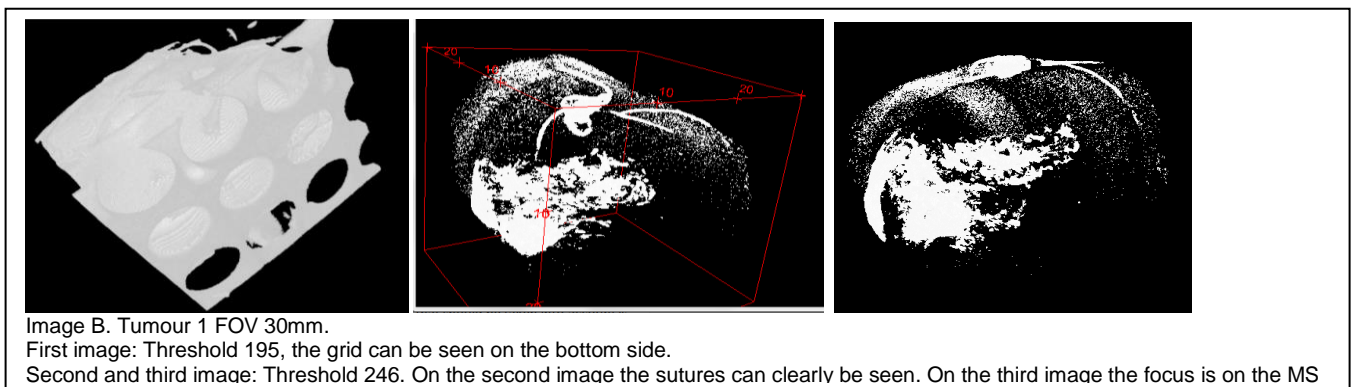Forth image: normal picture of the tumour for comparison.

Image B. Tumour 1 FOV 30mm.
First image: Threshold 195, the grid can be seen on the bottom side.
Second and third image: Threshold 246. On the second image the sutures can clearly be seen. On the third image the focus is on the MS
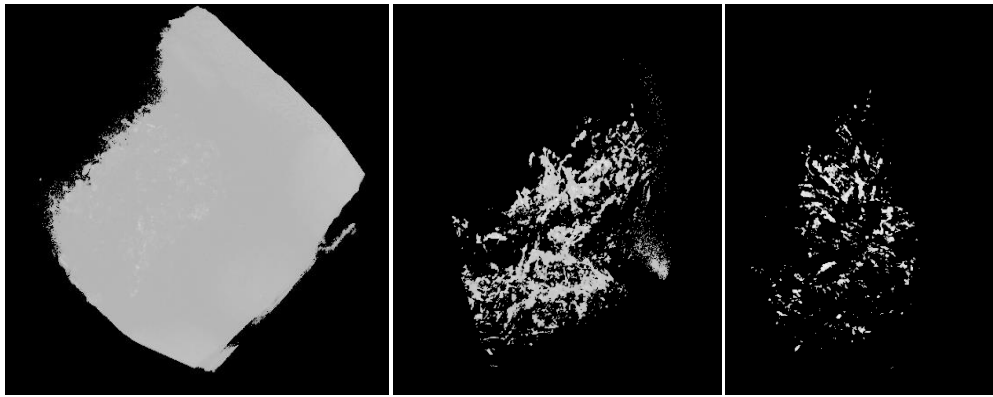
Image C Tumour 1 FOV 20mm.
First image: Threshold 180. Second image: Threshold 195. Third image: Threshold 210.
Pay attention to the different visibilities of tissue and spheres with different thresholds. (in steps of 15)
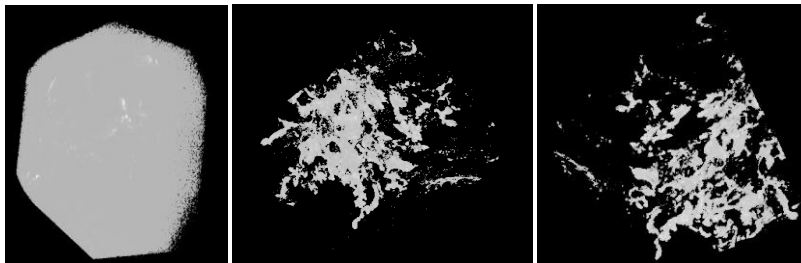

Image D Tumour 1. FOV 10mm.
First image: Threshold 180. Second image: Threshold 190. Third image: Threshold 195.
The tissue starts to disappear at threshold 180 and is almost gone at 190
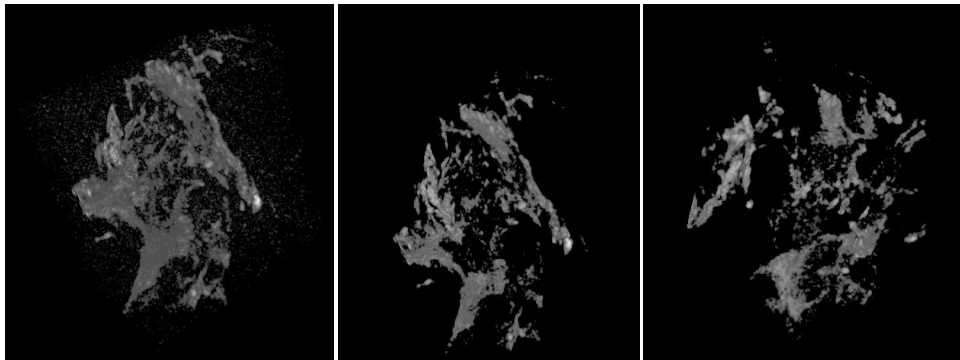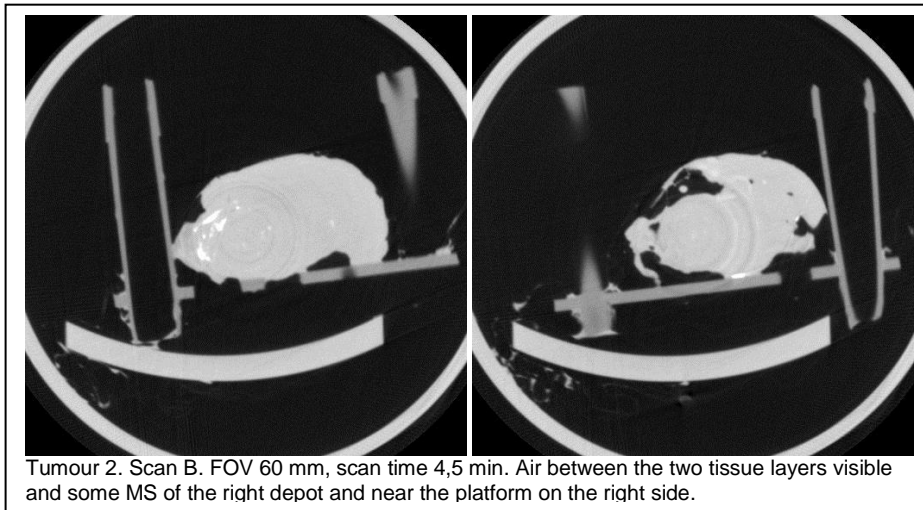(hereafter it is not clear whether the small dots are tissue or MS).

Image E. Tumour 1 FOV 5mm.
First image: Threshold 73. Second and third images: Threshold 90

Scans of Tumour 2

Tumour 2 was placed into the scanner. Again, first a rough scan A was made for a general topographic overview of the setting inside the scanner: (*voltage 90kV, current 80µA, FOV 60 mm and scan time 17 sec)*

Hereafter a scan B was made. *(current 160 µA, FOV 60 mm and a scan time of 4,5 min*) On the scan some air between the skin and the tumour can be seen (the tumour consists of two layers: one part with the main tumour and one part with the skin and a smaller tumour). Furthermore, two depots of MS can be seen. One is a large relatively well distributed depot on the left side of the images, comparable to the distribution of MS in the first and third tumour and the second a depot on the right side of the images. The depot on the right side of the images consisted of some white spots distributed at some distance from each other. The right depot was injected partly next to the tumour by free hand injections. Some larger depots of MS seem to be located in the muscle layer against the platform. In this scan, it is also evident that the platform has been overturned.

Tumour 2. Scan B. FOV 60 mm, scan time 4,5 min. Air between the two tissue layers visible and some MS of the right depot and near the platform on the right side.

Thirdly, a rough scan C was made *(90kV , 80µA, FOV 30mm and scan technique fine 3 min).* The depot on the lower part of the tumour (in muscle) is most probably the second injection site, because this injection was placed in the muscle instead of in the tumour due to needle instability.

Scan D and E were fast scans to focus on the first injection site. *(FOV 20mm and scan technique 17 sec)* The scanner table was moved to the left using the settings on the scanner. The movement to the left went a bit too far, which caused the platform to bump against the inner side of the scanner causing the platform to turn over slightly.

After the orientation scans, scans F and G showed the tissue was at the right position. *(FOV 20 mm and scan technique of 4,5 min)*Clusters of microspheres can be seen on the left side of the image. Remarkably on the scans two chord-like structures can be seen.
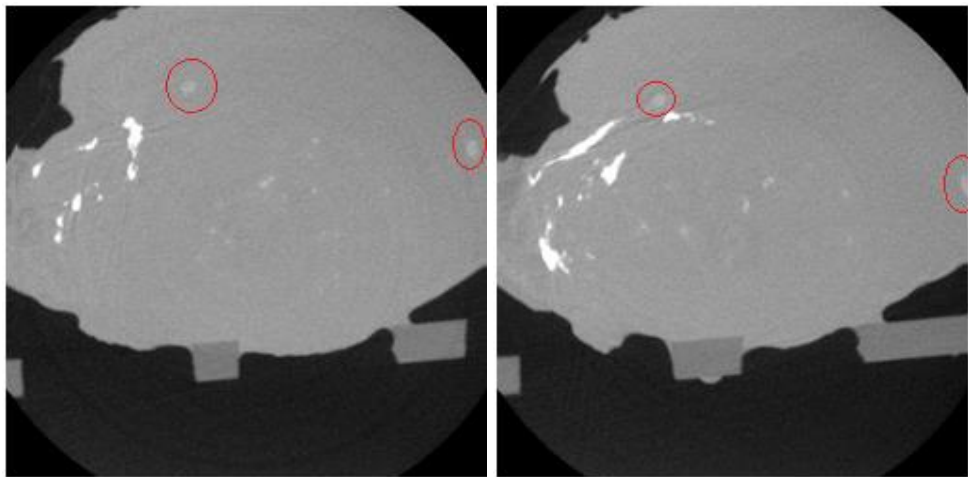
Tumour 2. Scans F and G. FOV 20mm, scan time 4,5 minutes. Two chordlike structures are visible, one slightly left from the middle and one on the right side of the images (encircled)

A planning scan H was made *(settings 90 kV, 80µA, FOV 10mm, standard 26 sec)*, then another fast scan I was made *(settings 90kV, 160µ, standard 26 seconds).* A bit more noise becomes visible. After the area was approved, a fine scan J of was made*.* With a higher resolution (around 20µm) smaller clusters become visible, but the noise becomes more apparent as well. Some MS are visible next to each other, individual MS/few MS together are more easily distinguished when the MS density is not too high. Some ring artefacts are visible as well, these are a property of the CT scanner and can sometimes be filtered out by software.
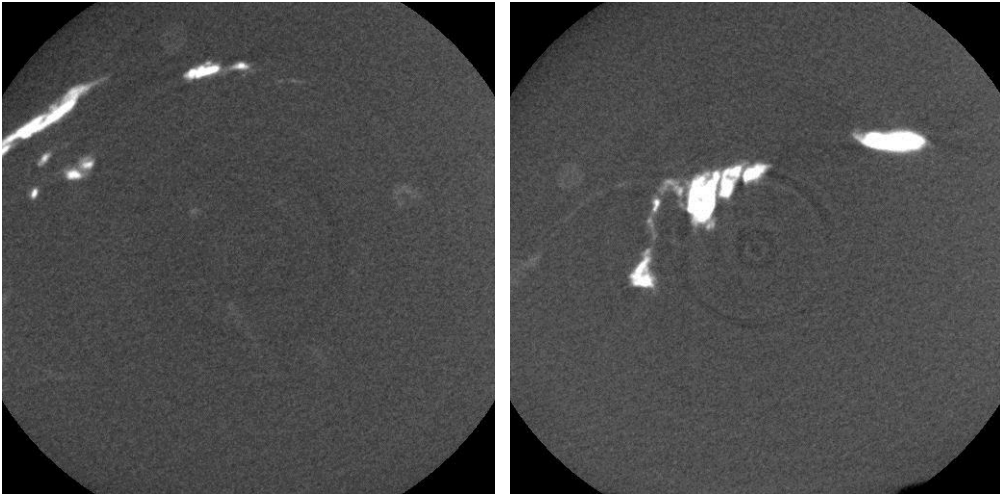

Tumour 2. Scan J. FOV 10mm scan time 3 minutes. Some large clusters and some smaller clusters are visible as well as a rope like structure. (left above MS cluster) and some ring artefacts.

Because the tumour was moved during the inzooming process (flipping over, while moving the platform to the side to concentrate on the region of interest in the middle of the scanner) an overall scan K was made for orientation to create an overview. (*FOV 60mm, scan time 4,5 min*) The images of these scan are already shown in scan B.

After the orientation scan K was made, scan I was made *(FOV 5 mm, 3 min).* A field of view of 5 mm provides a resolution of about 10µm! On these scans clusters of MS were clearly visible, but individual MS in the MS cloud were difficult to discover due to the huge amount of noise.
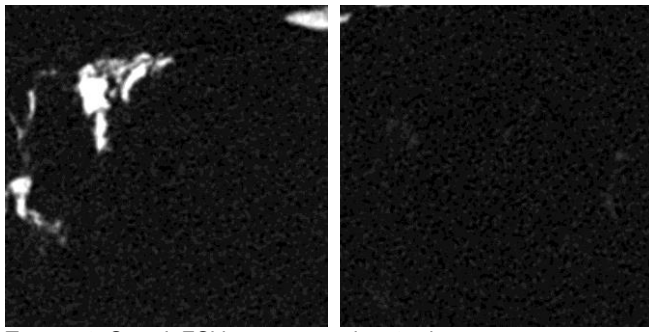
Tumour 2. Scan I. FOV 5 mm, scan time 3 min.
Some clusters are visible. Also a lot of noise can be seem in the remaining area.
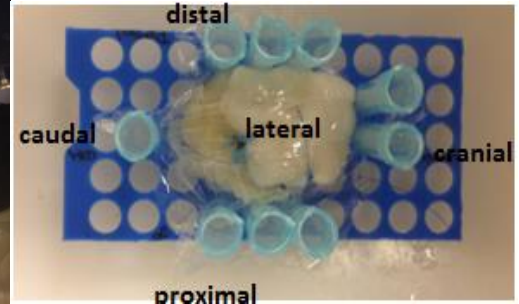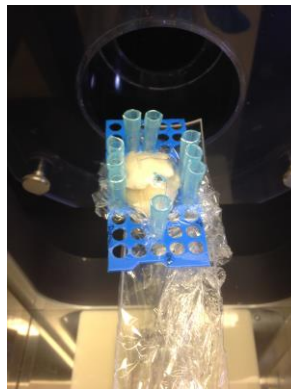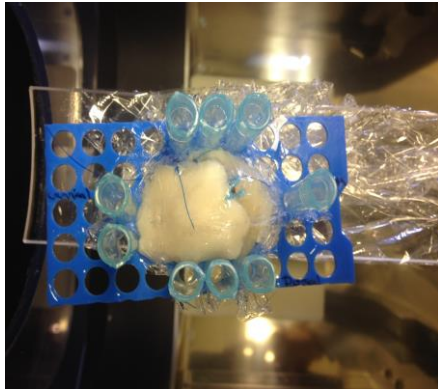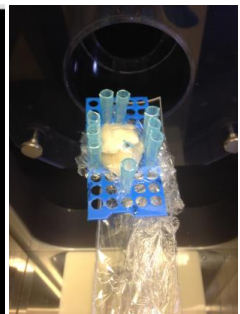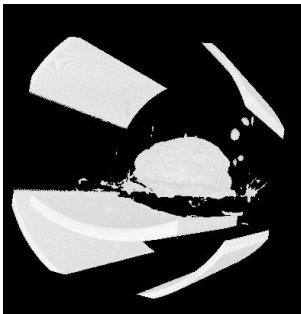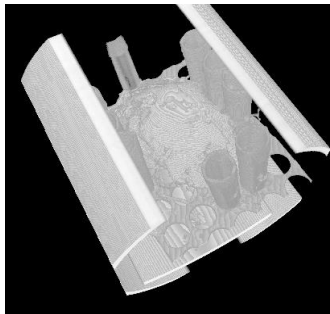

Tumour 2 in the scanner

Image F. Tumour 2 FOV 60mm.
First image: Threshold 155. Second image: Threshold 215. Third image: Threshold 255.
On the first image the complete setting can be visualized.
On the second image some strange white spots can be seen in the air, these are most probably some formalin drops.
On the third image some sutures and some white spots indicating MS clusters are visible.
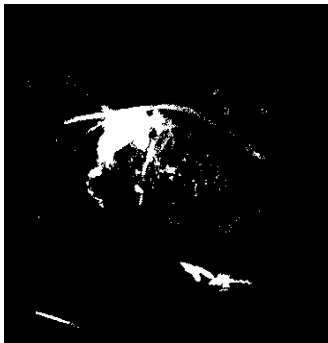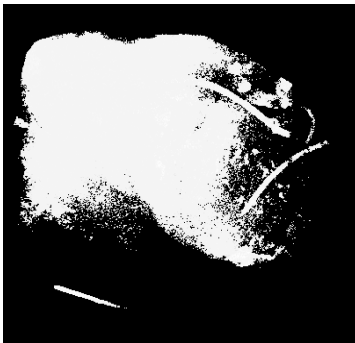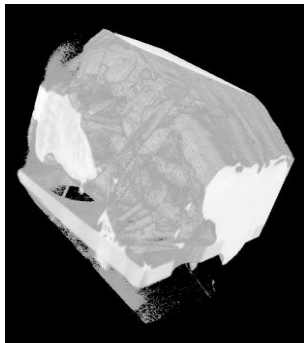

Image G Tumour 2 FOV 20mm.
First image: Threshold 167. Second image: Threshold 243. Third image: Threshold 253.
On the first image the edges of the tissue are visible. In the second image still a lot of tissue is present, some sutures are visible on the right side. On the third image a large white cloud is visible, but it is not clear whether this are all MS or that there is also some light tissue in between.
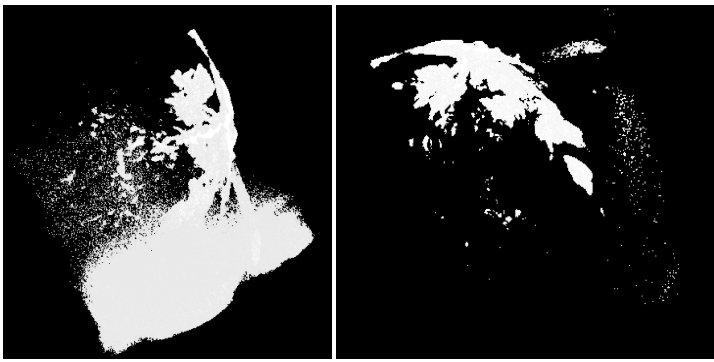
Image H Tumour 2 FOV 10mm
First image: Threshold 132. Second image: Threshold 243.
On the first image a large MS cluster may be seen, as well as a ropelike structure and a lot of scattering from some tissue. On the second image the MS cluster is more separate visible.
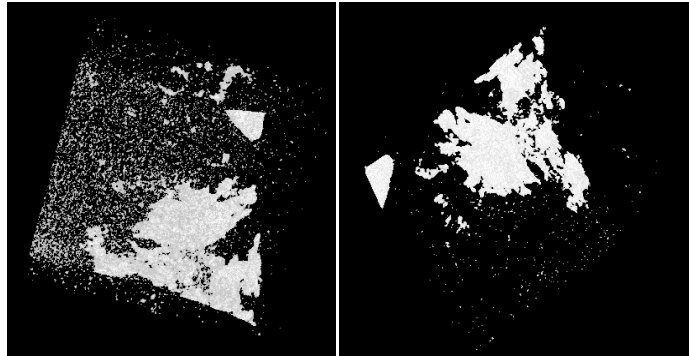

Image I Tumour 2 FOV 5mm.
First image: Threshold 190. Second image: Threshold 216.
A large accumulation of MS can be visualised at the edges of the image.
Some small white spots are distributed throughout the whole 3D reconstruction, most probably indicating a lot of noise, but it cannot be excluded that these might be some MS as well.

Scans of Tumour 3

This was the largest tumour with the broadest grid. Scan A was made to provide an overview of the whole setting. (*Settings: voltage 90 kV, current 80 µA, CT 160 live 80, FOV 60mm, scan technique standard 17 sec)*

Hereafter a more precise scan B was (*voltage 90 kV, current 160 µA, FOV 60 mm, scan technique 2 min)* This scan provided a good quality image of the whole setting, making everything in the setting visible (platform, pipette tips, cling film (or formalin on the cling film) and tumor). Some white clusters, containing the holmium microspheres, were visible as well. The only disadvantage is that it is not clear what tumor tissue is and what normal tissue, due to the little to no contrast between these tissue types.

Hereafter the field of view was narrowed and concentrated to an area were white spots were seen, this was scan C. (*FOV 24 mm)*. These settings showed a heterogeneous distribution of holmium microspheres in a kind of microsphere cloud. Furthermore, an area with a very dense concentration of microspheres (MS) could be seen on the upper side of the image. It looks more like a plaque of microspheres instead of an injection site. A dark spot could also be seen on the bottom side, which is most probably some air.
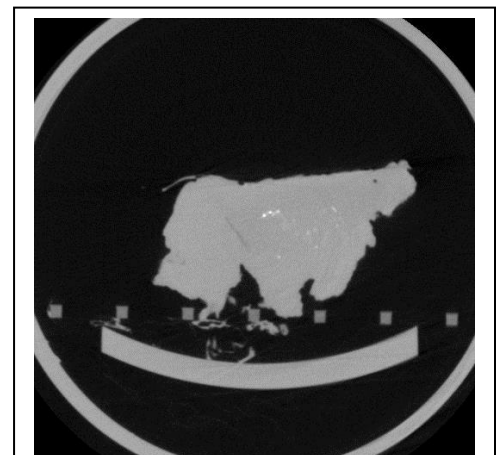
Tumour 3. Scan B. FOV 60 mm, scan time 2 minutes. Whole setting is visible.
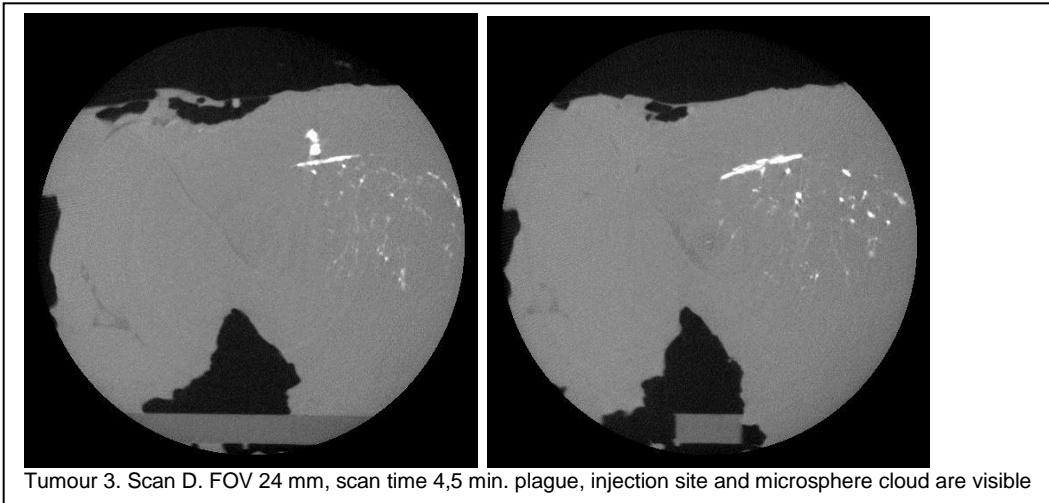
Tumour 3. Scan D. FOV 24 mm, scan time 4,5 min. plaque, injection site and microsphere cloud are visible

Scan D was made (*FOV 24mm and a scan technique of 4,5 min)* It became clear that a higher resolution also causes more noise in the tissue, when the scan time is insufficient (in this case 17 seconds). A longer scanning time results in longer reconstruction time.

After these scans, the tumour (including platform) was pushed to the left to put the region of interest (ROI) more to the centre of the scanner (the only area the scanner can zoom in to). First a rough scan E was made for orientation (*FOV 40mm, scan time 17 sec*). This scan showed that the MS seem to be located under the injection site, in a large plaque and furthermore that the microspheres seems to distribute heterogeneously through the (tumour)tissue. This is in agreement with the MRI images on which is visible that the microspheres distribute between the skin and the tumor.

The tissue was moved by moving the scanner table to the right and scan F was made. *(FOV 20mm and scan technique standard 17 sec)* It became clear that the tissue was moved in the opposite direction (the scanner mirrors the images) and now even more tissue was out of view.

The table was moved in the other direction and scan G was made to see if the tissue was moved in the proper direction now. *(FOV 40 mm and standard scan time of 17 seconds)*
On scan G, it became clear that the tumor was located correctly, so a fine scan H was made. (*FOV 10mm with scan technique fine 3 min)*
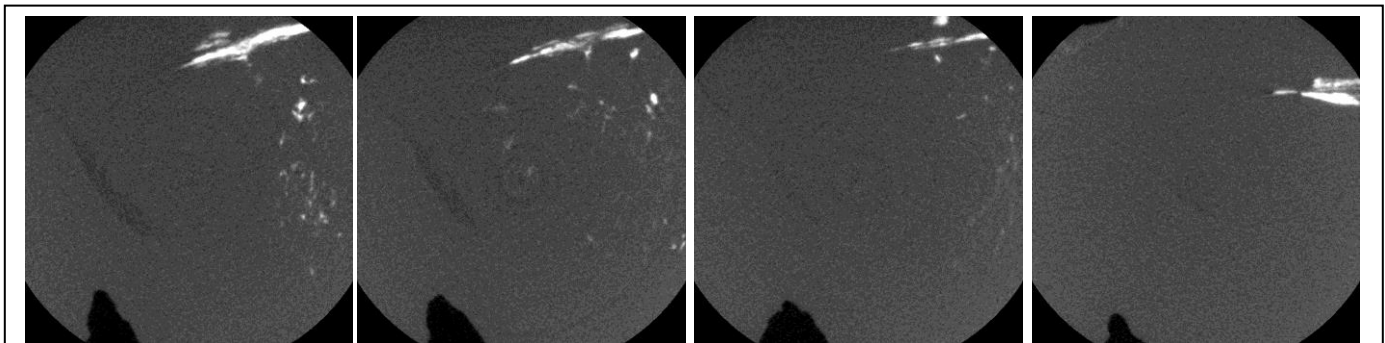
Tumour 3. Scan H. FOV 10 mm, scan time 3 minutes, plaque and MS cloud visible

Position of tumour 3 and platform in the scanner.

At the 3D reconstruction of tumour 3 the microspheres become visible only after a huge enlargement of the threshold at FOV 60mm. It can be seen that the MS are distributed in different ways, one layer of MS can be seen as well as MS diffused in a more extended way. Again the two purple sutures are clearly visible in the reconstruction.
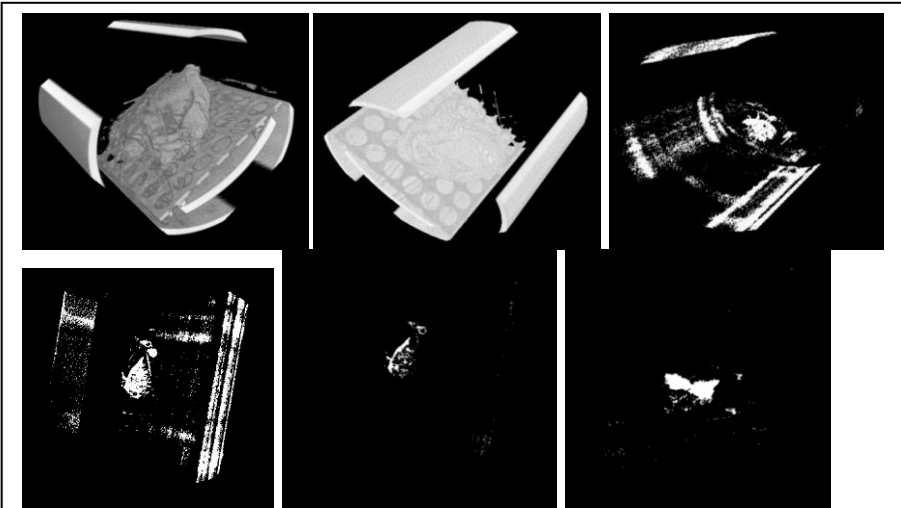

Image J. Tumour 3 FOV 60mm.
Threshold 88. Threshold 180. Threshold 246. Threshold 248. Treshold 253.
Two sutures are clearly visible on the last images.

At the FOV of 24mm the first and last images are missing in my dataset, therefore the 3D viewer displays a massive cylinder with no tissue, grid, MS or whatsoever visible. Therefore the image sequence was decreased from picture 66 till picture 473, including 372 images. With this smaller dataset it was possible to make a 3D reconstruction that did show all the elements of the setting. At the reconstruction of the images of FOV 10mm. A thick layer of MS becomes even more prominent. Under this layer some separate MS clusters can be seen. Individual MS are not visible.
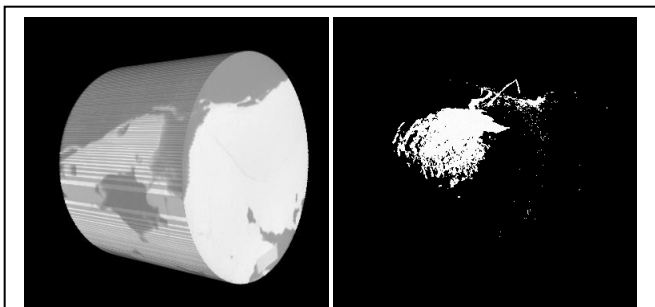

Image K Tumour 3 FOV 24mm.
Threshold 0. Threshold 247.
A suture can be seen, as well as a MS cloud under a region with a slightly more dense MS distribution. (adapted stock size)
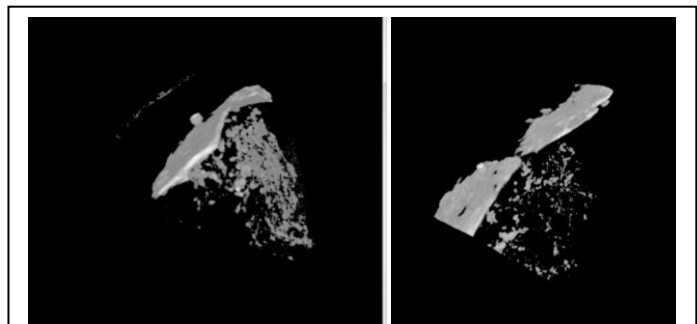
Image L Tumour 3 FOV 10mm.
Threshold 133. Threshold 148.

Substudy 4: CT

Introduction

The three tumours are attached to their grid and have been stored in formalin First the tumours were washed in saline solution to remove any excess formalin and the remaining moisture was dried by paper towels. Thereafter the tumours attached to their grid were positioned in a clinical CT scanner and scanned in whole, see image 1. The resulting images were evaluated using ImageJ.

Image 1: positioning of the tumours

Tumour 1   Tumour 2   Tumour 3

The biodistribution of holmium loaded microspheres in intratumoural injections   Sabine A. Peters   Master Research Internship

33

Scans of Tumour 1

Tumour 1: ROI 1
Left image: CT scan
Right images: microCT scan
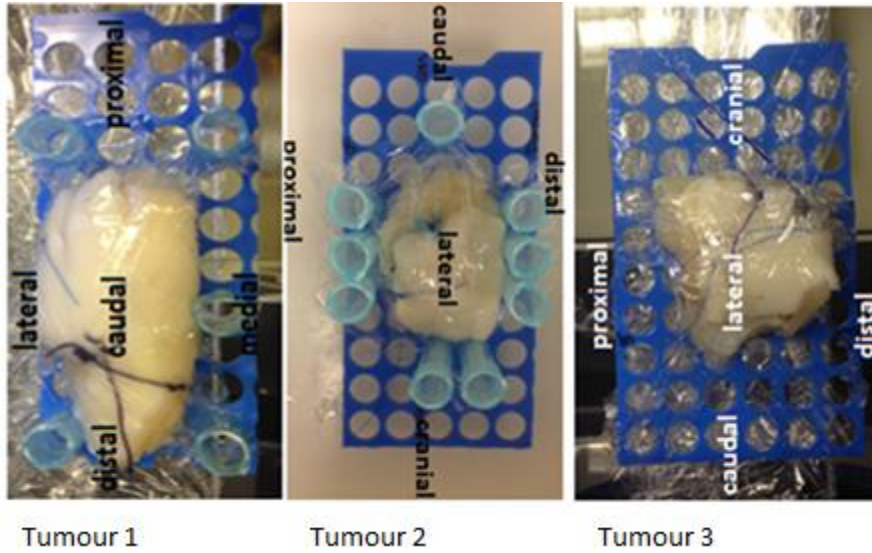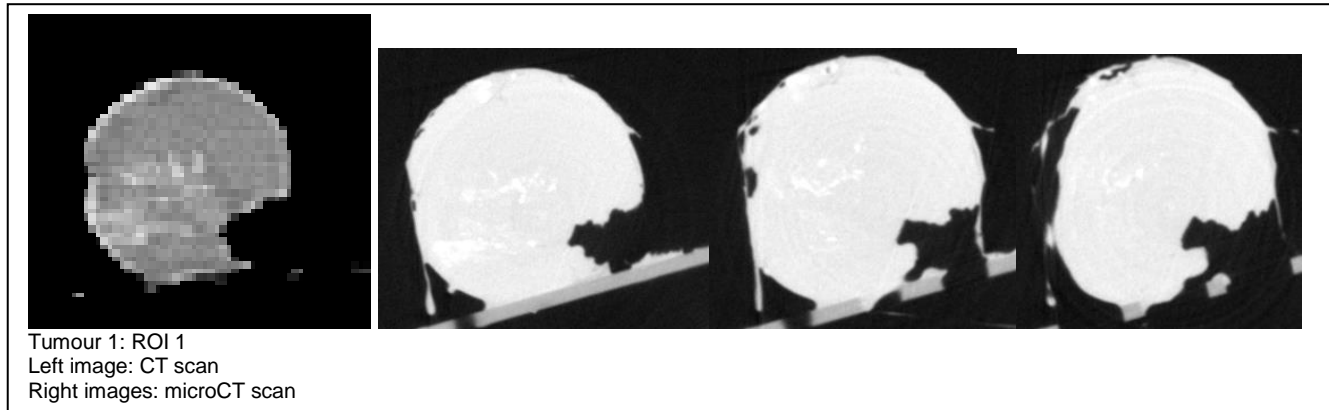
In tumour 1 scans, the holmium depots are not in the vicinity of the pipette tips, making it difficult to pinpoint their location. The contour of the tumour was used in this tumour to locate the exact placement. In these images, because the holmium is scattered, it proved more difficult to find the holmium in images without c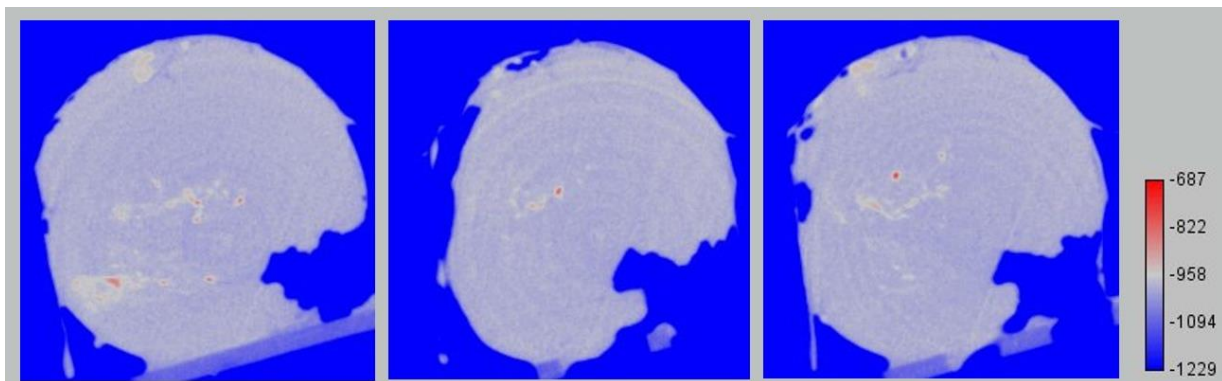ontrast through the ImageJ programme. It is clear that the scatter pattern of the three microCT images does fairly compare to the CT image.

In the image above, a concentration/dose map has been made using Image J with the microCT images. The redness indicates more concentration of holmium microspheres. The exact concentration cannot be attributed yet because the values are unknown. For example: -700 equals 1 mg Ho-MS per volume unit. The values need to be calibrated first for this to be possible. The concentration/dose image does prove once again that the holmium microspheres are in the redder areas. The brightness of the Ho-MS in the microCT images above clearly do not indicate the concentration of Ho-MS, because they are all white but in the image below only a few dots are red.

Scans of Tumour 2

Tumour 2: ROI 1
Left image: CT scan
Right images: microCT scan
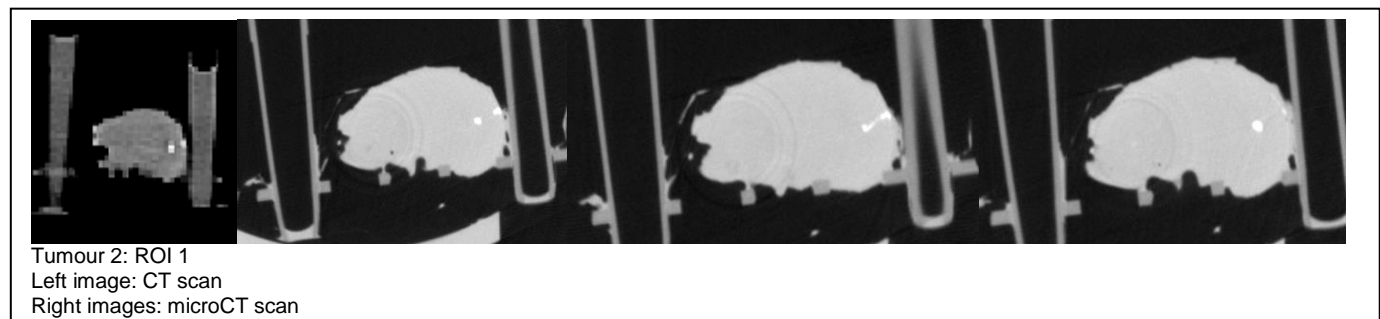
The first region of interest (ROI 1) was found by matching the tumour scans of the CT and microCT. The pipette tips were a useful marker to determine how far along the tumour the images were made. Holmium is visible on both CT and microCT as white spots and visually correlate well. The CT slices are 2 mm and the microCT slices are 0.6 mm. This means that the three microCT images together pr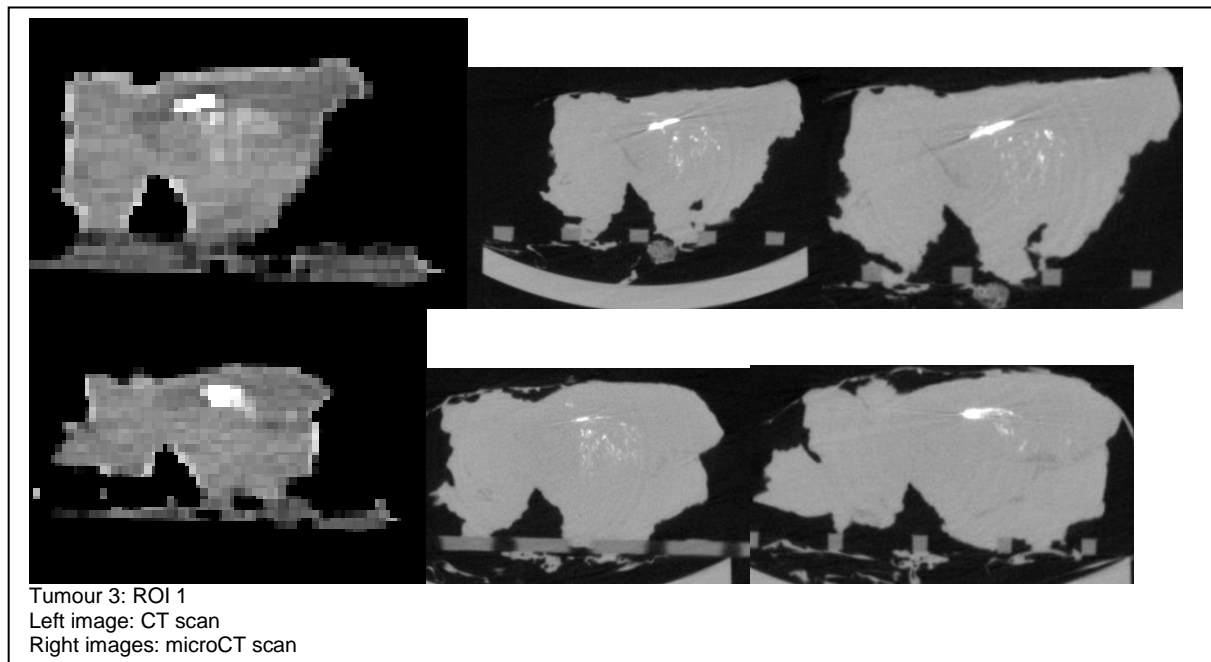oduce almost the same image on the CT. Note the added value of microCT in detecting connections or canals between holmium depots (third image)

In the ROI 2, the same is clearly evident but the depots are presented on the CT scan as two individual spots. The region was determined also using the pipette tips and the contour of the tumour. It is visible in the microCT images, which together comprise almost the same image on the CT scan, the smaller and connecting depots are not seen.

Tumour 2: ROI 2
Left image: CT scan
Right images: microCT scans

Scans of Tumour 3

Tumour 3: ROI 1
Left image: CT scan
Right images: microCT scan
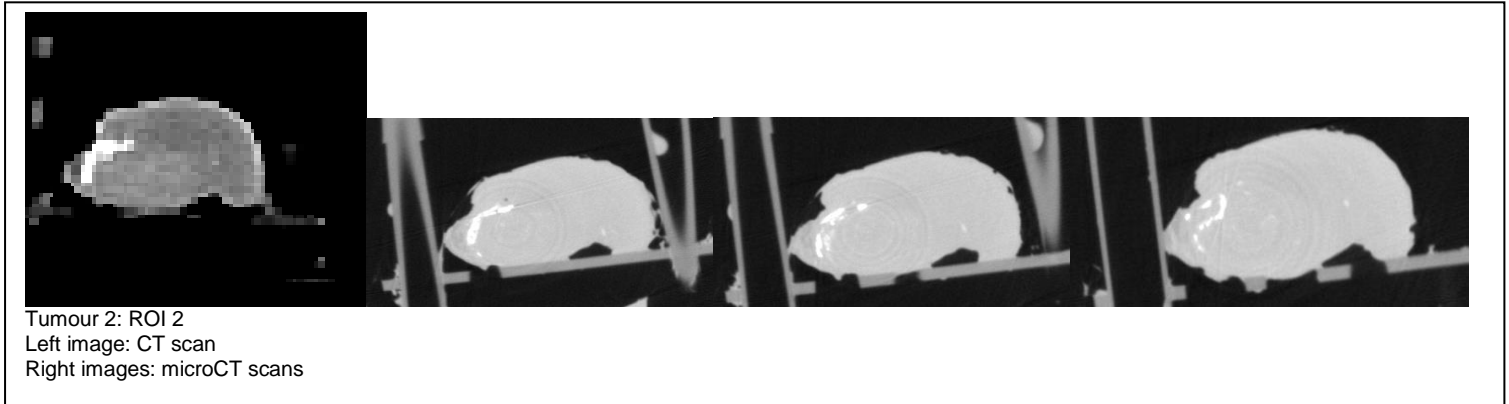
In the tumour 3 scans, point of orientations were difficult to find without the pipette tips. This only left the tumour contours to be used as points of reference. In both ROI's, it is evident that the holmium scatter is not clearly shown on the CT scans but are visible on the microCT scans.

Discussion

In this study, the feasibility of different image modalities to compare and illustrate the tumour and the biodistribution of holmium microspheres are evaluated.

Markers
Firstly, the use of markers to provide orientation of the tissue was tested in Substudy 1. The conclusion of these experiments is that paraffin and agar provided too little contrast to the tissue, so these cannot be used as markers themselves. However, the agar appeared more useful than paraffin because it was much easier to cut through, it had better fixating properties and transparency. Radio dense catheters pointed out to be the most suitable markers, because of the visibility on CT, by eye, the minimal dispersion and it is easy to use. As a conclusion to this Substudy, we used a blue plastic platform with holes in uniform design to fixate the tumour tissue upon. The use of air and plastic was theorised to be a better alternative, but because of time limitations, these were not tested beforehand and this setup was used in the subsequent substudies.
The grid, making use of a simple pipette tip tray, some cling film and cut pipette tips were sufficient in holding the tumour fixated in the same position. All the elements were visible on the scan images, which means that the grid can be used as a location marker to orientate in the 3D pictures. Maybe a finer grid would be more appropriate and some vertical markers may be handy as well (although the fixation properties should be kept in mind). Another aspect that should be taken into account is that the grid placed under the tumour should not be (much) larger than the tumour itself, because of the enlarging properties of the µCT scanner (only able to zoom into the centre of the scanner). To make an orientation in the µCT images (and MRI images) a marker can be placed on, for example, the proximal side of the tumour.

In vivo MRI
The MRI is used to give an anatomical overview of the tumour tissue in vivo and the (micro)CT for the imaging of the holmium-166 loaded microspheres on a very detailed level.
The in vivo MRI scans were obviously made when the tumour was still in the rabbit and not fixated upon the grid. The problem with this imaging is that the holmium microspheres can be found in huge clusters, but there is no detailed distribution visible because of the noise at a small resolution. This diminishes the visibility and shows the clusters as black clouds. The in vivo MRI scans prove to be useful to see if the injections were placed correctly, because tumour tissue is greyer than the surrounding tissue and the agar and bone are (nearly) white. This technique is however not useful for the mapping of the microspheres and their distribution. The volume measurement was quite difficult to perform and did not give accurate values. This may because the tumour and skin had the same colour which made the interpretation of the scans more difficult. It is better to calculate the tumour volume in an ex vivo MR image.

Ex vivo microCT and CT
The use of computer tomography is examined in microCT and CT imaging. The microCT images prove to be more detailed as thought, but the CT images do give a good overview of the holmium in the tumour and may be used in the clinic.
Holmium microsphere clusters are clearly visible on micro computed tomography images. The microspheres cannot be seen individually, because the noise at a small resolution is too great to see these. The contrast of the tissue is determined by the amount of tissue and air in the scan images. E.g. the CT intensity of the tissue was more dense when some air was also visible on the image. This made the MS more difficult to see on the images with FOV 60mm than on images with e.g. FOV 20mm. This is not a problem for the visualisation of the distribution of the MS, because a rough distribution is sufficient for the small resolution. The precise distribution is determined with a higher resolution.
The holmium microsphere distribution differs per tumour. This makes it impossible to already describe a standard distribution model. The most probable is that the MS diffuse through the tissue in a way the least resistance is met. This path of least resistance can be between two tissue layers, like in tumour 3, but can also be branched throughout the tissue like in tumour 1.

In the interstitial space, smaller and larger clusters are formed. It seems like the MS follow each other in a train like fashion or like a tree with dispersing branches.

The use of sutures may be investigated further, as these can be seen on the images and could be used theoretically as markers.

The concentration/dose map is a good model for distribution which could be used in the near future to test if the Ho-MS concentration is adequate and evenly distributed and in the right place. A step further is to check with this dose map if the whole tumour has been injected well enough with the concentration Ho-MS for the therapy to be successful. To validate the values of the concentration/dose map, future experiments should scan samples with known concentrations to predict values. The utility of this dose map is correlated with its precision and repeatability. Another aspect that should be looked at is the dispersion of/influence on the CT signal by the different surrounding tissues (effect on the visibility of the MS?) and the possibilities to correct for these dispersions.

Before quantification of the microspheres is possible, a threshold value should be developed for the holmium. This can be done making use of standard solutions of Ho-MS in for example an agar solution. The possibilities, added value and the requirements for this technique should be investigated. It may be useful when statistical analysis has to be performed, because quantifications can be put into statistical data analysis and only qualitative analysis is hard to compare when a lot of datasets are available. The use of ex vivo MRI may provide more information about the distribution in the tumour.

Histology
The next step, histology, is a step which should be further investigated to compare the histological coupes to the images. This should be done in the same direction and angle, the so called ROI (region of interest). When the ROI's are established, it may be possible to perform the microCT in the same fashion as to keep the orientation the same. The purpose of the histology in dose study is to examine the following:
- o Biodistribution of Ho-MS in tumours;
- o Reaction of tumour and tissue to the Ho-MS
    - o Necrosis and apoptosis (reaction) with markers
    - o Proliferation (no reaction) with markers
- o Link the reactions to the MR, CT and histology images

Transport
In future experiments, the tumours should be put in water before they are transported to any kind of scanner (μCT or MRI). This has two main reasons; first, our own health; second the formalin provides extra contrast on the CT (e.g. the different visibility of the cling film on the μCT images is most probably caused by the formalin still present on the cling film) and it is feared that it also affects the MR imaging.

Conclusion

This pilot study has demonstrated that it is possible to qualify the holmium microspheres but the quantification of the Ho-MS still proves to be difficult. The magnetic resonance and computer tomography imaging are valuable image modalities for the placing of in vivo injections, mapping the gross distribution of the Ho-MS in tumour tissue and providing a preliminary and minimal concentration/dose studies.

Further research is required to optimise the mapping of the biodistribution of holmium microspheres in intratumoural injections. With new technology and knowledge, it is possible to further develop intratumoural holmium treatment in veterinary and human medicine.

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