Supplemental Information

Simultaneous spatiotemporal tracking and oxygen sensing of transient implants in vivo using hot-spot MRI and machine learning

Virginia Spanoudaki[a,b], Joshua C. Doloff[a,b,c*], Wei Huang[a], Samuel R. Norcross[a,b], Shady Farah[a,b,c], Robert Langer[a,b,c,d,e], and Daniel G. Anderson[a,b,c,d,e#]

a. David H Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Cambridge, MA 02139

b. Department of Anesthesiology, Boston Children's Hospital, Harvard Medical School, Boston, MA 02115

c. Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139

d. Division of Health Science Technology, Massachusetts Institute of Technology, Cambridge, MA 02139

e. Institute for Medical Engineering and Science, Massachusetts Institute of Technology, Cambridge, MA 02139

* JCD is currently with the Departments of Biomedical and Materials Science Engineering, Translational Tissue Engineering Center, Wilmer Eye Institute, and the Institute for NanoBioTechnology, Johns Hopkins University School of Medicine, Baltimore, MD 21231.

email: dgander@mit.edu; Tel.: +1 617 258 6843; fax: +1 617 258 8827.

Materials and Methods

Preparation of the PFCE nanoparticle emulsion

The hydrophobic nature of PFCs requires that they are emulsified prior to injection or encapsulation. Several protocols for PFC emulsification have been published, all of which are based on the mixture of PFC with a lipid and a surfactant which is then emulsified by means of sonication, homogenization, or using a microfluidizer. We have created PFCE-lipid nanoparticle emulsions by mixing 600 ul PFCE (Oakwood Chemicals, 009312) with 300 ul of 10% m/v ethanol-dissolved 1,2-Dioleoyl-sn-glycero-3-phosphocholine (Sigma Aldrich, P6354) and 2.1 ml of deionized water**.** The end solution results in 17% v/v PFCE, 1% m/v DOPC mixture which is subsequently emulsified via sonication (Q125, QSonica) at 4 steps of 1 min duration each (3W sonication power, 75% amplitude). After sonication the emulsion goes through 5 stages of solvent exchange by means of stirred filtration (EMD Millipore, 5121) in order to remove the ethanol and replace it with 0.9% sterile saline aqueous solution. The emulsion's particle size is evaluated before and after filtration using dynamic light scattering (DLS, Malvern Zetasizer).

PFCE loaded alginate solution and islet microencapsulation

Rat islets were isolated by the Joslin Diabetes Center. Islets were put into equal suspension within culture media and aliquoted such that there were approximately 500 islet equivalents per 0.4mL of alginate. Islet aliquots were washed twice with Ca-free KREBS and centrifuged at 800 RPM. Alginate (SLG20, Novamatrix) mixed with the 17% PFCE solution was then dispersed onto the pellet of islets from a 5mL syringe. The islet alginate mixture was gently stirred before being re-drawn back into a 5mL syringe. Prior to encapsulation, a crosslinking solution of 2.4% BaCl and 4.5% mannitol in a 1:4 ratio was prepared in a sterile metal dish. A 5mL syringe filled with islet-alginate mixture was affixed to a Harvard Apparatus PicoPlus encapsulator pump and suspended at approximately 1.5" above the crosslinking solution. When creating 1.5 mm capsules, an 18G blunt tip needle was attached to the syringe. When creating 0.5 mm capsules, a 24G blunt tip needle was used. A power source was attached to the base of the needle and set to 8,200 V when making 1.5 mm capsules and 5,300 V when making 0.5 mm capsules. The encapsulator pump's diameter setting was set to 12.06 mm and the flow speed was set to 0.16mL/minute. Once the syringe was properly affixed to the encapsulator, the pump and the power source were turned

on. After the capsules finished forming in the cross-linking solution, they were transferred from the metal dish to a falcon tube using 25mL serological pipettes to reduce sheering forces. The capsules were then washed twice with HEPES and twice with cell culture media before being stored in cell culture at 37 °C. Prior to being implanted into mice, capsules were washed four times with sterile saline.

Toxicity study and leakage study

Although several studies have concluded that the effect of PFC emulsions on cell toxicity and viability is minimal, this study also monitors this effect in vivo. Islet containing PFCE-loaded alginate capsules were implanted through laparotomy in the IP space of diabetic mice and their blood glucose levels were monitored through sequential blood draws via the tail vein during a period of 3 months.

The stability of the PFCE emulsion inside the alginate capsules and potential PFCE leakage was also studied. PFCE capsules of two different sizes (0.5 mm and 1.5 mm diameter) were prepared as described above. The capsules were dispersed in a mixture of saline and IP fluid and were stored at 37 °C in order to emulate body conditions. At different time intervals the capsules were imaged using fluorine MRI against a standard sample containing 100% PFCE. A ratio of the signal intensity in the capsules versus the signal intensity in the standard was compared to the expected value of 17%.

In-vitro hypoxia experiment – GSIS assay

The effect of three different hypoxic conditions (10% pO_2, 8% pO_2 and 3% pO_2) on islet survival has been assessed in vitro. PFCE-loaded, islet-containing capsules of two different sizes (0.5 mm and 1.5 mm diameter) were prepared as described above. All capsules were kept in cell culture medium (DMEM) and split into 4 groups (500 islet equivalents each). The control group was kept in an incubator at 21% pO_2 for a duration of 4 days. Since it has been observed that encapsulated islets don't survive in cell culture longer than 2 weeks, a time window of 4 days was chosen to ensure that the assay is not biased by islet death for reasons unrelated to hypoxia. The second, third and fourth groups were kept at a separate incubator (Sanyo O_2/CO_2 incubator, MCO-18M) under hypoxic conditions (10%, 8% and 3% pO_2, respectively) for 4 days each. Each day an islet glucose stimulated insulin secretion (GSIS) assay was performed in a subset of each group that measured the insulin secreted by the islets in response to a

glucose stimulus. The same experiment was performed for pure alginate, islet containing capsules that were not loaded with PFCE.

Fluorine MRI and oxygen calibration measurement

MRI imaging was performed using a 7 Tesla Varian 7T/310/ASR-whole mouse MRI system (Varian/Agilent). A dual mode ($^{1}H/^{19}F$), 38 mm (bore diameter) x 40 mm (RF length), volume coil (Doty scientific, Litzcage) was used for all the imaging studies. The field of view (FoV) is typically selected to be 50 mm x 50 mm unless stated otherwise. The data matrix was 128x128 and the slice thickness was 1 mm unless stated otherwise. Two different imaging sequences were used: A fast spin echo (TR=2000 ms, TE=12 ms, 2 averages) with a slight T1 weighting that also shows the density of the ^{1}H or ^{19}F nuclei, and a slower inversion-recovery spin echo (TR=7820 ms, echo train length (ETL)=32, TE=9 ms, 2 averages) with 15 different inversion times (TI= [0.1, 0.137, 0.187, 0.256, 0.35, 0.478, 0.654, 0.894, 1.223, 1.673, 2.287, 3.128, 4.278, 5.85, 8] s) that is used to calculate the T1 relaxation times of the fluorine nuclei. The pixel intensity (or magnitude) of every image is declining for increasing TI until the "null point", TI_{null} for which the pixel intensity becomes zero. Further increasing the TI beyond the null point results in increased pixel intensity which becomes constant for very high TI values. The pixel intensity value for every pixel in the data matrix is plotted against the TI times. The plot (henceforth referred to as inversion time, TI, plot) is fitted with the following equation:

$$y = |a * (1 - b * e^{-\frac{x}{c}})| \qquad (1)$$

where y is the pixel intensity, x is the TI and the fit parameter c is T1, the PFCE relaxation time.
In order to map the PFCE T1 relaxation times, as calculated by MRI, to the different oxygen values a number of calibration curves were created ex vivo. PFCE-loaded, islet containing capsules of two different sizes (0.5 mm and 1.5 mm diameter) were prepared as described above and placed in Eppendorf tubes filled with saline. For each capsule size, five different samples were created with each sample being continuously flushed for at least 2 hours with balanced nitrogen gases of a different pO_2 concentration: 80%, 60%, 40%, 21%, 8%, 3%, and 1%. Oxygen partial pressure values were confirmed

using a portable optical-based oxygen microsensor inserted in a needle (PreSens, GmbH). The samples were imaged using the above pulse sequences with fluorine MRI at a temperature of 37 °C to emulate body conditions. The R1 relaxation rates (reciprocal of T1 times) corresponding to each sample were plotted against their known pO_2 value. Linear fits were applied to the plots and the slope and intercept were derived.

In-vivo hypoxia experiment – MRI imaging

All animal experiments were performed in accordance to detailed IACUC protocols as reviewed and approved by the Committee on Animal Care of the Massachusetts Institute of Technology. 10 diabetic (streptozotocin, STZ induced, Jackson Laboratories) and 10 healthy adult C57BL/6 mice were used in the in-vivo experiments. In each group of 10 mice, 5 of them had 1.5 mm capsules and the other 5 had 0.5 mm PFCE loaded capsules implanted. The volume of implanted capsules in each mouse was 400 ul which corresponds to approximately 500 islet equivalents per mouse. Diabetic mice were implanted with islet-containing capsules as described above, while healthy mice were implanted with equal volumes (400 ul per mouse) of empty capsules (no islets). At the day of imaging the food was retrieved from the mice for 2 hours and the glucose level of each diabetic mouse was monitored by acquiring a blood sample from the tail vein. During imaging mice were anesthetized with 2-3% isofluorine mixed with medical air (21% pO_2). The mouse breathing rate was maintained at approximately 20 respirations per minute in order to mitigate breathing motion artifacts in the reconstructed image. The vitals of each animal under anesthesia (temperature, breathing rate, heart rate) were continuously monitored. A warm air outlet maintained the temperature in the mouse bed at 30 °C. The animals were imaged with both the fast and the slow pulse sequences described above for a total imaging time of 25 min per mouse.

In-vivo hypoxia experiment – Optical oxygen microsensor

The sensor (PreSens, GmbH) was placed at 5 different locations in the IP space of mice (n=5) in the supine position. Using ultrasound guidance, we inserted the oxygen microsensor 5 mm deep into the IP space and recorded pO_2 measurements after the sensor readings were stabilized at each location in the IP cavity.

Blood glucose monitoring

Blood glucose levels for all diabetic mice studied were estimated using a commercially available, handheld glucometer (Clarity One). Prior to glucose measurements, mice were fasted for approximately 2 hours at approximately the same time of the day, after which a small volume of blood was collected from the tail. Blood sampling and measurement was repeated at least twice. Prior to any treatment the glucose levels of diabetic mice were confirmed to be consistently above 400 mg/dl for approximately 2 weeks.

Fibrosis quantification at terminal time point (ex-vivo)

Capsules were retrieved from euthanized mice 3 months after implantation. 100uL of capsules from each mouse were separated into 2 mL Eppendorf tubes and frozen over night at -80 ºC. The following day, 100 uL of water was added to each 100 uL sample of capsules which were then sonicated at 50% amplitude for three 10 second increments with a 10 second pause in between each increment. While being sonicated, the Eppendorf tubes were kept on dry ice to ensure the capsules remained frozen until the moment of sonication. After the samples were fully sonicated, 100 uL of 37% HCl was added to each one. Each was then vortexed, wrapped in parafilm and heated at 105 ºC for 2 hours. The samples were cooled to room temperature and then centrifuged at 13,000xg for 2 minutes.

50uL of supernatant was collected from each sample and loaded into separate wells of a 96 well plate in duplicate. Hydroxyproline standards were created by diluting a 1 mg/mL standard with DI water to generate 0, 0.2, 0.4, 0.6, 0.8 and 1ug/well standards. 50 uL of each standard were loaded into the 96 well plate in duplicate. The plate was then incubated at 60 ºC overnight to dry the samples in each well. The following day, a Hydroxyproline Assay kit (MAK008, Sigma-Aldrich) was used to prepare the samples in each well for quantification. To each well, 100 uL of chloramine T oxidation buffer was added and the plate was incubated at room temperature for 5 minutes. Next, 100 uL of diluted DMAB reagent was added to each sample and standard well and the plate was incubated for 90 mins at 60 ºC. Following this incubation, a plate reader was used to measure the absorbance of each well at 560 nm. The value collected from the blank standard was used as a background reading and subtracted from all readings.

To calculate the hydroxyproline in each sample, the readings from the standards were used to create a standard curve from which the amount of hydroxyproline in the experimental samples was calculated.

Data visualization, capsule clustering, and statistical analysis

All MRI images were acquired using the Vnmrj software (Agilent). The raw data was converted to DICOM format and was visualized using MATLAB. The T1 maps were calculated for every animal and every time point on all imaging slices using a custom-made software developed in Julia (https://julialang.org). The T1 maps were subject to edge detection to suppress noise. Region of interest analysis, and quantification were performed in MATLAB. Capsule dispersity was evaluated by calculating the position r of each pixel in a 2D MRI image that corresponds to capsule signal using the following equations for 128x128 pixel (50 mm x 50 mm) images:

$$x = (pixel_x - 65) * 0.39mm + 0.195mm$$
$$y = \left(pixel_y - 65\right) * 0.39mm + 0.195mm \qquad (2)$$
$$r = \sqrt{x^2 + y^2}$$

The inter-quartile-range (iqr) was calculated as the difference between the 25th and the 75th percentile of the r distribution. Classification of capsules into spatial groups (or clusters) was performed in MATLAB and Python using either hierarchical (single linkage) or density-based (DBSCAN) clustering algorithms based on the euclidean distance criterion and a minimum pixel neighborhood of 3 pixels. The classification quality of every pixel i in each image slice was assessed using silhouette scores for every image slice per the following equation:

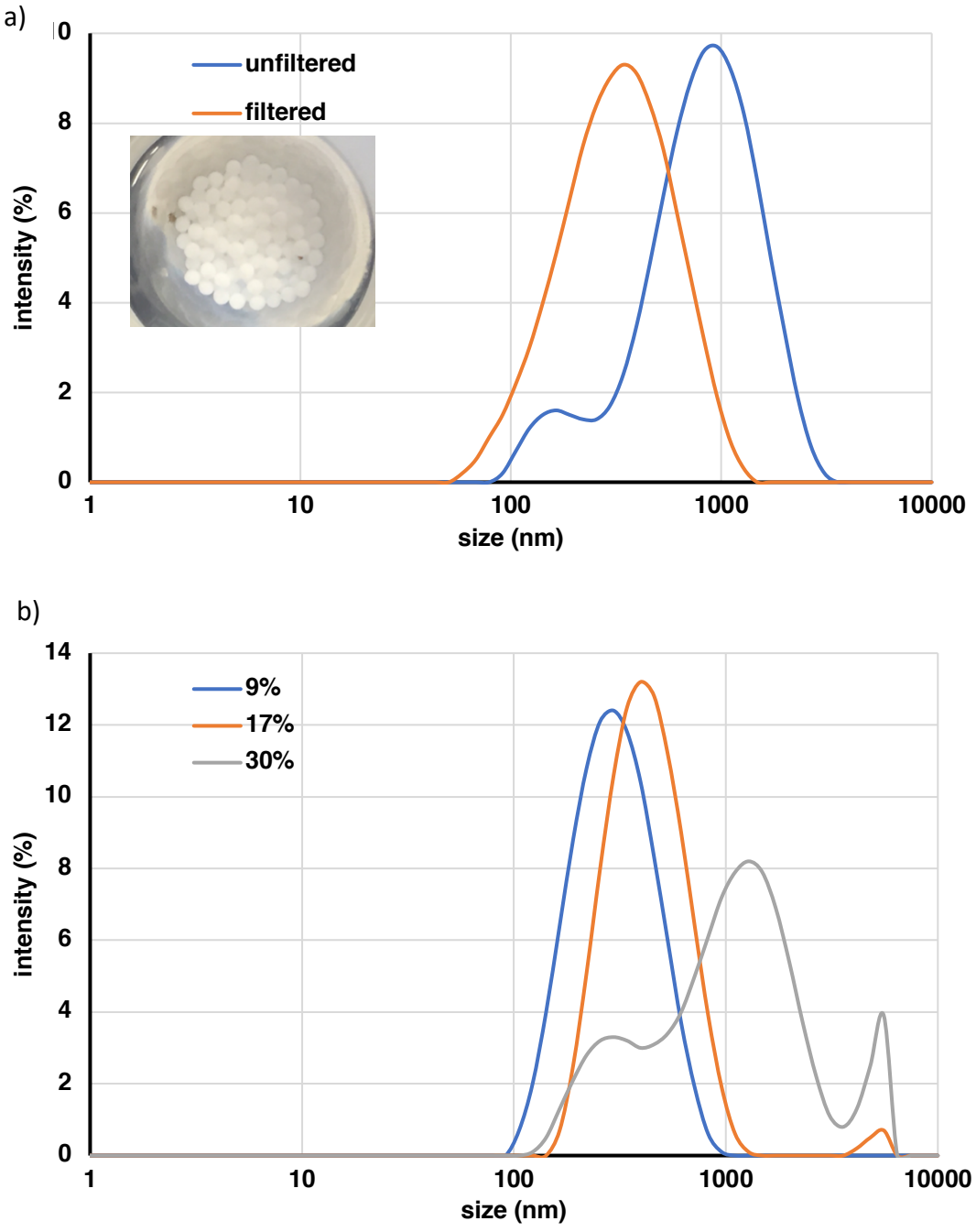
$$silhouette\ score = \frac{bi-ai}{\max(ai,bi)} \qquad (3)$$

where ai is the average distance of pixel i to every other pixel in the same cluster and bi is the average distance of pixel i to every other pixel of different clusters.

Statistical analysis was performed using RStudio. Statistical significance was assessed with Student's t-test or Wilcoxon non-parametric test and results with a p-value smaller than 0.05 were considered statistically significant.

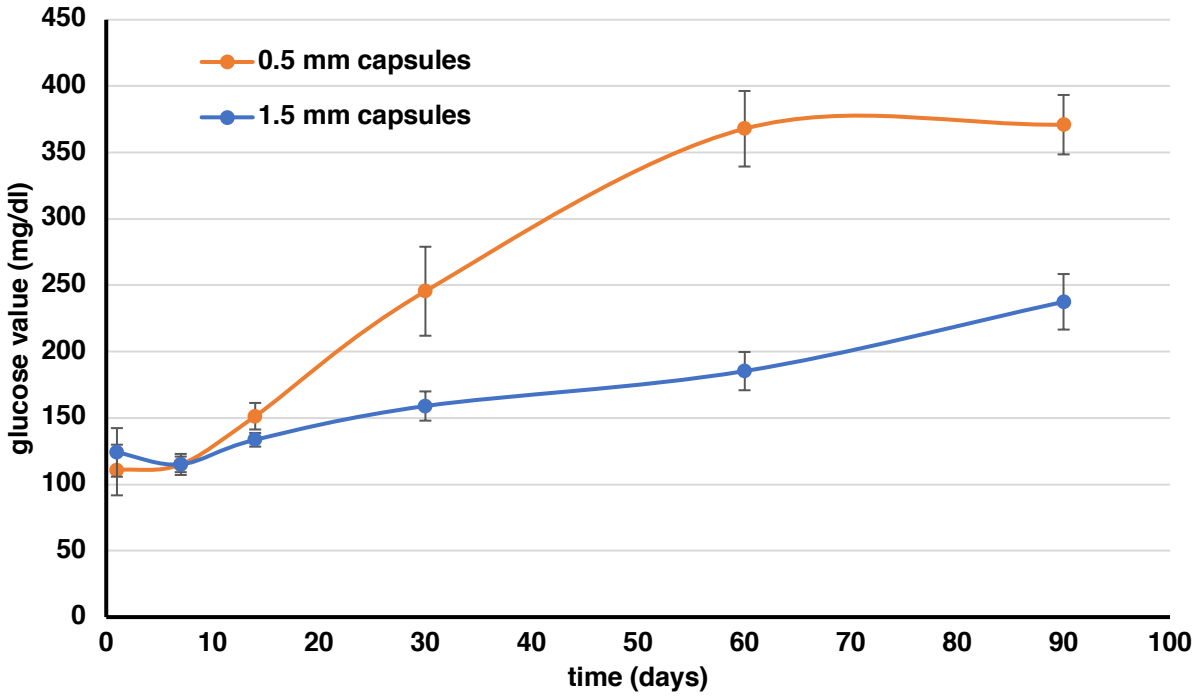
Supplemental Figures

DLS results



Supplemental Figure S1. Dynamic light scattering measurements of the PFCE emulsion for a 17% v/v PFCE concentration before and after filtration of a 3 ml volume using stirred filtration (top) and for three different PFCE concentrations (9%, 17%, and 30% v/v) (bottom). After filtration, we measure lipid nanoparticles with a diameter between 400 and 600 nm. Measurements were repeated at least twice.

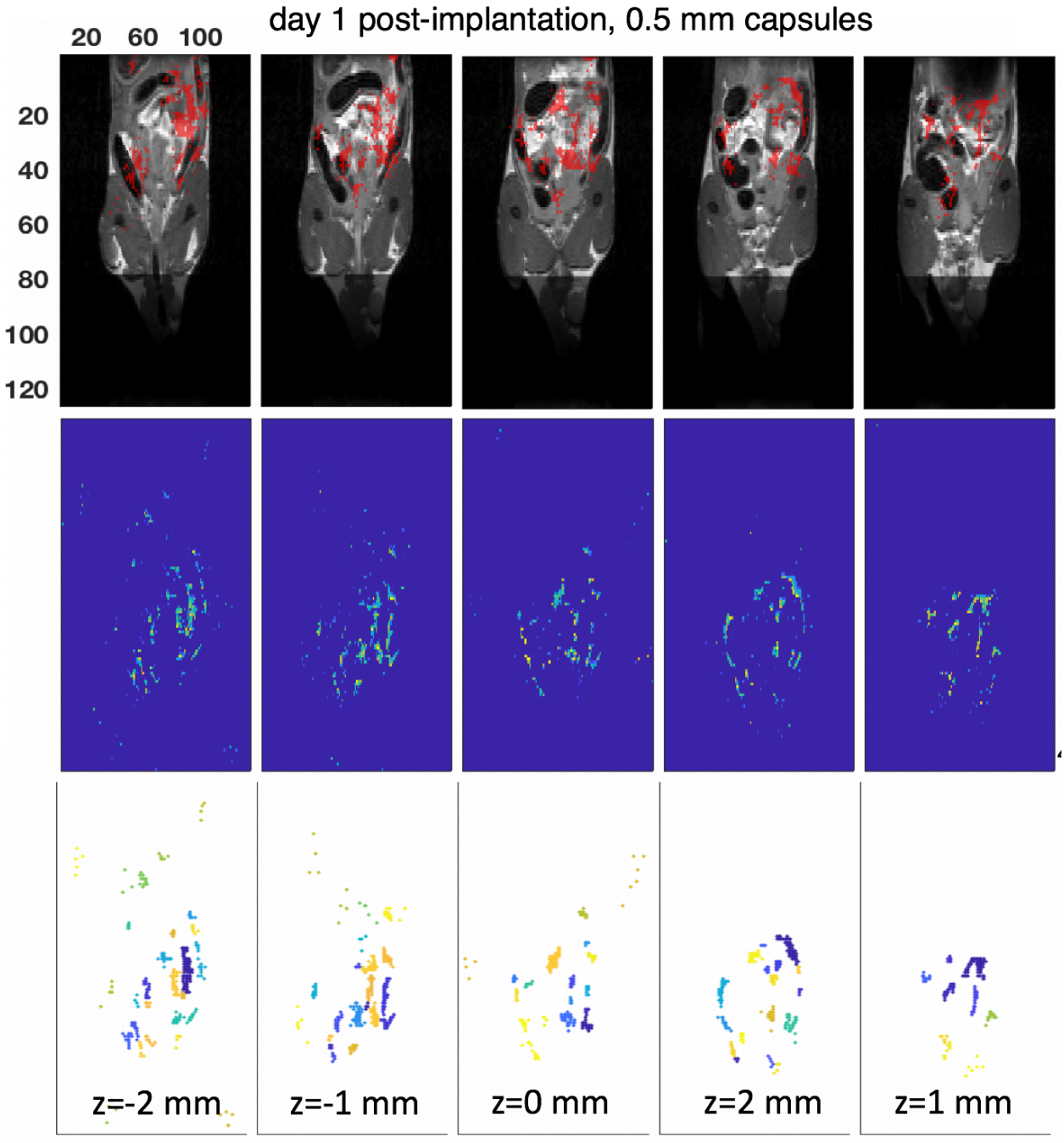
Glycemic control



Supplemental Figure S2. Average blood glucose trends for 0.5 mm (orange) and 1.5 mm (blue) capsules implanted in diabetic mice for a period of 3 months. N=15 for all data points and experiments were repeated at least twice. Values are mean ± s.e.m.

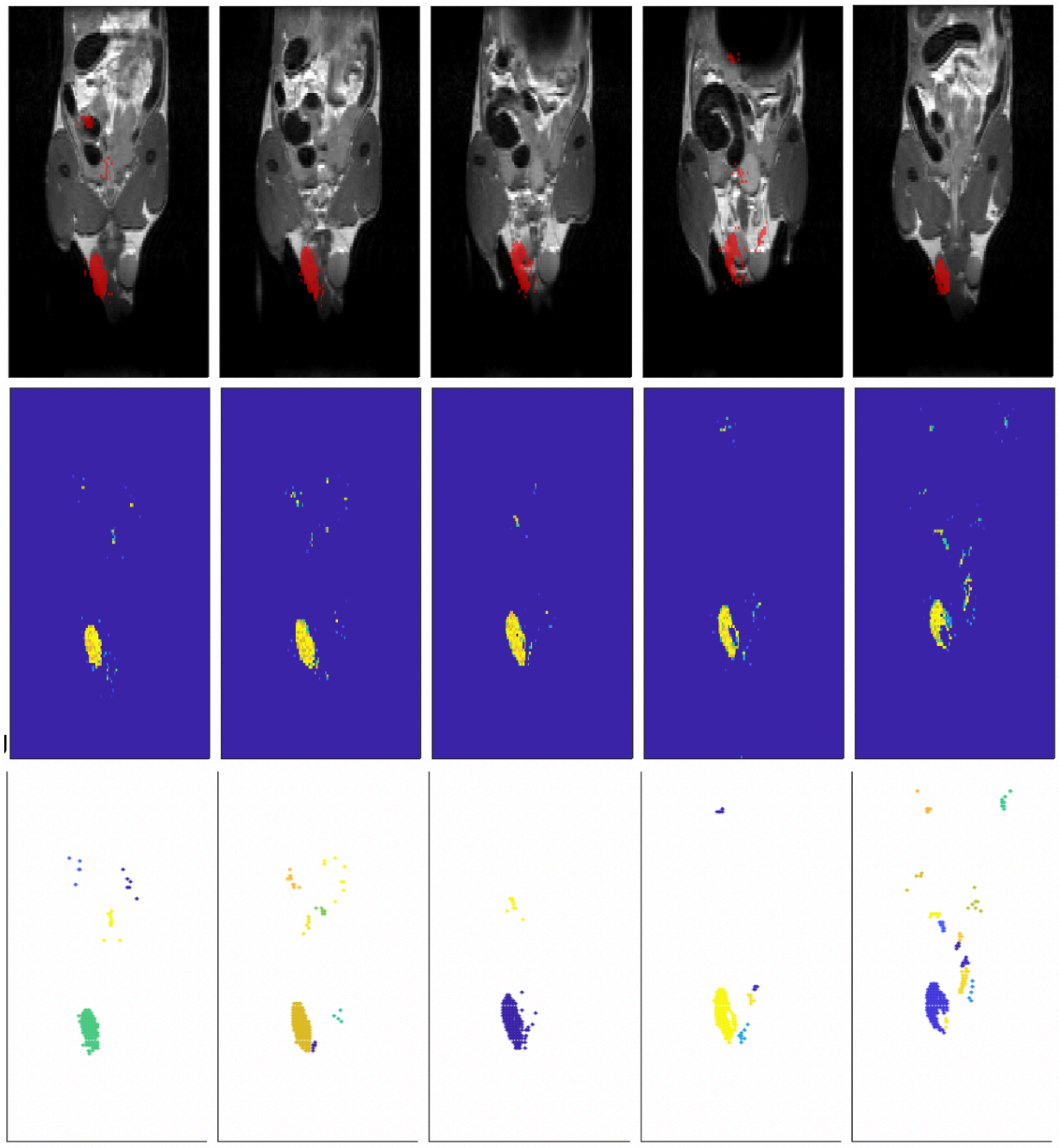
in vivo MRI imaging

a)

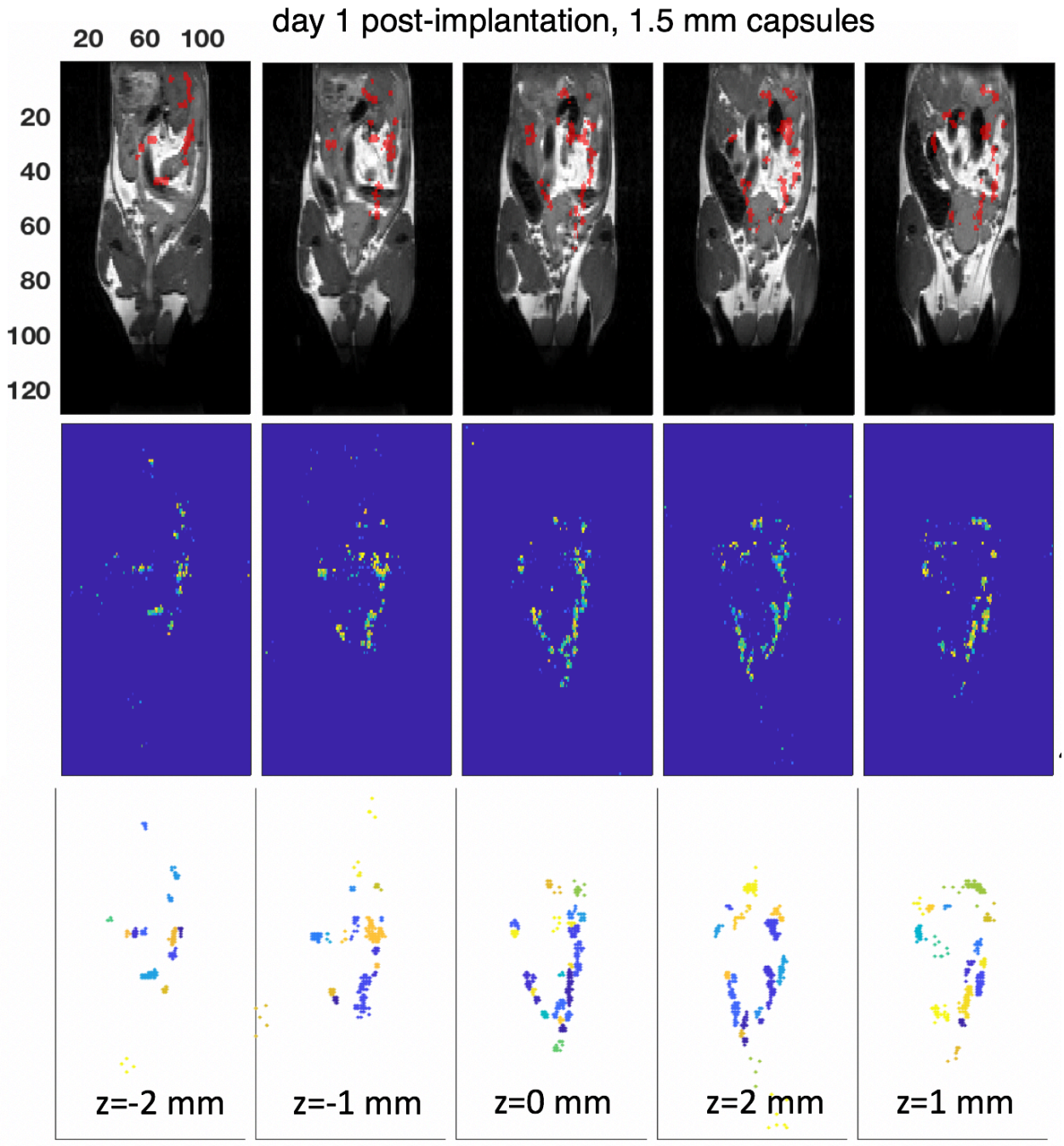


b)

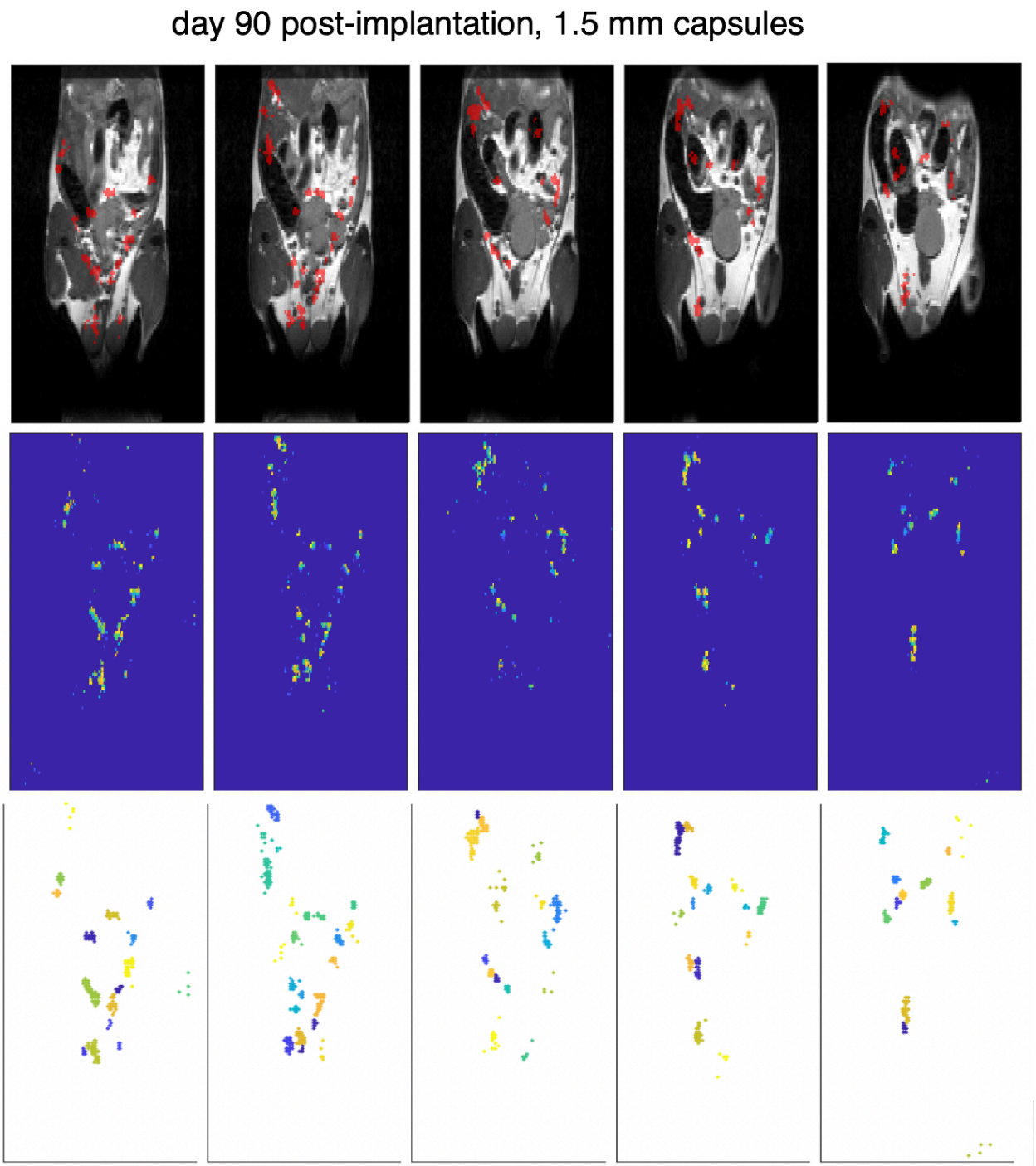
day 90 post-implantation, 0.5 mm capsules



c)

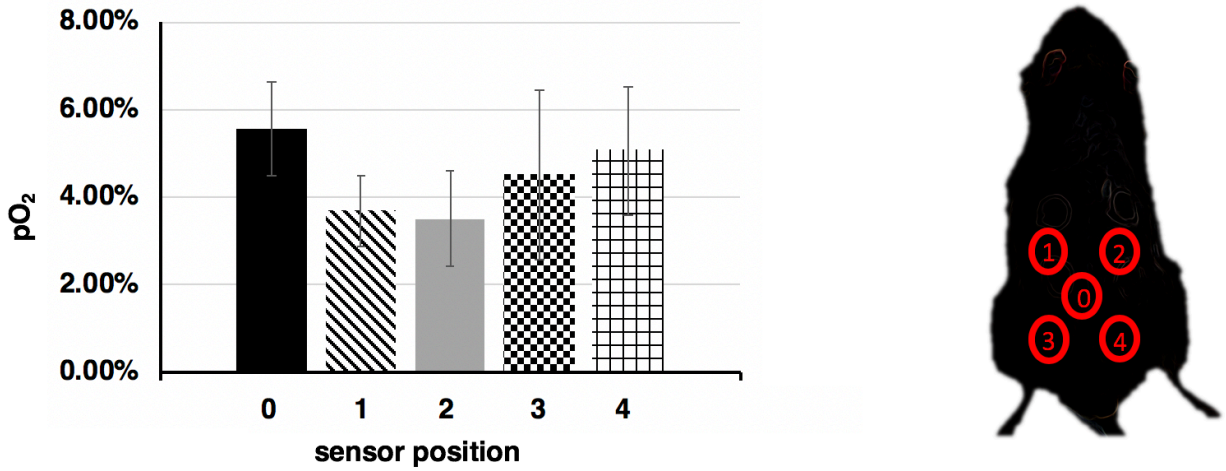

day 1 post-implantation, 1.5 mm capsules
20 60 100
20
40
60
80
100
120
z=-2 mm
z=-1 mm
z=0 mm
z=2 mm
z=1 mm

d)



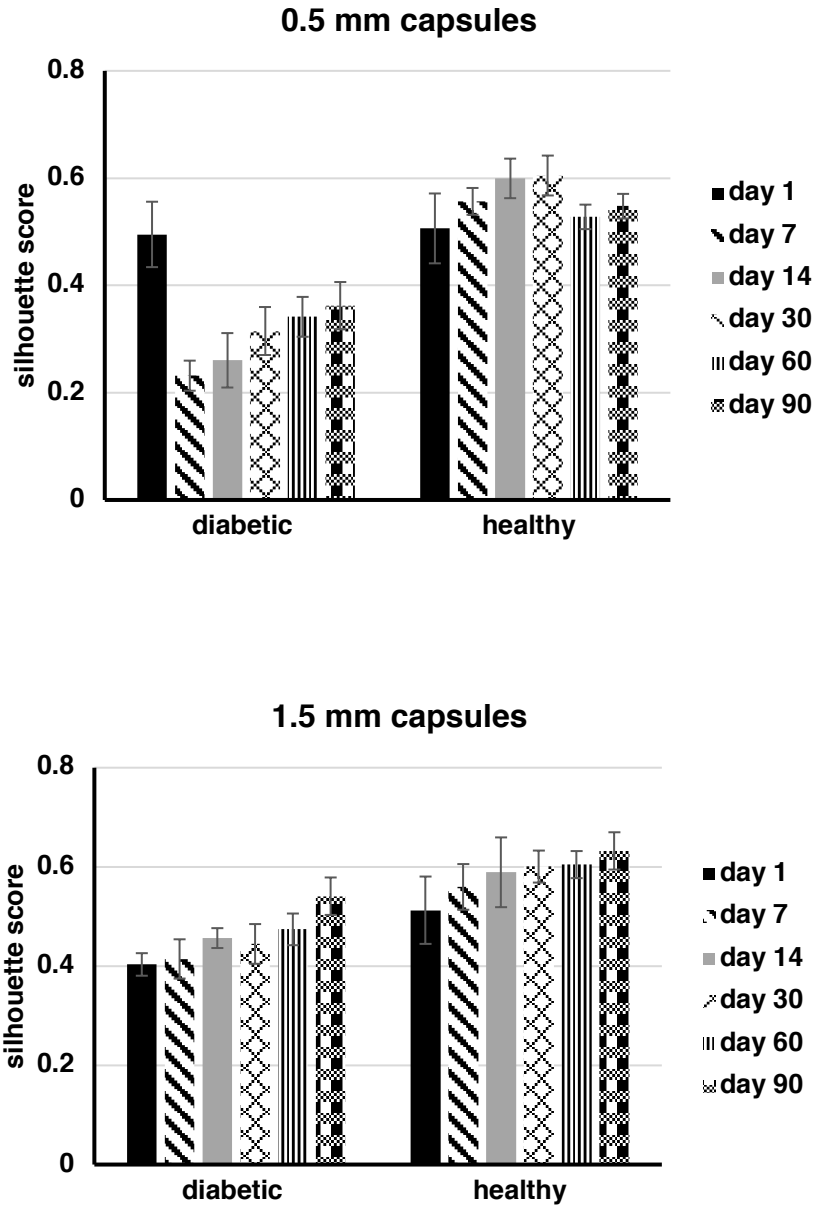
Supplemental Figure S3. Expanded view of **Fig. 5** for 0.5 mm capsules 1 day (a) and 90 days (b) post-implantation, and for 1.5 mm capsules 1 day (c) and 90 days (d) post-implantation.

In-vivo pO_2 monitoring using an oxygen microsensor



Supplemental Figure S4. Average pO_2 values (left) measured in vivo in the IP space of mice (n=5) at 5 different positions indicated by red circles in the cartoon (right). The measurements were performed with an optical, portable oxygen microsensor (PreSens GmbH). Ultrasound needle guidance ensured that the sensor was inserted 5 mm deep into the IP space. Each measurement is the average of at least two readings. Values are mean ± s.d.

Clustering validation



Supplemental Figure S5. Average silhouette scores for all image slices and mice within each group (STZ-induced or healthy C57BL/6 mice) at each time point. Silhouette scores fall within the [-1,1] range with higher values indicating tighter cluster assignment. Positive values indicate less ambiguity in the assignment of an image pixel to a cluster, as is the case for all animal groups shown in **Figure 5a-b (bottom panels)**. With the exception of one group (diabetic mice with 0.5 mm capsules) the silhouette score is around 0.6 indicating well separated clusters that maintain high dispersity over time. In the case of the diabetic mice with implanted 0.5 mm capsules the large, non-spherical aggregates forming within the first month past implantation results in significantly lower silhouette scores.

Supplemental Video 1. 3D volume rendering of a dual fluorine-hydrogen MRI image of a mouse implanted with 0.5 mm fluorinated alginate microcapsules. The gray scale tissue backround was acquired with the MRI tuned in the hydrogen mode and the colored scale capsule distribution image was acquired with the MRI tuned in the fluorine mode. For the volume rendering the slice thickness was reduced to 0.5 mm. The image corresponds to a time point of 1 week after implantation. The movie was produced with the Osirix image processing software.