Supplementary Material for Systematic comparison of methods for determining the *in vivo* biodistribution of porous nanostructured injectable inorganic particles

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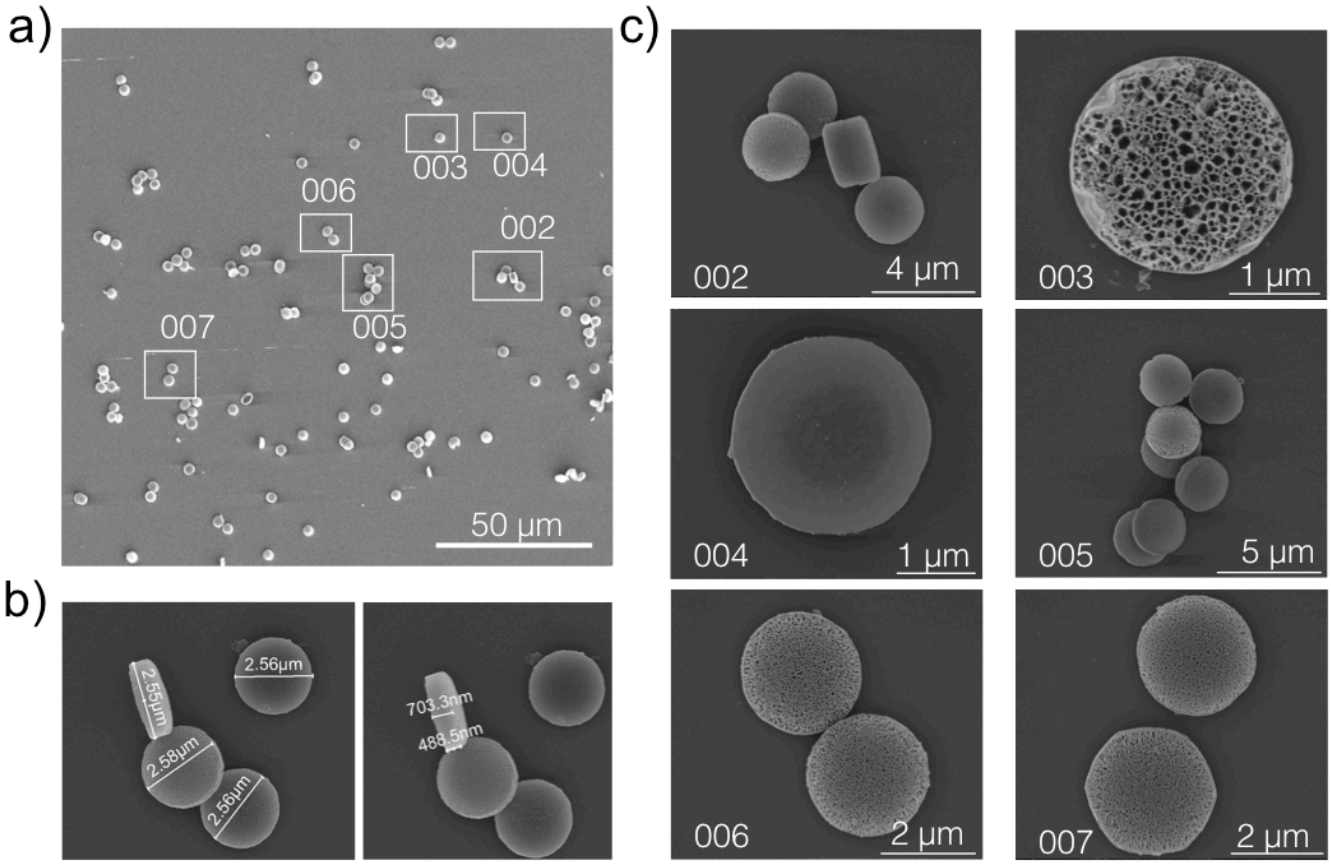
S1. Particle morphology characterization

Particle size and shape was characterized with scanning electron microscopy (SEM)



Supplementary Figure 1. Morphological characterization of PSP. a) Bright field microscopy, b) Representative SEM images used to measure size distribution. c) SEM images correlated with bright field.

Particles SEM characterization confirmed discoidal shape with average diameter of 2.5 +/- 0.1 µm and height of 616.2 +/- 138.4 nm. Dynamic light scattering (DLS) characterization of particles in solution confirms material homogeneity: a single narrow peak (583.0 =/- 0.670 nm) in size was obtained with DLS. It should be noted that the numerical size estimate obtained with DLS is only accurate for spherical particles, and scanning electron microscopy remains the most accurate size characterization method for discoidal particles.

S2. Surface charge characterization

Supplementary table ***1*** reports surface charge characterization of PSP in all formulations used for biodistribution studies: APTES-PSM, Alexa 647-PSM, and DFO-chelated PSM.

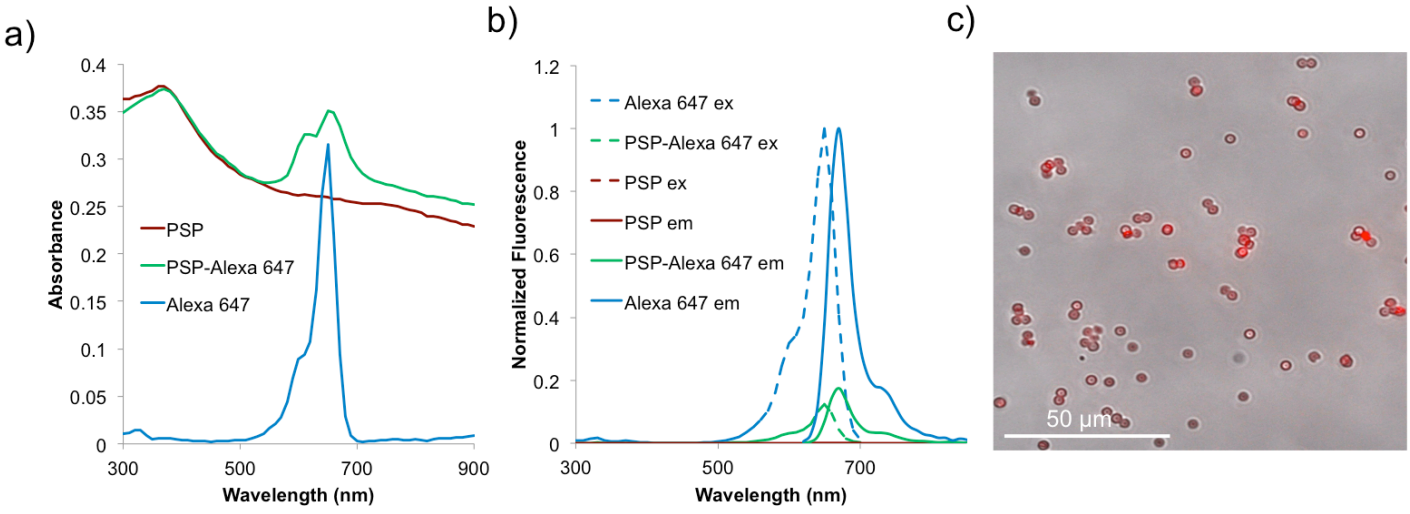
Supplementary table 1: Surface charge characterization for PSP

Material	**Solvent**	**Zeta potential (mV)**
APTES-PSP	Phospate Buffer (pH = 7.2)	0.15 +/- 6.6
Alexa 647-PSP	Phospate Buffer (pH = 7.2)	0.13 +/-5.1
DFO-PSP	Phospate Buffer (pH = 7.2)	0.14 +/- 5.2

No significant change is seen in the surface charge comparing unlabeled particles (APTES functionalization) with fluorescent (Alexa 647 conjugation) and radiolabeling (DFO conjugation). Because of the high sensitivity of radio detectors, only a small amount of Zr89was added to DFO-chelated PSP (0.1 uCi/ million particles). Thus, we do not expect significant change of in vivo biodistribution caused by radiolabeling –induced surface charge change [1].

S3. Particle fluorescence characterization

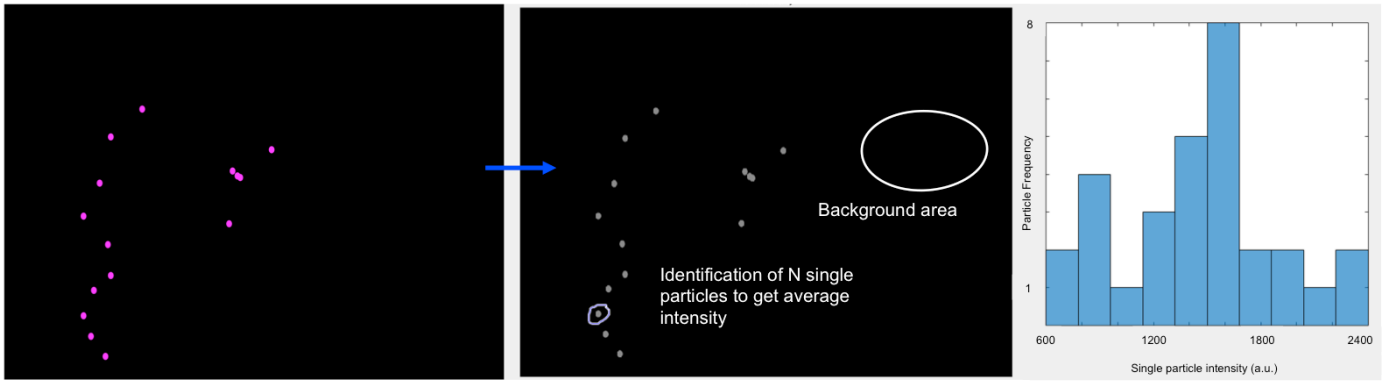
Particles were labeled with Alexa 647-NHS. **Error! Reference source not found.** reports fluorescent PSP optical characterization.



Supplementary Figure 2. Optical characterization of fluorescently labeled PSP. a) Absorbance spectra of PSP, PSP-Alexa 647, and Alexa 647 in PBS. b) Excitation (--ex) and emission (-em) spectra of PSP, PSP-Alexa 647, and Alexa-647 normalized to max ex and em of Alexa 647-NHS. c) Bright field and fluorescence overlap image of PSP-Alexa-647.

Fluorescent particle absorbance (PSP-Alexa 647) was measured and compared with absorbance of bare particles (PSP) and Alexa-647 alone (Supplementary Figure 2a). Excitation spectra (λ_{em} = 720 nm) and emission (λ_{ex} = 600 nm) spectra were measured as well (Supplementary Figure 2b), confirming successful fluorescent labeling of PSP. No spectral shift was detected upon Alexa 647-PSP conjugation. In addition, Supplementary Figure 2c reports overlapped bright field and fluorescence imaging of PSP-Alexa 647, thus confirming successful labeling of PSP.

S4. Confocal image processing algorithm

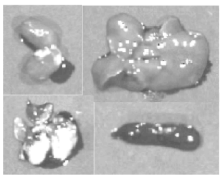


Supplementary Figure 3. Working principle of MATLAB algorithm used to count particle number from confocal images.

A single channel RGB image is extracted from each confocal image. That image is then converted in grayscale image to allow for intensity calculation. The background area is selected by the user and used to calculate and subtract the background. Upon background filtration, the total integrated intensity per field of view (FOV) is calculated, which is proportional to the number of particle per FOV. To estimate the number of particle per image, the user is asked to select N single particles. Intensities from these particles are plotted as a histogram to visually verify a gaussian distribution. Then, the average intensity of a single particle is calculated and the image integrated intensity is then divided by the single particle intensity to estimate the #/FOV. It must be noted that particle number estimate can only be performed when single particle can successfully be identified in each image/organ. Care must be applied to avoid over saturation and under saturation. Both issues will underestimate the intensity/number of particles. Code is attached as open source resource.

S5. Inductively Coupled Plasma Organ Processing Procedure

a)



+

Digestion solution (table b)

1

1 min 25000 rpm (590 xg)

2

48h/72h digestion @ room T

3

30 min @ 5000 xg

4

Supernatant dilution (table c) and measurement @ 250.69 nm, 251.61 nm, and 288.158 nm

5

b)

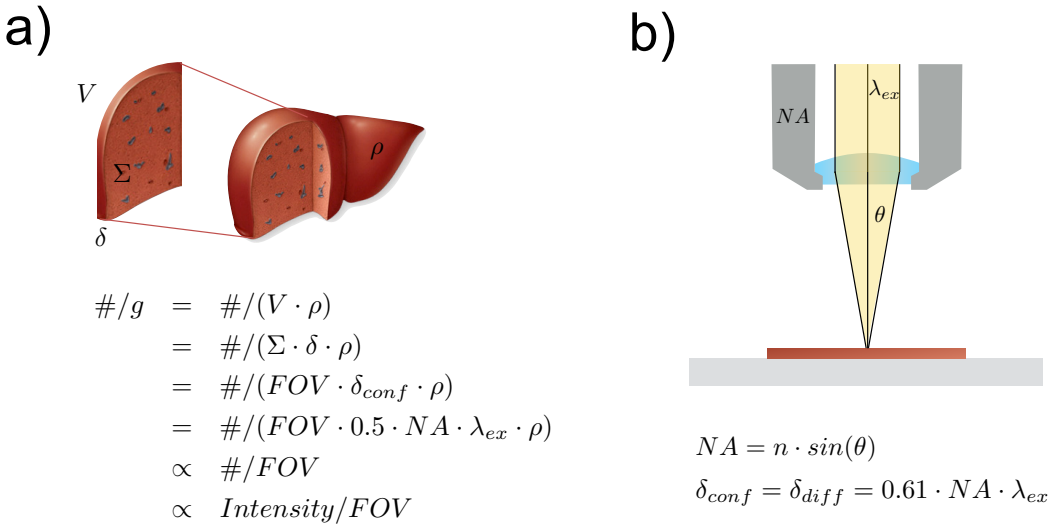
	Digestion ratio [organ:NaOH/ETOH]
Liver	1:3
Spleen	1:11
Lungs	4:20
Blood	1:1
Kidneys	1:2
Brain	1:2

c)

	Digested organ supernatant volume [µL]	NaOH in supernatant [µL]	Organ volume in supernatant [µL]	Tot volume desired [µL]	Water to add to supernatant [µL]
Liver	467	350	117	3000	2533
Spleen	382	350	32	3000	2618
Lungs	420	350	70	3000	2580
Blood*	700	350	350	3000	2300
Kidneys	525	350	175	3000	2475
Brain	525	350	175	3000	2475

Supplementary Figure 4. ICP biodistribution protocol. a) Schematic of processing procedure of organs for ICP measurement. b) Dilution factors per organ for digestion. c) Digested supernatant dilution for measurement.

S6: Mathematical considerations on surface vs volumetric optical methods



$$
\begin{aligned}
\#/g &= \#/(V \cdot \rho) \\
&= \#/(\Sigma \cdot \delta \cdot \rho) \\
&= \#/(FOV \cdot \delta_{conf} \cdot \rho) \\
&= \#/(FOV \cdot 0.5 \cdot NA \cdot \lambda_{ex} \cdot \rho) \\
&\propto \#/FOV \\
&\propto Intensity/FOV
\end{aligned}
$$

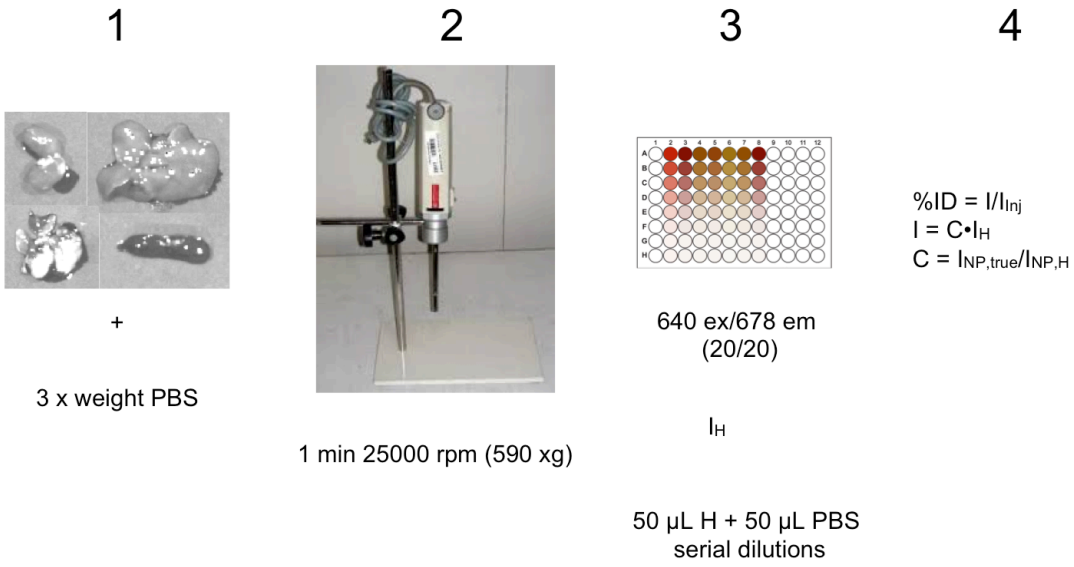
$$NA = n \cdot sin(\theta)$$

$$\delta_{conf} = \delta_{diff} = 0.61 \cdot NA \cdot \lambda_{ex}$$

Supplementary Figure 5. a) Mathematical demonstration of correspondence between particle per gram (#/g) and particle per field of view (#/FOV) measurements. The thickness of tissue analyzed with imaging techniques is limited by penetration depth. b) For confocal microscope, the thickness of analyzed tissue per image is proportional to the wavelength of excitation used (Abbe's diffraction limit).

The different nature of the three fluorescent methods compared (IVIS, confocal imaging, fluorescence of homogenized organs) results in a different effect of tissue attenuation. For IVIS measuremnets, particles emit from a densely packed and thick tissue, making it practically extremely challenging to determine the exact depth of tissues attenuating particle emission in IVIS measurements. Thus, IVIS biodistribution results are not adjusted for light attenuation by tissues. For confocal imaging analysis, a surface imaging method, only particles emitting within a tightly confined tissue section of height proportional to the wavelength of light used through the Abbe diffraction limit (~ 0.61 • NA • λ_{ex}) are detected. Since the effect of light attenuation in this tissue depth is negligible, no attenuation correction is necessary. Finally, by measuring fluorescence in homogenized tissue it is instead possible to account and correct for tissue absorption with the use of a calibration curve.

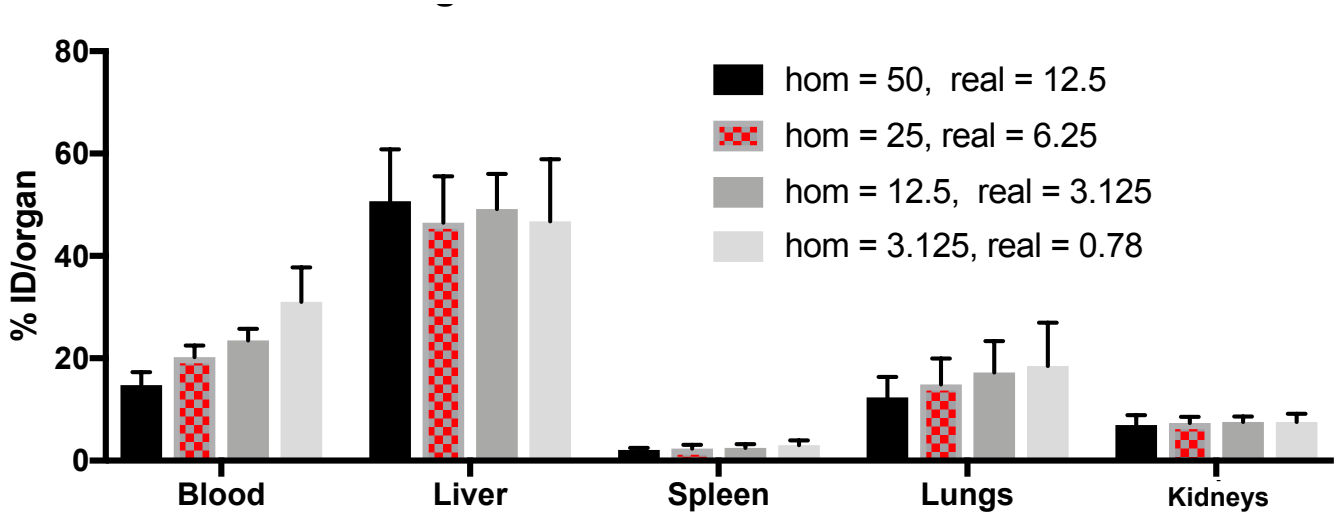
S7. Homogenized Organ Processing Procedure



Supplementary Figure 6. Schematic of processing procedure of organs for fluorescence measurement in organ homogenates.

First (1), 3x organ weight of PBS is added to whole organs, to facilitate homogenization (2). Homogenized organs are then diluted further in PBS (3) for measurement with a plate reader. %ID is then reconstructed as follows (4). I_H represents the background-subtracted fluorescence intensity measured from organ homogenates without correction. Correction factors are calculated as the ratio between intensity of a known amount of particles dispersed in PBS, and the intensity of the same amount of particles dispersed in organ homogenates. The %ID is calculated as the ratio between the corrected intensity and the intensity of the total injected amount of particles.

S8. Independence of biodistribution in organ homogenates from concentration



Supplementary Figure 7. Biodistribution as estimated through fluorescence measurements of serial dilutions of organ homogenates.

S9. Matlab script developed to process confocal images and count particles.

```
clear all
close all
%cd Images;
N_single = 10;
cd ./Images;
%cd Test; change to png
myDir = uigetdir; %gets directory
myFiles = dir(fullfile(myDir,'*.tif')); %gets all wav files in struct
S = zeros(length(myFiles), 1);
S_single = zeros(N_single,1);

for k = 1:length(myFiles)
  image = myFiles(k).name;
  fprintf(1, 'Now reading %s\n', image)
  %[wavData, Fs] = wavread(baseFileName);
  % all of your actions for filtering and plotting go here
  rgbImage = imread(image);
grayImage = rgb2gray(rgbImage);
figure, imshow(grayImage), title('Gray');
%   new
message = sprintf('Select background area. Left click and hold to begin
drawing.\nSimply lift the mouse button to finish');
uiwait(msgbox(message));
hFH = imfreehand();
% Create a binary image ("mask") from the ROI object.
binaryImage = hFH.createMask();
```

```
% Display the freehand mask.
subplot(2, 3, 2);
imshow(binaryImage);
title('Binary mask of the region');
% Calculate the area, in pixels, that they drew.
numberOfPixels1 = sum(binaryImage(:))
% Another way to calculate it that takes fractional pixels into account.
numberOfPixels2 = bwarea(binaryImage)
% Get coordinates of the boundary of the freehand drawn region.
structBoundaries = bwboundaries(binaryImage);
xy=structBoundaries[2]; % Get n by 2 array of x,y coordinates.
x = xy(:, 2); % Columns.
y = xy(:, 1); % Rows.
subplot(2, 3, 1); % Plot over original image.
hold on; % Don't blow away the image.
plot(x, y, 'LineWidth', 2);
drawnow; % Force it to draw immediately.
% Burn line into image by setting it to 255 wherever the mask is true.
burnedImage = grayImage;
burnedImage(binaryImage) = 255;
% Display the image with the mask "burned in."
subplot(2, 3, 3);
imshow(burnedImage);
caption = sprintf('New image with\nmask burned into image');
title(caption);
% Mask the image and display it.
```

```
% Will keep only the part of the image that's inside the mask, zero
outside mask.
blackMaskedImage = grayImage;
blackMaskedImage(~binaryImage) = 0;
subplot(2, 3, 4);
imshow(blackMaskedImage);
title('Masked Outside Region');
% Calculate the mean
meanGL = mean(blackMaskedImage(binaryImage));
% Report results.
message = sprintf('Mean value within drawn area = %.3f\nNumber of pixels
= %d\nArea in pixels = %.2f', ...
meanGL, numberOfPixels1, numberOfPixels2);
msgbox(message);
% Now do the same but blacken inside the region.
insideMasked = grayImage;
insideMasked(binaryImage) = 0;
subplot(2, 3, 5);
imshow(insideMasked);
title('Masked Inside Region');
% Now crop the image.
topLine = min(x);
bottomLine = max(x);
leftColumn = min(y);
rightColumn = max(y);
width = bottomLine - topLine + 1;
height = rightColumn - leftColumn + 1;
```

```
croppedImage = imcrop(blackMaskedImage, [topLine, leftColumn, width, height]);
% Display cropped image.
subplot(2, 3, 6);
imshow(croppedImage);
title('Cropped Image');
%IM2 = imcomplement(grayImage);
%h_im = imshow(grayImage);
%draw ellypse section for bkg
%e = imellipse(gca,[55 10 12 12]);
%mask = createMask(e, h_im);
% integrate intensity on area
%IntLin0 = sum(mask);
%Int0 = sum(IntLin0);
%N = size(mask);
%Area = N(1)*N(2);
%threshold = 0.8*Int0/Area; %  average intensity
% refine mask --
%find edges within mask
%BW = edge(1, method, threshold); outputs 1 in edges.
% generate image based on edges
% take bigger intersection
%--
%subplot(2,2,2);
%imshow(mask, []);

% Now get some threshold for the black
```

```
%Bkg =
%meanBkg = mean(blackImage(:))
%noiseThreshold = meanBkg
Threshold = meanGL; % Threshold defined as the mean of the black area
ThresG = grayImage > Threshold;          %simple thresholding--adjust for
your needs
 maskedImage = grayImage;       %copy image
 maskedImage(~ThresG) = 0;     %set all pixels that don't pass threshold
to zero
 S(k) = sum(maskedImage(:));
% Calculate intensity of single particle;
if k == 1
message = sprintf('Select particle area. Left click and hold to begin
drawing.\nSimply lift the mouse button to finish');
uiwait(msgbox(message));
for i = 1:size(S_single, 1)
figure, imshow(maskedImage), title('thresholdGray');
%   new

hFH = imfreehand();
% Create a binary image ("mask") from the ROI object.
binaryImage = hFH.createMask();
% Calculate the area, in pixels, that they drew.
numberOfPixels1 = sum(binaryImage(:))
% Mask the image and display it.
% Will keep only the part of the image that's inside the mask, zero
outside mask.
```

```
blackMaskedImage = maskedImage;
blackMaskedImage(~binaryImage) = 0;
S_single(i) = sum(blackMaskedImage(:))
Av_int_single = mean(S_single(:))
end
end
end
nbins = 4;
h = histogram(S_single(:),nbins);
N_part = S./Av_int_single;
savefig('histogram.fig');
save('Av_int_single.txt','Av_int_single','-ascii');
save('N_part.txt','N_part','-ascii');
save('Single_int.txt','S_single','-ascii');
save('Intensities.txt','S','-ascii');

fid = fopen('Names.txt', 'wt');
fprintf(fid, '%s\n', 'File names:');
fclose(fid);

for k = 1:length(myFiles)
    fid = fopen('Names.txt', 'a');
fprintf(fid, '%s\n', myFiles(k).name);
fclose(fid);
end
```

References

1. Vosjan, M.J., L.R. Perk, G.W. Visser, M. Budde, P. Jurek, G.E. Kiefer, and G.A. van Dongen, *Conjugation and radiolabeling of monoclonal antibodies with zirconium-89 for PET imaging using the bifunctional chelate p-isothiocyanatobenzyl-desferrioxamine.* Nat Protoc, 2010. **5**(4): p. 739-43.
2. Gerber, J.G., D.W. Kitch, C.J. Fichtenbaum, R.A. Zackin, S. Charles, E. Hogg, E.P. Acosta, E. Connick, D. Wohl, E.M. Kojic, C.A. Benson, J.A. Aberg, and A.C.T.G.A. Team, *Fish oil and Fenofibrate for the treatment of Hypertriglyceridemia in HIV-Infected subjects on antiretroviral therapy - Results of ACTG A5186.* Jaids-Journal of Acquired Immune Deficiency Syndromes, 2008. **47**(4): p. 459-466.