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Immunostaining and Toxicity Analysis of Fluorescent Nanodiamonds in Precision-Cut Mouse Liver Slices

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Abstract

Fluorescent Nanodiamonds (FNDs) are an upcoming research phenomenon and potentially useful for medical applications. Because of their highly useful characteristics, such as chemically inertness, biocompatibility, alterable surface, and fluorescent property, they can be used for cell labelling, imaging, and tracking. Before FNDs can be used in the clinic, research needs to be done to determine their toxicity as well as their behaviour within cells and tissues. This, because not enough is known about FNDs behaviour yet, and unwanted effects need to be excluded before using them in vivo. In this experiment the immunostaining process of precision-cut mouse liver slices was analysed and tried to be optimized using a confocal microscope three times. For this the antibodies CD68, CD31, and SE-1 were used as well as DAPI. Confocal images showed DAPI staining to be successful every time, however the antibody staining was not successful and was not able to be optimized within the time frame of this research. The toxicity of FNDs on liver tissue was tested by testing the viability of liver cells using an ATP, and protein assay on four different groups. Data analysis showed no significant difference in viability between cells without FND uptake and cells with FND uptake. Data analysis also showed that immobilization of cells decreased the viability of the cells. Therefore, it can be concluded that FND uptake is not toxic to mouse liver tissue, while immobilization is. Knowing this brings research a step closer to applying FNDs in medical applications.

Introduction

In the world of medicine, research on new medical applications is done continuously. The use of fluorescent nanodiamonds (FNDs) is such a potential new application. Nanodiamonds (NDs) have distinct properties useful for the medical and research field. Firstly, they are chemically inert, and biocompatible. In addition to this, the surface of a nanodiamond can be altered using various functional groups. Lastly, nanodiamonds have structural defects that are highly fluorescent and photostable. FNDs, in comparison to NDs, have an additional property containing a nitrogen-vacancy colour centre. This causes the FNDs to have a bright and stable fluorescence which can be used for cell labelling, imaging, and tracking (Chang et al., 2018). The combination of these properties make FNDs ideally suitable in, for example, disease development analysis. When talking about fluorescence, photobleaching is a closely associated term. Photobleaching is the process of photo-induced chemical destruction of fluorochromes. This process is stimulated by the excitation radiation. Since the excited molecules permanently lose their fluorescent capacity this process is irreversible and may cause potential useful information to be eliminated during imaging (Nathalie et al., 2007). However, when FNDs are exposed to green-yellow light, they emit non-photobleaching tissue-penetrating red photons, which make them very suitable for imaging applications (Chang et al., 2018).

One application of FNDs is using them to visualise the amount of free radicals within cells. So far there are multiple techniques to do this, however most of them with limitations. Electron spin resonance spectroscopy (ESR), for example, can detect free radicals due to its ability to detect the presence of unpaired electrons. The limitation of this technique is that the levels of unpaired electrons has to be altered to get detectable levels (Armstrong & Whiteman, 2007). Another technique is using fluorescence, where certain compounds will react with specific target molecules, thereby creating a detectable fluorescent molecule. Limitations of this technique are that the fluorescent dye can diffuse, measurements are not done in real time, and photobleaching can occur (Armstrong & Whiteman, 2007). Lastly, another commonly used technique for detecting free radicals is using fingerprinting. Fingerprinting is the approach where instead of the free radicals themselves is measured, their damage is measured in the form of lipid peroxidation, DNA damage, and protein alterations (Potter et al., 2010)(Hemnani and Parihar, 1998)(Halliwell & Whiteman, 2004). Free radicals can be visualised with FNDs by using the spin dephasing time (T_2*), the spin echo decay time (T_2), and the spin-lattice relaxation time (T_1). With these, the magnetization of the FNDs can be detected which are affected by the free radicals present in the cells. Therefore the amount of T_2*, T_2, and T_1 gives information about the amount of free radicals present (Schirhagl et al., 2014). The advantages of this technique are that no target reactions need to take place which makes it very specific, as well as the fact that it can be done in real time. Knowing the amount of free radicals within cells is important. When excessive radicals and reactive oxygen species are generated within a cell, for example after an ischemic period, they cause oxidative stress and tissue damage (Khalil et al., 2006). Therefore, knowing the amount of radicals within cells and where they reside within the cells can potentially help find solutions for decreasing these radicals or repair tissue damage caused by them.

In order to use FNDs in medical applications, their toxicity to cells needs to be known. This can be done by testing the cell's viability. In vitro testing of precision-cut tissue slices is an ideal substitution for testing in vivo, and is a commonly used method for testing the mechanisms, and toxicity of small compounds and chemicals. An example of testing a cell's viability is by executing an ATP and protein determination assay. Since the ATP and protein levels within cells are related to their metabolic activity, the ATP content normalized by the total protein content is an excellent indicator of the cells viability (Bartucci et al., 2020) (Castaño & Tarazona, 1994). Aside from knowing the toxicity of the FNDs to cells, it is also important to analyse where the FNDs reside within the tissue after uptake. This will give information about the dynamics of the FNDs when taken up by tissue which is important to know when FNDs are used as a medical application. A way of visualising where the FNDs reside within tissue is by using antibodies to stain certain cells. When using mouse liver tissue as a model, the antibody CD68 stains mouse Kupffer cells, CD31 stains mouse vascular and lymphatic endothelial cells, and SE-1 stains mouse hepatic sinusoidal endothelial cells (Slevin et al., 2020). When combined with FND uptake, a confocal microscope would show exactly in which cells the FNDs reside.

Research on the dynamics and toxicity of FNDs has been done already, however still more needs to be done before FNDs can be used in clinical practice. Bartucci et al. (2020) has researched the behaviour of nanoparticles, as well as analysed the toxicity of FNDs in rat liver slices. Their research served as inspiration for the research done in this article. The research consisted of two parts. During the first part the immunostaining process for mouse liver slices was analysed and attempts were made to optimize the protocol. The immunostaining of precision-cut mouse liver slices was done using CD68, CD31, and SE-1, and confocal microscope images were taken for analysis. The second part of the research was analysing

the toxicity of FNDs on precision-cut mouse liver slices by analysing the viability of the mouse liver tissue using an ATP, and protein determination assay.

Unfortunately, after multiple attempts, the immunostaining process was not successfully optimized, since no distinct cells were seen. More research needs to be done in order to fully optimize the protocol. The ATP, and protein assays showed no significant changes between 24h control and 24h after FND uptake. Therefore, FND uptake by cells does not affect the viability of mouse liver tissue in a negative way. FND uptake by cells in combination with 30 minutes immobilization showed lower ATP levels and may decrease the viability of mouse liver tissue.

Materials and Methods

Preparation of mouse liver cryosections

Materials

- Mouse liver cylinders
- Liquid nitrogen
- Starfrost® 76x26mm adhesive slides

Protocol

1. The pre-prepared mouse liver cylinder cryo blocks were taken out of storage, and put in liquid nitrogen until use.
2. Sample slices were cut using a cryotome with a cutting temperature (CT) and operating temperature (OT) of -20°C, and a slice thickness of 5µm.
3. Sample slices were collected using the Starfrost® adhesive slides and kept at -80 C°.

Immunostaining protocol for cryosections of mouse liver

Materials

- 4% formalin
- PAP Pen
- 0.2% Triton *(see appendix 1)*
- PBS
- PBS/5% Normal Mouse Serum (NMS) *(see appendix 2)*
- CD68 (KP1) Alexa Fluor 488, AF488 conjugated. Company: Santa Cruz Biotechnology
- Anti-Mo CD31 (PECAM-1). Company: Invitrogen
- Hepatic Sinusoidal Endothelial Cells Antibody (SE-1) [Alexa Fluor® 488]
- Antibody solutions *(see appendix 3-5).*
- DAPI *(see appendix 6)*
- ProLong™ Diamond Antifade Mountant. Company: Invitrogen
- 0.0375% H_2O_2 in PBS

Protocol using 4% formalin

1. Slides were taken out of storage and let to adjust to room temperature.
2. Slides were fixed for 15 minutes with 4% formalin..
3. Slides were washed three times for 5 minutes with PBS.
4. From this point on, the slides were never let to dry out.
5. A circle was drawn around the tissue using the PAP Pen.
6. Tissue was permeabilized for 15 minutes with 0.2% Triton.
7. Slides were washed three times for 5 minutes with PBS.
8. 50µl of antibody solution was added to the slides (except for the control) and incubated for 1.5 hours in darkness at room temperature.
9. 50ul of PBS/5% NMS was added to the control slide and incubated for 1.5 hours in darkness at room temperature.
10. Slides were washed three times for 5 minutes with PBS.
11. Slides were stained 50ul DAPI for 30 minutes.
12. 2 drops of mounting media were added to the slides and covered with a coverslip.
13. The slides were stored at 4°C.

Protocol using acetone

1. Slides were taken out of storage and let to adjust to room temperature.
2. Slides were fixed for 10 minutes with acetone.
3. From this point on, the slides were never let to dry out.
4. Slides were washed three times for 5 minutes with PBS.
5. A circle was drawn around the tissue using the PAP Pen.
6. 50µl 0.0375% H_2O_2 in PBS was added to the slides for 20 minutes.
7. Slides were washed three times for 5 minutes with PBS.
8. 50µl of antibody solution was added to the slides (except for the control) and incubated for 1.5 hours in darkness at room temperature.

9. 50µl of PBS/5% NMS was added to the control slide and incubated for 1.5 hours in darkness at room temperature.
10. Slides were washed three times for 5 minutes with PBS.
11. Slides were stained with 50µl DAPI for 30 minutes.
12. 2 drops of mounting media were added to the slides and covered with a coverslip.
13. The slides were stored at 4°C.

Obtaining images using confocal microscopy

Materials

- Pre-prepared immunofluorescent cryosections oof mouse liver
- LEICA TCS SP8X dls confocal microscope

Protocol

1. Samples were taken out of storage
2. Using the confocal microscope images of the samples were obtained using the following settings:
    a. 40x Oil 21°C
    b. Blue channel (DAPI) excitation at 405nm
    c. Green channel (FITC) excitation at 405nm

Tissue viability test preparation

Materials

- WEGG medium: 0.276g glucose, 100µl glutamicin, and 100ml WE medium.
- 24 well plate
- NDNV70nmHi

Protocol

1. 500µl WEGG medium was added to each of the wells in the 24 well plate.
2. The plate was prewarmed in an 37°C incubator with 5% CO2, 80% O2, and 50 rpm.
3. 25µl nanodiamonds was added to all walls except for the control group (*see appendix 7*).
4. A pre-prepared mouse liver slice was added to each well.
5. The slices were divided into 4 different groups:
    Group A – 0h control
    Group B – 24h control
    Group C – 24h FNDs
    Group D – 24h FNDs + 30 minute immobilization
6. Group A slices were put into a safelock vial with 1ml SONOP as well as 1 cup of minibeads (see *Tissue viability – ATP assay* section*)* and into liquid nitrogen immediately.
7. The plate was then incubated for 24 hours at 37°C.
8. Group B,C,D slices were transferred to new wells with WEGG medium, and incubated again for an additional 2 hours.
9. The slices were rinsed 3x with WEGG medium, and once with PBS.
10. Group B, and C slices were put in safelock vials and into liquid nitrogen.
11. Group D slices were put in a drop of WEGG medium in a small petri dish, and PDMS pillars were placed around it. A cover glass was put on top of the pillars and more WEGG medium was added to submerge the slice.
12. The dishes were set aside for 30 minutes on a heating pad.
13. Group D slices were then put into safelock vials and into liquid nitrogen.
14. Samples were kept at -80°C.

Tissue viability - ATP assay

Materials

- White 96-wells plate
- Minibead-beater
- Repetitive pipet with 50 µl tip
- Synergy HT plate reader
- ATP positive control (P) (-80°C) (1 aliquot/plate)
- SONOP (Sonification Solution), Ethanol (70% v/v) containing 2mM EDTA (M=372.24 g/mol) with pH=10.9 (*see appendix 8*).
- 100mM Tris-HCl, 2mM EDTA buffer (pH 7.6-8.0) (*see appendix 9*)
- ATP Bioluminescence assay kit Roche. Contents:
    - Luciferase reagent lyophilized (white cap)
      (Dissolve lyophilized luciferase in exactly 10.0 ml MQ-water and mix by swinging. Do not vortex)

- ATP-standard ± 10mg lyophilized (red cap) (aliqouts stored in -80°C)
  (Dissolve the ATP-standard from the kit to exactly 10mg/ml (= 16.5mM) with MQ-water)

Protocol

1. After the incubation 1 slice was put in 1 ml SONOP in a safelock vial as well as 1 cup of minibead and snap frozen in liquid N_2.
2. A new set of 1.5mL tubes was labeled (equal to the amount of ATP-samples).
3. The samples were homogenized with the minibead-beater for 2x 45sec with 2 minutes in between.
4. The samples were centrifuged homogenate for 5min at 13.000 rpm. The supernatant was transfered into the new tube and kept on ice. The tube with precipitate was dried at 37°C (1 day).
5. A calibration curve was prepared and the tubes were stored on ice:

Dilution	Amount (µl)	Tris/EDTA Buffer (µl)	Conc. (M)
A	10µl ATP-standard (S)	***90***	1.65 x 10^{-3}
B	50µl [A]	450	1.65 x 10^{-4}
C	50µl [B]	450	1.65 x 10^{-5}
Cal 1	50µl [C]	450	1.65 x 10^{-6}
Cal 2	**200µL [Cal 1]**	**300**	0.66 x 10^{-6}
Cal 3	**100µl [Cal 1]**	**400**	*3.30 x 10^{-7}*
Cal 4	**50µl [Cal 1]**	**450**	1.65 x 10^{-7}
Cal 5	**50µL [Cal 2]**	**450**	0.66 x 10^{-7}
Cal *6*	**100µl [Cal 4]**	**400**	*3.30 x 10^{-8}*
Cal 7	**50µl [Cal 4]**	**450**	1.65 x 10^{-8}

Dilution A, B and C are only to prepare the calibration curve and are not used for measuring. Calibration samples (a,b,c, Cal 1-7) should not be stored to reuse.

6. 5µl Blanc (Tris/EDTA), 5µl positive control (-80°C), and 5µL supernatant of each sample was pipetted in duplo in the designated wells of the white 96-wells plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blanc	Blanc	A7	A7	A15	A15	A23	A23	A31	A31	Cal 1	Cal 1
B	Positive	Positive	A8	A8	A16	A16	A24	A24	A32	A32	Cal 2	Cal 2
C	A1	A1	A9	A9	A17	A17	A25	A25	A33	A33	Cal 3	Cal 3
D	A2	A2	A10	A10	A18	A18	A26	A26	A34	A34	Cal 4	Cal 4
E	A3	A3	A11	A11	A19	A19	A27	A27	A35	A35	Cal 5	Cal 5
F	A4	A4	A12	A12	A20	A20	A28	A28	A36	A36	Cal 6	Cal 6
G	A5	A5	A13	A13	A21	A21	A29	A29	A37	A37	Cal 7	Cal 7
H	A6	A6	A14	A14	A22	A22	A30	A30	A38	A38	A39	A39

7. 45µl Tris/EDTA buffer was added to all wells containing blanc, positive control or sample.
8. 50µl diluted calibration curve was pipetted in duplo in the plate.
9. 50µl luciferase (stored at 4°C, leave for 30min on bench to reach RT before use) was added to every well using a repetitive pipet.
10. The plate was measured after 0min and 5 min using the luminometer (standard settings of the SynergyHT ATP protocol).

Important: The ATP in the slices is sensitive for breakdown by present enzymes. Therefore store samples at -80°C and keep tubes at 4°C throughout the determination.

Tissue viability - Protein estimation

Materials

- BSA stock solution 3.2 (A)
- Water bath with shaking function
- Minibead beater
- Transparent flat 96-wells plate
- Multichannel pipet
- Synergy HT plate reader (at 650nm)
- 5M NaOH solution (20g sodium hydroxide/100mL MQ-water)
- Reagent A and B of Biorad kit.

Protocol

1. 200µl 5M NaOH was added per tube (including pellet and beads).
2. The tubes were incubated for 30min at 37°C (shaking, high speed) in the waterbath.

3. Meanwhile, an aliquot of BSA (3.2 mg/ml) (-20°C) was thawed and the following calibration curve was prepared:

Dilution (conc.)	Volume (µl)	1M NAOH(µl)
A1 (1.6)	50µl [3.2 mg/mL BSA stock]	50
A2 (0.8)	50µl [A1]	50
A3 (0.4)	50µl [A2]	50
A4 (0.2)	50µl [A3]	50
Blanc (0.0)		50
B1 (1.2)	30µl [3.2mg/mL BSA stock]	50
B2 (0.6)	50µl [B1]	50

4. After incubation, 800µl MQ-water was pipetted in each tube (5x dilution, equal to SONOP volume of samples)
5. The tubes were homogenized with the minibead-beater for 40 seconds
6. 5µl of calibration standard or sample was pipetted in a transparent 96-wells plate as followed:

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blanc	0.2	0.4	0.6	0.8	1.2	1.6		A37	A37		
B	A1	A1	A2	A2	A3	•	A4	A4	A5	A5	A6	A6
C	A7	A7	A8	A8	A9	A9	A10	A10	A11	A11	A12	A12
D	A13	A13	A14	A14	A15	A15	A16	A16	A17	A17	A18	A18
E	A19	A19	A20	A20	A21	A21	A22	A22	A23	A23	A24	A24
F	A25	A25	A26	A26	A27	A27	A28	A28	A29	A29	A30	A30
G	A31	A31	A32	A32	A33	A33	A34	A34	A35	A35	A36	A36
H	Blanc	0.2	0.4	0.6	0.8	1.2	1.6		A38	A38	A39	A39

7. 25µl of reagent A was added in each well (standards and samples).
8. 200µl of reagent B was added to each well (standards and samples).
9. The plate was incubated in the dark (at RT) for 15 minutes
10. The absorbance was measured at 650nm (standard settings of Synergy's Lowry Assay protocol)

Results/Discussion

Optimization of immunostaining protocol of mouse liver tissue

The goal of this experiment was to analyse and optimize the immunostaining protocol for mouse liver tissue. During the initial experiment, the immunostaining protocol for cryosections of mouse liver slices was done using 4% formalin with the antibodies CD31, and CD68 and no control slide. Figure 1 shows the obtained confocal microscopy images. As can be seen in figure 1, the DAPI staining was successful and according to expectations. However, the CD31 and CD68 antibody staining were not as expected and showed no specific cells or structures (Bartucci et al., 2020). It was speculated that this had occurred due to the antibody staining going incorrectly, and that the fluorescence that was seen was due to autofluorescence. Since the results of the immunofluorescence staining were not as desired the experiment was done a second time.

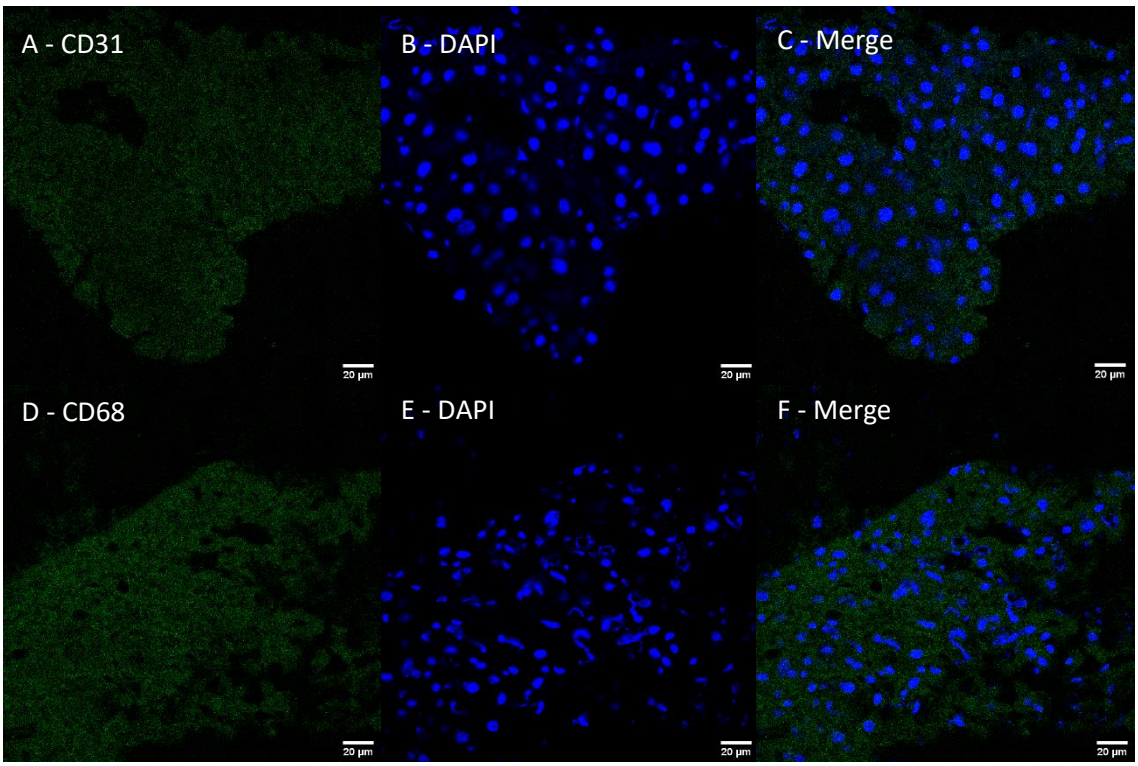


Figure 1: Confocal fluorescence imaging of mouse liver slices. Slices were exposed to CD31 (A) and CD68 (D) antibodies as well as DAPI (B+E). C and F show the merged images. Blue: DAPI-stained nuclei. Green: antibody staining. CD68: Kupffer cell staining. CD31: vascular and lymphatic endothelial cell staining. All images were processed at the same brightness values.

During the second experiment, the immunofluorescence protocol for cryosections of mouse liver was again done using 4% formalin. This time, aside from the antibodies CD31, and CD68, the antibody SE-1 was used to stain hepatic sinusoidal endothelial cells as well. This time a control slice was used to analyse the autofluorescence of the tissue. Figure 2 shows the obtained confocal microscopy images. As can be seen in figure 2, the DAPI staining was again successful and according to expectations. The control slides show some autofluorescence, but not as significantly as was expected after the first experiment. This means that the colours seen in figure 1 are most likely from a combination of autofluorescence and antibodies. Figure 2 also shows that all three antibodies show up in all cells and at accumulate at the same place, close to tissue borders and around vascular tissue. Since CD31 stains mouse vascular and lymphatic endothelial cells, this location does not seem illogical. However, for the CD68, and SE-1, this was not expected. Since all three antibodies were accumulating at the borders, the immunostaining protocol was altered and was done a third time.

During the third experiment two variables of the protocol were altered and analysed. The first variable was the fixation medium, and the second was the antibody concentration. The protocol was executed on multiple slices where half was fixed with 4% formalin, and the other half was fixed with acetone. In each half there was a control slide, a slide with 5 µg/ml CD68, and a slide with 0.5 µg/ml CD68. Only one antibody was required this time, since the effect of different protocols and antibody concentrations was analysed. Figure 3 shows the obtained confocal microscopy images. As can be seen in figure 3, both fixation media showed again no specific cells or structures concerning the antibody. The DAPI was again successful. It can also be seen that the autofluorescence of both the formalin group as the acetone group is significantly higher than can be seen in figure 2. This is due to the difference in laser power used. As expected, the slides with 5 µg/ml antibody showed more fluorescence, due to the higher antibody concentration. This proves that the dye conjugated with antibody works well. It can also be seen in figure 3 (B+K) that the sample fixed with acetone has poor cell morphology.

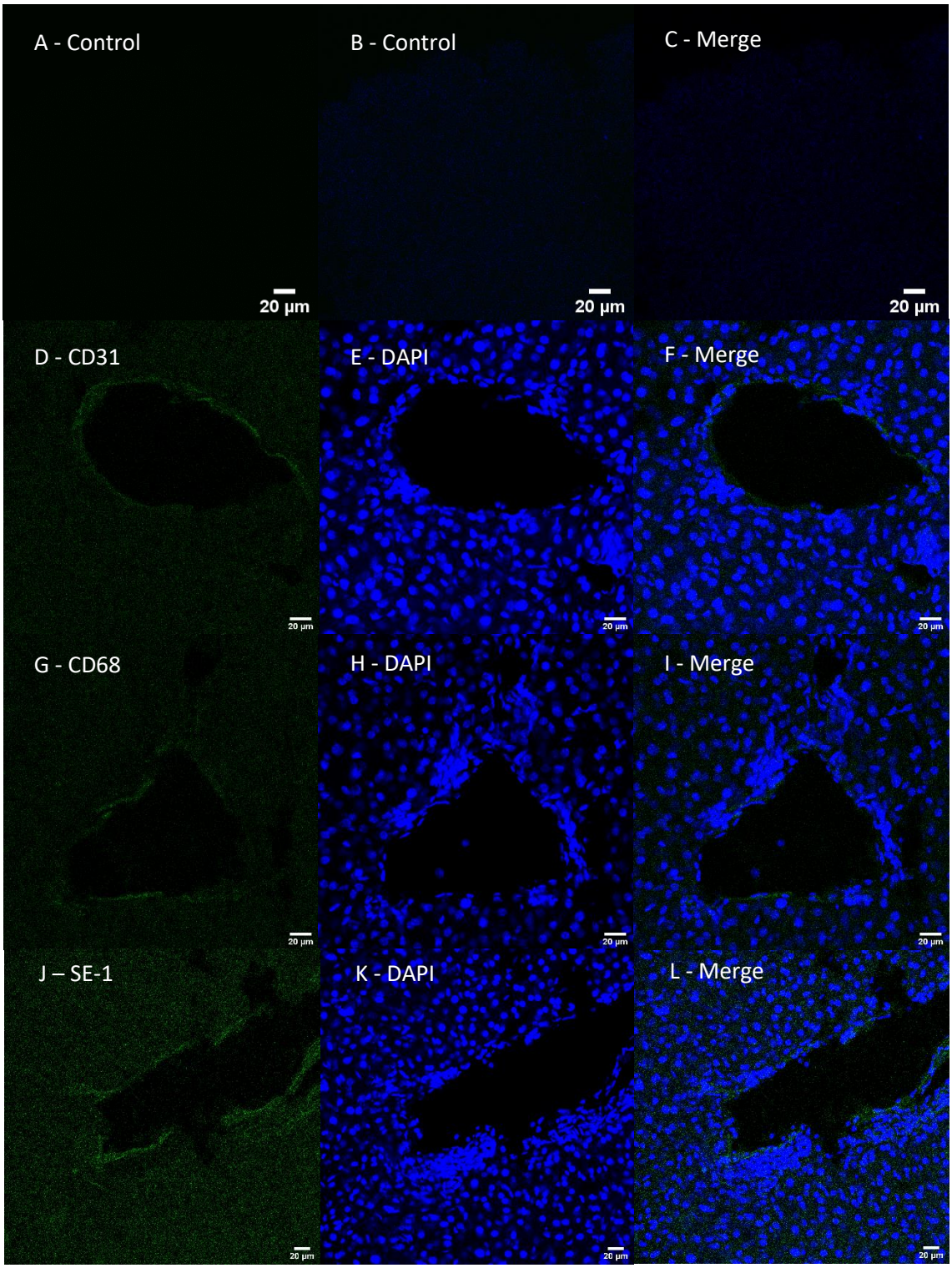
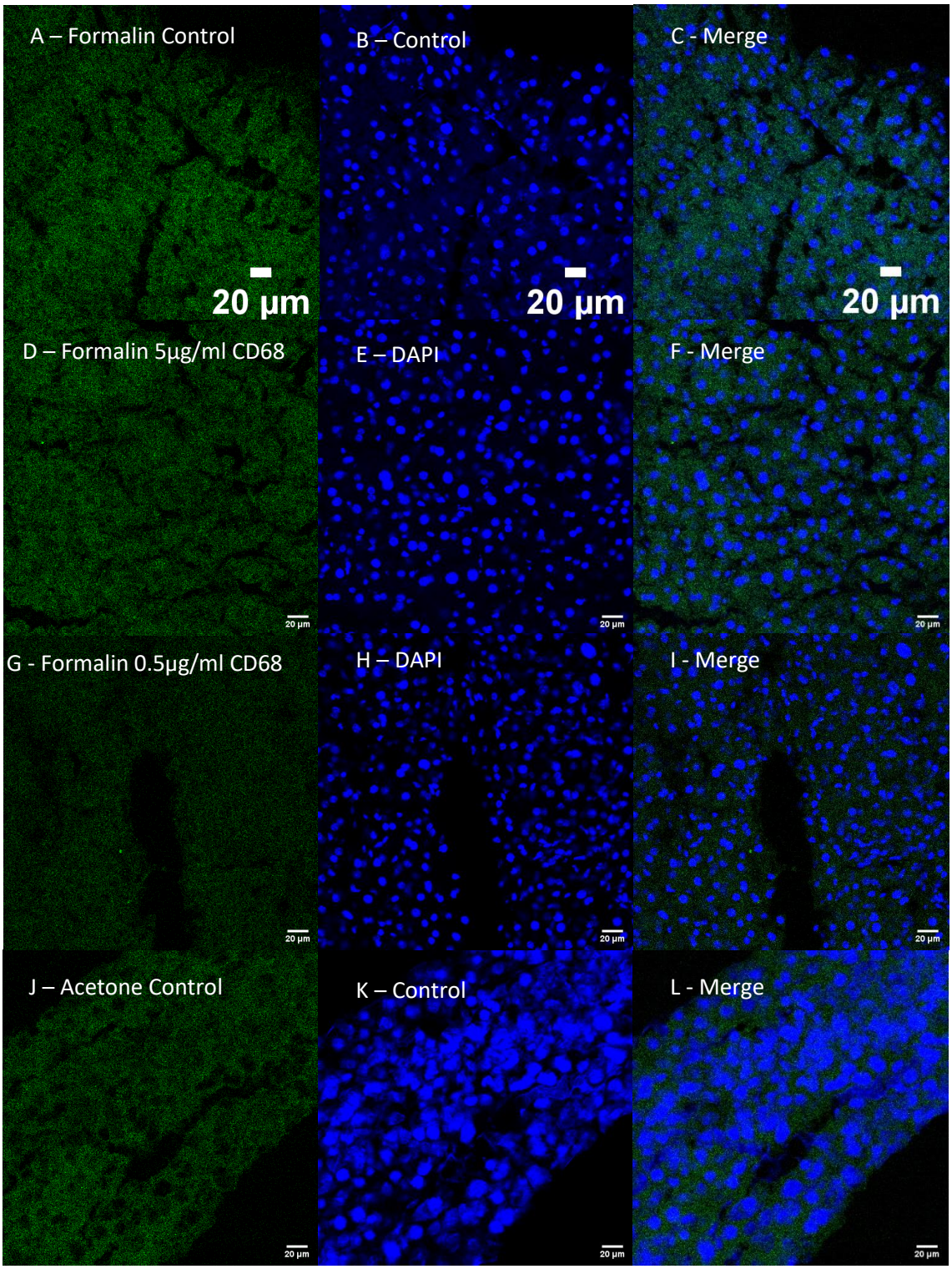


Figure 2: Confocal fluorescence imaging of mouse liver slices. Slices were exposed to only formalin (A-C), CD31 antibodies (D), CD68 antibodies (G), SE-1 antibodies (J) as well as DAPI (E,H,K). C,F,I and L show the merged images. Blue: DAPI-stained nuclei. Green: antibody staining. CD68: Kupfer cell staining. CD31: vascular and lymphatic endothelial cell staining. SE-1: Hepatic sinusoidal endothelial cells. All images were processed at the same brightness values.


A – Formalin Control
20 µm
B – Control
20 µm
C - Merge
20 µm
D – Formalin 5µg/ml CD68
E – DAPI
F - Merge
G - Formalin 0.5µg/ml CD68
H – DAPI
I - Merge
J – Acetone Control
K – Control
L - Merge

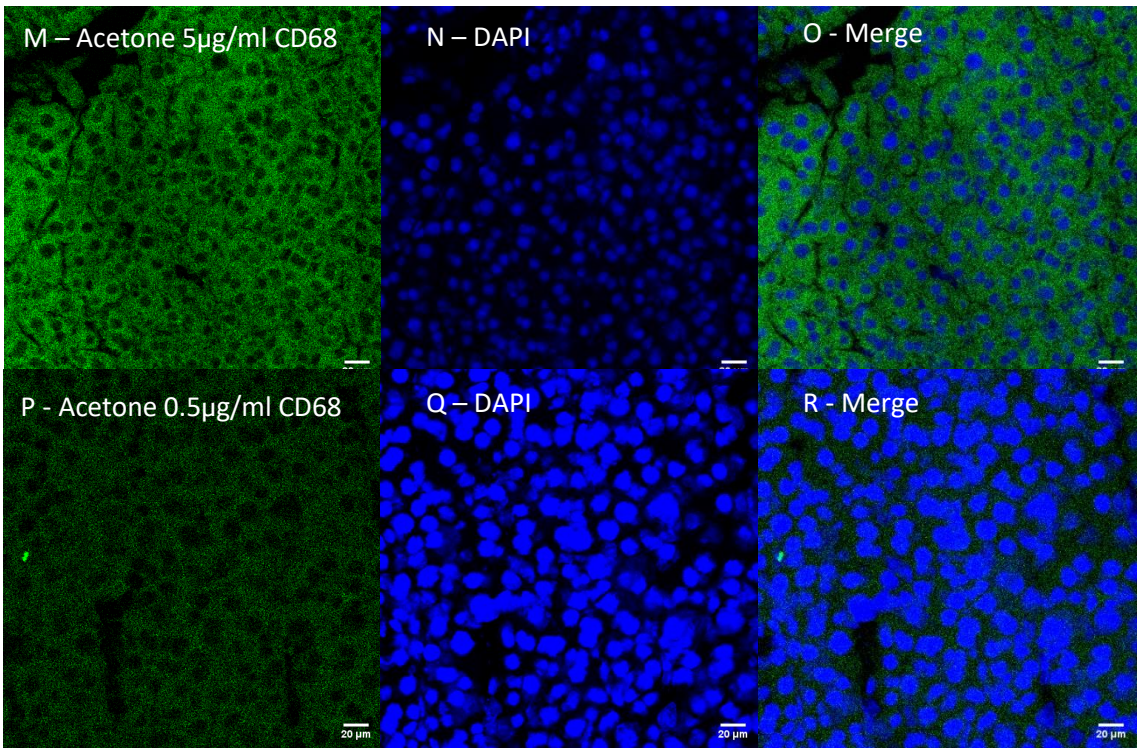


Figure 3: Confocal fluorescence imaging of mouse liver slices. Slices were exposed to only formalin (A-C), formalin with 5 µg/ml CD68 (D), formalin with 0.5 µg/ml CD68 (G), only acetone (J-L), acetone with 5 µg/ml CD68 (M), acetone with 0.5 µg/ml CD68 (P), and DAPI (B,E,H,K,N,Q). C,F,I,L,O,R show the merged images. Blue: DAPI-stained nuclei. Green: antibody staining. CD68: Kupffer cells. All images were processed at the same brightness values.

Viability testing of mouse liver slices

The goal of this experiment was to analyse the toxicity of FNDs to mouse liver cells using an ATP, and protein assay. During the experiment the viability tests were done twice and exactly as described in the protocol. There were 4 different groups of liver tissue. The four groups are 0h without FND uptake, 24h without FND uptake, 24h after FND uptake, and 24h after FND uptake with 30 minutes immobilization. Figure 4 shows the calibration curves as well as the graphs representing the ATP levels, protein levels, and ATP/protein values of the 4 different groups. As can be seen in figure 4, the calibration curves show straight lines which means they were done successfully.

Graph C and H show that the 24h control and 24h FND groups have similar and relatively higher ATP levels. The 24h FND + fixation has lower ATP levels. This would indicate that FND uptake does not affect the ATP levels of the cells, but that fixation does. The ATP levels in graph H are lower compared to graph C. This could be due to variation in time length before analysing the fluorescence or to the fact that liver slices from a different mouse were used.

Graph D and I show some differences. Graph D shows that both groups containing FNDs have significantly lower protein levels than the groups without FND uptake. This would indicate that FND uptake affects the protein levels of the cells. However, in graph D it can also be seen that the data points within the groups without FND uptake significantly differ from each other, and that the lowest point of these two groups do not differ much from the groups after FND uptake. Graph I shows the 0h control group to have higher protein levels than the other three groups, which are similar to each other. It can be seen that the protein levels in graph I are significantly higher than the protein levels in graph D. This can have multiple explanations. For example, the slices can differ from each other because they are from different mice.

When comparing the ATP/protein levels from graph E and J, it can be seen that in graph E the 24h FND group has the highest levels with one significant outlier. That a group with FND uptake would have a higher ATP/protein level than a group without FND uptake is against expectations since it would indicate that the FNDs would facilitate cell viability. In graph J it can be seen that the levels of the 24h control group and the 24h FND group are similar and that the 24h FND group with 30 minutes immobilization has slightly lower levels. The ATP/protein levels of graph E are higher than those in graph J, which could be due to the difference in protein levels seen in graph D and I, or it could be because of the different mice used. Overall it can be concluded that FND uptake by cells does not affect the viability of mouse liver tissue in a negative way. FND uptake by cells in combination with 30 minutes immobilization might affect the ATP levels and thereby the viability of mouse liver tissue in a negative way.

The goal of this experiment was to analyse and optimize the immunostaining protocol on mouse liver tissue, as well as analyse the toxicity of FNDs on mouse liver tissue. Unfortunately, optimization of the immunostaining protocol was not successful, and due to time restraints no other experiments were done to optimize the immunostaining process further. Therefore, more research needs to be done on this subject in order to be able to analyse the behaviour of FNDs within the tissue. The analysis of the toxicity of FNDs on mouse liver tissue resulted in the conclusion that

FNDs are not toxic to mouse liver tissue, and that immobilization may decrease the cell's viability. This brings research a step closer to using FNDs in medical applications.

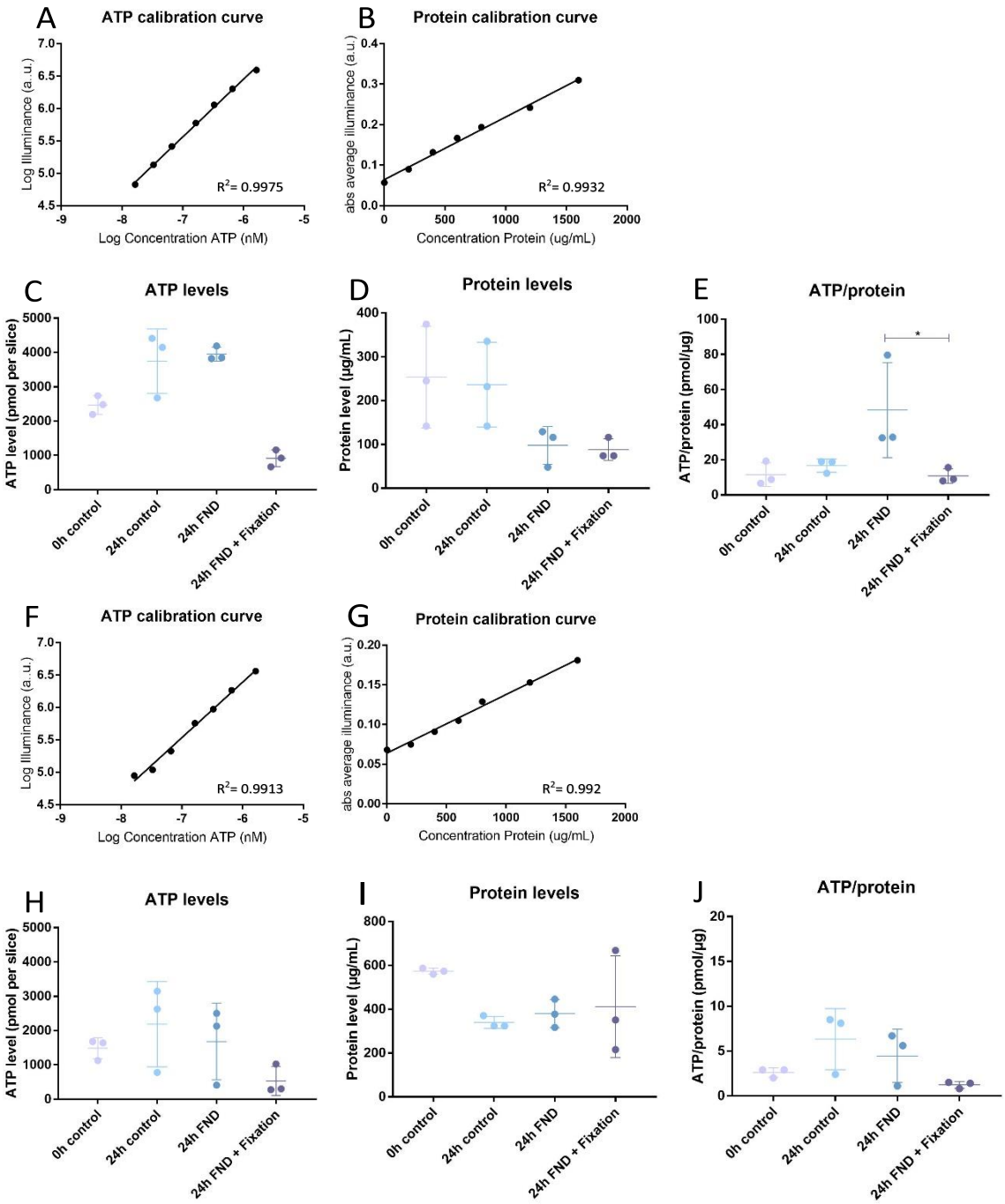


Figure 4: Viability testing of mouse liver tissue in duplo (A-E, and F-J). Using a viability test the calibration curves (A-B, F-G) ATP levels (C,H), protein levels (D,I), and the ATP/protein levels (E,J) were obtained of mouse liver slices after 0h without FND uptake, 24h without FND uptake, 24h after FND uptake, and 24h after FND uptake with 30 minutes immobilization. * means there is a significant difference. In this case between group 24h FND, and 24h FND + Fixation.

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Appendix

Appendix 1 – 50ml 0.2% Triton
0.2% of 50ml is 100µl.
Therefore to get 500ml 0.2% Triton, 100µl Triton was used combined with 49.900µl PBS.

Appendix 2 – 500µl PBS/5% NMS
5% of 500µl is 25µl.
Therefore to get 500µl solution, 25µl NMS was used combined with 475µl PBS.

Appendix 3 - 50µl CD68 antibody
Stock solution: 200µg/ml.
Working solution: 5µg/ml.
From 200 to 5 is 40x diluted.
To obtain 50µl, 50/40=1.25µl antibody was used combined with 48.74µl PBS/5% NMS.

Appendix 4 - 50µl CD31 antibody
Stock solution: 500µg/ml.
Working solution: 20µg/ml.
From 500 to 20 is 25x diluted.
To obtain 50µl, 50/25=2µl antibody was used combined with 48µl PBS/5% NMS.

Appendix 5 - 50µl SE-1 antibody
Stock solution: 670µg/ml.
Working solution: 10µg/ml.
From 670 to 10 is 67x diluted.
To obtain 50µl, 50/67=0.75µl antibody was used combined with 49µl PBS/5% NMS.

Appendix 6 - 500µl DAPI
Stock solution: 200µg/ml.
Working solution: 4µg/ml.
From 200 to 4 is 50x diluted.
To obtain 500µl, 500/50=10µl DAPI was used combined with 490µl PBS.

Appendix 7 – Nanodiamond solution
Stock solution: 1mg/ml (1000µg/ml).
Working concentration: 50µg/ml.
From 1000 to 50 is 20x diluted.
500µl medium was used per well so 500/20=25µl nanodiamonds was needed per well.

Appendix 8 – SONOP
For 1L: Dissolve 0.744g EDTA in ± 200ml of MQ-water, adjust pH with 5M NaOH to pH=10.9, add 60mL MQ-water and 740ml ethanol (96%).

Appendix 9 – Tris/EDTA buffer
For 500ml: Dissolve 6.0g Tris (M=121.14) (Tris(hydroxymethyl)amniophen; Merck) and 0.37g EDTA (Triplex III; M=372.24) in ± 300ml MQ-water, adjust pH with 6N HCl and fill up to 500ml total volume with MQ-water.

Appendix 10 - 50µl CD68 antibody lower working solution
Stock solution: 200µg/ml.
Working solution: 0.5µg/ml.
From 200 to 0.5 is 400x diluted.
To obtain 50µl, 50/400=0.125µl antibody was used combined with 49µl PBS/5% NMS.