# MEASURING OXIDATIVE STRESS IN ISOLATED ROUND SPERMATIDS

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# 1. ABSTRACT

With increasing parental age there is an increase in the risk for perinatal problems. One theory for the increase in this risk is the build-up of oxidative stress. Oxidative stress is the imbalance between oxidants and antioxidants. Oxidants include reactive oxygen species (ROS), among ROS free radicals contribute to this imbalance resulting in oxidative stress. The purpose of this study was to measure oxidative stress in round spermatids. A novel technique that enables the measurement of free radicals in germ cells is diamond magnetometry. To be able to perform these measurements different techniques were used including: enzymatic disaggregation, fluorescent-activated cell sorting (FACS), and purity analysis. Testis from mice were disaggregated through enzymatic disaggregation creating a testicular cell suspension, with FACS cells were sorted and a cell suspension with 90% round spermatids was obtained. Cells were used to perform T1 measurements. The hypothesis of increasing oxidative stress with increasing age could not be proven, however, this research shows that evaluation of oxidative stress in germ cells is within the contemporary possibilities of science. With control of the biological sample and a standardized protocol a definitive conclusion can be drawn.

# 2. INTRODUCTION

Spermatogenesis is a complex process in which spermatogonial stem cells (SSCs) differentiate into spermatozoa. This process consists of three phases, proliferation of spermatogonia by mitotic division, meiotic division of spermatocytes, and spermiogenesis where round spermatids transform into spermatozoa [1]. Throughout these phases male germ cells undergo specific modifications in DNA content, chromatin structure, size, and shape. Based on these characteristics it is possible to distinguish between cell types in different stages of spermatogenesis. The focus of this study lies on isolating round spermatids from the testis and measuring free radicals in/on those cells. Free radicals such as superoxide anions ($O_2^-$), hydrogen peroxide ($H_2O_2$), peroxyl (•ROO), and hydroxyl (•OH) are responsible for creating an imbalance resulting in oxidative stress [2,3]. A small amount is necessary for the physiological function of sperm, including capacitation, hyperactivation, acrosomal reaction, and sperm-oocyte fusion [4,5,6]. However, when ROS levels increase above a certain threshold it may affect the quality of the male germ cells, resulting in defective spermatozoa. The same approach using different cells from the spermatogenesis process will give an overview of how oxidative stress evolves throughout spermatogenesis. To be able to perform free radical measurements, isolation of these cells is necessary.

A technique that is widely used to isolate populations of specific cell types is fluorescence-activated cell sorting (FACS) [7]. The principle of this technique is based on the detection of light after a laser excites single cells. Three things are needed to use FACS, a cell suspension, the combination of DNA staining with fluorescent dyes and light scattering. In order to obtain a cell suspension with the different male germ cells testicular disaggregation is needed, this is the process of breaking down the testis [8]. This can be done through mechanical dissociation and/or enzymatic dissociation.

In our study we used the enzymatic dissociation as described in section 3.1.1. To stain the cell suspension multiple DNA staining dyes can be used, one that has been frequently used in flow cytometry analysis of testicular cells is Hoechst-342 (Ho342) [9]. Ho342 is a cell-permanent dye that does not require permeabilization. When Ho342 is excited with UV-light it emits blue fluorescence which is proportional to the DNA content, on top of this red fluorescence is emitted that reflects variability in chromatin structure and compaction. These properties enable the isolation of cells from different spermatogenesis stages based on specific emission and scatter patterns [9]. After isolation of the round spermatids free radical measurements can take place. Various techniques can be used to measure free radicals, these include: direct measurement of lipid peroxides in seminal plasma, chemiluminescence, and flow cytometry. All these methods have some downfalls. They are direct methods that require compounds to react with the free radicals, most of these compounds are nonspecific and usually react with all kinds of reactive species. The more specific probes used in flow cytometry, for example, have other downfalls. It is only possible to measure the amount of free radicals produced between adding the compound and performing the measurement, which means that these probes inform about the history of the sample but not about the real-time behavior of radical levels. On top of this photobleaching of fluorescent probes, this limits the number of measurements possible on the same sample. A novel technique that can be used to measure in real time without bleaching is diamond magnetometry [11].

Diamond magnetometry uses certain properties of nanodiamonds in order to measure free radicals. Most noticeably these properties include defects in the nanodiamond structure, the so-called nitrogen-vacancy (NV) centers. The use of NV centers enables the detection and measurement of a number of physical quantities, magnetic and electric fields for example [6]. The surrounding of the diamond determines the diamond's optical properties, since this technique uses an optical signal for measurement that enables nanoscale sensitivity. A specific type of diamond magnetometry is relaxometry, this method uses spin noise created by the free electron spin of free radicals. When the diamond is excited using a laser the NV center polarize into the ms = 0 of the ground state. With measuring after a certain amount of dark time you can see whether the NV centers are still in this state or have returned to equilibrium between ms = 0 and ms = +1/-1. The time it takes to reach the equilibrium gives a quantitative measurement of the radical concentration in the nanodiamonds surrounding [11].

The combination of various techniques makes the measurement of oxidative stress in germ cells a complex and interesting challenge. The importance of performing these experiments lies in the fact that there is little known about the amount of oxidative stress in different spermatogenic cell types, let alone measurements that give information about this.

# 3. MATERIALS AND METHODS

## 3.1 TESTICULAR DISAGGREGATION AND PREPARATION FOR FACS

### 3.1.1 Testicular Disaggregation

Mouse testes were extracted in The Central Animal Facility (CDP) of the University Medical Center Groningen (UMCG). Testes were placed one by one in a 35mm Petri dish with 1 ml room temperature Gey's Balanced Salt Solution (GBSS) to remove the tunica albuginea. This was done by puncturing the middle of the testis with a sharp-tip tweezer. With a second pair of tweezers an edge of the hole was pulled apart while the opposite edge was held by the sharp-tip tweezers. After this the tunica albuginea was pinched with the tweezers and the seminiferous tubules were squeezed out with the sharp-tip tweezers. Seminiferous tubules from 1 mouse (2 testicles) were incubated with 10 ml of pre-warmed GBSS with 0.5mg/ml collagenase type II and 4μg/ml DNase at 33 ºC for 15 min in constant agitation (60 rpm). Samples were manually mixed by gentle inversion before incubation and every 5 min during incubation. Tubules were allowed to sediment for 4min inside the incubator and the supernatant was discarded. After this the whole process of incubation with GBSS, collagenase and DNase was repeated. After this the seminiferous tubules were incubated with 9 ml of pre-warmed GBSS with 0.5 mg/ml collagenase type II, 10X commercial trypsin, and 4 μg/ml of DNase at 33 ºC for 15 min at 60 rpm. Following incubation 1ml of 10X commercial trypsin was added and incubated at 33 ºC for 15 min at 60 rpm. The mix was pipetted for 3 min with a Pasteur pipette in order to break the cell aggregates. After this 500 μl of Fetal Bovine Serum (FBS) was added to inactivate the trypsin. The suspension was filtered with a 70 um cell strainer into a 50 ml tube. This was centrifuged for 3 min at 960 rcf and 25 ºC. Supernatant was discarded and the volume was topped up until the 5 ml line. After resuspension of the pellet 40 ul of DNase was added. 5 μl of this cell suspension was taken for counting.

### 3.1.2 Cell counting

The 5 μl from the cell suspension was mixed with 25 μl GBSS and 30 μl trypan blue. Cells were counted using a light microscope. Based on this counting the amount of Ho342 was determined. 6 μg of Ho342 per million of cells was added and this is incubated for 1h at 33 ºC at 60 rpm. After incubation 40 μl of DNase was added, this suspension was ready for transport to the FACS.

## 3.2. FLUORESCENCE-ACTIVATED CELL SORTING (FACS)

### 3.2.1 FACS

After the testicular disaggregation is completed the sample was run through the fluorescence-activated cell sorting machine. Ho-342 staining is used to differentiate between different stages of spermatogenesis based on chromatin quantity and structure. Blue fluorescence is proportional to DNA content and an increase in red fluorescence represents less condensed chromatin and structural variations. A gating strategy was determined based on literature on round spermatids see Figure 1. With the use of this gating strategy round spermatids were sorted and collected in a vial with 200 µl GBSS/FBS 5%. For purity control 50k cells and for magnetometry another 50k cells were collected in a separate vials.

### 3.2.2 Post-FACS

All vials collected in FACS are centrifuged for 3 min, 960 rcf, and 25 ºC. Following this the supernatant was pipetted out, for magnetometry samples the pellet was resuspended in 80 µl of 5% GBSS/FBS + 4 ug/ml FNDs. The pellet in the vial for purity control was resuspended in 100 µl of 5% GBSS/FBS .

## 3.3 PURITY CONTROL

Microscope slides were prepared using the sample obtained from the FACS. With a grease pen a rectangle was drawn on the slide. The sample was pipetted in the rectangle on the slide. To fix the cells 100 µl of 4%formaldehyde solution was added and mixed by pipetting. The slide was incubated at room temperature until completely dry. Then after the dry slide was washed three times with Photo-flo 1% in DEMI water for 2 min each and under gentle shaking (240/min). In the next step the slide was washed with Phosphate Buffered Saline (PBS) for 5 min under gentle agitation (240/min). When the slide has dried 20 µl of mounting medium (Vectashield) was added and covered with a coverslip. Using a confocal microscope pictures were taken to determine a purity percentage (Figure 5). The purity control was done to check whether the gating strategy used at the FACS was correct.

## 3.4 DIAMOND MAGNETOMETRY

### 3.4.1 Sample preparation

The sample obtained from the FACS, dedicated for the nanodiamond magnetometry, was plated in a quarter of a 35mm 4-quartered glass dish, coated with 0.4% gelatin. The dish was incubated for 1h at 33 ºC.

### 3.4.2 T1 measurement

To study the free radical production in spermatids in real-time T1 measurements were performed in mice and compared with the results of mice with different ages. For relaxometry experiments (T1) the samples obtained from the FACS were used and treated as described in section 2.2.2.

The relaxometry experiments were performed using a homemade magnetometry setup available in the UMCG. A description of the setup used for the T1 measurements can be found elsewhere [10]. The first step is to localize the cells and pick a cell with the desired morphology, this was done using a brightfield microscope. T1 parameters were set: 10000 repetition, and the 5s re-centering function on. FNDs must be at least 1 million photon counts per second and show no sign of bleaching. When the FND was identified using these criteria the automatic T1 measurement was performed. The measurement consists of polarizing the NV centers with a laser pulse into the ms = 0 state of the ground state. Then we measured again after specific dark times to see whether the Nv centers were still in this state or had returned to equilibrium. The time that it takes to reach the equilibrium gives a quantitative measurement of the radical concentration in the surrounding of the nanodiamond. Raw T1 data was analysed using LabVIEW, MATLAB R2021b, and graphpad prism 5. Significance was determined by the One-way ANOVA test together with Tukey's Multiple Comparison Test.

# 4. RESULTS

## 4.1 FLUORESCENCE-ACTIVATED CELL SORTING

We isolated round spermatids from mouse testicular cell suspensions obtained from the testicular disaggregation. The gates used for sorting were defined based on observed clusters of cells and taking into account the expected location of the round spermatids. Round spermatids have haploid non-replicated (1C) DNA content and show a compact chromatin structure (chromocenter). These characteristics result in low Hoechst blue fluorescence and a narrow range of Hoechst red fluorescence. On top of this round spermatids are small cells that as the name suggests are round in shape.

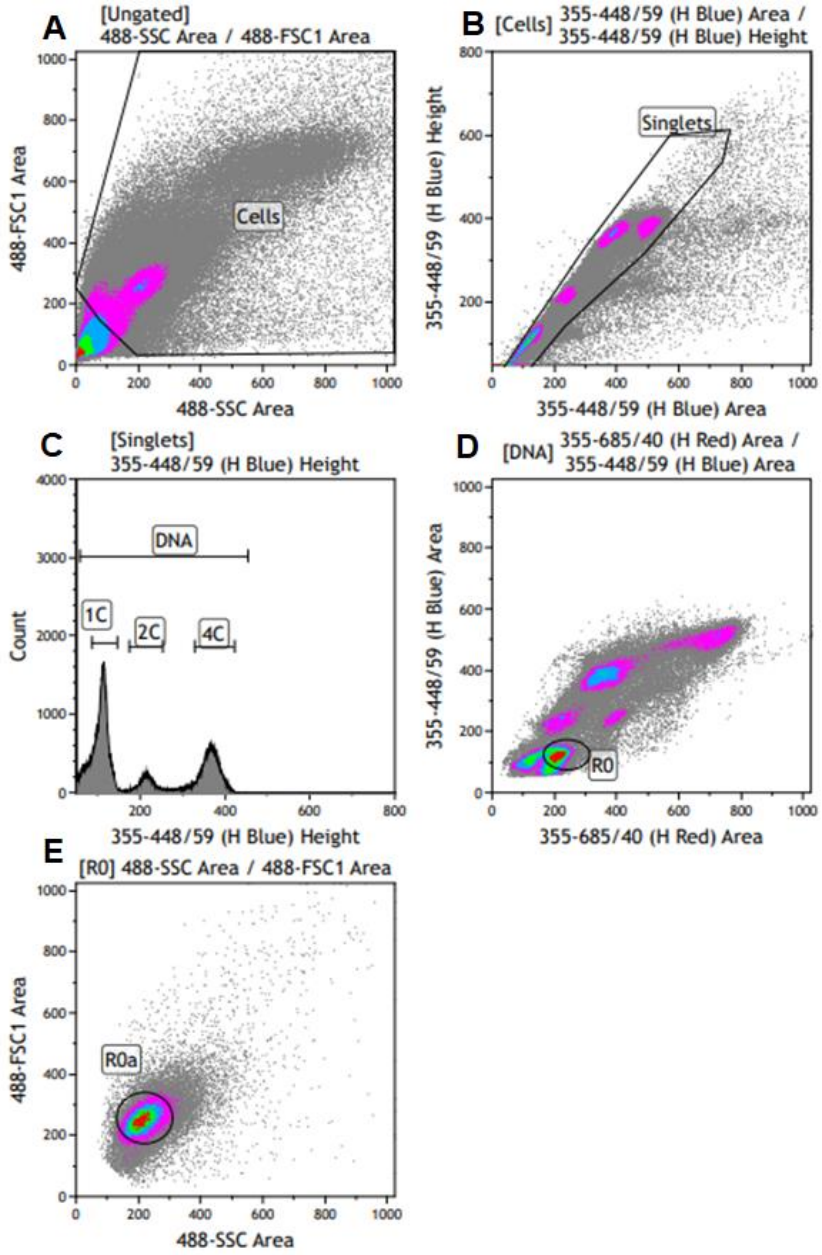


**Figure 1. FACS gating strategy used to sort round spermatids. A) The FSC/SSC plot is used to gate everything except cells/particles with a very small size such as, debris and early spermatids. B) Singlets are gated by following the diagonal of the cell population in the H-blue area/width-plot. C) Intensity in blue hight reflects the DNA content, round spermatids are haploid (1C). D) Based on H-blue and H-red fluorescence, different cell populations could be distinguished. E) Gating on the R0 population of figure 1D further sorts the R0 population.**

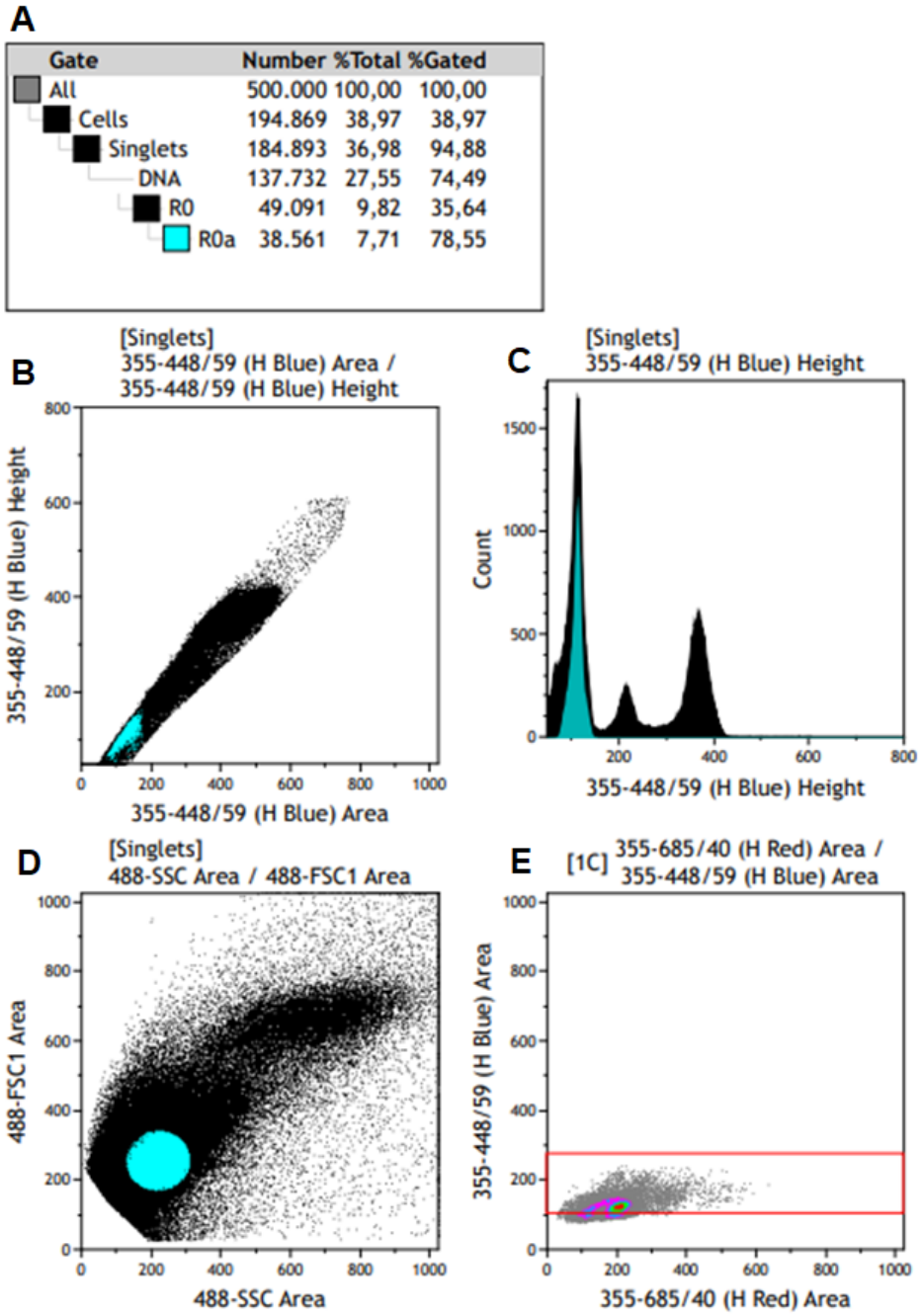


**Figure 2. FACS Gating strategy used to isolate round spermatids. A) Statistics about sorted cells. B,C,D) Blue area reflects where the R0a population of figure 1E originate from. E) Area between the red lines is where haploid cells are expected.**

The first gating was based on forward and side scattering and was used to filter out debris (Figure 1A). After this only single cells were gated based on the height and area of the blue fluorescence (Figure 1B). DNA content based on the singlets that have been gated (Figure 1C). Based on the area of the blue and red fluorescence different cell populations have been seen, the R0 population represents the round spermatid population (Figure 1D). To further specify the R0 population the gating of R0a is projected in Figure 1E. Figure 2 shows where the cells that were labelled as R0a originate from. As expected these cells were all in the area of low blue fluorescence (Figure 2B), and as mentioned before they showed a narrow range in the red fluorescence (Figure 2E). They all were characterised by the 1C DNA content (Figure 2C). Forward and side scatter gives an indication of size (Figure 2D). To validate the gating strategy and to determine the specificity a purity control was performed, results are shown below.

## 4.2 PURITY CONTROL

Purity of the isolated cells was determined by confocal microscopy assessment. Figure 3 shows a representative image of round spermatids with their characteristic chromocenter, and the population that expected for the majority of contamination, the elongated spermatids. To differentiate between them is very easy based on their morphology. Multiple pictures were taken so that approximately 100 cells were captured, based on cell counting a percentage was calculated. Figure 4 shows one of the pictures used for purity control. Table 1 shows the purity percentages from different experimental days, including the percentage of the expected contamination population (elongated spermatids) and other unidentified cells.

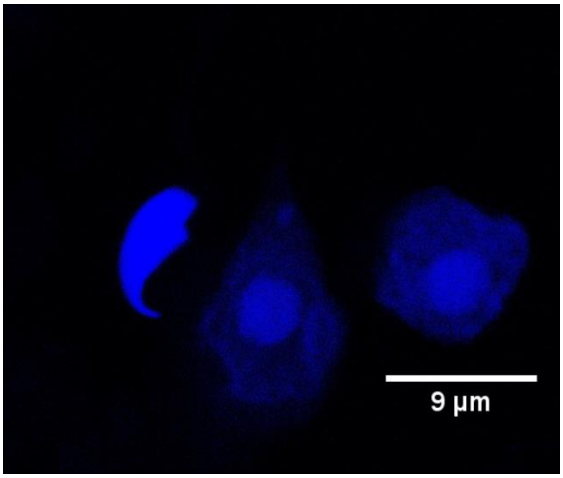


**Figure 3. Representative image round spermatid. Cell on the left is an elongated spermatid. Picture was taken with a confocal microscope 63x magnification, zoom: 5.5. DNA staining with Ho342.**

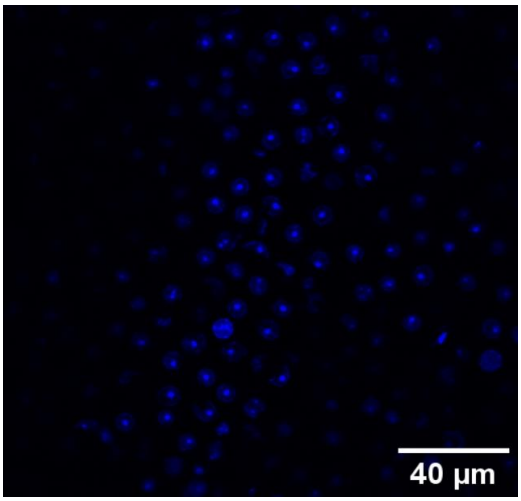


**Figure 4. Picture used for purity counting (20/06/2022). Picture taken with confocal microscope 63x magnification, zoom: 1. DNA staining with Ho342.**

| Date | # Total cells | % Round spermatids | % Elongated spermatids | % Unidentified cells |
|---|---|---|---|---|
| 11/05/2022 | 86 | 93 | 6 | 1 |
| 07/06/2022 | 105 | 90 | 7 | 3 |
| 13/06/2022 | 116 | 84 | 12 | 4 |
| 16/06/2022 | 254 | 87 | 9 | 4 |
| 20/06/2022 | 133 | 93 | 2 | 5 |

**Table 1. Data obtained from purity pictures. As expected the round spermatids account for the highest fraction in the sorted cells.**

Data analysis of the purity control verifies that the gating strategy used in FACS was correct. Round spermatids accounted for almost 90% of all the cells that were sorted with the FACS strategy. As expected the major contamination came from elongated spermatids, besides this a number of cells could not be identified resulting in a third subpopulation.

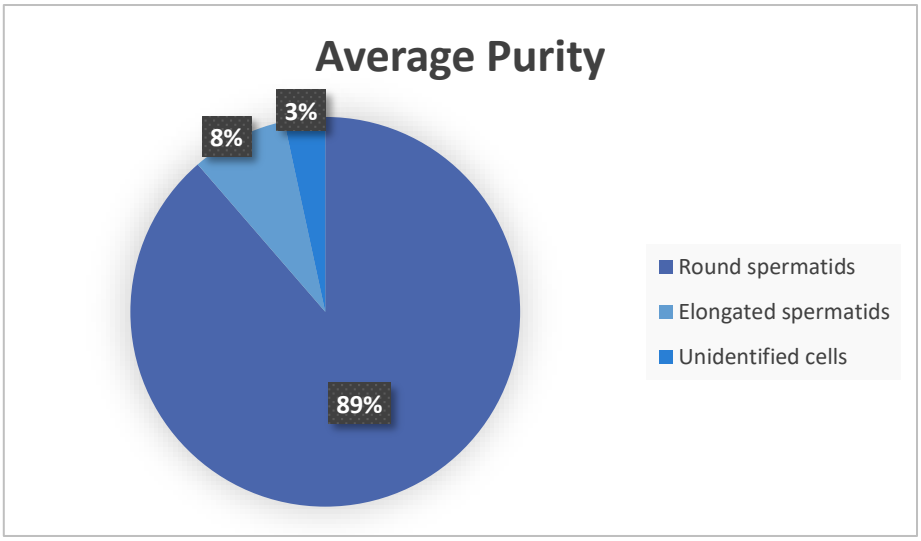


**Figure 5. Chart with average percentages of different subpopulations in the sorted cells**

## 4.3 DIAMOND MAGNETOMETRY

Results of the T1 measurements is shown in Table 2. For roughly every animal the measurement was repeated 12 times (Animal 2 has only 3 measurements). Raw T1 data was analysed using labview, matlab R2021b, and graphpad 5 in order to create a scatter plot that is shown in Figure 6. Averages of animals 1,3, and 4 consist of 10 measurements due to the removal of outliers using the interquartile method. The high T1 values of animal 3 may very well be due to fitting errors from the machine, this could mean that significance are not very reliable. Measurements of oldest animal (number 3) shows the least spread in T1 values. Based on this data the oxidative stress due to free radicals is the highest in animals 2 and 4, animals with a completely different age, based on these values no conclusion relating to the age of the animal can be drawn.

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| Animal number | Date of measurement | Age of the animal in days | Average T1 measurement | Standard Deviation | Standard Error |
|---|---|---|---|---|---|
| 1 | 23.05.2022 | 67 | 188.50 | 65.95 | 20.86 |
| 2 | 24.05.2022 | 347 | 98.07 | 17.15 | 9.90 |
| 3 | 09.06.2022 | 273 | 313.10 | 189.7 | 59.99 |
| 4 | 13.06.2022 | 84 | 95.67 | 63.60 | 19.17 |

**Table 2. Animals used for T1 measurements. Information is given about the age and mutation of the animal, together with the average, standard deviation, and standard error of the T1 measurements.**

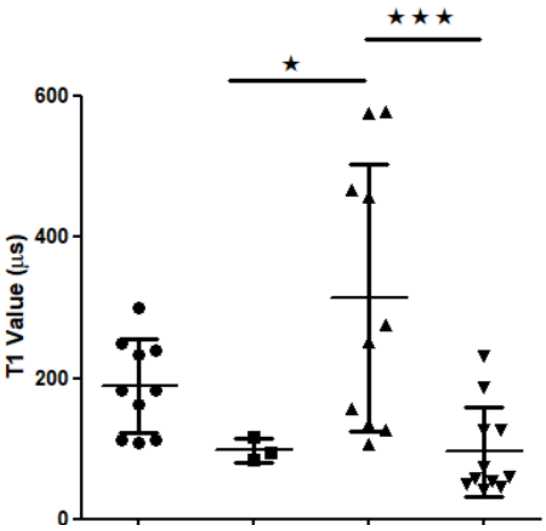


**Figure 6. T1 values (µs) from repeated measurements per mouse. Horizontal line reflects the mean and the error bar the standard deviation. One-way ANOVA together with Tukey's Multiple Comparison Test resulted in significance for the difference between 2 and 3 with *P<0.05 and 3 and 4 with ***P<0.001.**

# 5. DISCUSSION AND FUTURE PERSPECTIVES

The use of FACS to isolate specific cells from the spermatogenesis process is proven to be successful. Round spermatids were isolated from a testicular cell suspension with a 90% purity. This is in line with earlier strategies used for round spermatid isolation [9]. Performing the T1 measurements turned out to be the most challenging part of this study. To be able to compare results between animals a standardized protocol was used, however the biological sample can have a big impact on the measurements. Animals were used with different mutations that were not active, however to conclude that they are genetically similar cannot be done. Due to the fact that we were dependent on the sample obtained from the animal facility of the UMCG there was a great variance in the biological sample to start the experiment with. Due to this it was hard to interpret the results and draw conclusions based on the measurements performed. However, we expect that due to optimization of the protocols that results will be more significant when repeated in the future under more controlled circumstances.

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