Diamond magnetometry to measure oxidative stress in FACS-based purification of mouse spermatogonia

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Abstract

The age of fathers is increasing worldwide and so is the knowledge about the downside effects of advanced paternal age on fertility and child outcome. One of the factors that may play a part in this, is oxidative stress. In this experiment, the levels of oxidative stress are measured in the unipotent stem cell of the mouse germ line, spermatogonia. Oxidative stress is known to increase during aging and therefore we hypothesize that aging mouse spermatogonia show elevated levels of free radical load. Fluorescent activated cell sorting (FACS) is used to purify cell population while free radical loads are measured with a novel technique called *diamond magnetometry*. Diamond magnetometry focuses on the relaxometry time; a faster relaxometry time is associated with increased magnetic noise and elevated free radical load. In this experiment we successfully sorted spermatogonia with purities of at least 80 percent in all samples. Two younger populations were compared with an intermediate population and an aged population. Diamond magnetometry showed significant differences between one younger and older population (higher T1 values in younger model, $P<0,001$), and between the younger and intermediated population (higher T1 values in younger model, $P<0,05$). However, difference account for just one younger animal and the two younger population also differed statistically ($P<0,001$). Thus, we succeeded in sorting a purified population but to establish our hypothesis, more research is necessary.

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Advanced paternal age has been associated with both infertility[1] and multiple diseases in offspring including, autism[2], schizophrenia[3], and achondroplasia[4]. This could be explained by the fact that multiple parameters in sperm change as men age, such as, an increase in DNA errors [5], reduced motility [6], and altered morphology [7]. Oxidative stress might be one of the most important factors affecting sperm quality [8]–[11]. Since the age of fathers is increasing worldwide [12], and oxidative stresses are known to increase as we age [13], it is important to understand the potential harmful effects of aging on fertility and offspring. In this study, we focus on the levels of oxidative stress in mouse spermatogonia, the unipotent stem cells that give rise to all sperm cells.

Oxidative stress in cells is caused in part by the increase in reactive oxygen species (ROS). In most cases, ROS are radicals that have an extra electron in their outer shell. As a result, they are highly reactive molecules that can induce damage in macromolecules such as DNA, lipids, and proteins [14]–[17]. The radical load of ROS can be measured with a novel technique known as diamond magnetometry. Diamond magnetometry is based on the use of fluorescent nanodiamonds (FNDs) as markers for live measurements of free radical load. FNDs are diamond particles that are greatly appreciated due to their biocompatibility and optical properties. The fluorescence of FNDs comes from their nitrogen vacancies (NV-centre). Nitrogen vacancies are crystal defects that causes the FNDs to emit a stable, non-bleaching red light after exposure to green/yellow light. What makes FNDs interesting for measuring radical loads, is the fact that the emission from FNDs depends on the magnetic noise in their environment. When

FNDs are hit with a green/yellow laser, they are pushed into a bright state. The fluorescence of the NV-centres is then measured during so-called dark times. During these dark times, the system relaxes from the bright state to a lower equilibrium, thus becoming dimmer. By varying these dark times, it is possible to measure the systems natural relaxation time, also known as 'T1 relaxometry' or 'T1 measurement'. Relaxation of the system occurs faster in the presence of high magnetic noise. That means that elevated levels of free radicals induce a faster dimming emission of NV-centres, and thus smaller T1 values. In contrast to other techniques, this technique makes it is possible to measure oxidative stress in real-time.

The deleterious effects of oxidative stress are mainly observed in mature spermatozoa. However, it is unknown whether oxidative stress levels are already elevated in the earliest stages of the highly complex process of spermatogenesis. During spermatogenesis, undifferentiated spermatogonia divides and proliferates to eventually become future sperm cells[18]. Because of the highly dynamic nature of spermatogonia – spermatogonia undergo several mitotic divisions before differentiating - they are vulnerable to oxidative damage to DNA, lipids, and proteins. As a result, radical-induced damages could potentially accumulate in the stem cell population and result in unhealthy spermatozoa.

To study the levels of free radicals in spermatogonia, Fluorescence-Activated Cell Sorting (FACS) is used to purify a spermatogonial population from mouse testis. FACS is a specialized type of flow cytometer that makes it possible to distinguish individual cell types based on parameters such as cell size, relative complexity, and fluorescence intensity[19]. On top of that, FACS has proven to be effective in the purification of mouse germ cells [19]. To distinguish between the different cell types, the nucleic acid-binding dye Hoechst 33342 (Ho-342) helps to highlight the specific heterogeneity in the male germline. With the Ho-342 dye,

germ cells can be enriched based on DNA content, complexity, size, chromatin state, and morphology.

In conclusion, oxidative stress might be one of the most important factor in age-related infertility and offspring diseases. In this study we try to measure the levels of free radicals in spermatogonia since they are the origin of all sperm cells. To accomplish this, diamond magnetometry is combined with FACS-based cell type purification.

Materials and Methods

Animals

In this project black … mouse have been used to study the effects of aging on oxidative stress levels in spermatogonia. In this phase of the project, wild type mice have been used with different unexpressed mutations due to the unavailability of other options.

Table 1. Classification of mice.

Mouse No.	Age (days)	Mutation
1.	81	*Tg (H2B-GFP)*
2.	84	*WASHC1loxp;VillinCre*
3.	103	Tg(p16-3MR); Cyp2c70-KO
4.	150	*C57BL*

Collection and Disaggregation of Testicular Tissue

Prior to the testis disaggregation collagenase type II (0,5 mg/ml) was added to Gey's balanced salt solution (GBSS).

1. Testes of one mouse were decapsulated and placed in two 15 ml conical tubes containing 10 mL pre-warmed collagenase solution and DNase (4 µg/ml) (33 ºC). The sample was incubated at 33 ºC for 15 minutes at 60 rpm horizontal agitation. Every 5 minutes, the sample was manually mixed. After 15 minutes, tubes were placed vertically for 4 minutes to sediment. The supernatant was discarded and the procedure was repeated but without manual mixing every 5 minutes. Then the sample was incubated in 9 mL pre-warmed collagenase solution, DNase (4 µg/ml), and 1 mL 10x trypsin at 33 ºC for 15 minutes at 60 rpm horizontal agitation. 1 ml of 10x trypsin was

added before the final incubation at 33 ºC for 15 minutes at 60 rpm. After the final incubation, samples were pipetted for 3 minutes with Pasteur pipettes to break down cell aggregates in the solution.

2. 500 mL fetal bovine serum (FBS) was added per 15 ml tube to inactivate trypsin and the suspension was filtered through a 70 µm cell strainer into a conical 50 ml tube. The suspension was centrifuged for 3 min at 960 rcf and 25 ºC. The supernatant was removed and the pellet was resuspended in enough GBSS/FBS 5% to top up the volume until 5 ml. To prevent clumping of cells, 20 µl DNase (10 ug/mg) per testicle was added to the solution.
3. Based on the number of cells, Hoechst (Ho-342) (stock solution 10 mg/ml) per million cells was added before incubation for 1 h at 33 ºC under constant agitation (60 rpm). For counting, 5 µl of cell suspension was mixed with 25 µl GBSS and 30 µl trypan blue. An additional 20 µl DNase (10 ug/mg) per testicle was added to the solution to prevent clumping during incubation.

Hoechst Staining and Fluorescence-Activated Cell Sorting

Cell sorting was performed with the Beckman Coulter MoFlo Astrios cell sorter. The Hoechst stain was excited with a UV-laser and scatter was triggered with a 488-nm blue laser. To detect Hoechst-blue and Hoechst-red, a 463/25-nm band-pass filter and a 680-nm LP band-pass filter were used, respectively. Because the emission of Ho-342 shifts from blue to red with an increased dye concentration, fixed cells/dye ratio was maintained during the project as described in the ‘testis disaggregation’ section. For analysis of the sample, a 70 nm nozzle and a sorting flow rate roughly between the 5000 and 15000 events/second was used. Prior to cell sorting,

cells were filtered through a 0,35 µm filter and 500000 events were detected to set the gating strategy. The gating was performed as follows:

1. Cell debris was excluded based on forward- versus side-scatter.
2. Singlets were gated by narrowing the width of the (H0-342) blue-height and -area scatter.
3. On the Ho-342-blue histogram, a DNA gating was created to distinguish haploid (C), diploid (2C), and tetraploid (4C) cells.
4. R6 (spermatogonia) were identified based on the Ho342-blue/Ho342-red plot.
5. To justify the R6 identification, backgating was used in the forward-side scatter plot, the blue width-area plot, the DNA histogram, and the 2C- Ho342-blue/Ho342-red plot.

After gating, cells were collected in low-binding collection vials with 200 µl GBSS/FBS 5%. 50k of purified cells were used for diamond magnetometry and an additional 50k for microscopic evaluation.

Microscopic Evaluation of Purified Cells

To justify the gating, a confocal microscope was used to identify spermatogonia based on their nuclear structure and cell morphology. After FACS, the collection vials were centrifuged in a microcentrifuge for 3 minutes at 25 ºC and 960 rcf. The supernatant was removed and the cells were resuspended in 100 µl GBSS/FBS 5%. 100 µl of cell suspension was mounted on slides and fixed with 4% PFA. After washing steps with Photo-flo 1% and PBS, slides were stored in the

dark at 4 ºC (same-day microscopy) or -20 ºC (long-term storage). To determine purity, at least 100 cells were counted and analyzed based on nuclear structure (Hoechst stain) and cell morphology (in spermatogonia, the size of the nucleus was compared to the bright-field image of the cell).

Diamond magnetometry

For magnetometry, the collection vials from FACS were centrifuged in a microcentrifuge for 3 minutes at 25 ºC and 960 rcf. The supernatant was removed and the cell pellet was resuspended in 80 µl of GBSS/FBS 5% + 4 ug/ml FNDs (120 nm). 70 µl of the suspensions was added to gridded glass-bottom dishes coated with 0,4% gelatin. For T1 relaxometry measurements, a homemade magnetometry setup was used that allowed NV-centers to be brought into a bright ground state with laser pulses in varying time intervals, at the same time the response of these centers could be measured at specific time points. To data was processed by using MATLAB 2021, T1 viewer and excel. A … test in GraphPad 5 was performed to check statistical significance.

Results & Discussion

Fluorescence-Activated Cell Sorting

During spermatogenesis, male germ cells undergo various changes in terms of morphology, DNA content and chromatin structure. Therefore, the use of the DNA-binding dye Ho-342 in FACS is functional for distinguishing these cell types. Much can be said about the morphology of the cell by forward scattering (FSC) and side scattering (SSC). Since forward scattered light is refracted by a cell in the flow channel and remains in the light path, this tells us something about the size of the cell. Side scattered light, on the other hand, is refracted by cells in a direction outside the original light path and is more an indicator for the complexity of a cell. For this reason, in Figure 1.a, the lower left area in the graph has been removed from the gating. This material is considered very small in size and complexity and might be debris or early spermatids. Figure 1.b show the attempts to exclude doublets by narrowing the gating in the H-blue height/area plot.

Further specialization with FACS can be done based on DNA content and chromatin structure. When Hoechst is excited with UV light, it can emit both blue and red fluorescence. While blue fluorescence is proportional to DNA content, red fluorescence is an indicator of chromatin structure. An increase in H-blue is caused by a higher DNA content (e.g., primary spermatocytes (4C) have higher H-blue fluorescence than spermatogonia (2C), while increased values in H-red are due to a less condensed chromatin structure. In Figure 1.c, a H-blue/H-red plot is used to distinguish between cell types. Since spermatogonia has a DNA content of 2C, Figure 1.d separates cell types based on DNA content. This confirms that the selected spermatogonia population is in the 2C region. Two populations can be distinguished in the 2C

region, the spermatogonia and the secondary spermatocytes. However, the latter are haploid cells that have less chromatin density than the diploid spermatogonia and will therefore be more red-shifted. However, the gating of spermatogonia cannot completely rely on the DNA content as these cells are able to efficiently efflux the Hoechst dye and therefore this gating is optional[20]. An additional gate in Figure 1.e was used to counteract any contamination. The gating was shifted to the left to avoid contamination with secondary spermatocytes, while cells with a higher FSC were excluded to remove pre-leptotenes.

Although several studies have used PI to represent the life/death ratio of cells, we chose not to do so because PI can be toxic to the cells. However, since FACS and the total duration of the experiment stresses the cells a lot, it would be beneficial to consider approaches to displaying the life/death ratio in a follow-up study. Especially if these cells will be used for subsequent research. This could involve the use of PI or a life/death assay post FACS. It could also be beneficial to culture purified cell populations to later perform studies under more controlled conditions. For this, probably an other media should be used and usage of PI is not recommended due to its toxicity.

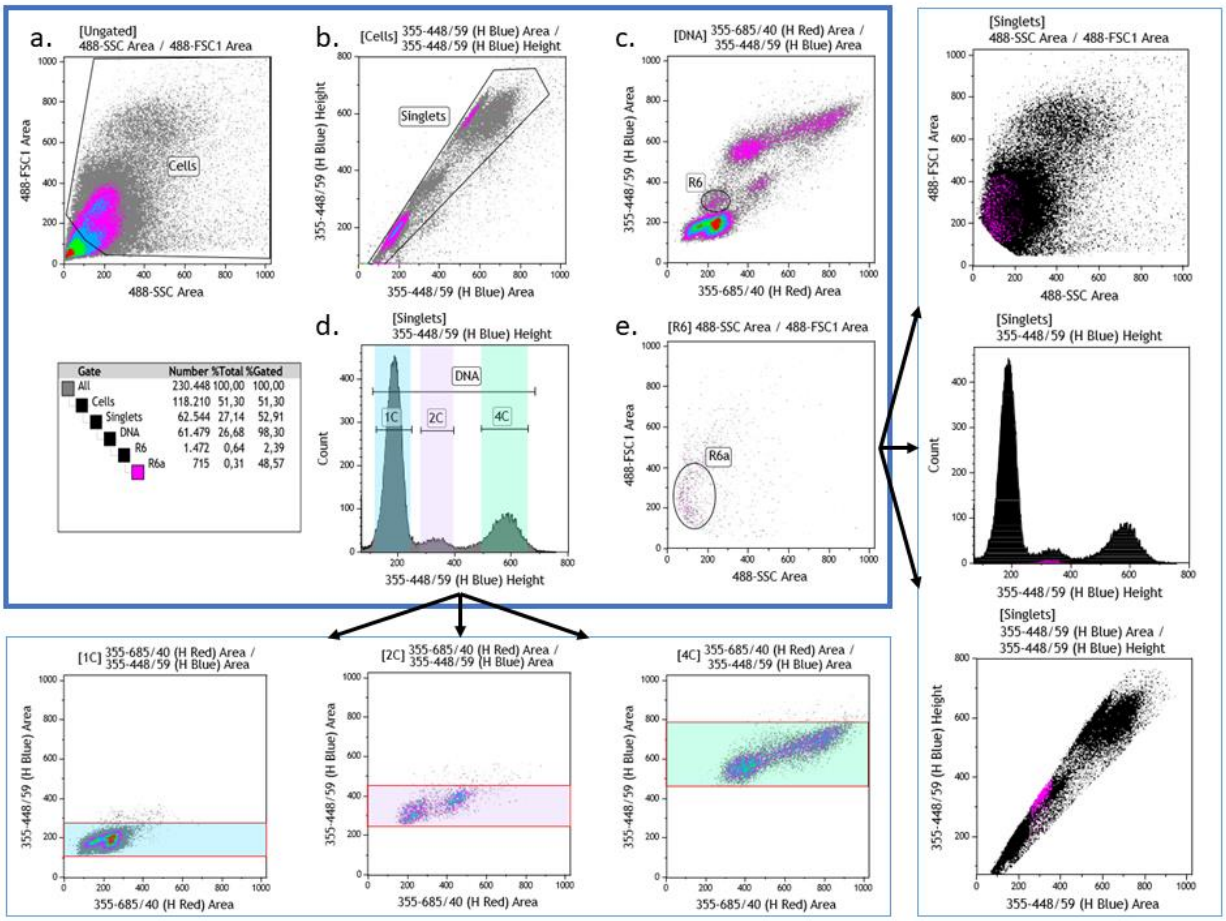

Figure 1. Representation of FACS-based purification of mouse spermatogonia (R6): **a**) The FSC/SSC plot is used to excludes materials with a very small size such as, debris and early spermatids. **b**) Singlets are discriminated by narrowing down the width of the cell population in the H-blue area/width-plot. **c**) Based on H-blue and H-red fluorescence alone, different cell populations could be distinguished. **d**) Gating in 'c' was proved to be correct in relation to DNA content of spermatogonia (2C). Next to the R6 population, Meiosis II spermatocytes are red-shifted. **e**) Extra R6 gate to exclude potential contamination with cells that are similar to R6, such as round spermatids (R0) and Meiosis II spermatocytes (R1).

Purity analysis

For purity analysis, the cells were judged based on Hoechst staining and bright field. Spermatogonia have a typical pericentric heterochromatin structure (Figure 2). In addition, the nucleus of spermatogonia is relatively large in relation to the total cell size. The cells were counted and are divided into "spermatogonia", "secondary spermatocytes" and "spermatids" in Table 1. The latter two because they are most likely contaminants. Other cells are not included in the table.

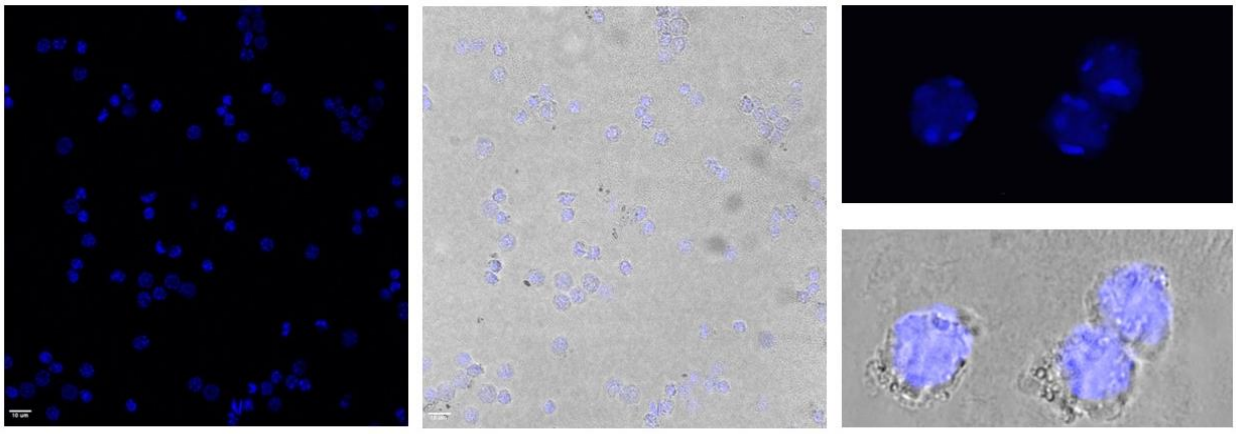

Figure 2. Morphology of spermatogonia. This figure shows the Hoechst staining and bright field of FACS sorted spermatogonia. The figure shows the pericentric heterochromatin structure and nucleus/cell wall-ratio that characterizes spermatogonia.

Table 1. Purity of FACS-sorted cells. Both secondary spermatocytes and spermatids potentially represent the greatest contamination. Cells not included in the total are considered "other".

Mouse no.	Cells counted (total)	Spg	Spc II	Spd
1.	100	80	16	2
2.	100	92	4	1
3.	100	88	4	2
4.	55	55	-	-

Based on the purity analyses, it seems that we succeeded in sorting pure populations with a purity of at least 80%. When counting the cells we aimed for 100 cells, but in the fourth sample we counted only 55. Although it is more difficult to draw conclusions in this sample, the purity of the first 55 cells was 100%. The lower number of cells counted may be a result of the purity, since very strict gating in FACS also means that many cells are filtered out.

In this experiment, we used Hoechst staining to assess the cells. It is not impossible, but very difficult, to assess cells based on this alone. Nakata et al. show in their experiment that the fluorescence of spermatogonia is very similar to that of pre leptotene, some primary spermatocytes and secondary spermatocytes[21]. Due to the H-blue/red gating, contamination with primary spermatocytes would be very unlikely but because of the possible presence of preleptotene and secondary spermatocytes, an additional means of identification may be appropriate. Nakata et al. used multiple staining to differentiate cells and for this experiment that could also be a way to exclude contamination.

Diamond magnetometry

Diamond magnetometry allows live monitoring of oxidative stress in a cell. In this experiment, we succeeded in making T1 measurements. For each cell, 12 measurements were taken. Using the interquartile method, outliers were filtered out, providing a reliable data set. However, since we do not know whether the diamonds were inside the cell or on the outside, the reliability of these measurements is debatable. As a matter of fact, cells in the male germline did not appear to be very good at taking up diamonds; after all, they are not cancer cells or phagocytic cells. Figure 3 presents T1 measurements found in spermatogonia.

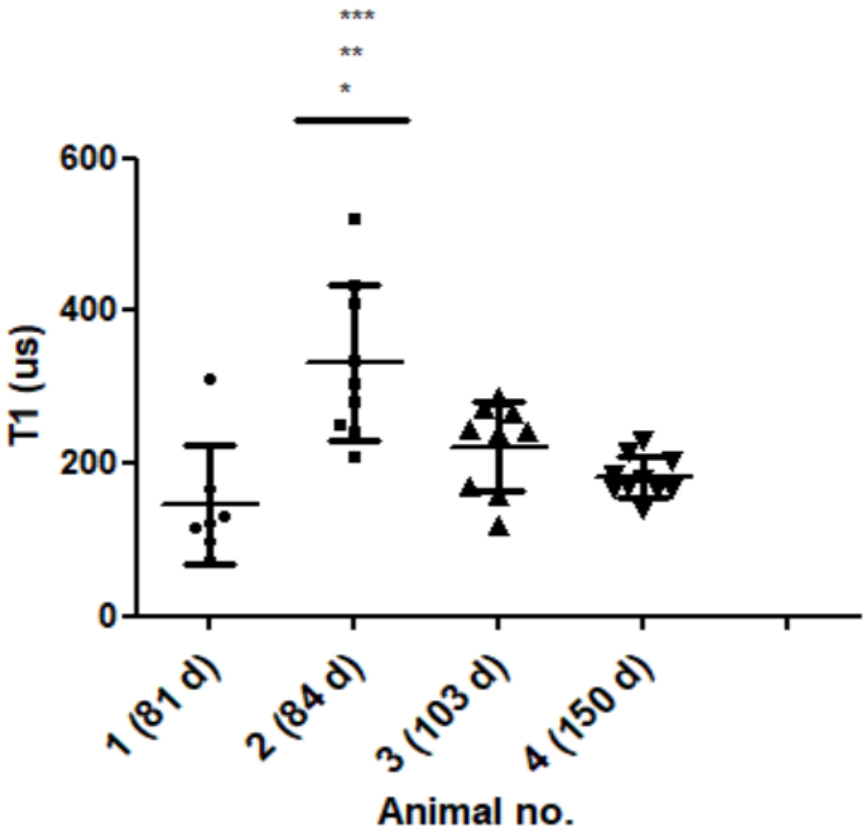

Figure 3. T1 measurements in mice. Statistical analysis was performed by a one-way ANOVA test followed by Tukey's Multiple Comparison Test. Significance was proven for the difference between 1 and 2 and, 2 and 4 with ***P<0,001. Differences between two and three proved significance of *P<0,05.

The magnetometry values of mouse 2 were significantly higher than those of the other mice. This means that the relaxometry time was longer in this mouse, and thus a lower magnetic noise around the diamond. Therefore, it is likely that the free radical count was lower in mouse 2. Based on mice 2,3, and 4, one could argue for a trend in decreasing T1 values, meaning increasing radical loads as mice age. However, the significant differences between the two younger mice counteracts this statement. Further research is necessary to accept or reject the trend seen in 2,3, and 4. Another noticeable finding is the fact that the measurement appeared to standardize as the mouse aged. For example, the standard deviations in mice 3 and 4 are smaller than the values of 1 and 2. Whether this can be explained by age is difficult to say.

Based on the results, it cannot be concluded that aging has a positive correlation with oxidative stress. Firstly, because only mouse 2 deviates significantly from the others. Although the data might suggest a trend, that cannot be stated, especially with a small sample size. Secondly, because this experiment was not yet optimal and too many factors may play a role in the measurements of oxidative stress. For example, there are large differences in the mice due to different mutations, inactive or not. On top of that, we were not certain whether the diamonds were incorporated into the cells or attached to the cell walls. In a follow-up to this study, it will be important to establish a structured approach that generates more reliable results. Several suggestions to reach this goal are:1) use of a single mouse population. 2) Use of control groups. 3) Extra validation of purity. 4) life/dead assay after FACS. 5)incorporation of diamonds into the cell and monitoring them.

In summary, this experiment demonstrated good purification of spermatogonia with FACS and purity analysis but diamond magnetometry, like many other factors, should be

optimized to generate reliable measurements. We are convinced of the potential of this study and believe that it can contribute to a better understanding of aging germ cells.

Future perspectives

As the age of fathers is increasing worldwide, it is important to study the effects of advanced age on child outcome and fertility. To this end, investigation of oxidative stress throughout the germline is essential to explain the underlying mechanisms of disease and infertility. Moreover, measuring oxidative stress in the male germ line may help to rule out potentially diseased sperm, which may be applicable in new techniques such as assisted reproductive technology. With this experiment, we hope to have set a foundation for future studies that can together contribute to new techniques and improved insights about aging germ cells.

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