Automatized cytogenetic screening of male breeding animals

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First year (2013.10.01. – 2014.10.30.)

The main aim of the first year of the project was to find the most suitable DNA-specific fluorochrome which can be applied on commercial benchtop flow cytometers equipped with a 488 nm laser line. Since the original protocol by Larsen et al (Larsen, J, Christensen, K, Larsen, J, Jensen, P, Gustavsson, I & Christensen, I. High resolution DNA flow cytometry of boar sperm cells in identification of boars carrying cytogenetic aberrations, Theriogenology, Vol. 3-4, pp. 501-511., 2004) applied UV-excited DAPI as DNA-fluorochrome, the main goal was to find the best alternative. So far I tested the following fluorochromes: SYBR- 14 (excitation maximum: 489 nm; emission maximum: 520 nm), propidium iodide (PI, excitation maximum: 535 nm; emission maximum: 614 nm) and acridine orange (excitation maximum: 500 nm; emission maximum: 526 nm when bound to intact double stranded DNA).

Fluorochrome characteristics (source: www.lifetechnologies.com):

SYBR-14 is a membrane-permeant nucleic acid stain developed at Molecular Probes (now Life Technologies) and available as Component A of the Live/Dead Sperm Viability Kit. It belongs to the SYTO dye family, a group of cell-permeant cyanine dyes. Unfortunately, the supplier does not provide exact information about the DNA-binding characteristics of the dye.

Propidium iodide (PI) is a popular red-fluorescent nuclear and chromosome counterstain. Since propidium iodide is not permeant to live cells, it is also commonly used to detect dead cells in a population. Propidium iodide is intercalating between the bases with little or no sequence preference and with a stoichiometry of one dye per 4–5 base pairs of DNA.

Acridine Orange is a cell-permeant nucleic acid binding dye that emits green fluorescence when bound to dsDNA and red fluorescence when bound to ssDNA or RNA. This unique characteristic makes acridine orange useful for cell-cycle studies. Acridine orange is an intercalator when bound to double stranded DNA and a stacking dye when bound to single stranded DNA or RNA.

Experiments were carried out on boar and bull spermatozoa. Since from the point of view of instrument setting and assay optimalization, boar and bull spermatozoa do not differ, and

I had access to bull samples with known cytogenetic defects, I included bull samples in the initial phase of the project.

Flow cytometric measurements were done on a Beckman Coulter FC 500 flow cytometer equipped with a 488 nm 20 mW Ar ion laser. Fluorescence intensity of SYBR-14 was collected with the FL 1 (525 nm BP) detector, PI was detected on the FL 3 (620 nm SP), acridine orange signals were collected with both FL 1 (green signals) and FL 3 (red signals). Spermatozoa and debris were distinguished based on light scatter (forward scatter and side scatter) properties. Ten thousand sperm events were recorded per sample. Acquisition files were stored as list mode (LMD) files. List mode files were analyzed with Flowing free flow cytometry data analysis software (version 2.5.1., www.flowing.com). Coefficients of variation values for DNA histograms were calculated as described by Larsen et al. (2004). Fluorescent labelling patterns were regularly monitored with an Olympus epifluorescent microscope equipped with corresponding FITC and PI filter sets).

Experiment 1. Boar spermatozoa labelled with SYBR-14 and propidium iodide.

A total of 94 boar sperm samples were analyzed. All boars were cytogenetically healthy. The arithmetic mean of the SYBR-14 histogram CV-s was 4,9 (SD +/- 1,22, minimum 3,23, maximum 8,22; dimensionless values). The arithmetic mean of the propidium iodide histogram CV-s was 3,7 (SD +/- 0,22, minimum 3,14, maximum 4,33; dimensionless values).

As the results revealed, propidium iodide showed lower histogram CV-s than SYBR-14, indicating better DNA-resolution (the difference was significant at $p<0,01$, T-test).

Experiment 2. Bull spermatozoa labelled with SYBR-14 and propidium iodide.

A total of 14 bull sperm samples were analyzed. The cytogenetic status of the bulls was unknown at the time of quantitative DNA-analysis. The arithmetic mean of the SYBR-14 histogram CV-s was 5,3 (SD +/- 0,76, minimum 4,46, maximum 7,09; dimensionless values). The arithmetic mean of the propidium iodide histogram CV-s was 3,5 (SD +/- 0,3, minimum 3,19, maximum 4,17; dimensionless values).

As the results revealed similarly to the boar experiment, propidium iodide showed lower histogram CV-s than SYBR-14, indicating better DNA-resolution (the difference was significant at $p<0,01$, T-test).

Experiment 3. Bull spermatozoa labelled with acridine orange.

A total of 49 bull sperm samples were analyzed. The cytogenetic status of the bulls was known at the time of quantitative DNA-analysis – two bulls had aneuploidy (ejaculates contained haploid and aneuploid spermatozoa), one bull had a reciprocal (2;4) translocation and one bull showed chromosomal breakages. The arithmetic mean of the acridine orange green histogram CV-s was 24,4 (SD +/- 1,86, minimum 22,63, maximum 33,13; dimensionless values).

As the results revealed, acridine orange provided unacceptably wide histograms as indicated by the high histogram CV-s. Moreover, the individual samples with known cytogenetic defects did not provide different results from the healthy animals.

The conclusions of the three experiments were the following: acridine orange failed to produce acceptably narrow histograms. SYBR-14 also provided unacceptably wide histograms (although much more narrow than the acridine orange labelled samples). We have to keep in mind that - according to the manufacturer - SYTO dyes do not act exclusively as nuclear stains in live cells and should not be equated in this regard with DNA-selective compounds such as DAPI or the Hoechst 33258 and Hoechst 33342 dyes, which readily stain cell nuclei at low concentrations in most cells. SYTO dye–stained eukaryotic cells will generally show diffuse cytoplasmic staining, as well as nuclear staining. Among the tested three fluorochromes, propidium iodide produced the lowest histogram CV-s. These values are still higher than the suggested threshold values of Larsen et al. (CV<1,3), therefore further optimization steps are needed. We have to keep in mind that DAPI is a minor groove binding dye with A-T preference, while propidium iodide has no sequence preference and an intercalating dye. However, according to my knowledge there are no other fluorochromes available with similar binding profile to DAPI and excitation around 488 nm (a prerequisite to the routine application with commercial benchtop flow cytometers). Therefore I decided to focus on the improvement of the propidium iodide labeling.

Experiment 4. applying the Nicoletti protocol on bull spermatozoa

A further pilot study was conducted to test a propidium-iodide based labeling protocol to measure nuclear DNA status, including DNA condensation as our previous studies suggested that spermatozoa carrying chromosomal defects may show abnormal condensation, too (Revay et al. Macrocephaly in bull spermatozoa is associated with nuclear vacuoles, diploidy and alteration of chromatin condensation. Cytogenetic and Genome Research, 126:202-209. 2009. and Revay et al. Diploid spermatozoa caused by failure of the second meiotic division in a bull. Theriogenology, 73:421-429. 2010). The experiment was presented as a poster at the annual ESDAR meeting in Helsinki, Finland. The aim of this pilot study was to test the applicability of the so-called „Nicoletti assay" previously used to measure human sperm DNA fragmentation (Winkle et al., 2009, J Assist Reprod Genet 26:41–46) and condensation (Vicari et al., 2002, Hum Reprod, 17:2128-2133) on bull spermatozoa. Frozen-thawed semen samples of 15 bulls were used in the study. Fixed human lymphoma cells from a commercial TUNEL kit (APO-BrdU™ TUNEL Assay Kit, A23210, Life Technologies) were used as positive control. Events with lower PI fluorescence than the main haploid peak were classified as DNA-fragmented cells; events with higher PI fluorescence were considered as spermatozoa with incomplete chromatin condensation.

The mean percentage of sperm cells with incomplete DNA condensation was 1.6% (SD: ±0.4%; min-max values: 0.5 – 6.7%). The arithmetic mean of the propidium iodide histogram CV-s was 2,6 (SD +/- 0,23, minimum 2,47, maximum 3,24; dimensionless values). The protocol showed narrower histograms than the previously used propidium iodide labeling protocol.

Second year (2014.11.01. – 2015.12.31.)

Based on the experiences gained during the first year of the project it became clear that with the current fluorochromes available for the excitation with 488 nm laser lines it is rather not possible to reach or exceed the CV% threshold values suggested by Larsen et al. Therefore instead of focusing on only low CV values, the main aim was to test DNA histogram profiles of cytogenetically healthy and translocation carrier animals. During the second year we screened the majority of the Hungarian Simmental bulls used during this time in Hungary (17 of the 22 bulls) using standard microscopic cytogenetic methods, but none of them showed translocations or other cytogenetic defects. Nevertheless we had access to frozen semen samples from known carrier bulls (described below).

It is known that spermatozoa show a distinctive „L-shaped" DNA histogram profile due to the random orientation of the flat sperm heads passing through the laser beam. We carried out pilot experiment with sperm head decondensation in order to make sperm heads rounder and therefore more uniform, but the results did not show any improvement in histogram CV-s or histogram profiles, moreover, decondensation protocols would make the cytogenetic analyses more difficult, time consuming and less practical under the routine conditions of artificial insemination centers. As an alternative we tested the histogram modeller option of the free flow cytometric software, Cyflogic (www.cyflogic.com) which allows to identify the main DNA peak of the histogram, which contains spermatozoa with similar head orientation.

Experiment 1. SYTO dye family and histogram modelling

In this experiment we tested a series of green SYTO dyes namely SYTO 9, 11, 12, 13, 14, 16, 21, 24 and 25 on five cytogenetically healthy bulls in three replicates to find the dye which has the strongest signal and highest DNA resolution. Main histogram peaks were identified with the histogram modeller option of Cyflogic. Signal intensity was expressed as the histogram mean and resolution as histogram main peak CV. Statistical analysis was done with R Commander and the different dyes were compared with Kruskal-Wallis rank sum test. SYTO 21 showed the highest signal intensity (mean channel: 277) and the lowest main peak CV (13). Since even after the main peak identification, this CV is much higher than the suggested threshold and our findings from the first year with other dyes, the conclusion was to reject the SYTO green dye family. The DNA binding mechanism of SYTO dyes is unknown and although they seem to show some minor groove selectivity, they are not binding as other nuclear stains in live cells such as DAPI. SYTO dyes tend to show some cytoplasmic staining as well (www.thermofischer.com) – theoretically, as spermatozoa have only minimal cytoplasm this should not mean a problem, but the wide histogram profiles indicate that these dyes are rather useless for sperm DNA analysis.

Experiment 2. FXCycle PI/RNase kit with histogram modelling

Note: this experiment was presented at the XXV Jubilee International Congress of the Hungarian Association for Buiatrics and appeared in the 2015. 137 (Supplement 1) issue of Magyar Állatorvosok Lapja (pages 87-90.)

In the present study sperm samples of a healthy breeding bull – served as control - three translocation carrier bulls and a bull with meiotic defects were studied with flow cytometry

after labelling with the commercially available FXCycle PI/Rnase kit which contains propidium iodide as DNA stain. Histogram main peaks were identified with Cyflogic software. Histogram profiles of every carrier bull were significantly different from the control male (Kolmogorov-Smirnov test, p<0,000001). The conclusion was that analysis of DNA-histogram profiles may be a useful tool to detect cytogenetically abnormal animals and/or semen doses. However, the histogram modeller option did not result in lower histogram CV values and moreover, the developer of the software stopped updating the website and the installation software was not available for download from the website, therefore the use of histogram modelling was stopped in the following experiments of the projects as such histogram modelling option is not available in the commercially available cytometry softwares.

Experiment 3. Direct labelling of the DNA of viable cells with Vybrant Green dye

Note: this experiment was presented as poster at the 21st Annual Meeting of the Hungarian Association of Animal Reproduction and was submitted for publication to Állattenyésztés és Takarmányozás which will publish the poster section materials as full papers. The manuscript is under review.

The present study was intended to test a DNA staining method, which allows direct labelling, without sample preparation steps. DNA histograms of sperm samples from a cytogenetically healthy control bull, three translocation-carriers and from a breeding bull producing diploid sperm were compared using Vybrant Green labelling. Histogram profiles were significantly different from the control in all cases (p <0.000001, Kolmogorov-Smirnov test). Our results show that direct DNA labelling with the Vybrant Green fluorescent probe is a promising alternative to tests that require complex sample preparations, but the evaluation of the diagnostic value of this assay requires further, larger scale studies.

Final experiment: FXCycle PI/RNase kit with raw histogram profile analysis

Note: this experiment was submitted as a manuscript „Quick cytogenetic screening of breeding bulls using flow cytometric sperm DNA histogram analysis“ to Acta Veterinaria Hungarica (manuscript no. 133/2015) on 08. 12. 2105. The manuscript is under review.

Although the Vybrant Green probe showed promising results in the previous experiment, working with unfixed, viable cells may be actually more difficult even if it sounds somewhat contradictory. Cell fixation means extra steps in the laboratory protocol, but working with fixed cells mean more flexible time schedule. Therefore we decided to return to the previously tested FXCycle PI/RNase (which is available as a ready to use kit) along with cell fixation. This time the histogram analyses focused on the raw histogram profiles without histogram modelling.

The aim of the final experiment was to test FXCycle PI/RNase kit which is properly excited with the 488 nm laser line and therefore would be suitable for routine DNA analyses aiming to detect breeding males and/or insemination doses carrying cytogenetic aberrations. In a series of experiments first we established basic DNA histogram parameters of cytogenetically healthy breeding bulls by measuring the intraspecific genome size variation of three animals (each of them three times – as suggested by Dolezel and Bartos: Plant DNA flow cytometry

and estimation of nuclear genome size. Ann. Bot. 95, 99-110. 2005), then we compared the histogram profiles of bulls carrying cytogenetic defects to the baseline values.

Materials and Methods

Semen samples

In experiment 1. we used frozen-thawed semen samples from three bulls free from cytogenetic defects. In experiment 2. one of the bulls from exp. 1. was used as control (bull A) and bulls with the following cytogenetic aberrations were tested: bull B was a carrier of 2;4 reciprocal translocation (Switonski et al., Identification of a new reciprocal translocation in an AI bull by synaptonemal complex analysis, followed by chromosome painting. Cytogenet Genome Res. 121, 245-248. 2008); bull C was a carrier of 20;24 reciprocal translocation Andersson et al., Embryo quality and andrological study of two subfertile bulls versus five control bulls with normal fertility. Theriogenology 38, 623-631. 1992); bull D produced approximately 20 – 25% diploid spermatozoa (Revay et al., Diploid spermatozoa caused by failure of the second meiotic division in a bull. Theriogenology 73, 421-429. 2010); bull E had a complex translocation between chromosomes 6 and 29 (Venhoranta et al., Ectopic KIT copy number variation underlies impaired migration of primordial germ cells associated with gonadal hypoplasia in cattle (Bos taurus). PLoS One 26, e75659. 2013).

DNA labelling

Sperm DNA was labelled with propidium iodide (PI) as a part of the FXCycle PI/RNase kit (F10797, Molecular Probes).

Labelling followed the protocol suggested by the manufacturer: spermatozoa were fixed in 70% ethanol then were washed from the fixative (centrifuged at 400 x g for 10 min). Five hundred µl of FXCycle PI/RNase staining solution was added to the cell pellets (approximately 1 x 10^6/ml spermatozoa) and after vortexing the samples were incubated for 30 min at room temperature in the dark before flow cytometric analyses.

Flow cytometry

We measured the DNA quantity expressed as PI fluorescence intensity with a Beckman Coulter FC500 flow cytometer, equipped with a 488 nm 20 mW argon ion laser. PI fluorescence was detected on FL3 detector (655 nm LP) in linear mode. Doublet discrimination was done according to the suggestions of the manufacturer (Beckman Coulter). Five thousand events per sample were recorded and scatter and fluorescence intensities were stored in list mode files. The flow cytometer was calibrated daily with FlowCheck fluorospheres (6605359, Beckman Coulter), moreover, in order to monitor random drift in fluorescence intensities between samples, every sample contained 10 µl FlowSet fluorospheres (6607007, Beckman Coulter) serving as internal control.

Analysis

In experiment 1. one-parameter histograms were drawn and individual histogram profiles were compared with the Kolmogorov-Smirnov option in the CXP Analysis software (Beckman Coulter) of the cytometer. This analysis assesses the difference between two populations and shows the maximum difference between two cumulative frequency

distributions expressed as Dmax value (Young, Proof without prejudice: use of the Kolmogorov–Smirnov test for the analysis of histograms from flow systems and other sources. J. Histochem.Cytochem. 25, 935-941. 1977, Watson, Proof without prejudice revisited: immunofluorescence histogram analysis using cumulative frequency subtraction plus ratio analysis of means. Cytometry 43, 55-68. 2001). Dmax values were calculated for paired histograms (three bulls, three repeats: n=36 pairs) and these values were checked for normality using Lilliefors test and descriptive statistics were established with Statistica for Windows (version 8., Statsoft Inc.).

In experiment 2. the histogram profiles of the carrier bulls were compared to the control bull using Kolmogorov-Smirnov test as described above. The threshold Dmax value to differentiate healthy and possibly defective animals was derived from the descriptive statistics in experiment 1. and was calculated as mean + 2SD (Indrayan, Medical Biostatistics. Chapman & Hall/CRC Press, 1024 pp. 2012).

Results and discussion

Kolmogorov-Smirnov test revealed significant differences between bulls or even between repeated measurements of the same individual ($p<0,05$) with the exception of Bull 2 where the repeated measurements were not significantly different. However, since Kolmogorov-Smirnov test is reported to be oversensitive (Parikh et al., Evaluation of an alternative to the Kolmogorov-Smirnov test for flow cytometric histogram comparisons. J. Immunol Methods 229, 97-105. 1999, Lampariello, On the use of the Kolmogorov-Smirnov statistical test for immunofluorescence histogram comparison. Cytometry 39, 179-188. 2000) we did not consider this as a flaw of the data analysis approach.

Dmax values showed normal distribution ($p>0,2$, Lilliefors test) and the descriptive statistics were the following: mean: 0,087; SD: 0,037; variance: 0,001; minimum: 0,018; maximum: 0,155. The calculated Dmax threshold value was 0,161 (calculated as mean + 2SD).

The histogram profiles of the carrier bulls differed significantly ($p<0,01$) from the control bull (A) and the Dmax values were the following: bull A vs B: 0,245; A vs C: 0,128; A vs D: 0,416; A vs E: 0,166. With the exception of Bull C, every carrier bull showed higher Dmax value than the threshold value established in Experiment 1. This can be explained as in that case smaller chromosomes (chromosomes 20 and 24) were involved so probably only a very small amount of DNA was missing or added in the affected sperm cells.

As a part of the experiment we analyzed another DNA probe, SYTOX Green which showed better histogram resolution than propidium iodide (the dye in the FXCycle PI/RNase kit) on other cell types (https://www.thermofisher.com/hu/en/home/references/molecular-probes-the-handbook/assays-for-cell-viability-proliferation-and-function/assays-for-cell-enumeration-cell-proliferation-and-cell-cycle.html#head3). Experimental settings were exactly the same as described above. SYTOX Green is not available as a ready to use kit, therefore a working solution was prepared with Triton-X and RNase. Contrary to the information from the manufacturer's website, SYTOX Green did not perform better than the FXCycle kit: the mean Dmax value was 0,14 while the FXCycle kit had a mean Dmax value of 0,087 ($n=36$; $p<0,001$, T-test), therefore the conclusion was that SYTOX Green is not as useful for sperm DNA analyses as the FXCycle kit – besides the less uniform histogram patterns (indicated by the higher Dmax values), the SYTOX Green dye is not available as a ready to use kit.

In conclusion, the quick flow cytometric cytogenetic screening approach has several advantages:

- The fluorescent labelling FXCycle PI/RNase kit is easy to use, does not require excessive sample preparation, quick, and the fluorochrome can be analyzed with the standard laser line and optics of every benchtop flow cytometer.
- Ethanol fixation makes sample collection, handling and the time management of laboratory analyses more flexible and relaxed.
- The Kolmogorov-Smirnov option is available in the software of most flow cytometer brands therefore no additional software purchase is needed.
- Individual males or even insemination doses can be evaluated quickly so it can be applied to test the animal even if he is not physically present (like in the case of semen import).
- The test fits to the laboratory routine where flow cytometry is applied for semen quality control, and this analysis should be carried out only once in the lifetime of the animal.