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Fluorescent labelling of boar spermatozoa for quantitative studies on competitive sperm–oviduct binding

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Abstract. *In vitro* sperm–oviduct binding assays enable assessment of the capacity of spermatozoa to form a 'reservoir' in the oviduct. Competitive approaches, such as experimental set-ups that test multiple males or semen samples simultaneously on the same tissue explants, are desirable because they reduce the likelihood of bias when using material from different females. Therefore, we established a fluorescent labelling technique that allows tagging and storage of spermatozoa before competitive studies of sperm–oviduct binding *in vitro*. Fluorescent markers were tested for reliability and compatibility with parameters of boar spermatozoa viability. The addition of seminal plasma after density gradient centrifugation was essential to counteract centrifugation stress during the labelling procedure. It was demonstrated that sperm tagged with MitoTracker Green FM or MitoTracker Red FM can be successfully used in competitive sperm–oviduct binding studies. The assay was sensitive enough to indicate subtle effects of semen storage temperature on the ability of the spermatozoa to contribute to the female sperm reservoir.

Additional keywords: sperm competition, sperm reservoir.

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Introduction

Although arrays of semen tests are available, assessment of male fertility or the effects of semen treatment on the fertilising capacity of spermatozoa remains challenging. This is largely because the female reproductive tract is not a passive vessel, but instead selects potentially competent spermatozoa from the different subpopulations present in a semen sample (Amann *et al.* 2018). The ability of the female genital tract to select and store spermatozoa in a so-called 'sperm reservoir' is a conserved feature in non-vertebrate (e.g. insects; Heifetz and Rivlin 2010) and vertebrate (e.g. reptiles, birds, marsupials, mammals; Holt and Fazeli 2016) animal species. The sperm reservoir in most mammalian species is located at the uterotubal junction and the caudal isthmus of the oviduct, as reported for the pig (Fléchon and Hunter 1981; Hunter 1981), cattle (Hunter *et al.* 1991) and rabbit (Overstreet and Cooper 1978).

The ability of spermatozoa to transiently bind to the epithelial lining of this sperm reservoir is hypothesised to serve at least four purposes, namely the selection of viable sperm, reducing the number of spermatozoa to avoid polyspermy, sperm storage until ovulation occurs and control (delay) of capacitation. Therefore, sperm–oviduct binding *in vitro* has been studied as a potential means of quantifying the fertilisation-competent sperm population (e.g. Gualtieri and Talevi 2003; Daigneault *et al.* 2015; Winters *et al.* 2018). To this end, spermatozoa are coincubated with either intact pieces of oviduct tissue (so called explants; Suarez *et al.* 1991; Waberski *et al.* 2006), aggregated vesicles of epithelial cells (Rottmayer *et al.* 2006) or monolayers of cultured oviduct epithelial cells (Thomas and Ball 1996). More recently, *in vitro* assay systems were extended to include Transwell (Corning) or membrane-based culture systems (Chen *et al.* 2013; Gualtieri *et al.* 2013), which offer the opportunity to more closely mimic an epithelium with an *in vivo*-like morphology. For all these systems, the ability to quantify viable spermatozoa bound to oviduct epithelial cells is critical. Using light microscopy, only spermatozoa showing flagellar beating or projecting at right angles from the explant surface can reliably be identified at ×400 magnification (see Movie S1, available as Supplementary Material to this paper). Therefore, labelling with fluorescent dyes has been used to facilitate sperm detection during interaction with various types of female cells both *in vivo* and *in vitro* (Table S1). Although such stains should meet a range of criteria for suitability, supporting evidence that they do so is often missing. Most importantly, all spermatozoa must be stained, but tagging should not interfere with either general sperm function (e.g. sperm motility) or with the specific sperm

Table 1. Criteria for fluorescent dyes to validate their use for studying sperm–female genital tract interactions

Feature	Minimum requirement
Staining efficiency	All spermatozoa should be tagged
Persistence of tag during semen processing	No removal of dye during density gradient centrifugation or dilution
Persistence of tag during semen storage	Spermatozoa should remain tagged for at least 72 h during *in vitro* storage
Sperm motility	No effect of the dye on sperm motility
Sperm morphology	No effect of the dye on sperm morphology
Sperm binding to oviduct cells	No effect of the dye on the ability of spermatozoa to bind to oviduct epithelium
Bleeding of the dye	No bleeding of the dye to spermatozoa or other cells during coincubation at 38°C
Bleaching of the dye	No bleaching of the dye during recording of live (or fixed) bound spermatozoa in video sequences
Additional features (not tested in the present study)	
Sperm migration in female genital tract	No effect of the dye on sperm distribution and migration towards fertilisation site in female genital tract
Preparative steps for fertilisation	No effect of the dye on sperm viability in the oviduct or preparative steps for fertilisation (i.e. capacitation, acrosome reaction, zona penetration)
Fertilisation and early embryo development	No effect of the dye on fertilisation and early embryo development

attribute under investigation (e.g. the ability to bind to oviduct epithelial cells). A detailed list of criteria that fluorescent dyes should conform to before being used in studies of sperm–female tract interaction is presented in Table 1.

Tests that aim to assess sperm fertilising potential will be most meaningful when competitive approaches are used, because this helps remove any bias stemming from using tissue from different females (Amann *et al.* 2018). In a competitive assay, two (or more) males or semen treatments are compared by including spermatozoa from both (or all) in equal numbers, and the ratio of the desired outcome is then determined for each individual or treatment (e.g. the proportion of bound spermatozoa or of piglets sired). Competitive assays for sperm–oviduct binding capacities will be especially valuable when they use freshly collected oviduct tissue with its characteristic folds, crypts and grooves. Competitive approaches that allow quantification of the ability of spermatozoa from different sources to bind to the same oviduct specimen would minimise the female factor and other interassay variations. Such approaches have been described by creating transgenic male mice or fruit flies that express fluorescent proteins in their sperm acrosome or midpiece. These spermatozoa can be used to visualise sperm–oviduct binding, sperm migration and sperm competition within the female genital tract (Lüpold *et al.* 2012; Muro *et al.* 2016). In farm animal species, only a few transgenic males with fluorescently labelled spermatozoa have been generated, such as the pig (Garrels *et al.* 2012) and rabbit (Hoffmann *et al.* 2016). Instead, labelling ejaculated spermatozoa with fluorescent dyes is the method of choice for visualising spermatozoa during binding studies.

The aim of the present study was to establish a fluorescent labelling technique that allows sperm tagging and storage before competitive *in vitro* oviduct binding studies. In screening trials, we evaluated potential candidate dyes that included DNA-binding dyes and other cell tags (e.g. lipid analogues, non-specific amine reactive dyes and thiol-reactive dyes with a specificity for mitochondria; Table 2). Based on their staining characteristics and absence of detrimental effects on sperm motility, the results of the most promising candidates only, namely Hoechst 33342, fluorescein isothiocyanate (FITC), tetramethylrhodamine isothiocyanate (TRITC), MitoTracker Green FM (Life Technologies) and MitoTracker Red FM (Life Technologies), are presented in detail. In the first trial of a competitive sperm–oviduct binding assay using MitoTracker FM dyes, we examined the effects of boar semen preservation on sperm binding capacity. Semen samples from this species exhibit decreases in motility, viability and mitochondrial function as a function of storage duration and temperature (e.g. Schulze *et al.* 2013). Because removal of excess dye from labelled spermatozoa by (density gradient) centrifugation is essential before interaction with other cells, we also examined the importance of seminal plasma for minimising sperm damage during centrifugation and subsequent dilution.

Materials and methods

Labelling of spermatozoa and semen processing

Semen was collected from mature, fertile boars (Landrace, Duroc and cross-bred animals) housed at the Unit for Reproductive Medicine. Boars were kept and handled according to the European Commission directive for pig welfare. Ejaculates were collected and the raw semen evaluated as described previously (Nguyen *et al.* 2016). Normospermic semen samples were diluted in prewarmed (32°C) Beltsville thawing solution (BTS; Minitube) to a concentration of 100×10^6 spermatozoa mL^{-1}. In Experiments 4 and 5, a modified Androhep extender (Minitube) without EDTA was used instead of BTS. In Experiments 1, 2 and 3, subsamples of extended semen were stained with either Hoechst 33342 (Life Technologies), FITC or TRITC (Sigma-Aldrich), respectively, at final concentrations of 1.25 μg mL^{-1} for each dye by incubating the samples with the dye for 30 min at room temperature. In Experiments 4 and 5, subsamples of extended semen were stained with either Hoechst 33342 (as described above), MitoTracker Red FM or MitoTracker Green FM, the latter two dyes at a final concentration of 200 nM. Semen samples were incubated with MitoTracker dyes for

Table 2. Overview of dyes tested for sperm labelling

DiI, $DiIC_{18}(3)$ (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate); DiD, $DiIC_{18}(5)$ (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate); FITC, fluorescein isothiocyanate; RT, room temperature; TRITC, tetramethylrhodamine isothiocyanate

	Sperm concentration ($\times 10^6$ mL^{-1})	Incubation time (min)	Incubation temperature (°C)	Minimum concentration for visual detection with fluorescence microscope	Staining of all spermatozoa	Persistence of tag during semen processing	Bleaching during imaging	Bleeding of dye	Effect on sperm motility	Effect on sperm morphology	Persistence of tag during 72 h storage	Effect on sperm binding to oviduct explant
Non-DNA-binding dyes												
CellTracker Green (Invitrogen)	100	30	32	1.5 μM	Yes	Yes	No	Yes	Yes	No	Yes	–
CellTracker Red (Invitrogen)	100	30	32	1.5 μM	Yes	Yes	No	Yes	Yes	No	Yes	–
CYTO-ID Green (Enzo Life Sciences)	20	5	RT	According to manual[A]	No	–	–	–	–	–	–	–
FITC (Sigma-Aldrich)	100	30	RT	1.40 μg mL^{-1}	Yes	Yes	No	No	No	No	Yes	Yes
MitoTracker Green FM (Life Technologies)	20	15	38	200 nM	Yes	Yes	No	No	No	No	Yes	No
MitoTracker Red FM (Life Technologies)	20	15	38	200 nM	Yes	Yes	No	No	No	No	Yes	No
TRITC (Sigma-Aldrich)	100	30	RT	1.25 μg mL^{-1}	Yes	Yes	No	No	No	No	Yes	No
VyBrant DiI (Invitrogen)	5	20	38	20 μL mL^{-1}	No	Yes	–	–	–	–	–	–
VyBrant DiD (Invitrogen)	5	20	38	20 μL mL^{-1}	No	Yes	–	–	–	–	–	–
DNA-binding dyes												
Acridine Orange (Polysciences)	100	30	RT	1.5 μM	Yes	Yes	Yes	Yes	No	No	Yes	–
CyTRAK Orange (BioStatus)	100	30	RT	5 μM	Yes	Moderate	No	–	Yes	–	–	–
DRAQ5 (BioStatus)	100	30	RT	5 μM	Yes	Moderate	No	–	Yes	–	–	–
Hoechst 33342 (Life Technologies)	20	30	RT	1.2 μg mL^{-1}	Yes	Yes	No	Yes (weak bleeding)	No	No	Yes	No
Nuclear Green (AAT Bioquest)	100	30	RT	250 nM	Yes	Yes	No	–	Yes	–	–	–
SYBR-14 (Sigma-Aldrich)	100	30	32	2 μM	Yes	Yes	No	–	Yes	No	Yes	–
SYTO 16 (Invitrogen)	100	30	32	1 μM	Yes	Yes	No	Yes	Yes	No	Yes	–

[A]Enzo Life Sciences (http://www.enzolifesciences.com/fileadmin/files/manual/ENZ-51036_insert.pdf, accessed 15 April 2019).

15 min at 38°C to ensure a high mitochondrial transmembrane potential during the labelling procedure. To remove excess dye, 45 mL stained sample was centrifuged at room temperature for 10 min at 300*g*, followed by 10 min at 750*g* through a 5-mL layer of either 35% Percoll (GE Healthcare; Experiments 1–3) or 20% Percoll (Experiments 4 and 5). Working solutions of Percoll were prepared using a 10× concentrated stock solution of HEPES-buffered saline (1.37 M NaCl, 0.2 M HEPES, 0.1 M glucose, 0.025 M KOH), as described by Vincent and Nadeau (1984). The Percoll working solutions had a mean (± s.d.) pH of 7.45 ± 0.05 and osmolality of 300 ± 5 mOsmol kg^{-1}. The supernatant was aspirated to a residual volume of 0.5–1.0 mL. The sperm concentration was then adjusted to 20×10^6 sperm mL^{-1} using isothermic extender and the spermatozoa examined under a fluorescence microscope for staining and motility.

When semen was stored for 24 h or more (Experiments 2 and 5), the semen extender was supplemented with 10% fresh homologous seminal plasma (v/v). Seminal plasma was prepared by repeated centrifugation of non-stained raw semen (3370*g*, 10 min, room temperature) until the supernatant appeared microscopically sperm free.

Semen samples were maintained at room temperature until further use (Experiments 1, 3, 4). In the case of semen storage (Experiments 2, 5), samples were held for 90 min at room temperature before being stored at 17°C. For storage at 10°C, samples were transferred to a storage unit set to 10°C after 1 h equilibration at 17°C.

Preparation of oviduct explants

Oviducts from healthy sows (parity ≥1) were transported in ice-cold phosphate-buffered saline (PBS; 150 mM NaCl, 11.7 mM Na_2HPO_4, 2.5 mM KH_2PO_4, 50 μg mL^{-1} streptomycin und 75 μg mL^{-1} penicillin, pH 7.4, at room temperature; 295 ± 15 mOsmol kg^{-1}) from a local abattoir to the laboratory. Oviduct explants were prepared as described by Petrunkina *et al.* (2001). Briefly, after removal of connective tissue and a longitudinal incision into the lumen, 0.5- to 1.0-mm lengths of tissue (explants) were cut from the longitudinal folds of the oviductal isthmus and stored in Tyrode's medium (100.5 mM NaCl, 20 mM HEPES, 15 mM $NaHCO_3$, 5 mM glucose, 3.1 mM KCl, 2 mM $CaCl_2$, 0.4 mM $MgSO_4$, 0.3 mM KH_2PO_4, 1.0 mM sodium pyruvate, 21.7 mM sodium lactate, 100 μg mL^{-1} gentamycin sulfate; SERVA), 20 μg mL^{-1} phenol red, 3 mg mL^{-1} bovine serum albumin (BSA; Cohn's Fraction V, fatty acid free), pH 7.60 ± 0.05 at 20°C, 300 ± 5 mOsmol kg^{-1} for a maximum of 2 h at 4°C. One explant was prepared for each time point of incubation. Tissues from each sow were processed separately and only explants exhibiting rapidly beating cilia on an intact explant ridge were used.

Coincubation of spermatozoa and explants

Two to three explants, each from a different sow, were placed in prewarmed and equilibrated Tyrode's medium (38°C; equilibrated in an atmosphere of 5% CO_2, 100% humidity). One well of a 24-well plate (Greiner Bio-one) was prepared for each time point. Stained spermatozoa were added to each well (final concentration 2×10^5 spermatozoa mL^{-1} from each of the competing samples, final volume 500 μL) and coincubated with explants at 38°C, in an atmosphere of 5% CO_2 in air and 100% humidity. Thereafter, explants were transferred to fresh, prewarmed, equilibrated Tyrode's medium and gently agitated to remove loosely bound spermatozoa. Washed explants were held in a fresh drop of Tyrode's medium (maximum 15 min) before mounting to record sperm binding.

Recording sperm binding

To assess sperm–explant binding, a single explant was mounted in a drop of Tyrode's medium (60 μL) within a silicon grease frame (silicon stopcock grease; Dow Corning) on a prewarmed slide and sealed with a 20 mm × 20 mm coverslip (#1; Menzel-Gläser). Samples were examined under an Olympus BX41 microscope equipped with a heated stage and a mercury lamp (100 Watt) as an excitation source.

Hoechst 33342 (Experiments 1 and 2) was detected through a single-band filter (excitation H350/50 nm, ET 400 nm long-pass (LP), emission E420 nm LP; AHF Analysentechnik). For simultaneous recording of multiple colours (Experiment 3), a triple-band filter was used (F66–412, 4′,6′-diamidino-2-phenylindole (DAPI), FITC and TRITC; AHF Analysentechnik). For comparison of sperm binding (Experiment 4), two recordings were made at the same location in quick succession using a filter set for Hoechst 33342 (excitation AR350/50 nm, T400 nm LP, emission ET460/50 nm; Chroma Technology) followed by a filter set for MitoTracker Green (excitation 460–490 nm, dichroic mirror 500 nm, emission BA520IF; Olympus). An optimised filter set for simultaneous detection of MitoTracker Red and MitoTracker Green signals (FH1–012, HQ-SET FITC, excitation HQ480/40 nm, Q505 nm LP, emission HQ510 nm LP; AHF Analysentechnik) was used to simultaneously record differentially labelled spermatozoa (Experiments 4 and 5).

A video of each explant was recorded using a long-distance lens (LUCPlanFL N, 20x/0.45 Ph1 ∞/0–2/FN 22) on a DP72 camera (Olympus) and Cell^P version 3.4 software (Olympus). Three locations with dense sperm binding on the intact explant surface were evaluated, thereby avoiding the cut edge of the explant because high sperm binding density to the damaged epithelial surface was regularly observed. The recorded explant area covered at least 75% of the field of view. At each location, all layers of the explant were slowly brought in to focus. Video files were analysed after the experiment using Cell^P software. The explant area was measured and the number of fluorescent spermatozoa for each dye counted.

The mean number of bound spermatozoa per square millimetre was defined as the binding index (BI), analogous to Petrunkina *et al.* (2001):

$$BI(EI) = (N_{11} + N_{12} + N_{13})/(A_{11} + A_{12} + A_{13})$$

where *E1* is the explant from Sow no. 1, A_{11}, A_{12} and A_{13} are the areas Locations 1, 2 and 3 respectively and N_{11}, N_{12} and N_{13} are the number of spermatozoa bound to Locations 1, 2 and 3 respectively. The BI was calculated separately for each colour.

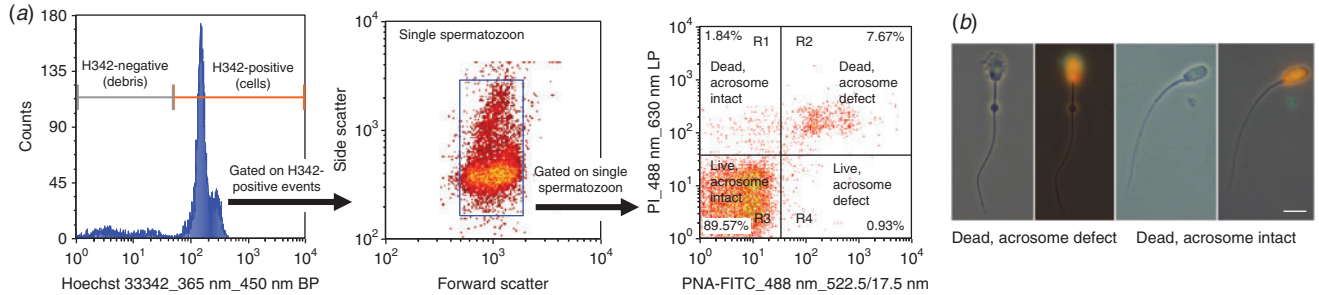


Fig. 1. Gating strategy for flow cytometry. (*a*) Gating strategy for identifying live, acrosome-intact spermatozoa by flow cytometry. First, DNA-containing events are separated from non-DNA containing debris (left histogram). Next, events with forward and side scatter characteristics of a single boar spermatozoon are selected (middle plot). Finally, the population of interest is evaluated for fluorescence intensity of propidium iodide (PI) and fluorescein isothiocyanate-conjugated peanut agglutinin (PNA-FITC) to determine the percentage of live, acrosome intact (PI and PNA-FITC negative) spermatozoa (right plot). H342, Hoechst 33342; LP, longpass; BP, bandpass. (*b*) Representative images illustrating the staining pattern for PI and PNA-FITC. Scale bar = 10 μm.

When differently labelled spermatozoa were used on an explant (MitoTracker Red, MitoTracker Green), the ratio of bound spermatozoa to that explant was also calculated.

Because competitive assays can also be biased by a specific male–female interaction, it is advisable to test any set of competing samples on material from several different females (Amann *et al.* 2018). Therefore, each sperm competition assay was performed on tissue from at least two sows, and the mean of the results was calculated to minimise the effect of any individual female on the results.

Assessment of sperm quality

A subsample of diluted semen was fixed in buffered formaldehyde (10 mM citric acid in demineralised water with 1.5% formaldehyde) and sperm morphology was classified according to a simplified scheme based on Waberski *et al.* (1990). Sperm motility was examined in extended non-stained and stained semen samples after various storage times at room temperature, 17°C or 10°C. An aliquot of diluted semen, was incubated for 15 min (Experiments 1, 3, 4, 5) or 30 min (Experiment 2) in a water bath at 38°C and motility was assessed using a computer-aided sperm analysis (CASA) system (SpermVision v3.5; Minitube; Experiments 1, 3, 4, 5) as described by Henning *et al.* (2012). In Experiment 2, AndroVision v9.1 (Minitube) was used. The AndroVision system consisted of a 20× objective, camera adaptor (UPMTVC tv-0.75; Olympus) and a camera (avA1000-100gm, 1024 pixels × 1024 pixels; Basler). Video recordings (0.8 s, 60 frames per second (f.p.s.)) were made of five successive fields in the central axis of a chamber. AndroVision software considered spermatozoa to be motile when their amplitude of lateral head displacement (ALH) was greater than 1.0 μm and their curvilinear velocity (VCL) exceeded 24.0 μm s^{-1}. Spermatozoa were considered to be progressively motile when the radius was <5.0° or >30.0°, rotation was <0.8, VCL exceeded 55.0 μm s^{-1} and straight line velocity exceeded 10.0 μm s^{-1}.

Plasma membrane and acrosome integrity were analysed using a Galaxy Flow cytometer (DAKO) on non-fixed spermatozoa. A 5-μL subsample of diluted semen was transferred to 995 μL HEPES-buffered saline medium (HBS; 137 mM NaCl, 20 mM HEPES, 10 mM glucose, 2.5 mM KOH, 3 mg mL^{-1} BSA (Cohn's Fraction V), pH 7.4 at 20°C, 300 ± 5 mOsmol kg^{-1}) containing propidium iodide (PI; final concentration 5 μg mL^{-1}), FITC-conjugated peanut agglutinin (PNA; final concentration 3.0 μg mL^{-1}) and Hoechst 33342 (final concentration 0.75 μg mL^{-1}). After incubation (15 min, 25°C), 10 000 events were analysed using FloMax v2.4 software (Partec). Cells were excited at 488 nm (argon ion laser, 20 mW) and approximately 365 nm (mercury lamp). Signals were detected using a 537.5/22.5-nm filter (PNA-FITC), a 630-nm LP filter (PI) and a 455/10-nm filter (Hoechst 33342). Overlap of emission spectra between PI and PNA-FITC was compensated after acquisition. Data were evaluated as illustrated in Fig. 1*a*. Representative images for plasma membrane damaged (= dead) spermatozoa with a damaged or reacted acrosome (PI and PNA-FITC positive) or dead spermatozoa with an intact acrosome (PI positive and PNA-FITC negative) are shown in Fig. 1*b*. The interpretation of the staining patterns is in agreement with other publications (Flesch *et al.* 1998; Hossain *et al.* 2011).

Experimental design

Experiment 1: oviduct binding kinetics of Hoechst 33342-labelled spermatozoa

Hoechst 33342 is a DNA-binding dye that penetrates the plasma membrane of intact spermatozoa and provides clear labelling of the sperm head. Hoechst 33342 labelling does not interfere with fertility (Vazquez *et al.* 2002; Parrilla *et al.* 2004) and labelled, sex-sorted spermatozoa are used successfully for commercial breeding (Rath and Johnson 2008; Garner 2009).

Semen samples ($n = 6$ boars) were stained with Hoechst 33342 (Life Technologies) at a final concentration of 1.2 μg mL^{-1} and evaluated for motility (CASA), plasma and acrosome membrane integrity and sperm morphology. Stained spermatozoa were coincubated with oviduct explants. After 15, 30, 45, 60, 90 and 120 min, the binding indices were determined.

Experiment 2: stability of Hoechst 33342-labelled boar spermatozoa during semen storage

Semen samples ($n = 8$ boars) were stained with Hoechst 33342 (1.2 μg mL^{-1}) and diluted in semen extender with or

without 10% homologous seminal plasma. The proportion of seminal plasma was similar to that in commercially diluted boar semen (Johnson *et al.* 2000). Motility and plasma and acrosome membrane integrity were evaluated after 24, 48 and 72 h storage at 17°C. Stability of staining with Hoechst 33342 was examined after 24 and 72 h. Subsequently, the oviduct explant BI was evaluated after 15 min coincubation. Control samples were either directly diluted with semen extender to 20×10^6 spermatozoa mL^{-1} (routine processing for AI) or processed with the solvent in Hoechst 33342 (Aqua Dest; final addition 0.4% (v/v)).

Experiment 3: FITC and TRITC labelling as an alternative to Hoechst 33342

As an alternative, non-DNA-binding dyes have been described for sperm labelling, such as the amine reactive probe FITC (Mellish and Baker 1970; Table S1).

Semen samples ($n = 3$ boars) were split and equal portions stained with either FITC (final concentration 1.25 μg mL^{-1}), TRITC (1.25 μg mL^{-1}) or Hoechst 33342 (1.25 μg mL^{-1}). Semen treated with Dulbecco's PBS (Sigma-Aldrich; pH 7.4), the solvent used for FITC and TRITC, served as a control. Equal numbers of tagged spermatozoa from given boars were coincubated for 45 min with three oviduct explants ($n = 9$ explants). The effects of the different tags on sperm motility and the number of bound spermatozoa were examined. Fluorescently labelled spermatozoa were shielded from light before coincubation and analysis of sperm binding in order to avoid irreversible immobilisation (Mellish and Baker 1970).

Experiment 4: MitoTracker FM dyes as an alternative to Hoechst 33342

In a two-step approach, the effects of labelling spermatozoa with MitoTracker dyes on their motility and oviduct binding capacity was examined. First, spermatozoa labelled with MitoTracker Green FM were compared with Hoechst 33342-tagged spermatozoa. Semen from two boars was used, and the binding to three explants was assessed after a 45-min coincubation period ($n = 6$ explants). Second, binding of MitoTracker Red FM- and MitoTracker Green FM-labelled spermatozoa was compared using the same approach ($n = 6$ explants). In both experiments, semen was processed on the day of collection and resuspended in Androhep (Minitube) without EDTA and without the addition of seminal plasma.

Experiment 5: using MitoTracker labelling to test the maintenance of oviduct binding capacity in samples stored at different temperatures

Aliquots of semen ($n = 6$ boars) were stained either with MitoTracker Red FM (final concentration 200 nM), MitoTracker Green FM (200 nM) or processed with the appropriate solvent (i.e. dimethylsulfoxide (DMSO); control; final concentration 0.02%). Samples were then diluted in semen extender (Androhep without EDTA) including 10% homologous seminal plasma before being stored at 17°C or 10°C. After 24 h storage, motility and sperm binding to oviduct explants were assessed. Semen samples were incubated with explants for 45 min in a cross-over design. First, red-tagged spermatozoa from 10°C storage were tested against green-tagged spermatozoa stored at 17°C. Next, the opposite combination was tested with other explants from the same sow. The mean sperm BI for samples stored at 10°C and 17°C and the mean ratio of bound spermatozoa were calculated.

Statistical analysis

Data were analysed using SAS version 9.1 (SAS Institute) and SigmaPlot for Windows version 13 (Systat Software). Data for motility, membrane integrity and BI were tested for normality (Shapiro–Wilk test) and comparisons made using Student's one-sample *t*-test (PROC UNIVARIATE). Linear and non-linear regression curves for changes in mean sperm BI with coincubation time were calculated using PROC GLM and PROC NLIN. Data from kinetic analysis were tested for normal distribution and equivalence of variance. The effect of incubation time on the sperm BI was assessed using a one-factor analysis of variance (ANOVA) with a post hoc Tukey test for multiple comparisons. Correlations between the BI and selected sperm parameters were examined using Pearson's correlation coefficient.

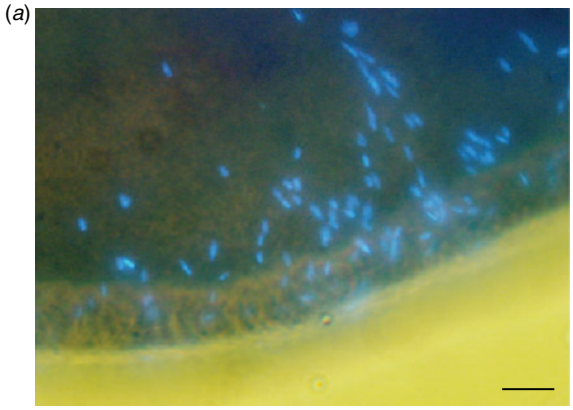
Unless stated otherwise, data are presented as the mean ± s.d. Two-sided $P < 0.05$ was taken to indicate statistical significance.

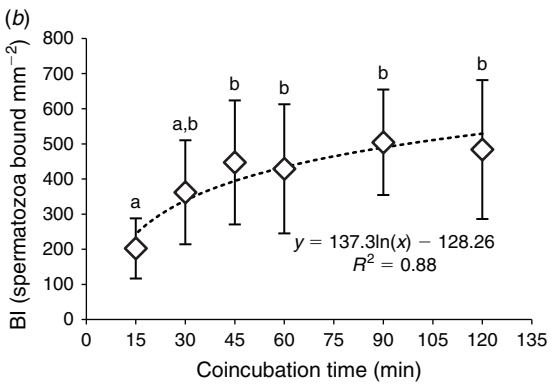
Results

Hoechst 33342 labelling for sperm binding studies

Counting boar spermatozoa bound to the surface of oviduct explants using phase contrast optics is challenging. Only spermatozoa with beating tails or tails protruding perpendicularly from the explant surface are readily identified at ×400 magnification (Movie S1). In contrast, the heads of Hoechst 33342-labelled boar spermatozoa were readily visible on the explant surface at only ×200 magnification (Fig. 2*a*; Movie S2). Areas with densely bound spermatozoa and areas almost devoid of sperm alternated. Sperm binding was recorded only for densely populated areas. The BI (i.e. number of bound spermatozoa per square millimetre), was lowest after 15 min coincubation (203 ± 86 spermatozoa mm^{-2}) and increased in a logarithmic fashion (Fig. 2*b*). Values plateaued after 45 min (447 ± 177 spermatozoa mm^{-2}). These kinetics correspond with those recorded in earlier studies using non-labelled spermatozoa (Waberski *et al.* 2006).

The BI after 15 min incubation was negatively correlated with the percentage of morphologically abnormal spermatozoa (Fig. 2*c*; Table S2). Binding indices after 15 and 45 min incubation were positively correlated with the percentages of spermatozoa with intact plasma and acrosome membranes ($r = 0.92$ and $r = 0.85$ respectively; both $P > 0.05$; Fig. 2*c*). In line with previous studies, correlations between the BI and sperm motility were not evident (Petrunkina *et al.* 2001; Khalil *et al.* 2006). Taking all observations together, we conclude that Hoechst 33342-labelled spermatozoa are an appropriate control when evaluating the effects of other fluorochromes on sperm–oviduct binding.





(c)

	Morphologically abnormal spermatozoa	PI and PNA-FITC negative	Total motility	Progressive motility
BI				
15 min	−0.99 **	0.92 **	0.29	0.39
45 min	−0.69	0.85 *	0.55	0.59

Fig. 2. The binding index (BI) for Hoechst 33342-labelled spermatozoa depends on coincubation time and sperm quality. (*a*) Hoechst 33342-labelled spermatozoa bound to the epithelial lining of an oviduct explant. This image was taken from Movie S2. Scale bar = 50 μm. (*b*) The BI for freshly collected and stained spermatozoa increased in a logarithmic fashion with coincubation time. Data are the mean ± s.d. ($n = 6$ boars). Different letters indicate significant differences ($P < 0.05$) between time points). (*c*) There were significant correlations observed between the BI and sperm morphology and the percentage of live, acrosome intact spermatozoa ($n = 6$ boars), but not between BI and total or progressive motility. Data show Pearson's correlation coefficients (r). *$P = 0.05$; **$P < 0.01$. PI, propidium iodide; PNA-FITC, fluorescein isothiocyanate-conjugated peanut agglutinin.

Seminal plasma stabilises fluorescently tagged spermatozoa during storage

The motility of stored boar spermatozoa was dependant on the presence of seminal plasma in the extended semen (Fig. 3*a*). After 24 h storage, motility in the absence of seminal plasma had dropped below 40%. Total motility in the standard processed variant (i.e. with seminal plasma) was more than twice as high (85 ± 7%; $P < 0.05$; Fig. 3*a*). The addition of distilled water

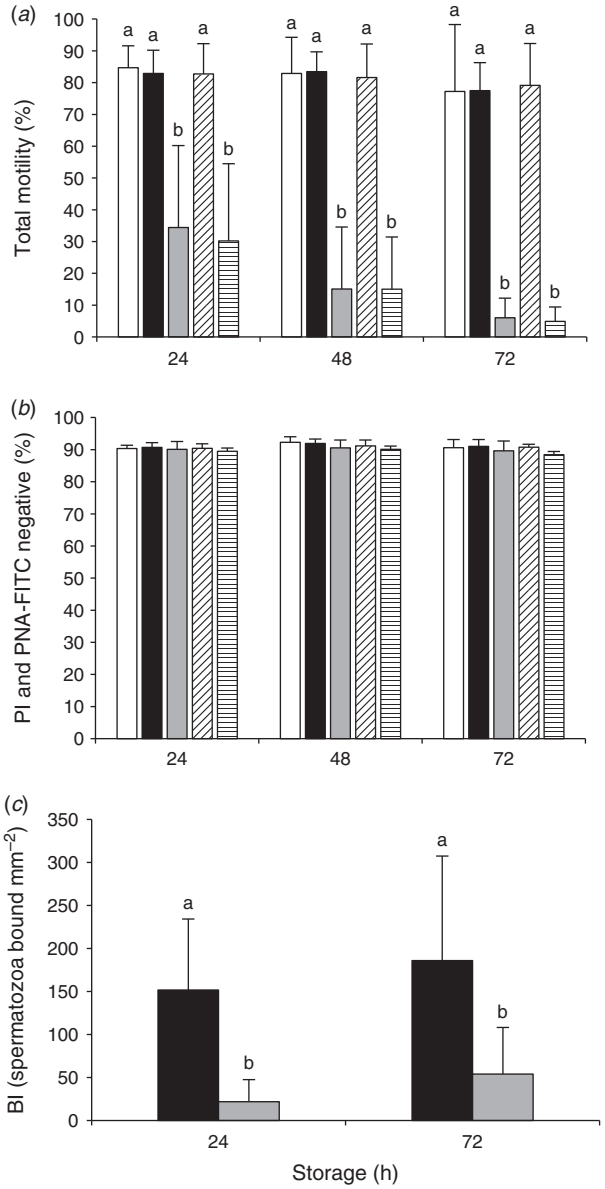


Fig. 3. Sperm motility and the ability to bind to oviduct epithelial cells are better preserved in the presence of seminal plasma. (*a*) Hoechst 33342-labelled spermatozoa remained motile when stored in the presence of 10% homologous seminal plasma for up to 72 h at 17°C. (*b*) The percentage of live acrosome-intact spermatozoa was not affected by seminal plasma. (□), standard processed samples (control); (■), Hoechst 33342-labelled spermatozoa stored with 10% seminal plasma; (▩), Hoechst 33342-labelled spermatozoa stored without seminal plasma; (▨), Aqua Dest-treated spermatozoa stored with 10% seminal plasma; (▤), Aqua Dest-treated spermatozoa stored without seminal plasma. Data are the mean ± s.d. ($n = 7$ boars). Different letters above columns indicate significant ($P < 0.05$) differences between samples for a given storage time. (*c*) Storage time had no effect on the binding index (BI) of spermatozoa stored with 10% seminal plasma (■; $P > 0.05$). However, the BIs for spermatozoa stored without seminal plasma (▩) were lower at both time points. Data are the mean ± s.d. ($n = 7$ boars). Different letters above columns indicate significant ($P < 0.05$) differences in BIs.

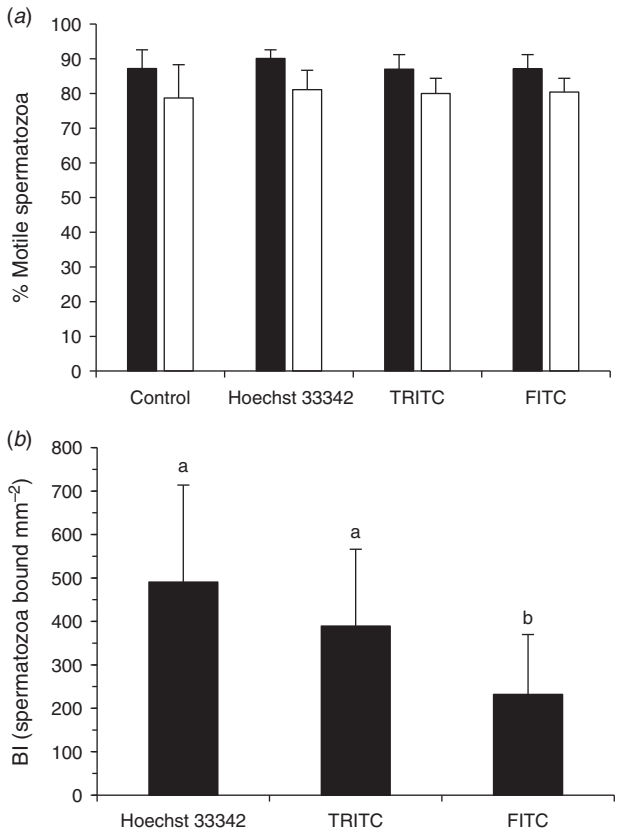


Fig. 4. Evaluating the suitability of labelling sperm with fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRITC) for competitive sperm–oviduct binding studies. (*a*) Labelling of spermatozoa with either Hoechst 33342, TRITC or FITC had no effect on total motility (■) or progressive motility (□) of spermatozoa. (*b*) However, the number of bound spermatozoa per explant area (i.e. binding index (BI)) was significantly reduced for FITC-labelled spermatozoa after 45 min coincubation. Data are the mean ± s.d. ($n = 9$ explants). Different letters above columns indicate significant ($P < 0.05$) differences in BIs.

and/or Hoechst 33342 in the presence of 10% seminal plasma did not affect total or progressive motility, sperm velocity, linearity of sperm movement or beat cross frequency at any storage time (Fig. 3*a*; Table S3). The percentage of viable and acrosome-intact spermatozoa was not affected by the presence of either seminal plasma or Hoechst 33342 in the semen samples (Fig. 3*b*).

Omitting seminal plasma resulted in lower percentages of motile and oviduct-bound spermatozoa, regardless of the duration of storage. The BI did not decrease when semen storage was extended to 72 h (Fig. 3*c*).

FITC and TRITC for differential labelling of spermatozoa

Motility of boar spermatozoa was not affected by FITC or TRITC labelling (Fig. 4*a*; Table S4). However, the BI for FITC-labelled spermatozoa was less than half of that for Hoechst 33342-labelled spermatozoa ($P < 0.05$). The BI for TRITC-tagged spermatozoa was not significantly lower than for Hoechst 33342-labelled spermatozoa ($P = 0.24$; Fig. 4*b*).

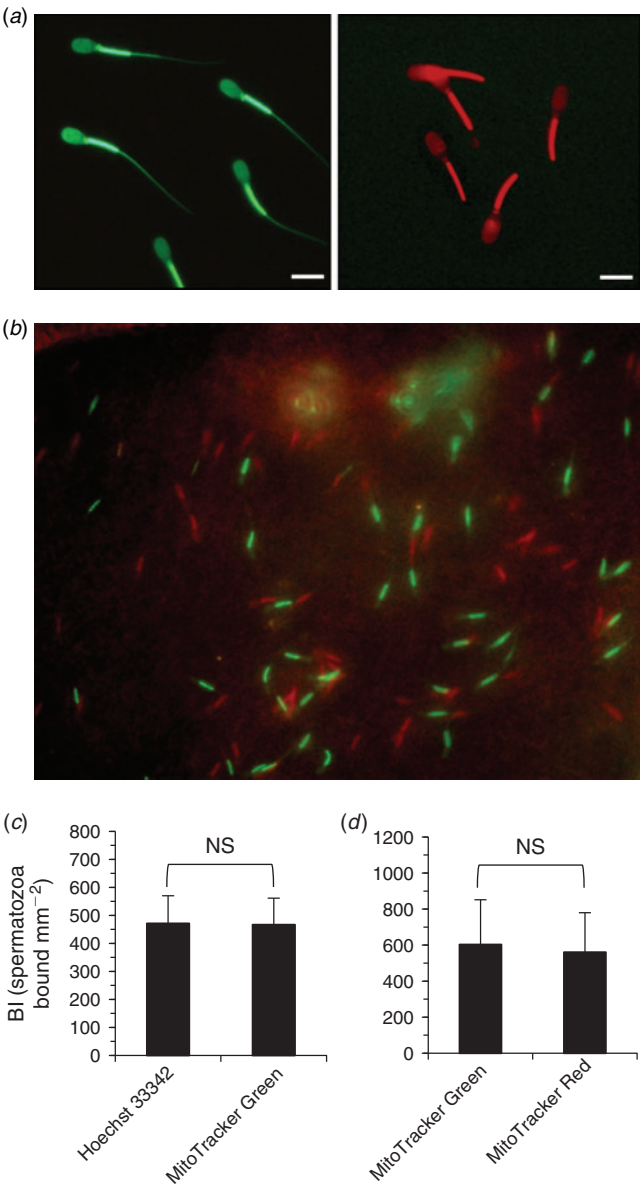


Fig. 5. Evaluating the suitability of sperm labelling with MitoTracker Green FM or MitoTracker Red FM for competitive sperm–oviduct binding studies. (*a*) Representative images of spermatozoa labelled with MitoTracker Green or MitoTracker Red. Scale bars = 10 μm. (*b*) Image of MitoTracker Green- or MitoTracker Red-labelled spermatozoa bound to the epithelial lining of oviduct explants. The image is taken from Movie S3. (*c*, *d*) Binding indices (BIs) for spermatozoa labelled with either Hoechst 33342 or MitoTracker Green (*c*) or MitoTracker Green or MitoTracker Red (*d*) did not differ after 45 min coincubation. Data are the mean ± s.d. ($n = 6$ explants).

MitoTracker FM dyes for differential labelling of spermatozoa

MitoTracker Red- and Green-labelled boar spermatozoa showed strong staining of the midpiece and weaker staining of the sperm head (Fig. 5*a*). Recordings of spermatozoa bound to

Table 3. Motility of MitoTracker FM-labelled (Life Technologies) and non-labelled spermatozoa stored at either 10°C or 17°C

Comparison of computer-aided sperm analysis (CASA) parameters for semen samples stored at 17°C or 10°C. Samples were treated with either MitoTracker Green FM, MitoTracker Red FM (Life Technologies) or dimethylsulfoxide (DMSO; control). Data are the mean ± s.d. ($n = 6$ boars). No significant differences between stained samples and the control were detected at any given storage temperature ($P > 0.05$). VAP, average path velocity; VCL, curvilinear velocity; VSL, straight line velocity; STR, straightness (VSL/VAP); LIN, linearity (VSL/VCL); WOB, wobble (VAP/VCL); ALH, amplitude of lateral head displacement; BCF, beat cross frequency

	10°C			17°C		
	MitoTracker Red	MitoTracker Green	DMSO	MitoTracker Red	MitoTracker Green	DMSO
Total motility (%)	82.8 ± 1.7	82.3 ± 2.1	82.3 ± 2.6	89.6 ± 1.2	90.1 ± 1.5	90.1 ± 2.4
Progressive motility (%)	77.1 ± 3.4	76.6 ± 4.6	75.4 ± 3.3	83.4 ± 1.7	84.1 ± 4.0	84.2 ± 4.1
VAP[A] (μm s^{-1})	71.7 ± 16.2	71.7 ± 15.9	75.6 ± 16.2	75.6 ± 17.9	77.4 ± 19.6	75.6 ± 16.2
VCL[A] (μm s^{-1})	94.2 ± 21.1	93.0 ± 21.3	89.5 ± 19.6	102.2 ± 25.5	103.0 ± 27.3	102.2 ± 25.0
VSL[A] (μm s^{-1})	62.5 ± 14.5	62.7 ± 14.3	58.2 ± 13.9	65.5 ± 15.7	67.1 ± 17.2	65.5 ± 14.0
STR[A]	0.87 ± 0.02	0.87 ± 0.02	0.86 ± 0.02	0.86 ± 0.02	0.86 ± 0.03	0.86 ± 0.02
LIN[A]	0.66 ± 0.05	0.67 ± 0.06	0.64 ± 0.06	0.64 ± 0.06	0.65 ± 0.06	0.64 ± 0.05
WOB[A]	0.76 ± 0.05	0.77 ± 0.05	0.74 ± 0.05	0.74 ± 0.05	0.75 ± 0.05	0.74 ± 0.05
ALH[A] (μm)	1.73 ± 0.32	1.67 ± 0.34	1.72 ± 0.29	1.89 ± 0.44	1.90 ± 0.45	1.92 ± 0.46
BCF[A] (Hz)	37.1 ± 4.4	36.7 ± 4.1	36.0 ± 4.8	38.7 ± 3.6	38.3 ± 4.6	39.1 ± 3.9

[A]Mean values for progressively motile spermatozoa.

oviduct explants demonstrated primarily labelling of the midpieces of the spermatozoa (Fig. 5*b*; Movie S3). Colour levels for red signals were manually adjusted during evaluation of image stacks to enhance the contrast between MitoTracker Red-tagged spermatozoa and the background fluorescence of the explants. BIs did not differ between the two MitoTracker dyes or from Hoechst-labelled control spermatozoa (Fig. 5*c*, *d*). Labelling did not have any measurable negative effects on sperm kinematics in fresh spermatozoa (Table S5) or in samples stored for 24 h at 10°C or 17°C (Table 3), suggesting that the dyes do not interfere with sperm motility.

Using a competitive sperm-binding assay

Boar spermatozoa stored at the commercially routine temperature (17°C) or subjected to mild hypothermic stress (10°C) were compared in a competitive oviduct binding assay. Most spermatozoa appeared to remain functionally intact during storage at both temperatures (on average >80% motile cells; Table 3). Although differences were small, samples stored at 17°C had a significantly higher total motility than samples stored at 10°C ($P > 0.05$; Fig. 6*a*). Two assay replicates were performed with differentially stained samples. The BIs for samples stored at 10°C were significantly lower than those for samples stored at 17°C for both MitoTracker colour-by-storage temperature combinations ($P < 0.05$; Fig. 6*b*). BIs tended to be lower in the second assay run. The direct comparison of red- and green-tagged spermatozoa on each explant demonstrated that the sperm binding ratio was significantly skewed in favour of samples stored at 17°C, regardless of the MitoTracker colour (58% bound spermatozoa from 17°C samples vs 42% from 10°C samples; Fig. 6*c*). There was no significant correlation between the binding ratio and the difference in the percentage of motile spermatozoa for the tested samples ($P > 0.05$).

Discussion

Using a series of experiments we have established an *in vitro* assay for competitively studying sperm–oviduct binding based on fluorescently labelled spermatozoa. This assay allows direct comparison of samples from two different males or semen treatments.

Attempts to label spermatozoa with fluorescent dyes to quantify sperm binding or sperm migration in the female genital tract date back more than 50 years (e.g. Mellish *et al.* 1968) and have been used extensively with different dyes, particularly for sperm–zona binding studies (Liu and Baker 1990; Braundmeier *et al.* 2004; Daigneault *et al.* 2015). However, systematic data on fluorochrome–sperm interaction are often lacking or incomplete. Minimum requirements for adequate fluorochrome labelling include: (1) all sperm are stained; (2) the staining is stable during semen processing and storage; and (3) sperm function is not impaired (c.f. Table 1). In addition, fluorochromes for competitive studies must differ in their light emission spectra. The increased availability of novel fluorescent probes for tagging different intracellular targets prompted us to examine a selection of dyes for their potential use in competitive sperm–oviduct binding studies.

Spermatozoa are transcriptionally inactive. Therefore, the DNA in the nucleus can be assumed to be a good first-choice target for a label that will not interfere with sperm function before fertilisation. Of the six DNA-binding fluorochromes tested for inclusion in this study, only Hoechst 33342 fulfilled the minimum requirements listed above. However, when used with unstained spermatozoa at 38°C, a gradual decrease in the percentage of non-labelled spermatozoa was accompanied by an increase in dimly Hoechst 33342-positive spermatozoa, probably resulting from 'bleeding' of Hoechst 33342 from stained, plasma membrane-damaged cells. Therefore, Hoechst 33342 is only suitable for competitive approaches alongside a stable counterpart dye, which was not present among DNA-binding probes we tested.

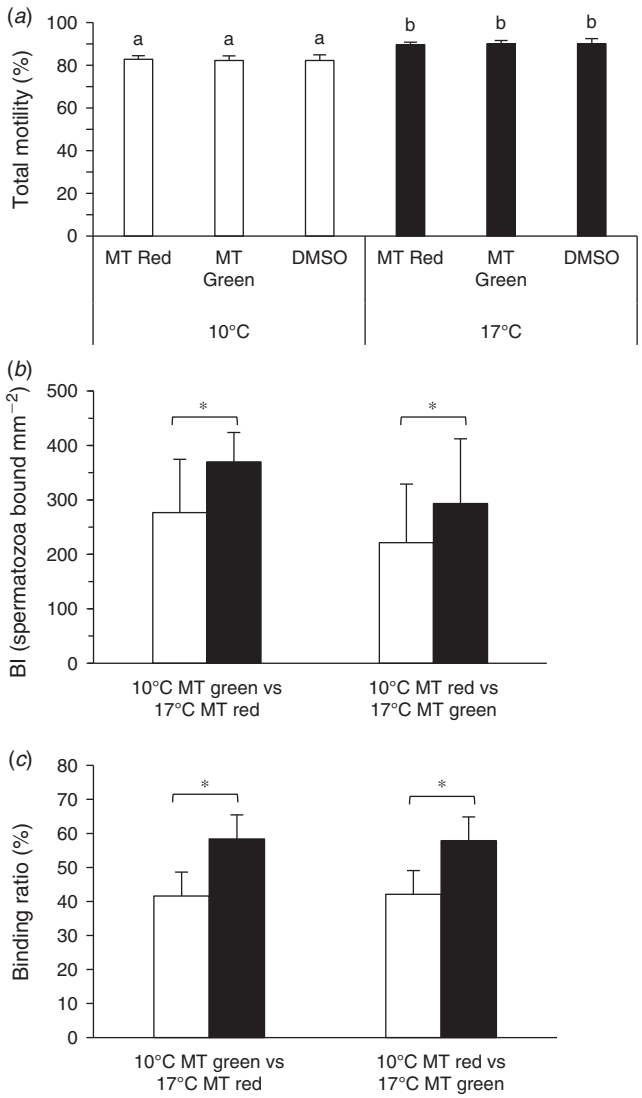


Fig. 6. Evaluating the suitability of labelling sperm with MitoTracker Green FM (MT Green) and MitoTracker Red FM (MT Red) for competitive sperm–oviduct binding studies. (*a*) The total motility of samples stained with MitoTracker dyes did not differ to that from control samples when stored at 17°C (■) or 10°C (□). Different letters above columns indicate significant differences in total motility between samples stored at 17°C and 10°C ($P < 0.05$). (*b*) The binding index (BI) for the competitive assay was significantly higher for samples stored at 17°C (■) than for samples stored at 10°C (□), regardless of the MitoTracker colour-by-temperature combination. *$P < 0.05$. (*c*) The ratio of bound spermatozoa was identical in both assay runs (i.e. more spermatozoa from samples stored at 17°C (■) than from samples stored at 10°C (□) bound to oviduct explants *in vitro*). *$P < 0.05$. Data are the mean ± s.d. ($n = 6$ boars). DMSO, dimethylsulfoxide.

Non-DNA-binding probes carry a higher risk than DNA-binding dyes of interfering with cell homeostasis and function, such as intracellular calcium chelation (Niu *et al.* 2006). The most frequently used tags for competitive sperm-binding assays in the past were FITC and TRITC. These may appear to be ideal tags because neither compromised fertilisation of rabbit oocytes, whereas the fertilising spermatozoon was readily identified by the presence of a fluorescent midpiece within the ooplasm at the pronuclear stage (Parrish and Foote 1985). In our experiments, the motility of boar spermatozoa was not affected by either FITC or TRITC, but binding to oviduct explants was significantly impaired in FITC-tagged spermatozoa. Theoretically, tagging spermatozoa with TRITC or FITC should have had a similar effect on sperm binding. The isothiocyanate group of both fluorochromes is supposed to cross-link with amino, sulfhydryl, imidazoyl, tyrosyl or carbonyl groups on sperm surface proteins. In this regard, a higher level of sulfhydryl groups on the sperm surface is associated with lower sperm binding to the oviduct epithelium in cattle (Gualtieri *et al.* 2009). Whether the same relationship holds true for pig spermatozoa and whether FITC or TRITC affected sulfhydryl group concentration on the pig sperm surface remains to be determined. Although we cannot explain the reason for the difference in sperm–oviduct binding, this example demonstrates why it is important to carefully evaluate fluorescent tags for compatibility with an experimental set-up. Species-specific differences in the biochemical composition of the spermatozoa and the target cells or substrates (e.g. oviduct epithelial cells, zona pellucida) need to be considered.

Despite their limited ability to stain spermatozoa, lipophilic fluorescent dyes such as 4-[4-(dihexadecylamino)styryl]-N-methylquinolinium iodide (DiQ) and 3,3′-dihexadecyloxacarbocyanine perchlorate ($DIOC_{16}$) have been used in heterospermic *in vitro* approaches to rank boars according to their sperm–zona binding capacity (Miller *et al.* 1998; Braundmeier *et al.* 2004). Staining of almost all spermatozoa was achieved only at the expense of inhibiting sperm motility. Accordingly, we precluded the lipophilic membrane stains Vybrant Dil and Vybrant DiD (Thermo Fisher Scientific) from oviduct binding experiments because of inadequate staining of spermatozoa.

MitoTracker Green FM and Red FM were the dyes best suited to establishing a competitive sperm-binding assay in the present study. The assay sensitively detected an 8% lower binding ratio for spermatozoa stored at 10°C compared with spermatozoa stored at 17°C. The differences in progressive or total motility were in a similar range (6.3% and 7.8% respectively), although they did not directly correlate to the binding ratio, thereby suggesting that the binding assay is an independent tool for assessing an essential sperm function.

The MitoTracker probes passively diffuse into cells and accumulate preferentially in the mitochondria, thus staining the same cellular target but exhibiting distinct emission spectra (Johnson and Spence 2010). They covalently bind to mitochondrial proteins by reacting with free thiol groups on cysteine residues (Presley *et al.* 2003). To date, MitoTracker Green FM has been successfully used for non-competitive sperm imaging *in vivo*, for example in the sheep (Druart *et al.* 2009; Rickard *et al.* 2014), as well as *in vitro* in the cow (Sutovsky *et al.* 1996) and horse (Leemans *et al.* 2015). Recent attempts to establish competitive MitoTracker-based sperm tracking in the blue mussel *Mytilus galloprovincialis* failed due to inconsistent labelling of spermatozoa exposed to MitoTracker Red FM (Lymbery *et al.* 2016). Here we demonstrate that both MitoTracker FM dyes can be used for safe labelling of porcine spermatozoa during semen storage at 17°C or 10°C and

subsequent quantification in a competitive sperm–oviduct binding assay. BIs *in vitro* did not differ between the two MitoTracker dyes, thus making competitive cross-over experiments based on reciprocal use of the dyes dispensable in the future. Theoretically, an extension to a competitive assay with three dyes is possible by adding MitoTracker DeepRed FM; however, a more sophisticated microscope set-up would be required for simultaneous excitation of all three dyes and collection of emitted light. MitoTracker-tagged mammalian spermatozoa have the potential to be used in competitive set-ups in a broad array of assays, including mucus penetration tests (Richardson *et al.* 2011), chemotaxis and thermotaxis-based sperm selection studies (Gatica *et al.* 2013; Pérez-Cerezales *et al.* 2018) or recently established microfluidic approaches to study sperm selection and function (de Wagenaar *et al.* 2015; Ferraz *et al.* 2017). However, validation of specific staining and sperm processing conditions is required for every species and assay system. It is also important to note that intensity of sperm staining with MitoTracker FM dyes is best when labelling is conducted at body temperature to ensure adequate mitochondrial activity. Special attention to sample preparation is required, especially if sensitive boar spermatozoa are used. To counteract the negative effects of centrifugation stress and high dilution rates on sperm function (Maxwell and Johnson 1999; Henning *et al.* 2015), the addition of seminal plasma to the resuspension medium is essential to stabilise sperm motility during storage of fluorescently labelled spermatozoa for 72 h.

Preliminary observations in gilts inseminated with MitoTracker Green FM- and Red FM-labelled, stored spermatozoa indicate that the dyes are stable in the female genital tract (Fig. S1). In this regard, spermatozoa were readily imaged *in situ* on tissue pieces recovered from the uterotubal junction and oviductal isthmus. Thus, MitoTracker FM-labelled spermatozoa are suitable for studying sperm distribution and survival in the genital tract in a competitive *in vivo* set-up. Assuming the MitoTracker tags remain stable for several days at body temperature, identification of the fertilising spermatozoon in early stage embryos could even be achieved (Sutovsky *et al.* 2003).

In conclusion, a labelling protocol with two different MitoTracker FM probes was established that enables efficient testing of how a sperm cell's capacity to bind to oviduct epithelium *in vitro* is affected by preservation conditions or intrinsic male factors.

Conflicts of interests

The authors declare no conflicts of interest.

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