Sperm gatekeeping: 3D imaging reveals a constricted entrance to zebra finch sperm storage tubules

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Short Title: Visualising female sperm storage
Abstract:

Females across many internally fertilising taxa store sperm, often in specialised storage organs in their reproductive tracts. In birds, several hundred sperm storage tubules exist in the utero-vaginal junction of the oviduct and there is growing evidence that sperm storage in these tubules is selective. The nature of the mechanisms underlying female sperm storage in birds remains unknown due to our limited ability to make three-dimensional, live observations inside the large, muscular avian oviduct. Here, we describe a new application of fluorescence selective plane illumination microscopy to optically section oviduct tissue from zebra finch *Taeniopygia guttata* females label-free, by harnessing tissue autofluorescence. Our data provide the first description of the 3D structure of sperm storage tubules in any bird, and reveal the presence of constricted openings to tubules, suggesting active entrapment and release of sperm.
Introduction:

Across many internal fertilisers, females have evolved the capacity to maintain viable sperm in specialised sperm storage organs in their reproductive tract as a strategy to maximise fertility. Sperm storage ensures the female has sufficient sperm for fertilisation when copulation and ovulation are not synchronised [1]. Since female promiscuity is common across taxa (e.g. birds [2], mammals [3–5], reptiles [6], fishes [7] and insects [8–10]), storage also provides the opportunity for females to exert control over post-copulatory processes [11–13]. Post-copulatory sexual selection has driven the diversification of sperm storage organs, which vary from single bean-shaped structures in many damselfly species [14,15], to one or more sac-like spermathecae in certain fly species (e.g. three spermathecae in Scathophaga stercoraria [16]; two spermathecae along with a seminal receptacle in many Drosophila species [17]), and multiple epithelial crypts in snakes [18], lizards [19], turtles [20] and birds [21,22].

In birds, epithelial sperm storage crypts are called sperm storage tubules (SSTs) and are located in the utero-vaginal junction (UVJ) of the oviduct [23]. The number of SSTs possessed by a single female ranges from around 500 SSTs in the UVJ of the budgerigar Melopsittam undulatus to 20,000 in the turkey Meleagris gallopavo [24]. A growing body of evidence suggests that avian SSTs may be an important site of sperm selection. Steele and Wishart [25] demonstrated experimentally that when chicken sperm were treated to remove surface proteins from their membranes, they could not enter the SSTs after normal intra-vaginal artificial insemination, even though sperm treated in the same way were capable of fertilising the ovum when inseminated further up the oviduct, past the vagina and UVJ. Bobr et al. [23] also noted a lack of abnormal sperm in the SSTs of domestic fowl Gallus domesticus, suggesting that abnormal sperm are unable to reach
enter sperm storage sites. The large number of SSTs present in the avian oviduct may also allow spatio-temporal segregation of sperm from competing ejaculates within the oviduct [26–28]. However, the mechanisms by which sperm are selected at the time of storage remain poorly understood, and how sperm enter and exit the SSTs is unknown.

The role of the SSTs in sperm storage and selection may be relatively passive. Froman [29] proposed a model where sperm motility, rather than SST function, is pivotal in sperm retention in SSTs. According to this model, sperm must maintain an optimum swimming velocity to maintain their position and counter a fluid current within the SST. This model was supported by evidence that faster sperm emerged out of SSTs later than slower sperm [30], and that passive loss of sperm from storage might be sufficient to explain last male precedence in the domestic fowl, turkeys, and zebra finches Taeniopygia guttata [31,32; but see 27]. However, there have been no observations of sperm swimming inside the SSTs, and other studies have detected the presence of sperm motility suppressors such as lactic acid in Japanese quail Coturnix japonica SSTs [33], calcium and zinc in the SSTs of chicken, turkeys and Japanese quail [34,35], and carbonic anhydrase in the SSTs of turkeys, common quail Coturnix coturnix and ostriches Struthio camelus [36–38]. The neurotransmitter acetylcholine, released by nerve endings detected in the vicinity of SSTs [39], has been shown to enhance sperm motility [40], implying a nervous control on sperm mobilisation at ejection from SSTs. Additionally, Hiyama et al. [41] presented evidence for the potential role of heat shock protein 70 (HSP70) [42] in enhancing sperm motility at the point of sperm release. HSP70 proteins were found to be localised on the surface epithelium of the UVJ and their levels were upregulated ~20h post oviposition in Japanese quail, coincidental with when sperm have been shown to be released from storage in these birds [41]. The presence of such sperm motility suppressors and
activators within or near the SSTs suggests storage may not be as passive as Froman [29] suggested.

It is possible that rather than acting as passive refugia, SSTs may be dynamic structures, capable of active constriction and dilation to mediate the entrance and exit of sperm. Although numerous studies have failed to find smooth muscle fibres or myoepithelial cells [39,43,44] around SSTs, Freedman et al. [39] detected fibroblast-like cells and an F-actin rich cytoskeletal mesh called the “terminal web” in turkey SST epithelia. The terminal web is composed of contractile proteins (actin and myosin) and has been shown to contribute to contractility in other tissues, such as intestinal brush border cells [45,46] and embryonic pigmented epithelia in chicken [47]. Freedman et al. [39] also found terminal innervations in the turkey UVJ, suggesting there may be some degree of nervous control over SST function. Recent evidence also suggests the possibility of SST contraction, influenced hormonally by the action of progesterone [48,49]. It is therefore possible that the passage of sperm into and out of storage is controlled, to some degree, by the physical structure of SSTs themselves.

Our understanding of how SST structure influences sperm storage is limited by our relatively basic knowledge of SST morphology. The avian oviduct is convoluted, with opaque, muscular walls, creating numerous practical limitations for making observations of tubules in living epithelial tissue using conventional microscopy techniques. Empirical studies of SST morphology have so far used histology [23,50,51] and electron microscopy [34,43,52] on fixed tissue sections, but these approaches not only remove functional information, but typically provide two-dimensional information only. Moreover, serial sectioning is laborious, and loss of material can be difficult to avoid. Commonly used light
microscopy techniques rely on thin sections and squash preparations [26,53], which are largely inappropriate for large tissue samples since they distort structures of interest and allow only limited imaging depths.

In this study we used selective plane illumination microscopy (SPIM), to develop a novel method for live, \textit{ex vivo} imaging and examining the 3D structure of avian SSTs for the first time. SPIM has been demonstrated to be highly suitable for imaging large samples at cellular resolution with lower phototoxicity levels than with other optical sectioning methods [54,55]. Using SPIM, we were able to optically section tissue up to depths of 100 µm, so that entire folds of UVJ mucosal tissue could be imaged live without distorting or damaging their structure. We provide the first true estimates of the 3D structure of avian SSTs in living tissue, including the relationship between SST length and diameter, and report the existence of a previously unreported physical constriction at the entrance to tubules that may act to regulate sperm transport into and out of storage. We also find no evidence of sperm motility inside or on release from sperm storage tubules, providing support for the idea that sperm motility is suppressed inside the SSTs.

\textbf{Materials and Methods:}

\textit{Animals}

Zebra finches were from a captive population kept at the University of Sheffield [56,57]. Females (all between one to three years old) were placed in unisex housing for at least two weeks before being paired with males, in cages (dimensions of each: 0.6m x 0.5m x 0.4m) placed side-by-side and separated by a wire-mesh with the male and female on either side. A modified nest-box with a wire-mesh partition was
supplied to each pair. This set up ensured that the male and female established a
normal breeding pair bond, leading the female to come into breeding condition, while
ensuring that she had no sperm in her SSTs (sperm can be stored for up to 12 days
after mating in zebra finches [24]). Only females from this no-copulation set-up were
used for SST structural imaging, and all imaging methods described below, unless
stated otherwise, refer to this part of the study. For a separate study, a small number
of females were allowed to copulate with males, and from these females we collected
data on sperm motility inside and on release from the SSTs using different methods
(see “Sperm motility” below). Females were only used once they had started to lay
eggs, to ensure their oviduct was in full reproductive condition. After the second egg
in the ovulatory cycle, females were euthanized (in accordance with Schedule 1
[Animals (Scientific Procedures) Act 1986]) and dissected immediately.

Sample preparation

The oviduct, including the cloaca, was immediately removed from the female and the
connective tissue surrounding it was cleared to uncoil and straighten the vagina and
the UVJ. The lower end of the oviduct was cut through the middle of the uterus to
obtain a segment that included the UVJ, vagina and cloaca. This piece of the oviduct
was then cut open lengthwise and pinned flat on a petri-dish filled with silicone
elastomer (SYLGARD® 184; Dow Corning). A sufficient quantity of Ham’s F10 Nutrient
Mix (Invitrogen, UK) was added to keep the tissue moist but not submerged. For SPIM
imaging, UVJ folds were cut individually with iris scissors and mounted one at a time,
on a custom-made sample holder (supplementary materials) using fine insect needles.
The sample holder, with the UVJ fold mounted, was immersed in phenol-free
DMEM/F12 media, at 37°C during imaging.
**SPIM imaging**

Live UVJ tissue samples, prepared as above, were imaged using a custom-built SPIM microscope (at the University of Sheffield) with laser excitation at 473 nm and a 520 nm long pass (LP) fluorescence emission filter (Semrock, Inc.). The microscope hardware and optical components are based on the OpenSPIM platform [58] but with modifications detailed in Mendonca et al. [59 supplementary materials]. The camera, detection and illumination objectives, and magnification are fixed for the system, ensuring that the imaging results are reproducible. The autofluorescence image stacks were acquired using 500 ms exposure, starting at the outer surface and moving up to 100 µm deep into the tissue fold.

**Characterisation of autofluorescence**

SSTs were clearly identifiable in live UVJ tissue during fluorescence imaging on the SPIM and had a punctate appearance on account of autofluorescent granules (Figure 1, (B and D)) which appeared to be mostly confined to SST epithelial cells and were present along the entire length of the SST from orifice to blind end. No other cell structure or organelle was visible in these autofluorescence images.

To determine the organisation of the autofluorescent granules in the SST epithelium, label-free images from live UVJ folds (n = 10 birds) were acquired on the SPIM were compared to those of fixed UVJ tissue folds, imaged on the SPIM after staining for nucleic acids (n = 3 birds), and on a bright-field microscope after histological sectioning and general histochemical staining (n = 3 birds) (Supplementary materials).
Figure 1: (A) Schematic of SST transverse section showing cellular polarisation with nuclei (N) towards the basement membrane (BM) and microvilli (MV) at the apical end of the epithelium. The punctate autofluorescence (PA) detected by the SPIM is present proximal to the nucleus but not at the apical end of the epithelium near the lumen (L). (B) Max intensity projection of a UVJ fold with multiple detectable SSTs imaged on the SPIM. (C)-(E) Cross section of SST from histology, autofluorescence imaged on the SPIM, and SYTO-13 labelled nuclei imaged on the SPIM respectively.

**Image Analysis**

The image stacks acquired using the SPIM were used to reconstruct 480 µm x 480 µm x 100 µm tissue sections containing 3D information on SST structure (n = 10 females, one SST per female). SST shape information was extracted by measuring the diameter
enclosed by the autofluorescence from images of live tissue, at ten equidistant points along the length of the SST.

UVJ tissue image stacks were first pre-processed in Fiji [60]. Individual unbranched SSTs were selected from each female such that the entire SST structure was included in the 3D image stacks. The SSTs follow convoluted paths through the UVJ fold tissue, so in order to measure cross-sectional diameter at multiple points, it was necessary to slice the image volume at arbitrary angles to ensure the measurement planes were perpendicular to the direction of the SST structure. This was accomplished by first tracing the direction of the SST structure using a dilated version of the SST image (generated using the ‘MorphoLibJ’ plugin [61] followed by the application of Gaussian blur), to smooth the punctate autofluorescence. Two outlines for each SST were then semi-automatically traced in 3D, from the orifice to the blind end and along opposite sides of the SST lumen, using the ‘Simple Neurite Tracer’ [62] plugin in Fiji.

The next stage of image analysis was performed using MATLAB® (2015b, version 8.6, MathWorks, Natick, MA). An average trace which passed through the SST lumen was computed from the two traces for each SST. SST lengths were measured from these average traces. For each SST (n = 10), the average trace was interpolated at ten equidistant points (the first at the orifice and the tenth point before the blind end of the tubule) and at each interpolated point a vector describing the direction of the SST at that point was computed using its nearest neighbouring points on the trace. By using these vectors and the interpolated points, slicing planes normal to the vectors were defined. The indices for these slicing planes were used to extract 2D image sections from the un-dilated original image stacks using the ‘ExtractSlice.m’ [63]
function. For every extracted slice, its distance from the orifice along the luminal trace of the SST was computed using the ‘Arclength.m’ [64] function, and the major axis diameter (d1) and the minor axis diameter (d2) of the SST (enclosed by autofluorescence) were measured.

Sperm motility

To test the hypothesis that sperm are motile in the SSTs [29], we used tissue from a subset of 13 females from a separate study, that had copulated with males prior to dissection (and therefore had sperm inside their SSTs). These females were dissected as described above, but instead of mounting UVJ folds for SPIM imaging, folds were dissected on a microscope slide under a Nikon SMZ25 stereomicroscope to isolate and open individual SSTs and release sperm. We identified one SST per fold (46 SSTs in total) with high numbers of stored sperm (mean = 33 per SST), isolated the SST using methods described in [28] and submerged it in warmed nutrient media (Ham’s F10: Invitrogen, UK; typically used for avian sperm motility analysis) on a microscope slide. The slide was then placed on a heated stage (set at 38º C) and observed under a 200X phase contrast microscope for 5 mins to screen for sperm motility inside the tubule. After this initial observation period, the SST was pulled open to release sperm using fine dissection needles, as described in [28], and sperm were gently shaken free of the tubule. The slide was then observed under a 200X phase contrast microscope for another 5 mins to screen for sperm motility after release from the tubule.

Statistical Analysis:

Data analysis was performed using the statistical package R, version 3.2.3 [65]. We tested whether SST diameter varied with SST length using a mixed effects model
with average SST diameter $[(d1 + d2) / 2]$ at the sampled point as the dependent variable, the distance of sampled point from SST orifice and the SST total length as fixed effects, and the bird ID as a random effect to account for repeated measures from each female.

We also assessed if the SST was elliptical or circular in cross-section (the former providing greater epithelial apical surface area for increased contact with sperm) and whether any such ellipticity changed in response to SST length. A circularity index was first calculated by dividing the major axis diameter ($d1$) by the minor axis diameter ($d2$), where a circularity index of one indicates a circular SST cross-section. Data were then analysed via a mixed effects model using the 'lmer' function [66], with the circularity index as the dependent variable and the total length of the SST as a fixed effect. The sum of the major and minor axis diameters ($d1 + d2$) was also incorporated as a fixed effect to account for magnitude of change in diameter along each axis, as well as the distance of sampled point from SST orifice, with an interaction term between them. As before, bird ID was included as a random effect to account for repeated measures from each female.

Results:
The diameter of SSTs was found to be notably constricted at their orifice, suggestive of a structural ‘barrier’ for entry and exit (Figure 2 (A)). SST diameter increased slightly increase along the SST’s length up to a mid-point, after which it decreased slightly towards the blind end of the SST, resulting in a significant quadratic relationship between SST diameter and the distance from the SST orifice (estimated
effect = -16.761, t = -3.085, p = 0.003; Figure 2 (B)). There was no relationship between SST length and either mean, maximum, or minimum diameter, so long SSTs were neither wider nor thinner than short SSTs (estimated effect = 0.038, t = 1.058, p = 0.319; Figure 4). The relationship between SST diameter and distance from orifice also held true with data from labelled tissue (Supplementary Figure S4), confirming that the shape measured from autofluorescence images was real and not an artefact resulting from the distribution of the autofluorescence granules.

Figure 2: (A) 3D rendering of an SST showing its constricted orifice (arrowhead). (B) SST diameter has a quadratic relationship with distance from the SST opening suggesting a constriction at the orifice and a slight increase in diameter along its length up to the middle of the SST. Each colour on the plot represents measurements from the same SST (n = 10). Scale on red bounding box is in microns.

SSTs were found to be slightly elliptical in cross-section, with the major axis diameter being 1.642 ± 0.168 times larger than the minor axis diameter. The circularity of the SST in cross-section did not vary significantly with SST diameter (estimated effect = 0.014, t = 0.979, p = 0.330), distance from orifice (estimated effect = 0.003, t = 0.587, p = 0.558), or the interaction between these two variables (estimated effect = -
Circularity was also not related to SST total length (estimated effect = -0.004, t = -1.14, p = 0.282).

Comparisons between the SST measurements from histology and SPIM images indicate that the autofluorescent granules are present in the supranuclear region of the SST epithelium (Figure 1). The size of the lumen diameter scaled linearly with the width of the SST (Supplementary Figure S4), indicating that epithelial cells remained the same thickness in cross-section with increasing SST diameter. This allowed us to extrapolate shape information from the above analyses to the SST lumen, and using this method, we estimated the diameter of the SST orifice to be 3.278 ± 1.115 µm (mean ± SD; Table 1).

Finally, we found no evidence of sperm motility either inside, or on release from, the SSTs. Of 1520 sperm across 46 SSTs (from 13 females), no sperm were observed actively swimming before or after release from the tubules.

Table 1: SST dimensions at orifice and widest section

| Diameter (mean ± SD)         | at orifice (µm) | at widest section (µm) |
|------------------------------|-----------------|------------------------|
| inter-nuclear diameter¹     | 12.060 ± 1.419  | 30.830 ± 11.055        |
| autofluorescence¹            | 10.065 ± 4.288  | 16.374 ± 6.610         |
| lumen diameter²             | 3.278 ± 1.115   | 9.128 ± 1.377          |

¹Measurements acquired from SPIM image z-stacks
²SST lumen diameter values were predicted from the model describing the relationship between lumen and inter-nuclear diameter.
Discussion:

Using novel 3D imaging methods, we have demonstrated for the first time the existence of a constricted orifice at the entrance/exit of avian SSTs. Although the precise function of this constriction remains to be identified, it is likely that it plays an important role in sperm selection and storage. Consistent with previous findings of an F-actin rich terminal web in the SSTs in turkeys [39], this constricted opening is suggestive of contractile activity for active entrapment and release of sperm. However, even without contractility, the narrow SST orifice (mean diameter of about 3 µm), with the added obstruction of microvilli (1-2 µm in length, [52]) could restrict the rate of sperm (mean diameter at mid-piece - ~0.6 µm [59]) entering and exiting the SST, thereby providing a mechanism by which sperm storage and release can be regulated. This, combined with the fact that sperm do not appear to actively swim inside or on immediate release from the tubules, supports the idea that avian SSTs play an active and selective role in sperm storage, regulating sperm uptake and release and suppressing motility [33–38]. The constricted orifice, together with its microvilli, may act as a valve, allowing unidirectional movement of sperm only, and preventing them from being flushed back out. The small luminal diameter along the SST (mean: 9 µm, Table 1) may also limit the ability of sperm to turn around inside the SST and swim out.

SSTs were also found to be slightly elliptical in cross-section, with the major axis diameter being about 1.6 times larger than the minor axis diameter. This ellipticity was independent of SST radius, the distance along the SST from orifice, or total SST length. Cross-sectional ellipticity increases the surface area of the SST epithelial apical
surface, allowing for a greater number of microvilli (as compared to a circular lumen with the same volume) for increased contact with sperm and optimum exchange of nutrients and waste.

SST diameter varied widely across SSTs. Birkhead et al. [68] suggested that some SSTs might remain inactive in the zebra finch UVJ even in its fully developed state. It is possible that some of the variation in SST shape that we observed can be explained by the presence of functional and non-functional SSTs, but it is unclear whether thinner, more uniform SSTs, or more distended morphs would represent active SSTs. Mero and Ogasawara [69], and Burke [70] described ‘swollen’ tubules in chicken and suggested swelling to be associated with sperm release. Such swellings might help explain the outliers in our data (Figure 2). It is possible that conformational changes in SST shape from functional to non-functional may be caused by neural stimulation [39,71] and/or hormonal effects [48,49], potentially aiding sperm release. Variation in SST shape might also be explained by factors not tested in this study, including age, hormone levels and location of the SST in the UVJ.
Figure 3: Sperm storage tubules (SSTs) imaged label-free using the SPIM. SST shape showed a constricted opening (arrowheads) but varied between more elliptical shapes (left) and thinner, more uniform shapes (right). Scale on red bounding box is in microns.

About 4 – 27% of all the sperm storage tubules in the zebra finch UVJ are branched [28,72]. These were not included in the present study, but individual branches are expected to show similar shapes as unbranched tubules. Hemmings and Birkhead [28] described sperm from different males differentially stored in separate branches of a branched SST (albeit a single observation, since in most cases sperm from different males were stored in different SSTs). Further study of the 3D structure of branched SSTs could shed light on the mechanisms that might prevent sperm mixing in branched tubules.

Our novel 3D data on SST structure was made possible by the presence of punctate/granular autofluorescence, confined to the SST epithelial cells and uniformly distributed throughout the SST's entire length. These granules were found to have a supranuclear localisation in the SST epithelial cells (Figure 1). Although
identifying the exact source of the autofluorescence was beyond the scope of this study, autofluorescence in a similar range has been noted in the ewe *Ovis aries* endometrium ($\lambda_{ex}/\lambda_{em} = 488/525-575$ nm) [73] and in human colonic crypts ($\lambda_{ex}/\lambda_{em} = 488/580$ nm) [74]. While such autofluorescence has been attributed to NADH metabolism in mitochondria [73,75], another likely source might be lipofuscin in lysosomes [74]. Mitochondria are not confined to the apical cytoplasm of SST epithelium as observed in turkeys [76] and domestic fowl [77] so it is unlikely that these granules represent mitochondria. Lysosomes on the other hand, are globular vesicles similar to the autofluorescent granules observed here in size (<1 µm) [78], and have been detected in the apical cytoplasm of turkey SST epithelia [76], and less abundantly in domestic fowl [77] and passerine alpine accentor *Prunella collaris* [78]. Multiple studies have also detected the presence of acid phosphatase, an enzyme found in lysosomes, in the supranuclear cytoplasm of SST epithelia in turkeys [21], quail [79], domestic fowl [80] and ducks *Anas sp.* [81], but not in the SST lumen, which corresponds with the autofluorescence pattern we observed here. Acid phosphatase has been implicated in autolysis associated with oviduct regression [82] as well as with sperm release [81]. If this is true, the label-free imaging methods developed here may provide exciting new means for investigating SST functional development throughout the reproductive cycle. Identifying the chemical nature of the autofluorescent substance present in SST granules therefore represents an important avenue for future research.

In summary, we have demonstrated that SSTs in living avian oviductal tissue can be imaged label-free using SPIM microscopy, and this novel 3D imaging technique has enabled us to produce the most detailed account of avian SST structure to date,
including the discovery of a previously unknown structural constriction at the entrance/exit of tubules that may play a key role in the regulation of sperm storage and selection. The imaging methods described here hold immense potential for studying SST function and interactions with sperm in real time.

**Ethics:**

This study was approved by the University of Sheffield, UK. All procedures performed conform to the legal requirements for animal research in the UK and were conducted under a project licence (PPL 40/3481) issued by the Home Office. Animals were humanely killed under Schedule 1 (Animals (Scientific Procedures) Act 1986).

**Competing interests:**

The authors declare no conflict of interest.

**Authors’ contributions:**

T.M. coordinated the study, conducted the experiments and analyses, and wrote the manuscript; N.H. conceived the study and advised on data analyses. All authors participated in the design of the study, helped draft the manuscript and gave final approval for publication.

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