

Streamlining the image capture and
analysis for high throughput unbiased
quantitation and categorization of
nuclear morphology

J.C Perry

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List of commonly used abbreviations

NMA – Nuclear Morphology Analysis described in (Skinner *et al.*, 2019)

WWLL- WSB X Lewes mouse cross

PPLL- PWK X Lewes mouse cross

UMAP – Uniform Manifold Aproximation Projection

DAPI - 4',6-diamidino-2-phenylindole

PFA – paraformaldehyde

1 ABSTRACT

The morphology of cells can be closely linked to their function, particularly in sperm, where it can affect movement and behavior, thus impacting fertility. Automated image analysis can quantify shape differences between cells and has demonstrated links between fertility and sperm shape, particularly in mice. However, a bottleneck in manually capturing images limits the detection of rare (both uncommon and abnormal) shape phenotypes. There are three main aims of this thesis: to develop an unbiased automated sperm imaging system and compare this system to manual imaging,

to increase the analytical capacity to identify sperm morphologies in a sample and to use the pipeline to characterise new rodent sperm morphologies. Automated imaging was implemented using a Nikon Ti fluorescence microscope with a focus on unbiased, fast, low effort image capture. In this project, imaging throughput was increased from 100 to 3000 images per sample, permitting analysis of tens of thousands of nuclei from a single sample. With these images the statistical analysis portion of the pipeline can be developed to segment different phenotypes in a sample of sperm. An automated R analysis pipeline was developed to characterise common morphotypes within a sample, providing biological insight into the region of the sperm head varying. Using this method, the structure of two species of hamster sperm has also been clearly categorised, demonstrating a conservation of hamster sperm structure over 1 million years of evolution. This conservation of hamster sperm shape is in stark contrast to the variety of sperm shapes in mouse subspecies. This system has potential to be used to identify clinically relevant data about morphology of human sperm in the analysis of infertility phenotypes.

2 INTRODUCTION

Morphology is the study of the shape and morphometrics is the study of morphology with numerical values. More specifically nuclear morphology and nuclear morphometrics focus on applying those methods of study to cell nuclei. From the literature it is clear there is potential for large improvements in the field of nuclear morphology (Mitteroecker and Schaefer, 2022). One of these improvements would be a more widespread usage of in-depth nuclear morphometrics. There are barriers to a more widespread usage of nuclear morphometrics such as the imaging training and time requirement. The streamlining of the sample processing pathway with the usage of automated image acquisition would increase the viability of using nuclear morphometrics by increasing the quantity of images acquired and allow for the detection of rarer morphological variations in samples.

2.1 WHY IS NUCLEAR MORPHOLOGY IMPORTANT?

The study of morphology of cells in general as well as nuclear morphology and morphometrics is a growing field that is valuable to the future of diagnostic medicine as well as understanding of cells, evolution, and inheritance. An example of this is the deletion of Y chromosome in mouse sperm long arm effecting sperm nuclear morphology and in turn sperm function reducing y chromosome inheritance resulting in sex skews (Ward and Burgoyne, 2006; Skinner and Johnson, 2017). The nucleus is one of the most important parts of the eukaryote cell and its structure can give valuable information about the structure and state of the cells.

2.2 NUCLEAR MORPHOLOGY OF CELLS WITH RELEVANT CHARACTERISTICS TO SPERM

2.2.1 Nuclear morphology and the immune system

Nuclear morphology is implicated as an important signifier of many biological functions. For example, the mammalian neutrophil is an important immune cell (Janeway *et al.*, 2001). Skinner and Johnson, (2017) explain that there is a large quantity of research on the nucleus of the neutrophil, especially its multi-lobed nuclei. The neutrophil passes through small gaps and the multi-lobed nuclei are implicated in this (Hoffmann et al. 2007). The flexibility of the neutrophil nucleus is thought to be increased by the nuclear lamina (Manley et al, 2018). The proposed function of the multi-lobed nuclei is shown in figure 1 where the nucleus deforms to allow passage through constriction points.

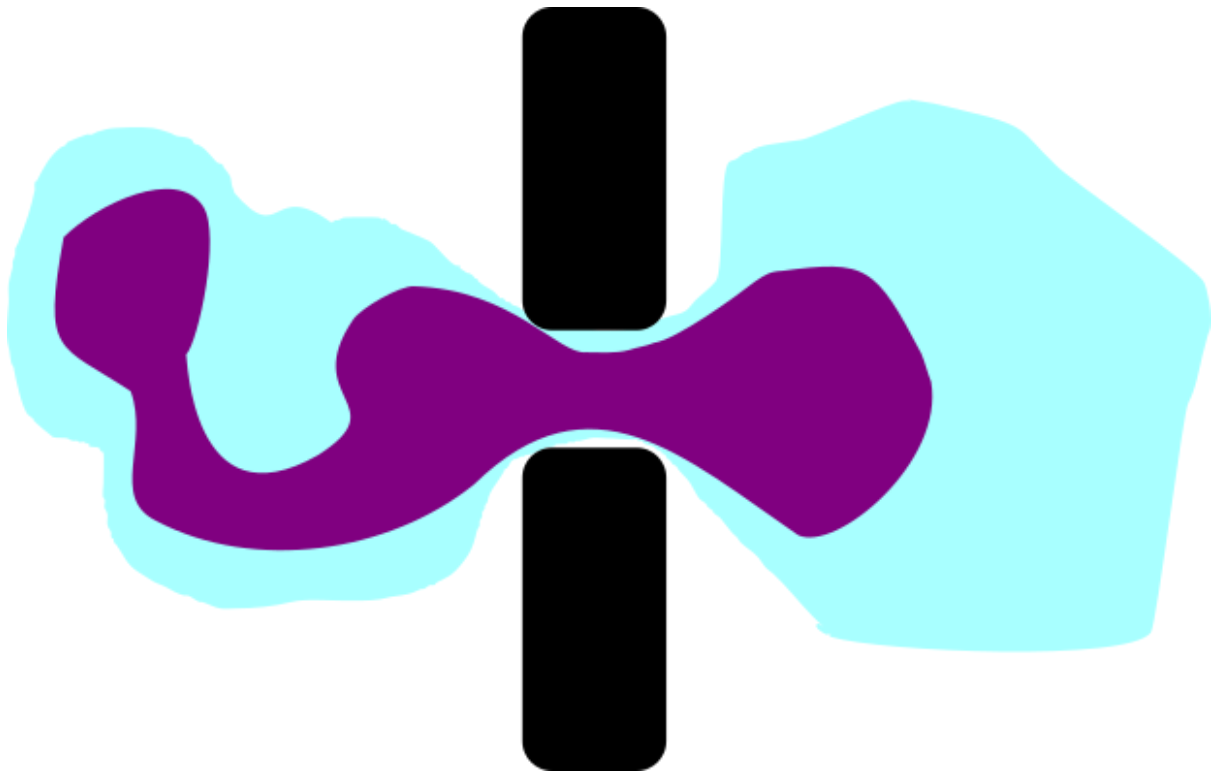


Figure 1 shows the mechanisms of neutrophil migration and the importance of the nuclear morphology.

This can be seen as the deformation of nucleus is vital in its migration (Manley et al, 2018). This diagram is simplified from Manley et al, 2018. The black boxes represent constriction sites, the purple is a nucleus and the blue is the cytoplasm of the cell.

Other cells of the immune system also have distinct nuclear shapes (Figure 2A-D): megakaryocyte, eosinophils, basophils, and giant multinucleated macrophages have characteristic morphologies linked to function as well as being used for identification.

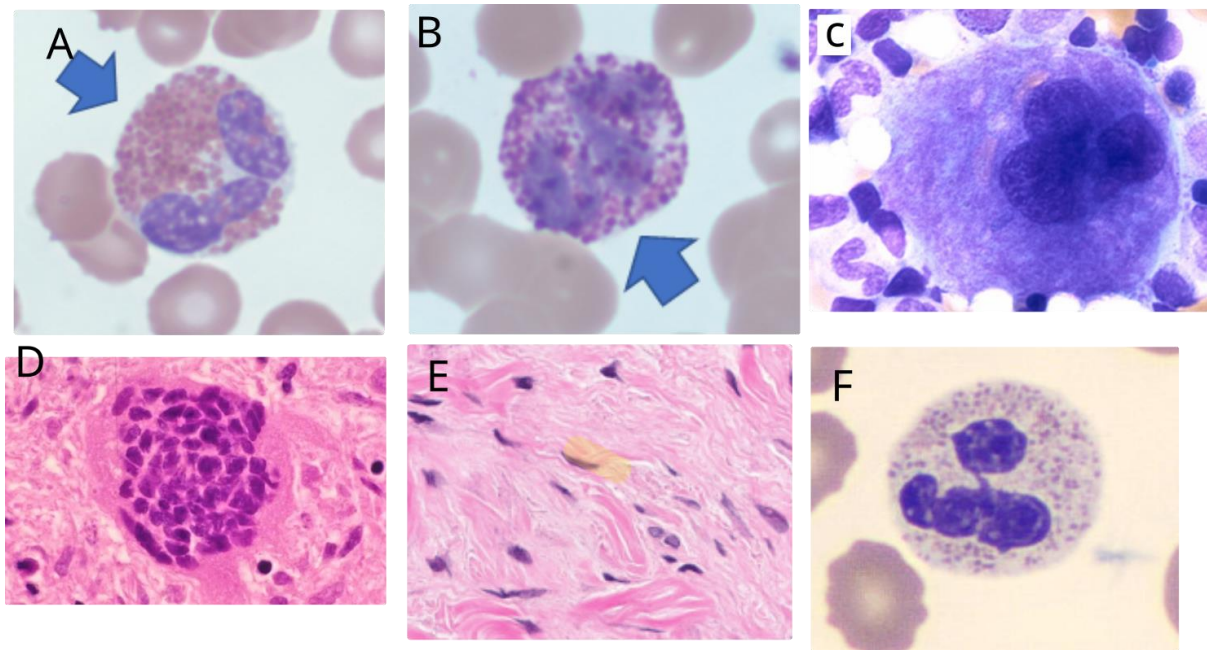


Figure 2 Morphology of key human immune cells.

Section A shows the eosinophil with the distinctive lobed nucleus and granules in it (Aird, 2021). Section B shows the basophil with its bean shaped nucleus and granules (Aird, 2021). C shows the megakaryocyte (Normal Histology, no date available accessed 2023). D shows a giant multinucleated macrophage ('Giant multinucleated cells', 2012). E shows many fusiform fibrocytes with its elongated nucleus, a central one highlighted in yellow to distinguish it from surrounding tissue (Cuffy et al., 2010). F shows the neutrophil and its distinct lobed nuclei (Segmented neutrophil, no date available accessed 2023). A , B, D,E and F are haematoxylin and eosin stained. C has no stain specified. None of the image sources have scales provided.

The Megakaryocyte (Figure 2C) is a cell found in bone marrow. Its function is the production of platelet components and organelles (Eldor *et al.*, 1989) and is implicated in Philadelphia-negative myeloproliferative neoplasms (MPNs) (Sirinukunwattana *et al.*, 2020). Young megakaryocytes have a large ratio of nuclear size: cytoplasm size and are the only cell that is commonly found in normal bone marrow with multiple lobes. The morphology of these cells has been studied for many years; in 1982 Levine, et al found a cell size threshold (using the diameter) for simple identification of megakaryocytes using photomicrographs and using that studied the multilobed nuclei finding support for the relationship between maturation stage and ploidy. More recently, Sirinukunwattana

et al.(2020) developed a morphological-based AI system for the identification of megakaryocytes with a focus on disease detection. This system can differentiate between a normal megakaryocyte and the megakaryocyte of an individual suffering from myeloproliferative neoplasms. They did this by first creating a megakaryocyte library of annotated images and found a series of megakaryocyte subtypes with features that were associated with myeloproliferative neoplasms that they segmented into two separate categories of megakaryocytes. This study is of significant relevance to this project as it shows the great potential of automated morphological analysis for use in a medical diagnostic environment.

Eosinophils (Figure 2A) are leukocytes involved in the inflammatory response (Hogan *et al.*, 2008). They also have a proposed role in disease processes such as infection, gastrointestinal disorders, and a non-allergen-based asthma known as intrinsic asthma. The mechanism of the implication of eosinophils in asthma is thought to be that they are involved in airway remodelling due to increased adhesion (Hogan *et al.*, 2008). An increase of eosinophils in tissue, blood and bone marrow are more easily detected due to their morphology and are characteristic of most asthma phenotypes. Mouse and human eosinophils have different morphology and are not functionally equivalent due to divergent evolution, their difference in morphology correlates with a difference in function and behaviour. There are a few different eosinophil abnormalities: an absence of peroxidase and phospholipids in them, large abnormal granules, grey inclusions, and granule deficiency are all inherited abnormality (Kuijpers, 2021) these are all morphologically visible. There are also abnormalities in the eosinophils of leukaemia patients, with the immature eosinophils an abnormal number of granules this could potentially be useful in diagnostics, as the increased granules would give a visible and measurable morphological change (Kuijpers, 2021).

The basophil (Figure 2B) was originally viewed as a redundant but are unique leukocytes with immunomodulatory functions (Shah *et al.*, 2021). They have lobed nuclei and are distinguished by

their purple granules in their cytoplasm. The basophil is the least abundant cell in the blood representing less than 1% of cells. Consequently, these cells are difficult to identify for novice histologists, yet they are important for the detection of basophilic leukaemia. For example, high basophil counts are a signifier for chronic myeloid leukaemia (Shah *et al.*, 2021). Despite this, modern digital morphology programs have yet to successfully produce a system for identifying and counting basophils to the degree that Hoffmann in 2021 wrote about potentially stopping the use of basophil counts in clinical diagnosis due to them being unreliable and potentially misleading. A reason for this is that it is especially difficult to detect excessive amounts of basophils as they can have aberrant morphology during basophilic leukaemia. This concludes that there is a definite need for better morphological systems for the identification of basophils, and In-depth nuclear morphometry could be of great use for this improving the reliability of basophil counts.

Giant multinucleated macrophages (Figure 2 D) originate from the fusion of monocytes that compete with macrophages and other monocytes to fuse into giant multinucleated macrophages (Dittmar and Zänker, 2011). The giant multinucleated macrophage is formed from monocytes and macrophages following chemoattractant cues which are caused by cytokine responses as part of processes like foreign body reactions (Brooks et al, 2019). The giant multinucleated macrophages also have crucial functions in a broad group of immunological processes that regulate responses such as inflammation, infection, and neoplasia. Another example of a disease that giant multinucleated macrophages are important are chronic obstructive pulmonary disease which was studied in Sato-Nishiwaki *et al.*(2013). The morphology of giant multinucleated macrophages is clearly relevant to their function, especially that of pseudopods which was found in Sato-Nishiwaki *et al.*(2013). This relevance of the morphology of giant multinucleated macrophages was found when studying transgenic mice with a mutation to the *MafB* gene, that caused the a reduction in the ability of pseudopod production, and considerably reduced their ability to perform phagocytosis. Another indicator of the importance of not only the mechanics of their morphology but the study of

their morphology is that the giant multinucleated macrophages have varying morphologies when found in the vicinity of different conditions. Examples of the varying morphologies of giant multinucleated macrophages include crescent or horseshoe shaped group of nuclei during chronic tuberculosis unlike in figure 2D there whereas for foreign bodies it is loosely grouped nuclei with some connected but most free floating as shown in figure 2D (Sato-Nishiwaki *et al.*, 2013).

Ulloa *et al.* (2022) showed the relevance of the nuclear morphology of B cells to their function. They found that the nuclear morphology of the B cells is important in immune synapse and reorient their nuclear grove (a nuclear feature thought to be implicated in the lysosome recruitment) towards the immune synapse as well as the fact this nuclear reorientation is cytoskeleton dependent.

2.2.2 Nuclear shape and nuclear skeleton.

Among other cell types that can have asymmetric nuclei, are the fusiform (spindle shaped) nuclei which are often heavily environmentally influenced by factors such as cell density and tissue state and can also be found with more spherical morphology. Some examples of this is macrophages and mesenchymal cells, which can have fusiform nuclei when under certain conditions, such as chemical signalling from cytokines but otherwise exhibit more standard nuclei (Weiss and Garber, 1952; Menzyanova *et al.*, 2020). Other examples include fibrocytes (Nagayama, Yahiro and Matsumoto, 2011), mesenchyme and endosperm in flowering plants.

Mesenchyme has fusiform nuclei that are twisted into a spring shape as the muscle contracts (Weiss and Garber, 1952). The morphological properties of these cells are interesting as they are found in other contexts especially that of cancer. Lam *et al* did a study in 2020 on morphological identification of cancer cells with mesenchymal features as this is relevant to cancer outcomes

(Kenny *et al.*, 2007). This is because the epithelial to mesenchymal transition is a vital part of the spread and metastasis of cancer and so being able to give it a quantitative value helps with determining diagnosis and treatment. They did this using machine learning algorithms applied with a high throughput imaging system, this system uses lasers to give a quantitative grade to each cell allowing for phenotypic classification. This test identified both cancer cells with mesenchymal features and epithelial features which can be used to determine cancer outcomes and was mainly tested with breast cancer cells.

Fibrocytes (shown in Figure 2F) are migratory cell implicated in healing (Metz, 2003) and their morphology is important in identification and potentially their migration. They migrate to wounds by following chemokine gradients and their distinctive morphology allows them to be identified at sites they have migrated for example Metz (2003) found the fusiform shaped cells with small, elongated nuclei and small dendrite-like projections with morphometric analysis in tumours that were fibrocytes. This morphological analysis was used to detect different fibrocytes present in different pancreatic diseases and proved its potential for use in pancreatic disease diagnosis.

The nuclear morphology of cells was also used in Niide *et al.* (2022) with the use of nuclear morphometrics to perform a study of the relationship of nuclear size : erythrocyte size scaling with frog erythrocytes. This was by taking images with a confocal microscope and using them to create z-stacked regions of interest in ImageJ then using those to give the 3D volume of the erythrocytes. They found a weak correlation between nuclear and erythrocyte size in individual frogs. This work shows that nuclear morphology and cell morphology are connected and that it can give us information about different mechanisms of how cells are structured.

2.2.3 Plant nuclear morphology

A further example of a fusiform nucleus in a flowering plant is *Coronopus didymus* (Brassicaceae) endosperm, which has a nucleus surrounded by a microtubule cage (Nguyen, Brown and Lemmon, 2001), though the functional significance of this is as yet uncertain. Formation of this endosperm starts by fusion with the central cell during double fertilisation (Boisnard-Lorig *et al.*, 2001; Li and Berger, 2012; Xi *et al.*, 2021). Endosperm develops in phases this starts with the immediate mitosis of the central cell which isn't followed by cell division this is followed by syncytial developmental phase which ends as the multinucleated syncytial endosperm into individual cells that differentiate. The final phase is arresting cell division and metabolic changes to produce a reserve in the seed. This endosperm development proceeds embryogenesis. This is clearly a complex process with a large amount of morphometric importance and variation.

However, by far one of the most diverse and distinctive group of nuclear morphologies is found in the spermatozoa, and we have yet only scratched the surface of the variation within species phenotypes. This variation is evident between taxonomic groups but there are many influences of sperm shape especially with fertility. The next section focuses on sperm morphology and their functional relevance.

2.3 MORPHOLOGY OF SPERM

There is a huge variety of sperm with it being nearly universal among modern eukaryotes (Goodenough and Heitman, 2014). There is also a huge amount of variation between species with it being a major factor in pre-zygotic isolation.

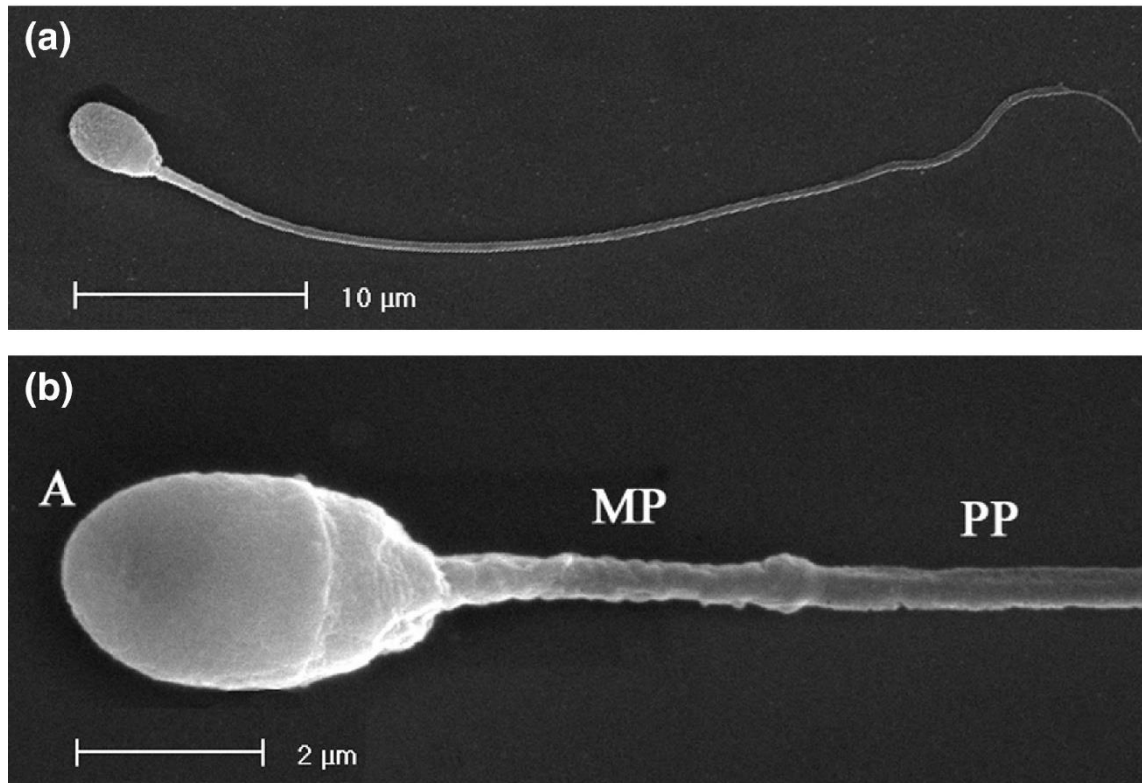


Figure 3 SEM images of human sperm.

(a) A whole spermatozoon with normal morphological features. (b) Morphological features of a normal sperm head, neck and midpiece. A: acrosome; MP, midpiece; PP, principal piece. Scale bar = 10 μm (a), 2 μm (b) (Zhu, 2018)

There is a huge variety of sperm with it being nearly universal among eukaryotes (*Goodenough and Heitman, 2014*). There is also a huge amount of variation between species with it being a major factor in pre-zygotic mechanisms of reproductive isolation between species. Figure 5 shows the huge variety of sperm among animal eukaryotes however animals are not the only examples of sperm morphological variety as can be seen in figure 4.

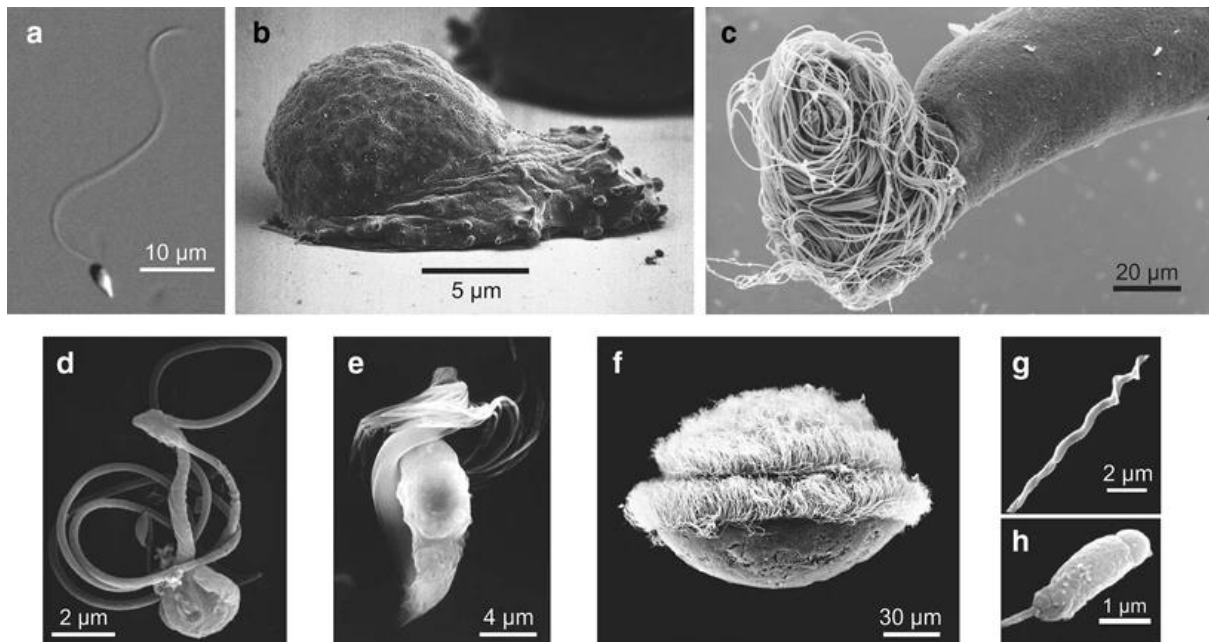


Figure 4 The variations found in sperm morphology from across both animal (non-mammal) and plant species.

a shows *Arbacia punctulata* sperm. **b** shows *Ascaris suum* sperm which noticeably has no flagella and moves by crawling. **c** shows sperm from *Drosophila bifurca* which is the longest known (the duct the sperm is emerging from is included). **d-f** are sperm from the plants: **d** - *Conocephalum conicum* (biflagellated), **e** - *Equisetum hyemale* (with at least 80 flagella) and **f** - *Cycas revoluta* (1000s of flagella). **G** Sperm from Eurasian bullfinch *Pyrrhula pyrrhula* (also shown in figure 5) and **g** the House sparrow *Passer domesticus*. (Alvarez, 2017)

Many distinct morphologies of spermatozoa (as can be seen in figure 5) have been studied, such as the sharp pointed and flattened sperm of the short tailed fruit bat (Beguelini *et al.*, 2014), the square headed orca sperm (Miller *et al.*, 2002), and the short rounded head of Eurasian Bulfinch sperm (Birkhead *et al.*, 2007). More spermatozoa morphologies could be listed however a lot can be gleaned about the uses of sperm morphology from just these three, for example in Beguelini *et al.* (2014) the morphological structure is compared to that of many other mammals and compared to multiple species of bats. The features of closely related bats were compared to infer whether some features of bats thought distantly related could have been caused by convergent evolution. In Miller *et al.* (2002) they did similar comparisons to other species but also studied the spermatozoa specimens that came from a deceased beluga which produced spermatozoa that had many morphological issues allowing them to infer treatments received before death and the illness it had likely affected the morphology of its sperm with support of earlier studies that showed infections and other illnesses as well as chemotherapy can influence the morphology of sperm. In Birkhead *et*

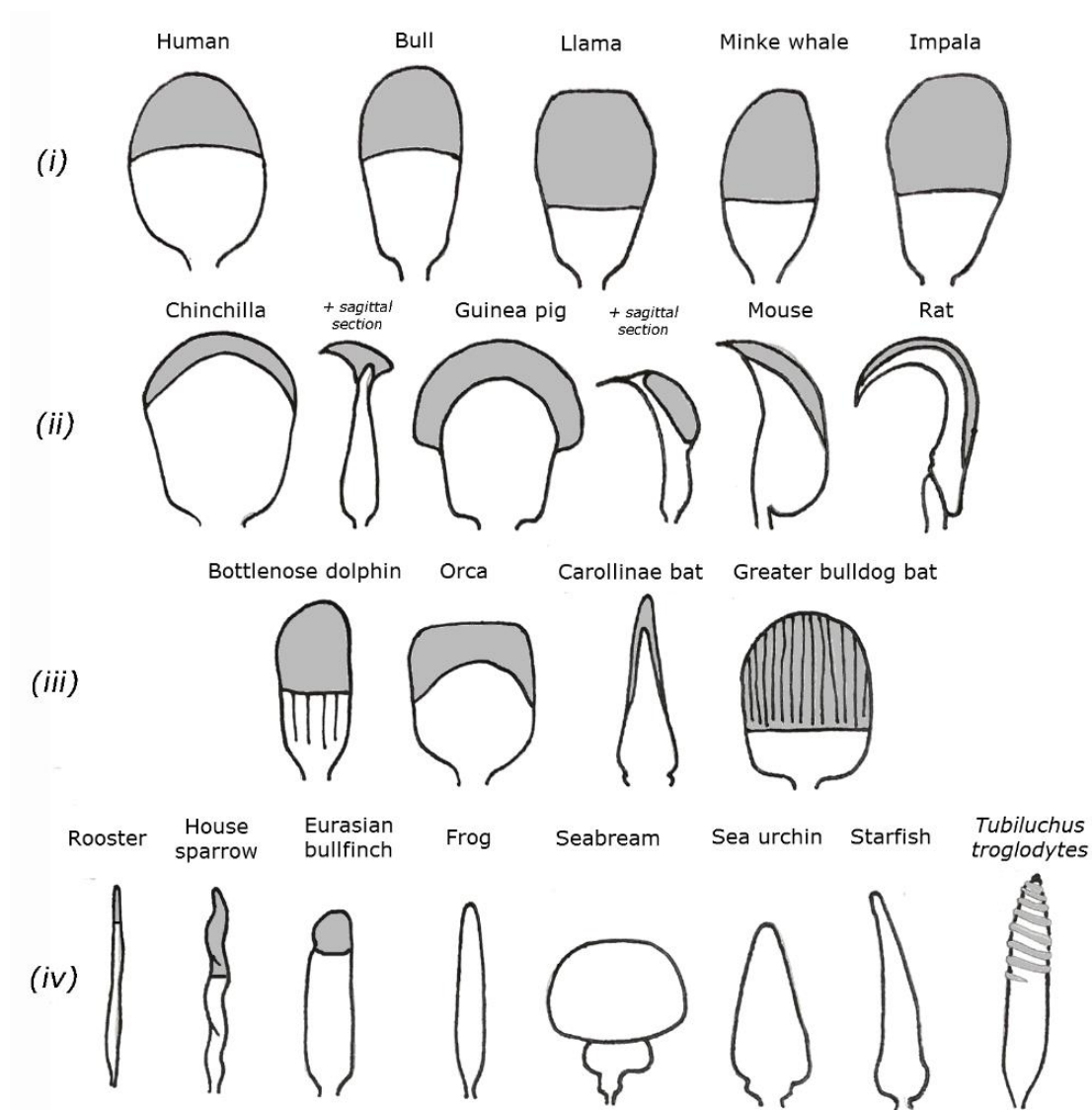


Figure 5 The morphology of a large variety of sperm from across the animal kingdom.

Row i, ii and iii showing mammals and the final row (iv) showing other species and the more extreme morphological phenotypes. The grey shading represents acrosomal regions. From Skinner and Johnson (2017).

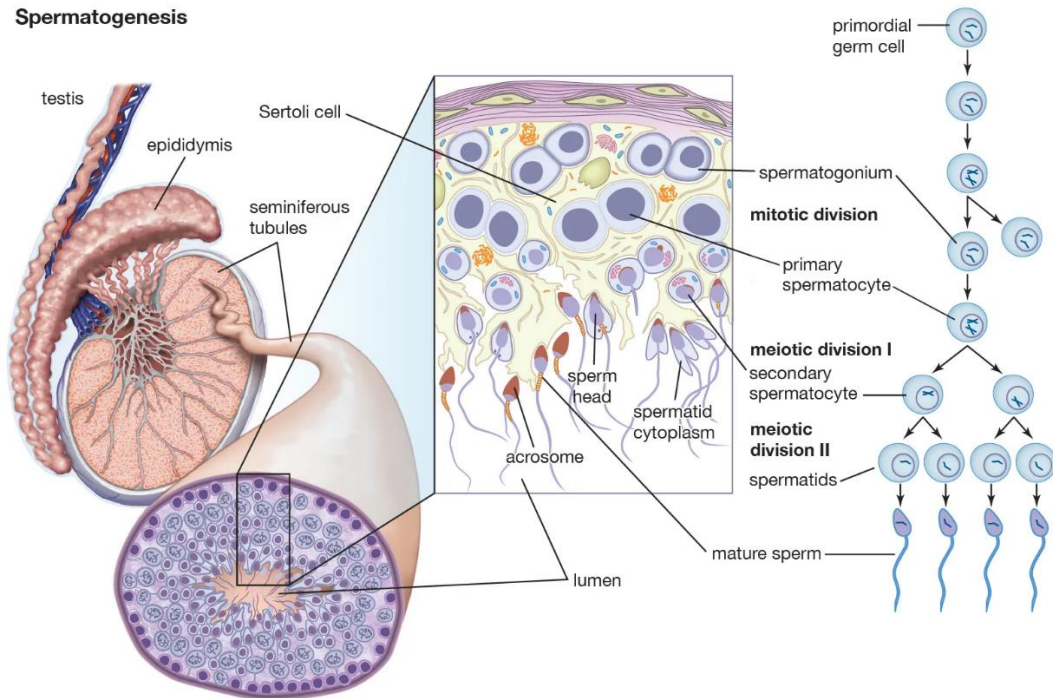
all (2007) they found the morphology of the Eurasian Bulfinch sperm showing it was potentially neotenic and had not fully undergone spermatogenesis resulting in it's short rounded head.

Female promiscuity plays a crucial role in selective pressures on sperm morphology. This promiscuity is one of the main drivers of sperm competition, resulting in the selection of many fertility characteristics, including morphology (Ramm *et al.*, 2014). Although it is not the only source of sperm competition, the correlation between female promiscuity and sperm quality is demonstrated in cichlids. (Fitzpatrick *et al.*, 2009)

2.3.1 Mammalian spermatogenesis

Sperm is produced in the seminiferous tubules of the testes (figure 6), and this section outlines the general process of spermatogenesis. Spermatogenesis is where spermatogonia the primordial spermatogenic cell forms spermatozoa the male gamete.

Spermatogenesis



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Figure 6 The sites and processes of Spermatogenesis (Spermatogenesis | Description & Process | Britannica, 2023)

This diagram shows spermatogenesis in the context of the testis(left), in the context of location relative to individual cells within the seminiferous tubules(middle) and in the context of the changes a single cell goes through(right).

Spermatogenesis can be split into three phases: the proliferative phase, the meiotic phase, and the spermiogenic phase or four if the spermiogenic phase is split into the differentiation and maturation phases (Russell *et al.*, 1993; Wistuba, Stukenborg and Luetjens, 2009). The first stage is the proliferative phase where the spermatogonia go through rapid mitosis to keep their population from depleting as a portion of them become spermatocytes and move onto the next stage which is the meiotic stage. Meiosis is required to produce the haploid cells required for fertilisation this stage is made up of two meiotic divisions. These meiotic divisions start as the spermatocytes enter the ad luminal compartment after reaching the Sertoli cell barrier (Suede, Malik and Sapra, 2023). The first division is of the diploid primary spermatocytes into haploid secondary spermatocytes. The second division is of the secondary spermatocyte into two haploid spermatids resulting in four haploid cells.

The spermiogenic stage follows where the spermatids gain their features and become spermatozoa (Suede, Malik and Sapra, 2023). Murat *et al*, (2023) discovered that the most significant evolutionary differences occurred in mammalian spermatogenesis due to molecular innovations during spermiogenesis, because many processes depend on the interactions between many protein complexes. There is a great deal of morphological and mechanical change in this stage such as the attachment of the acrosome (The cap-like structure containing enzymes that digest part of the outer membrane of the egg cell allowing fertilisation) and the flagellum and the condensation of the nucleus and removal of the cytoplasm that changes the spermatids into the mature spermatozoans.

2.3.2 Important structures in sperm shape determination

A large amount of the sperm studied among mammals with low levels of promiscuity (Ramm *et al*, 2014) (e.g. humans, and to a lesser extent lab mice however there are other factors also causing more variation) have a series of abnormalities and so the understanding of the structures within those cells and the relevance of those to the development of the sperm. There are four main structures that the literature suggests are of great importance to the structure of the sperm: the acrosome, the acroplaxome, the manchette and the LINC (Linker of Nucleoskeleton and Cytoskeleton) complex which binds the manchette to the nucleus (Kierszenbaum and Tres, 2004).

The nuclear structure of sperm goes through massive changes during spermatogenesis, the steps of spermatogenesis require a functional cooperation between the nuclear envelope proteins, the

acroplaxome and the manchette (Manfrevola *et al.*, 2021).

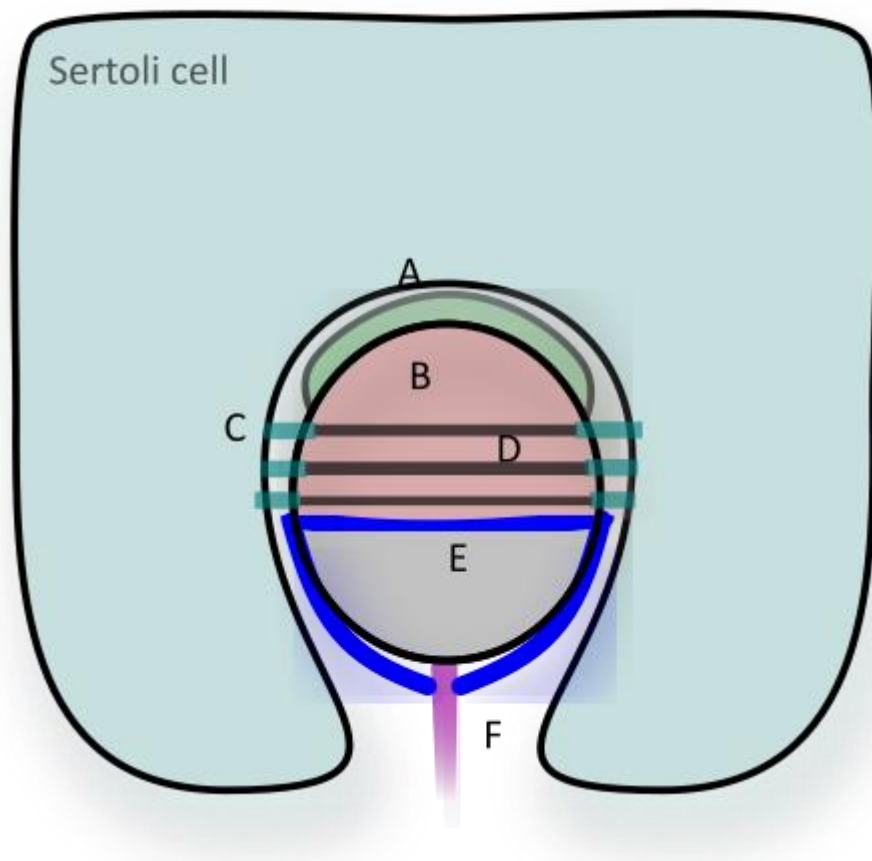


Figure 7 is a diagrammatic representation of the main structures involved in the shaping of the mammalian sperm head.

A is the acrosome. B is the acroplaxome. C is the connection between the ectoplasmic specialisation of the Sertoli cells. D is the f-actin loops that support elongation of sperm. E is the manchette. F is the tail. The diagram is not to scale and is before elongation.

The manchette is a perinuclear mantle formed of microtubules during acrosomal spermiogenesis and disassembles during maturation. It is very heavily implicated in the nuclear morphology of the sperm and head to tail coupling shown in figure 7 (Tapia Contreras and Hoyer-Fender, 2019). The head of the spermatid must be coupled to the tail via the manchette and groove belt to prevent globozoospermia (and other similar phenotypes such as the head failing to attach to the tail and midpiece and the sperm not being functional). It is also thought that the manchette may be used for storage of molecules needed for elongation of the nucleus (Lehti, Kotaja and Sironen, 2013). The

manchette stretches upwards to the acroplaxome and downwards in an open cage-like structure around the basal portions of the nucleus (Calvi *et al.*, 2015)

The LINC complex binds the manchette and the nucleus and can give insight into the importance of the manchette in nuclear function. It is not fully known specifically how the LINC complex connects to the nucleus either via nuclear lamina or directly by chromatin (Manfrevola *et al.*, 2021). The Linc complex is found inside the nuclear envelope and has two major parts; the SUN proteins in the inner nuclear membrane and the KASH proteins in the outer nuclear membrane, this links the nucleus to structures like the manchette (Kmonickova *et al.*, 2020). Several studies have been done mutating components of the LINC complex to see its importance and the mechanisms it is involved in. The main focus has been on SUN4 deficient strains of lab mice (Calvi *et al.*, 2015; Pasch *et al.*, 2015). An interesting result of these SUN4 deficient mutants is that the manchettes don't form correctly resulting in a globozoospermia-like phenotype where the nucleus isn't attached to the tail causing complete male infertility (figure 7 part E shows the area where the lack of SUN4 causes issues as the manchette to be unable to connect to the nucleus at).

Although not actually part of the spermatid the Sertoli cell is vital in the formation of sperm and is responsible for the important extracellular forces compressive forces from their actomyosin bundles that surround the spermatid as it forms guiding it into its final shape (Dunleavy *et al.*, 2019). Often referred to as 'nurse' cells they are situated in the seminiferous epithelium. They have a dynamic cytoskeleton made up of actin filaments and microtubules that is cyclically remodelled to accommodate spermatogenesis. This can be seen in figure 7 showing the Sertoli cell accommodating spermatogenesis in the basal and ad luminal compartment.

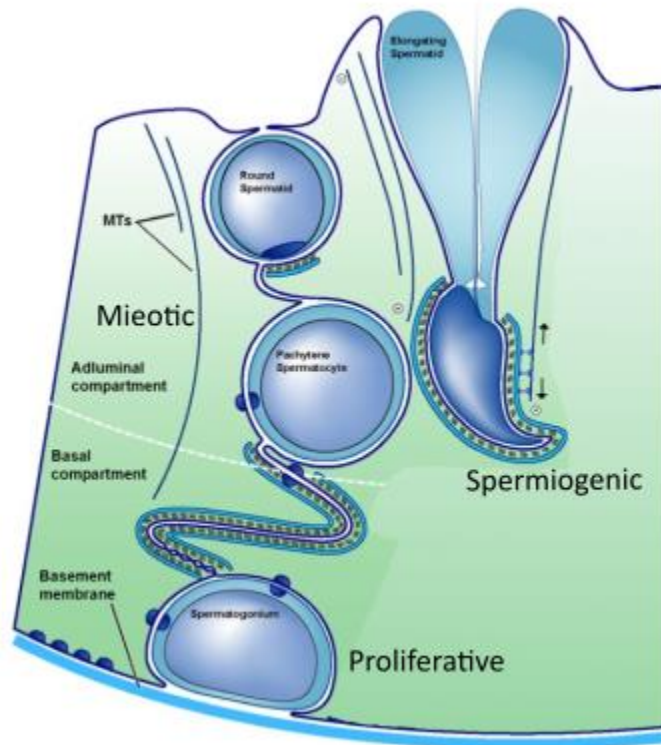


Figure 8 The cytoskeletal organisation of the seminiferous epithelium. This diagram shows rodent sperm in various stages of spermatogenesis. (Dunleavy et al., 2019) Proliferative is first then meiotic then spermiogenic.

The overall structure and function of the sperm is very heavily influenced by the acrosome-acroplaxome manchette complex shown in figure 8. The acrosome-acroplaxome complex has actin-based molecular motors and actin filaments (Zakrzewski *et al.*, 2020). In Kierszenbaum and Tres, (2004) two functions of the acroplaxome (a plate containing f-actin and keratin) are proposed: anchoring the acrosome to the spermatid head as it develops and functioning as a scaffolding plate for the manchette. It is proposed that the sperm head is mainly shaped by the contractile forces generated by F-actin hoops near the top of the spermatid nucleus around the acroplaxome. These contractile forces are from the Sertoli cells that create an exogenous clutch from F-actin and other proteins in the Sertoli cell ectoplasmic specialisation. Wei have detected at least 7 of the F- actin hoops that create the exogenous clutch that exerts pressure on the spermatid head (shown in Figure

9).

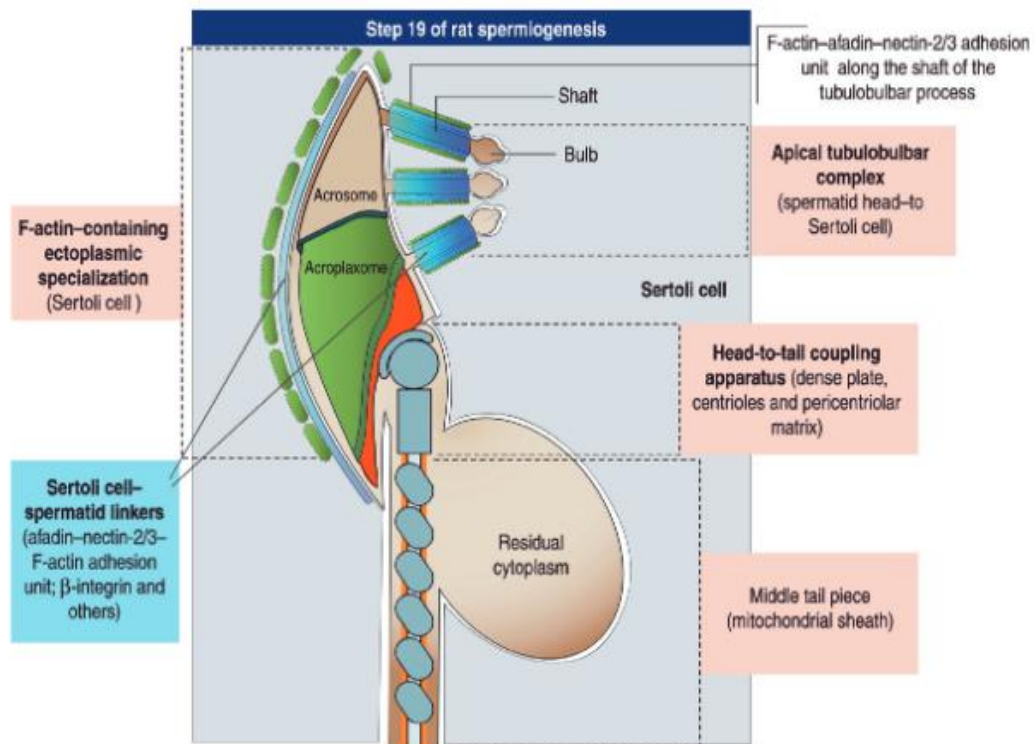


Figure 9 Diagrammatic representation of the acrosome and acroplaxome. This example is from rat spermiogenesis. The nucleus is in orange covered by the acrosome and acroplaxome. (Kierszenbaum and Tres, 2004)

2.3.3 Rodent sperm morphology in general

Rodent sperm is an interesting focus for the study of nuclear morphology as rodents are used as model animals in many studies and so sources of rodent sperm are plentiful and often go unused in animal studies. Wyrobek *et al.* (1976) shows with the studying of parameters of mouse sperm that there is potential for sperm filtration applied with nuclear morphometrics. The purpose of this sperm filtration would be for prezygotic prevention of diseases detectable with nuclear morphometrics that possess different enough physical parameters. Rodent sperm tends to possess a falciform head shape that is morphologically distinct as can be seen in figure 9-11.

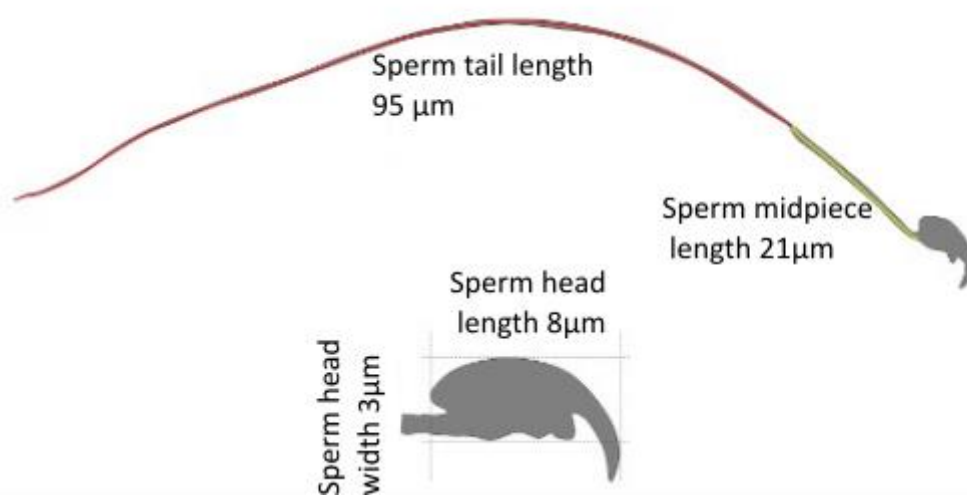


Figure 10 Overview of the morphology of rodent sperm showing the three main sections: the tail, midpiece and head. The sperm shown is specifically from a mouse and exhibits the distinctive falciform shape (Albrechtová *et al.*, 2014).

2.3.3.1 Mouse sperm morphology

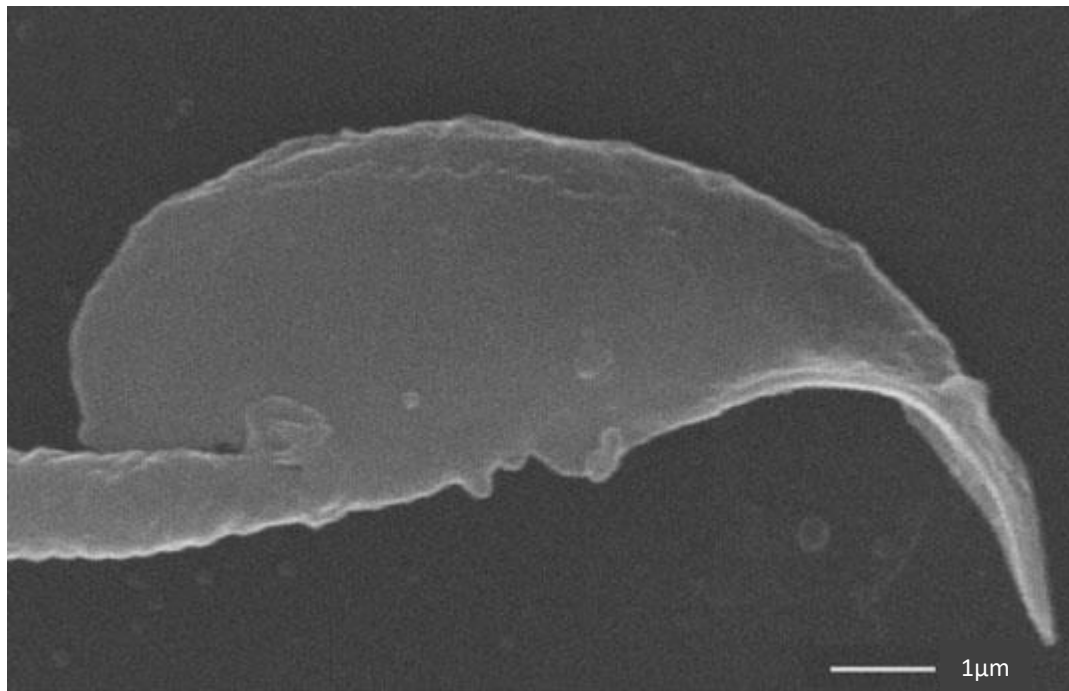


Figure 11 SEM image of mouse sperm head (Buffone, Foster and Gerton, 2008), demonstrating the falciform 'hook' shape

Mouse sperm is the primary focus of this study due to a series of factors. There are many reasons to use mouse sperm as a model to study nuclear morphometrics. *Mus musculus* is an incredibly well documented organism and so there is a great deal of literature entailing its evolution, genetics, and fertility. This literature allows for a great deal of different applications of the morphometric data to characterise the interplay between the evolution, genetics and fertility of the mouse and the morphology of its sperm. There is also the point of convenience that the morphology of the mouse sperm nucleus is beneficial to the process of studying it morphometrically. This is because the dimensions of the mouse sperm make it easier to image especially due to z depth when imaging being low (meaning it is considerably easier to image sperm on comparable planes of 3D orientation). Mouse sperm are normally around 3-4μm wide and 7-10μm long at the head as is evident in figure 10 (Albrechtová *et al.*, 2014) the z value is less than 0.5μm (relatively normal in width and height but their depth/z value is very low in comparison to humans). This results in only

the X and Y planes being relevant to the morphometrics and making it much easier to image. It is also readily available due to there being a large amount of research on mice that are vivisected, and the sperm is produced as a side product to make sure all the mouse is used. This also results in many specimens from interesting sources that results in new pathways of research. There are also many subtle differences between the sperm of different strains of mouse that allows the study of specific genetic differences that affect sperm morphology (Wyrobek *et al.*, 1976; Albrechtová *et al.*, 2014). Albrechtová *et al.* (2014) Studied variation between *Mus musculus musculus* and *Mus musculus domesticus*. The *musculus* and *domesticus* subspecies are used to categorise which mice are wild type mouse strains which are lab strains. Strains that have been inbred in labs and have started to accumulate mutation are referred to as domesticus. They found a significant difference in sperm morphology. They also found geographical variation compared this with the variation between the species finding that the species difference was down to more than just geographical variation. The study based around the central European hybrid zone which allowed them to study the difference in more depth with less geographical variation. They also noticed their findings could be strain specific instead of other factors as inbred strains offer only a snapshot of phenotypes due to only having a few individuals' genetic makeup.

2.3.3.2 Hamster Sperm morphology



Figure 12 A SEM image of Hamster sperm (Hamster Sperm Cell Sem by David M. Phillips, 2014).

No scale provided but assuming the image is comparable to the hamsters we have studied the image is approximately 40µm wide

The hamster species (*Phodopus sungorus* and *Phodopus campbelli*) diverged from their common ancestor between 0.1 and 1.7 million years ago in comparison to their divergence from *Mus musculus* 24.2 – 33.0 million years ago which puts them as close enough to be interesting for the potential development morphological differences but still distinct species. Hamster sperm (Figure 12) is a morphologically interesting as it is very similar to mouse sperm, but hamsters are considerably less documented. There is a very small amount of literature about their morphology and so performing nuclear morphometrics on them holds a value of its own. *Phodopus sungorus* and *Phodopus campbelli* are interesting as they behave reproductively in a similar manner to *Mus musculus musculus* and *Mus musculus domesticus* (Brekke and Good, 2014) making them a good outgroup to compare the mouse sperm to. We do not have *sungorus/campbelli* strains to compare

but Sokolov and Vasil'eva, (1993) show a similar pattern of hybrid sterility as some *Mus musculus musculus/domesticus* crosses.

2.4 FORMS OF MORPHOMETRIC ANALYSIS

2.4.1 Morphometric method and software review

There are many systems used to produce nuclear morphometric data which can be split into three different types of software: simple 2D morphometric programs, more complex 3D morphometric programs and programs using elliptical Fourier analysis which is a more complex kind of 2D reliant on Fourier transforms. Nuclear morphology analysis (NMA) is the system we currently use and so will be used to compare software to.

2.4.1.1 General 2D analysis

Some examples of simple 2D morphometric software are EImage (Pau *et al.*, 2010) and Morphoj (Klingenberg, 2011) as well as the program used by Filippi-Chiela *et al.* (2012) when they studied senescence, apoptosis and nuclear irregularities. The program covered in Filippi-Chiela *et al.* studies the circularity and bounding box of a nucleus and then uses a system of nuclear irregularity index to show how far from a manually selected control set the nuclei are. These parameters are analysed with principal component analysis (PCA) and the features Area box (Arbx), Aspect (Asp), Roundness (Rou) and Radius ratio (Rr) are combined in an index called Nuclear Irregularity Index ($NII = Asp * 2Arbx + Rr + Rou$) are used in a similar way to the variables detected in NMA. It was developed originally outside of ImageJ (Schindelin *et al.*, 2012) then converted into it (In much the opposite way NMA was developed in ImageJ then converted into separate software). Pau *et al.*'s 2010) EImage functions on multidimensional arrays of pixel values from images much in a similar way as NMA, however not using the same edge detection algorithms and not working with the images in

image format but instead processing them in R. It then uses sequential R functions to transform the arrays to extract the features from the data that are intended for use with machine learning functions that can be trained to determine the phenotypes of cells. This is useful and has better potential landmark detection than NMA however it does not provide the same number of variables to characterise the nuclei in the same level of detail as NMA as it is heavily reliant on the users selection of analysis system. Klingenberg's MorphoJ works directly from landmark data (points picked on a cell by an individual) and can produce graphical outputs of the cell with the landmarks displaying it's structure between the landmarks. It functions by measuring a set of shape variables such as width and height as well as circularity that can then be used for further analysis. It can do comparisons from these shape variables. EBIImage and MorphoJ cannot work directly from images and requires processing beforehand from external programs like ImageJ to change the images into an accepted format requiring users to have a decent understanding of those programs.

2.4.1.2 3D analysis

The more complex (than 2D) 3D morphometric software tends to have more overall functions due to the added complexity of the third dimension; some examples of these software are: the Parsimi image pathway (Chiang *et al.*, 2015), CellProfiler (Kamentsky *et al.*, 2011), CellClassifier (Rämö *et al.*, 2009) and TANGO (Ollion *et al.*, 2013).

Parsimi image pathway is made of four portions. The first being an interface to manually annotate cells in 3D image and to curate automatic detections if desired. The next step is an automatic cell detector that can be trained from manual annotations, which is performed by active contour implementation that produces cell segmentations and then uses several plugins for analysis of segmented cells to quantify their position. It has been used to examine morphological predictors of cell cycle stages in *C. elegans* germ line cells. (Chiang *et al.*, 2015)

CellProfiler (Kamentsky *et al.*, 2011) is designed to make modular processing pathways. It is standalone software (much the same as NMA) that has been built up using portions of other

morphometric software such as ImageJ (Schindelin *et al.*, 2012) and is often used in conjunction with it. The modular system works by allowing you to call functions from many other morphometric software systems and applying them in a single pipeline that can be called using only one piece of software. It is capable of processing 3d images and has a large variety of functions. The modular system makes setting up processing pathways easy but because the modules are so self-contained it is difficult to understand when pathways fail. It has a lot of utility but suffers from a hard to navigate UI. Although cell classifier is mentioned separately it is built around the usage of CellProfiler and functions as an additional way of acquiring morphological information. It facilitates the building of pipelines to measure sizes of portions of large quantities of images with tools like those present in ImageJ.

TANGO is comprised of two parts: an ImageJ plugin and a R package and functions based on making objects out of multichannel Z stack images. It is capable of statistical processing and many different measurements of 3D objects via many layers of combined modules. Parts of the program are no longer supported such as the imageware plugin. It is designed to study nuclear structure (Ollion *et al.*, 2013).

2.4.1.3 Elliptical Fourier Analysis

Elliptical Fourier analysis is a form of analysing complex shapes based on the concepts of Fourier transforms and Fourier Series. A Fourier transform is built up from the usage of a series of layered sine waves from a Fourier series to build up a shape. This is useful as it can describe a complex shape in a series of comparable coefficients, but it is very abstract conceptually which makes interpretation of data more difficult. Three morphometric analysis programs that have been used are SHAPE made by Iwata and Ukai, (2002), LOCO-EFA made by Sánchez-Corrales *et al.*, (2018) and DiaOutline made by Wishkerman and Hamilton (2018). SHAPE converts an object into its contours via edge detection and calculates area. This is then converted to a Fourier series and a discrete Fourier transformation.

The coefficients of the Fourier transform are then normalised (to stop them changing with size, rotation, and starting point) then an analysis is done to reduce the number of coefficients. These Fourier coefficients are stored as a matrix and can be used for further analysis, such as quantitative genetic analysis by comparing them to genetic information to try and associate genetic and morphological features. LOCO-EFA is a very interesting software as it combats one of the main downfalls of Fourier analysis which is the issues with losing data with complex cell shapes. This is solved by a system of identifying separate lobes of the cells (a lump or protrusion that cannot be fully characterised with elliptical Fourier analysis) and combines that with the elliptical Fourier data so that it can compare the lobes to prevent data loss. This was not designed with real cells to make it easier to develop. DiaOutline is made up of an outline detector made in MATLAB and an R package that performs elliptical Fourier analysis. It has been used to distinguish diatom taxa using their full cell outline. It is designed and tested around using light microscope images.

2.4.1.4 Analysis focused on Human sperm.

There are also functioning systems made for automated analysis of human semen. Agarwal *et al.* (2019) and Ilhan *et al.* (2020) have both produced similar automated systems for analysing human semen, however neither do complex morphometric analysis the focus is mainly on length and width measurements. Ilhan *et al.* made a hybrid system and can classify regions of interest as either sperm, abnormal sperm, or non-sperm. The program works on sperm morphology (whole sperm not just nucleus) and so is distinctly different from other programs however these programs have potential to be combined with nuclear morphometrics to increase the data the programs can use. The program has two steps segmentation and classification and acquires images by videoing them. The backbone of the identification and classification of sperm is done by neural nets (trained machine learning systems that follow complex decision trees to decide outcomes from inputs) and the highest accuracy achieved was 83.8% (of identifying abnormal sperm against manual identification). Agarwal *et al.* made a system that uses optical microscopes and artificial intelligence to produce a fully automated unlike the current conventional systems that are semi-automated. The aim of the

system is to overcome the shortcomings of the current system (manual analysis and identification of sperm via microscopy to identify and count abnormal sperm) which is: labour-intensive, subjective, expensive and laboratory based. They found their system is comparable to manual analysis of semen fertility parameters for finding concentration of sperm however it was significantly less effective at measuring motility. When finding data from the manual analysis for comparison they did find the issue of a lack of standardisation due to each hospital having their own procedures and criteria for sperm morphological analysis. This issue could be seen as another call for more complex morphometrics to be applied to this system and the system of semen analysis in general as it could be used to standardise criteria used in morphological analysis using unbiased systems. They also show a distinct advantage of their system which is that it is home-based meaning there is no social stigma or embarrassment for individuals using it.

2.4.1.5 Commercially available systems

There are many different types of software for nuclear morphometric analysis such as the Proiser Computer Aided Sperm Analysis system ('Proiser Computer Aided Sperm Analysis (CASA) Systems – ISAS and Sundries | Cryolab Limited', 2017) has been used for some time now and is relatively well established in the field as a commercially available system. This functions as 2D systems for the study of human sperm and although being commercially viable currently lack a lot of the information that NMA can potentially provide (such as full breakdown of the subtle morphological differences found with parameters such as angle profiles).

2.4.1.6 Nuclear Morphology Analysis (NMA)

The program I will be using Nuclear Morphology Analysis shown in Skinner *et al.* (2019) that solves a series of problems found by other programs in the literature by building on many of the functions of ImageJ (Schindelin *et al.*, 2012) to perform accurate unbiased nuclear morphometric analysis.

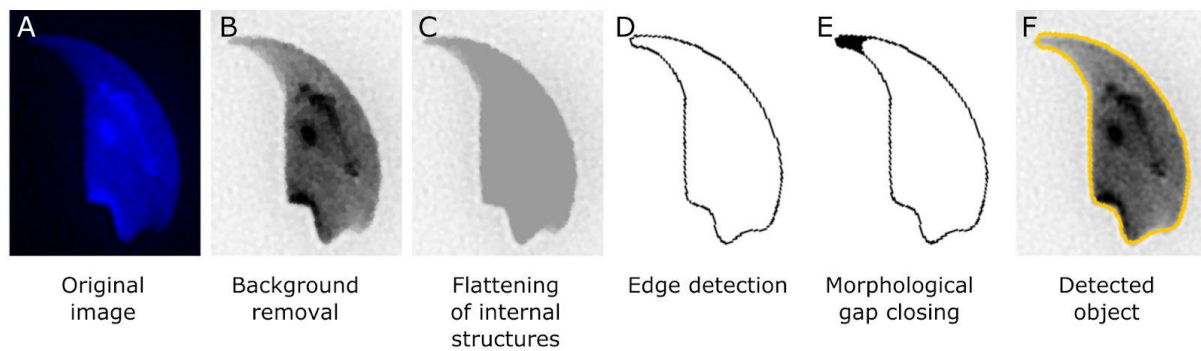


Figure 13 The process of detecting nuclei using nuclear morphology analysis.

A) Input; B) Background removal; C) Chromocenter flattening; D) Edge detection; E) Gap closing; F) detected object. Images B-F have been inverted for clarity. (from the supplementary figures of Skinner et al., 2019)

Figure 13 shows the system of object detection schematically between the various steps of the edge detection of NMA. Figure 13 A is the base image, normally a DAPI-stained nucleus. Figure 13 B shows the background removal by using only a single colour channel of the fluorescent image. The first filter used is called the Kuwahara filter (Kuwahara *et al.*, 1976), which functions by smoothing lines using a segment-based system of homogenising colours based on taking the average colour of the segment with the closest average to the original value of the pixel. This happens between Figure 13 B-C. Chromocenter Flattening (Figure 13 C) is also used to make the spermatozoa more uniform in pixel intensity, as the methods used to create the original images do not give the spermatozoa a uniform pixel intensity (this is important as it reduces the amount of edge detection errors). Chromocenter Flattening filters work by taking the one-dimensional slices of the image and taking a threshold at the first intensity raise within a maximum and minimum threshold and the last intensity drop within that same threshold, then homogenising the intensity between these points, which results in the spermatozoa being uniform intensity, allowing for much better edge detection. The program used for edge detection is the Canny edge (Figure 13 D) detection system, which detects gradients of pixels around each and uses these to find the edge. This edge detection sometimes has gaps in the edge; these gaps are filled by expanding the size of the lines in all directions, causing them to merge, then reducing the diameter of the lines to a single pixel. This process of expanding and eroding the lines fills most of the gaps in the lines, allowing for a well-defined edge (Figure 13 E). The program

functions almost entirely from readily available plugins for ImageJ, the part of it that is more difficult to replicate is the inbuilt analysis software which takes the array of X and Y coordinates from the region of interest of the spermatozoa found by ImageJ then taking every fifth pixel (by default) then calculates the angles between those points and uses them to make an angle profile of the sperm. The Angle profile is used to align the sperm outlines which are then interpolated into a set number of indices (100 as standard). These 100 indexes are then used to derive the diameter and radius profiles that are measurements of the radius and diameter through the centre of mass (Figure 14). The outlines are aligned using the features of the angle profile with all other profiles with flipping to get the best match around the profiles. These angle profiles represent the morphology of the sperm and can be used to find subtle differences between the sperm. One of the methods for this is using the consensus image function which shows an outline made from averaged outlines to give a representation of sperm from a specific sample or sections of one sample with different morphology. Another form of analysis done with this software is described in (Skinner *et al.*, 2019) known as nuclear cartography in which chromosome positions are recorded using FISH signals in combination with nuclear morphometry.

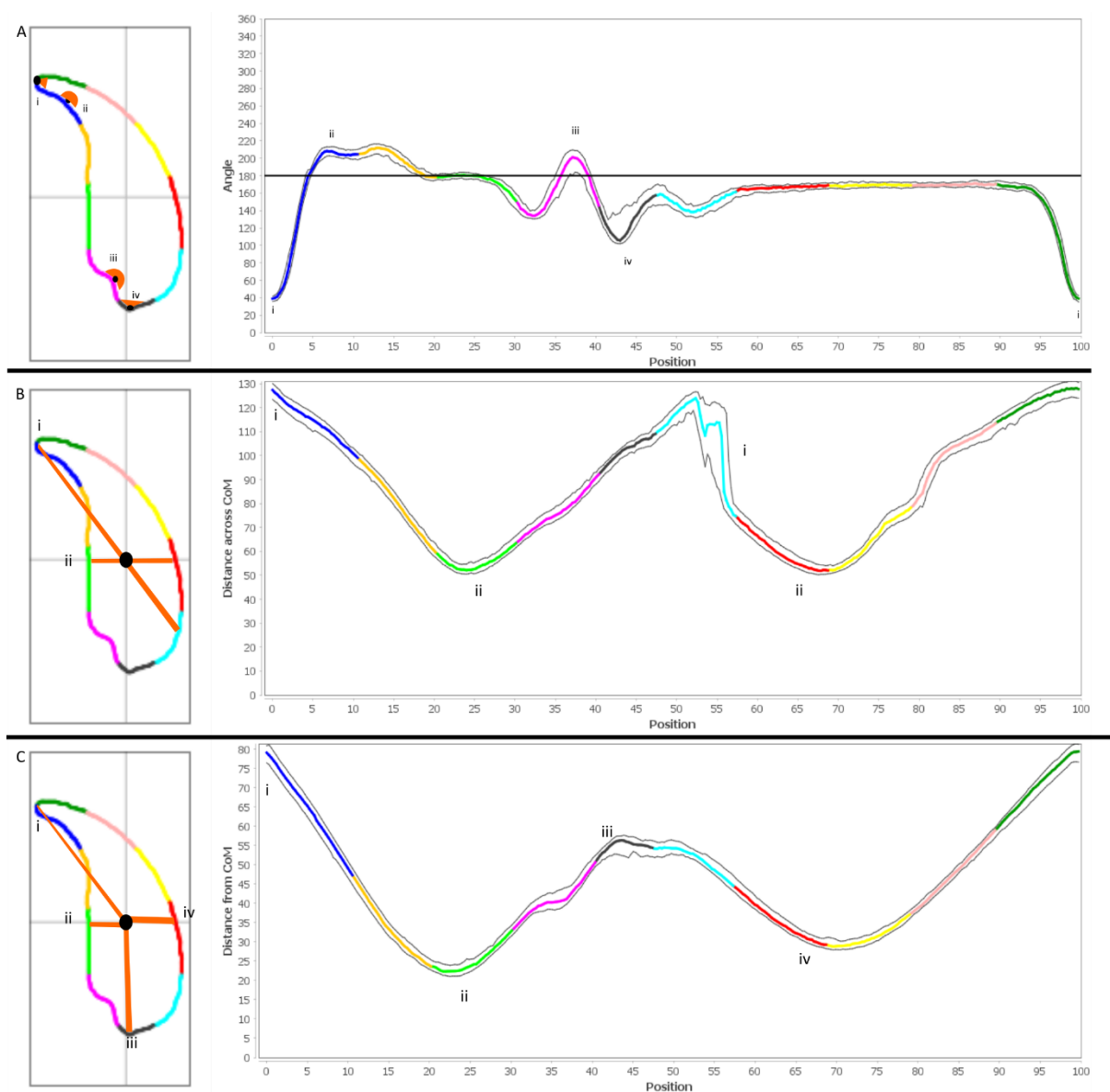


Figure 14 NMA Angle, Diameter and Radius profile diagram.

A) Shows a nucleus and profile with labelled internal angles and how they correspond to the features of the cells in the graph of measured interior angles at each index. i is the tip of the hook. ii is the middle of the bend of the hook. iii is the centre of tail attachment region. iv is the bottom of the nucleus.

B) Shows a nucleus and profile with labelled diameters and how they correspond to the features of the cells in the graph of measured diameters at each index (diameters are in arbitrary units). Each diameter corresponds to two different points on the nucleus, but those points are not necessarily direct mirrors due to the asymmetry of the cell. i shows the diameter from the tip which is different from which side cell the diameter is taken from as it is measured from the index across the centre of mass to the first edge of the cell across the centre of mass resulting in the asymmetrical profile. ii shows the minimum diameter which is useful as it helps determine the orientation of the cell.

C) Shows a nucleus and profile with labelled radii and how they correspond to the features of the cells in the graph of measured radii at each index (radii are in arbitrary units). i shows the tip of hook radius.

2.5 THESIS RATIONAL

From the literature it is clear there is potential for large improvements in the field of nuclear morphology. The ability to categorise in detail the morphological differences between two different types of sperm showing distinct regions of morphology that have different numerically categorised features. This will allow for firstly the detection and categorisation of phenotypes of sperm that correlate with medical conditions such as infertility phenotypes (Sharma *et al.*, 2020) then the ability to create a clinical test to detect those conditions automatically. One of these improvements would be a more widespread usage of nuclear morphometrics. One thing preventing widespread usage of nuclear morphometrics are the imaging training and time requirement. The streamlining of the processing pathway with the usage of automated image acquisition would increase the viability of using nuclear morphometrics for purposes such as categorisation of phenotypes within a sperm sample from single individuals. Another thing that would make the application of nuclear morphometrics more viable is increasing the quantity of images acquired as that gives more support to morphometric data and allow for the detection of rarer morphological variations in samples which is especially relevant for studying the sperm of animals with large morphological variation in their sperm. These rare morphological variants are not currently studied in depth and so little is known on what they are and where they come from biologically and so there is potentially much to be found with associating them to known sources of abnormal morphological features. The analysis will also have to be streamlined to keep up with these larger volumes of data.

2.6 AIMS

Specific Aim 1: To create an unbiased (not favouring imaging any cells over other cells) automated imaging pipeline that can determine the field for capture, focus, and acquire images without

supervision

Specific Aim 2: To assess the quality of automatically captured images against manually captured images, thereby ensuring the method is suitable for morphometric analysis pipelines

Specific Aim 3: To use previously manually imaged mouse sperm samples to develop and refine data analysis methods for identifying different morphologies in data

Specific Aim 4: To use the new imaging pipeline to perform biological comparisons of hamster and mouse sperm to characterise the development of the rodent sperm head shape across 30 million years of divergent evolution.

3 MATERIALS AND METHODS

There are two major sections of methods developed and refined in this project: image acquisition and image analysis. Section 3.2 is relevant to the methods used in chapters 4.1 and 4.2 and section 3.3 is relevant to the methods used in chapters 4.3 and 4.4.

3.1 SPERM SAMPLE ACQUISITION

The sperm samples were harvested and prepared in the same manner as described in Skinner *et al.* (2019) and Kopania *et al.* (2022). Briefly this entails sacrificing the rodents and dissecting out the testes and vas deferens (Figure 6 shows where this is), the sperm is then flushed from them, and

pieces of tissue are centrifuged out (as waste), then they are washed with PBS then the fixative (either 3:1 methanol:acetic acid or 2% PFA in PBS) is added filling 1ml microcentrifuge tubes of sample for storage in a -22°C.

3.1.1.1 Rodent strains

The rodent itself is relevant to the sperm it produces and helps give added context to some of the features of the sperm for example older lab strains that had been inbred for a longer time have more morphologically distinct sperm (they tend to build up abnormal traits due to the founder effect causing things like hook malformation or reduced size). Some of the mouse strains we have samples or images from are listed here and shown in Figure 15 such as Lewes and PWK as well as BALB/c.

PWK – A wild strain of *Mus musculus musculus* mouse trapped in Lhotka, Czech Republic in 1974 the founders were selected for their ability to produce sterile sons when mated with C57BL/10 mice (Jackson Laboratory strain id 003715). This mouse type has been studied due to its interesting crosses in Shao, Reed and Tordoff, (2007) which is also why it has previously been used in this lab and in Kopania *et al.*(2022). These crosses tend to produce sperm that is considerably different morphologically than both parent species such as the PPLL cross we use.

WSB – A mouse strain derived from wild mice trapped in Centerville, Maryland in 1976 that were selected for their dark agouti coat and white head blaze. (Jackson Laboratory strain id 001145). These mice were used in Kopania *et al.* (2022) to produce some of the crosses such as the WWLL cross we use.

LEWES – A wild derived strain of pure (have not been crossbred with *Mus musculus musculus*) *Mus musculus domesticus* that were trapped in Lewes, Delaware in 1996. (Jackson Laboratory strain id

002798) These mice were also used in Kopania *et al.* (2022) to produce some of the crosses such as the WWLL and PPLL crosses we use.

Balb/C – A laboratory bred balb (an old albino strain of mouse) derived inbred sub-strain of mice that are albino and are known for their production of plasmacytoma (used for cancer research) after mineral oil injection (Jackson Laboratory strain id 000651). They have very obscure morphology that cannot fully be shown in the Figure 15 consensus images as it has many different variants that have their own distinct morphology which can be seen by the high variation.

C57- The most widely used laboratory bred strain of mice that has many sub-strains due to its large amount of use it also is very well researched. A reason for their usage is that they breed well, have low susceptibility to tumours and live relatively long. (Jackson Laboratory strain id 000664)

FBXO7 Knockout C57– Although this is a knockout mutant and not a strain on its own it is useful to treat it as one for the purposes of this study as it gives sperm with a general shape but variation a low frequency phenotype with distinctly different morphology. It has been used previously in Rathje *et al.* (2019) in which it was found to have highly variable morphology.

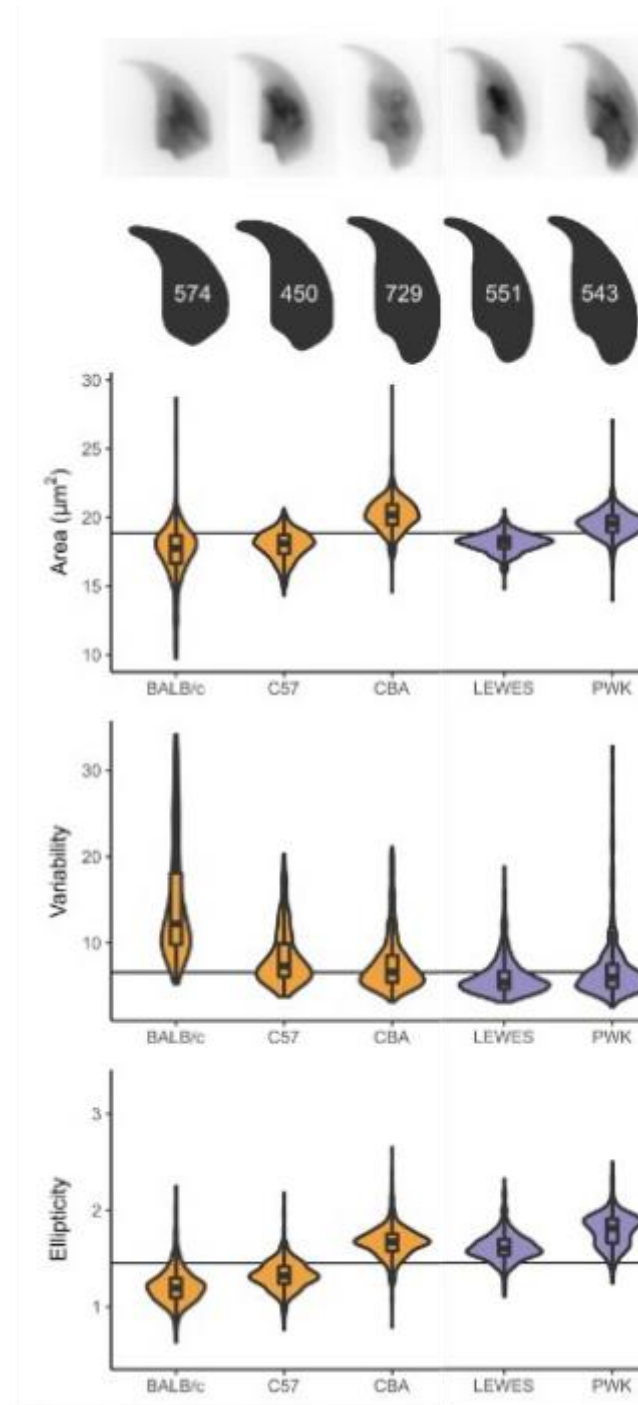


Figure 15 Morphology and morphometrics of mouse sperm from different strains.

The subtle differences between the different mouse strain sperm morphology are made clearer by the morphometric information (Skinner et al., 2019). This graph shows three different metrics from NMA with an example image and a consensus with the number of cells studied in the experiment on them. Yellow is domesticus strains blue is musculus.

Phodopus campbelli or the Cambell's dwarf hamster is a common pet hamster that is native to the deserts of mainland northeast Asia. These are closely related to mice (diverged 24.2 – 33.0 million years ago) and very closely related to *Phodopus sungorus* (diverged between 0.1 and 1.7 million years ago (Kumar et al., 2022)).

Phodopus sungorus or the winter white dwarf hamster is a common pet hamster that is found in the Kazakh steppes. These are closely related to mice (diverged 24.2 – 33.0 million years ago) and very closely related to *Phodopus campbelli* (diverged between 0.1 and 1.7 million years ago (Kumar et al., 2022)).

Table 1 Samples imaged in this thesis.

Samples imaged	Common Name	Characteristics	Species
PWK X Lewes (PPLL)	house mouse	Cross	<i>Mus musculus</i>
Fbxo7	house mouse	Mutant	<i>Mus musculus</i>
<i>Phodopus Sungorus</i>	winter white dwarf hamster	Wild Type	<i>Phodopus Sungorus</i>
<i>Phodopus Campbelli</i>	Campbell's dwarf hamster	Wild Type	<i>Phodopus Campbelli</i>
WSB X Lewes (WWLL)	house mouse	Cross	<i>Mus musculus</i>

3.2 EPIFLUORESCENCE IMAGING

All images were taken using the Nikon eclipse Ti-E inverted microscope using two separate filters the DAPI and the DIC filters. The images were taken using 90X magnification from a the same 60X objective lens (numerical aperture 1.49) and the internal microscope 1.5X setting (Tube lens) without over or under sampling. The camera used was the Andor Luca-R DL-626 with the pE300 system for controlling light intensity for fluorescence imaging. The images were taken with a resolution of 11.1 pixels per micron and scaled to 22.2 pixels per micron however those pixels were scaled for ease of analysis with the software (NMA works better at that scale).

3.2.1 Manual imaging method

The manual method is the first one we used for taking fluorescence images of the sperm samples and is by far the slowest. This method entailed manually finding and focusing each field of images used taken. This method is relatively skill based and time consuming as it requires understanding how to properly focus and select good fields of cells. This process is also quite mentally and physically taxing as it often requires long periods of time in a room with very little light and focusing the microscope can cause eye strain requiring frequent breaks. This method can be done with either PFA or 3:1 methanol: acetic acid and the distribution of cells on the slide is irrelevant to this imaging method. The fields are selected

based on having the most cells on the same focal plane that are of sufficient intensity. This method is performed by manually moving the slide using the x and y manipulation of the stage to take individual pictures of cells that are manually focused.

3.2.2 Semi-automated imaging method

This method requires the use of the large area scan function on an automated stage microscope with manual focusing but automatic field of view movement. This method removes the need to select good fields as it quickly moves from field to field without overlaps (preventing reimaging the same cells) and allows imaging at a much higher speed as all it requires is adjusting the focus with the change in focal plane of the cells. This method does not function well with PFA as it is difficult to manually focus with the field movement as automatic field change is faster than the exposure and so is jarring and hard to follow. The bias in field selection is removed with automatic field changes however this means all cells are imaged causing an increase in artifacts due to fields with many sources of them being included when they wouldn't be by a person.

3.2.3 Fully automated imaging method

This process streamlines imaging to simply selecting an area with at least one cell per field and then setting the area to image with automatic focus using the large area scan function then waiting for the imaging to complete as can be seen in Figure 16. This takes considerably less training and human time than the other methods and allows for higher volumes of data and so is the one standardised on for image acquisition. This method has the same advantages as semi-automated for field selection and an even greater increase in artifacts as it will sometimes image many cells slightly out of focus causing more issues.

More than just microscopy techniques were required for image acquisition, a series of methods were also needed for sample preparation as well as slide preparation. The samples were in a series of different physical conditions (different concentrations and some had leaching DNA from oxidative

damage), and a standard preparation was needed for the imaging method. 3:1 methanol acetic acid was decided on for the fixative, as the way it evaporates adheres the sperm to the slide evenly reduces the spread of the cells across the Z axis. The flat adhering of the cells reduces the number of different focal planes the cells will be spread over for imaging and therefore reduces artifacts.

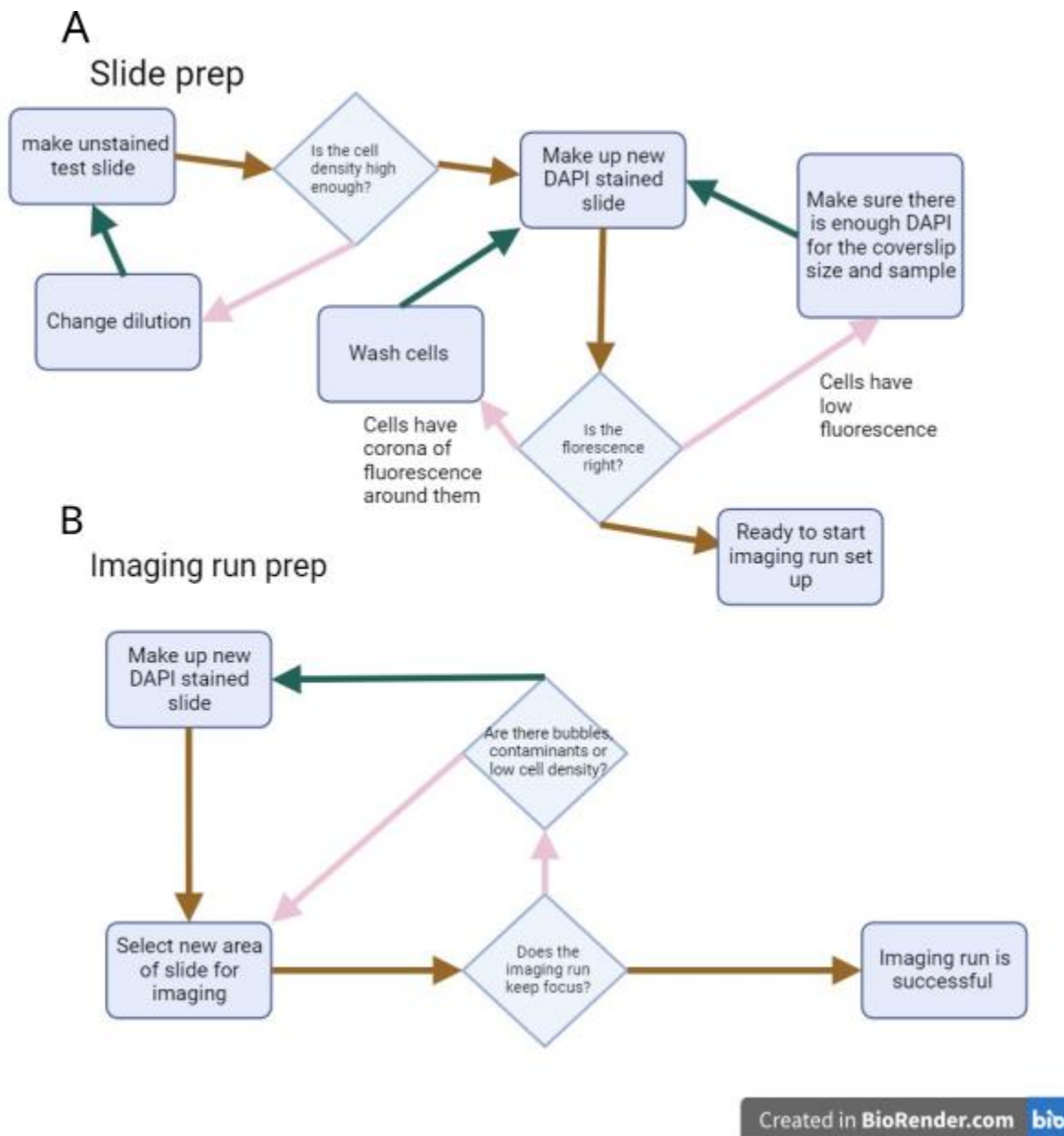
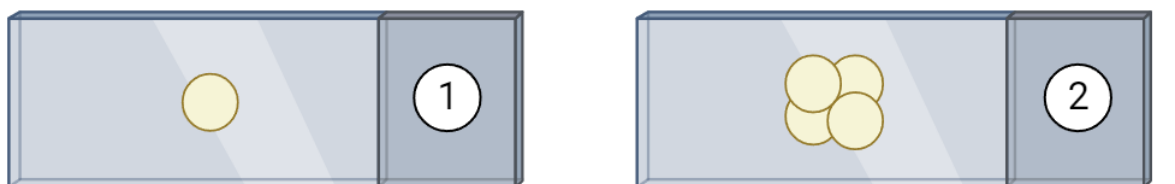


Figure 16 shows a simplified outline of the methodology for running an automated imaging run.

The brown arrows show the simplest route of imaging run set up, green arrows show yes/progress and the pink represent no/issues that can be solved. A) shows the troubleshooting process to make a cell that is ready for analysis. B) shows the process of setting up the imaging on the microscope.

The method of slide preparation was standardized based on the usage of 4µl droplets of sperm sample(as a single unit dropped represented by the circles in Figure 17)and a variety of patterns were tried resulting in a system where a slide is made up depending on the amount of sample on the slide that needs to be imaged. Form every slide imaged a series of preliminary slides were prepared following the procedure in Figure 16 to ensure all the parameters met. If only 2000-5000 individual images were desired to be imaged, then only a single droplet of sperm is used (as shown in fig 17 section 1) in combination with 16 µl of DAPI (VECTASHIELD® Antifade Mounting Medium with DAPI)and a 22x22mm coverslip (edges sealed with nail polish) this style of slide tends to take 12-18 hours to fully image all cells on the slides that the method can capture. If more than 5000 cells were imaged additional droplets were added in a grid pattern (as shown in fig 17 section 2) using a 50x22 coverslip with imaging time scaling linearly. 30µl of DAPI is used with the 50x22 coverslip as when incubating on the slide the DAPI (with vectashield) was scaled to the coverslip not the sample volume. The grid pattern overlaps to ensure that there are no large empty areas of the slide that will make imaging difficult.



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Figure 17 Examples of the dropping patterns used for samples.

3.3 ANALYSIS

The bulk of the analysis of this project has been within NMA and started in NMA version 1.180 and due to NMA being in development simultaneously with this project the version has updated to 2.1.0, improving the program with the advancements made in the project in mind. The R packages used to

build NMA companion were: UMAP 0.2.10.0 (McInnes, Healy and Melville, 2020), factoextra 1.0.7 (Kassambara and Mundt, 2020), ggplot2 3.4.3 (Wickham, 2016), shiny 1.7.5 (Chang *et al.*, 2023), stringr 1.5.0 (Wickham and RStudio, 2022), dplyr 1.1.3 (Wickham, François, *et al.*, 2023), tidyr 1.3.0 (Wickham, Vaughan, *et al.*, 2023), fossil 0.4.0 (Vavrek, 2020) and diptest 0.76-0 (Ringach and NYU.edu), 2021).

3.3.1 Increased data volumes from imaging

The massively increased data volumes (between 10-40 times more nuclei imaged per session depending on the session) have required some changes in the way the data is handled as the increase has resulted in reaching the limits of what NMA can work on with the resources of a standard commercially available computer. This increase resource requirement has opened new challenges with the usage of the data and pushed the processing towards the edge of having to use the University of Essex computing cluster. The reason high power computing (such as the University of Essex computing cluster) was not used for this project was the focus on trying to make a pipeline that can be used widely with low cost and resource requirements. This huge increase has also changed the objectives of some parts of the analysis as there is now the data to find not only the common phenotypes of sperm but also try and find the rare phenotypes of sperm it also will allow us to process more variable sperm and get enough data for informative and biologically relevant clusters. This required the additional use of R with NMA to perform analysis of the data.

3.3.2 Dimensionality reduction for visualisation of morphological relationships of sperm

The data acquired using NMA is highly multidimensional (it has 300 profile indexes at the default setting for every cell meaning a dataset with 5000 nuclei has 1.5 million datapoints) and therefore difficult to visualise in a way that can be easily interpreted by a human.

Reducing this data down to two dimensions produces graphs that are much easier to visualise the relationships between the morphological data. There are two main methods for

this used are (t-distributed Stochastic Neighbor Embedding) (Donaldson, 2022) and UMAP(uniform manifold approximation projection) (McInnes, Healy and Melville, 2020)and the method settled on was UMAP. The reason for using UMAP is that it has the advantage of being accessible by a singular function in R and performing it is as simple as inputting a dataset.

3.3.3 Clustering for characterisation of sperm morphology

This is done to separate out potentially interesting morphological phenotypes in the samples. There are multiple different methods that were explored for clustering; K-means, Hierarchical and a hybrid of the two. The hierarchical clustering was done for preliminary work with built in NMA functions and the NMA companion uses the `hkclustering` function in the `factoextra` package for R(Kassambara and Mundt, 2020). The R based clustering is considerably faster than the NMA clustering.

3.3.4 NMA companion

This currently entails combining clustering and UMAP to show of regions with different phenotypes or using R based Hartigan's dip (a test for unimodality) testing systems to separate out interesting regions of angle profiles and clustering on them which gives very clearly defined clusters that have improved detection of morphological features. It also contains the start of a process for further refining these clustering of interesting features however this lacks the implementation to be useful.

The analysis is started with simply running the images through NMA and then depending on the variety of phenotypes visible there are different analytical processes that can be done. The next step entails either performing clustering in NMA if the data set is small (2000-5000) or exporting the cell stats from NMA (this is a tab separated file that contains a data frame of all the cells and the values measured from them from NMA) and performing additional data analysis in R. If the dataset is large, (over 30000 nuclei) then clustering may have to be done on a computing cluster as the volume of

cells and features may be too much to compute on a commercially available computer. There was also the potential of using a K-means fuzzy clusterer like fclust (Ferraro, Giordani and Serafini, 2019) which would be for samples with massive amounts variation and so much larger quantities of nuclei would be needed for categorisation. A large portion of the analysis was done with NMA companion. The main design philosophy of which is based around having a large central function that processes the multimodal regions (sections of the nuclear profiles such as angle or diameter that are not unimodal representing an area of the nucleus with distinct features) and outputs a shiny interface of the data that can be read and breaks down the morphological phenotypes found in the data.

4 RESULTS

4.1 AN EFFICIENT AUTOMATED IMAGING PIPELINE

The building of the automated imaging pipeline is based around the Nikon eclipse Ti inverted microscope. The preliminary work was done simply experimenting with what can be done with the proprietary software NIS elements (installed on the Nikon microscope used). This entailed working with some of the previous techniques such as taking individual images and multipoint images and once a basic understanding of those is acquired the natural conclusion is to use the large area scan function which is based around the ability to select a region that the microscope will break down into individual fields of view then sequentially image them. As this now gives us a way to automatically move the slide however does not give us usable images. This loss of focus due to the slide not being perfectly flat lead to the challenges faced turning this system into a useable part the pipeline. This was achieved with a balancing game between slide preparation and software usage to solve the issue of the images being taken in the large area scan not being usable.

Nis elements (the software installed the university microscopes) has an inbuilt autofocus system however the system is based on contrast detection focusing and so cannot image areas of samples

with a large spread of focal planes that contain nuclei. This issue is because it does not have the ability to image two nuclei at completely different Z positions that are in the same field and so it can only image one of the nuclei. This leads to the first issue that needed to be tackled, Z position variance of the samples.

4.1.1 Z position variance of the samples

The field of the sample on a microscope slide can be broken down into optical sections. To get an image the microscope must be focused on the focal planes that encompass the optical section of the sample that is wanted for the image. Only one focal plane can be captured per image and so the cells not on that plane will come up blurry. Figure 18 shows what the effects of changing the fixative do to the position of the nuclei in Z space with methanol acetic acid flattening the cells to the surface of the slide as it evaporates making them more consistent.

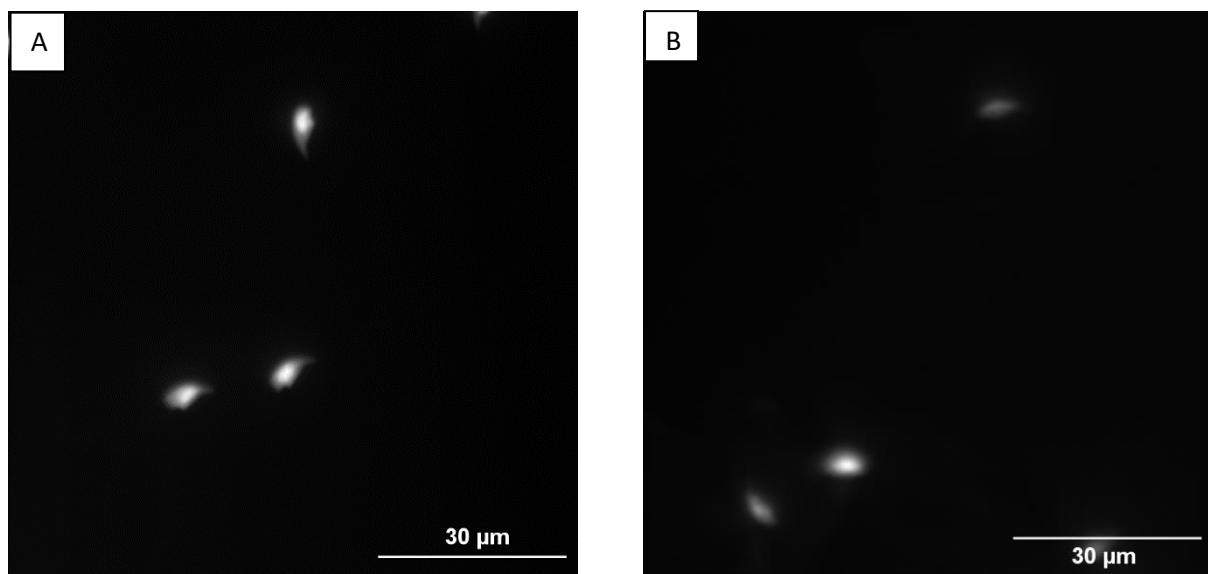


Figure 18 shows two fluorescence images with 3:1 Methanol acetic acid on the left and 2% PFA on the right.

This shows the differences between the fixatives. Methanol acetic acid evaporates resulting in cells lying flatter in relatively consistent orientation across the Z axis making it easier to focus on them because of them being at the same Z level.

Cells on different focal planes look like in the 2% PFA sample. Figure 19 also illustrates the differences between the two fixatives that are used as standard for sperm storage. Since the two fixatives are used as standard samples stored in both fixatives were available. In the standard

method used in Skinner lab in previous papers (Skinner *et al.*, 2019; Skinner *et al.*, 2019; Kopania *et al.*, 2022; Skinner, 2022) the fixative used is PFA and so preliminary experiments were performed with 2% PFA stored samples the move was made to 3:1 methanol: acetic acid as it has a lower variance of the focal planes of the samples allowing all the cells on single fields to be in focus. The focal plane variance is also a crucial factor in the function of the auto focus system as it has a limited range of focal planes it can scan through from field to field and so if it misses the focal planes, it can lose the focus on the samples and due to its simplicity, it cannot find them again. One solution for this would be the writing of a macro for the microscope to perform the same thing as the large area scan however the NIS elements macro system is relatively abstruse and has a high potential to damage the microscope so without access to NIS elements training it was not worth risking the microscope which leaves the solution down to sample preparation. The usage of 3:1 methanol: acetic acid solves this as it brings down that variation between where the cells are situated in the Z axis from $>50\mu\text{m}$ down to $<10\mu\text{m}$ allowing for automated imaging. This however has not completely

solved the problem of losing focus as although the microscope can reach the correct focal plane it may now face another issue, that of concentration.

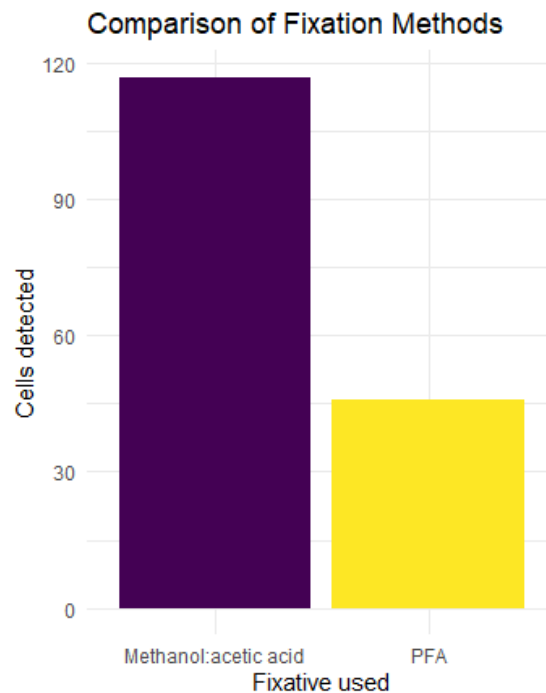


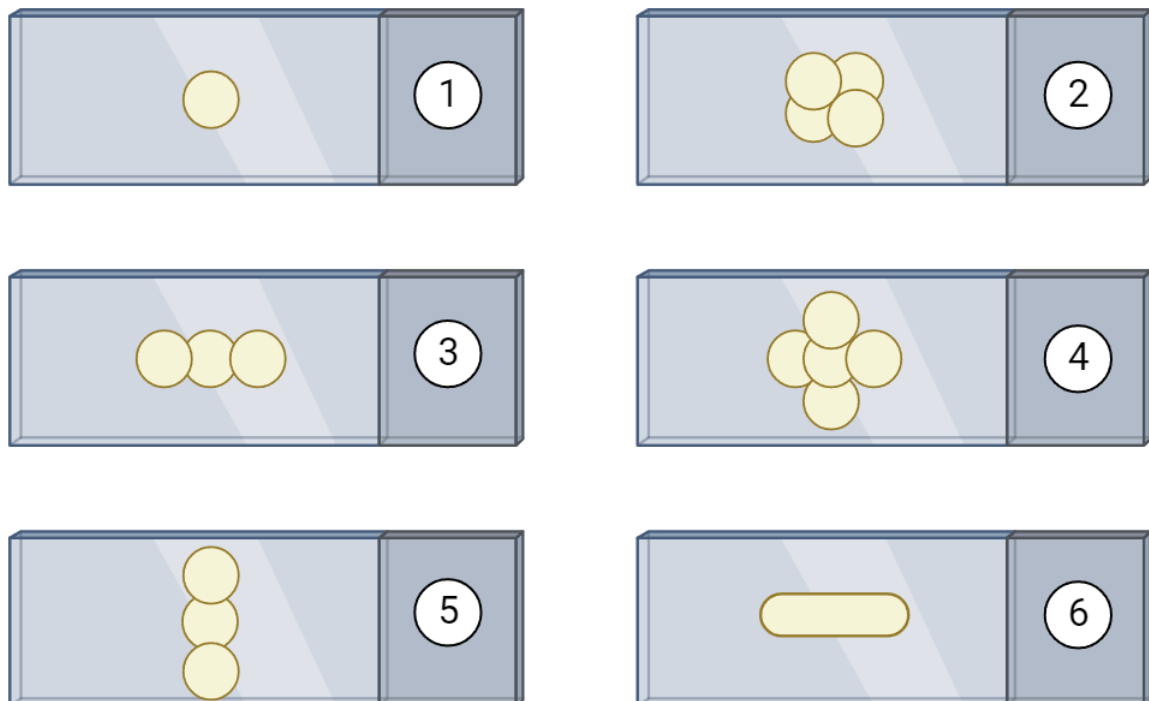
Figure 19 comparison of fixative type to cells detected over an imaging session with NMA over the same number of images with similar cell densities.

This difference is due to the lower variance of orientation and Z position with methanol:acetic acid

It is acceptable to substitute PFA for 3:1 methanol acetic acid as can be seen in the supplementary figures of Skinner *et al.*(2019) that show there is no significant difference in the profiles of cell fixed with PFA and 3:1 methanol acetic acid. This is further supported by a curated sample of 10 sperm from two mice strains with sperm with comparable features curated to find well orientated samples in the PFA that are directly comparable as shown in supplementary figures of Skinner *et al.*(2019). A 6 μ m difference in area between the PFA and 3:1 methanol acetic acid curated sample was found. There was no significant difference found between any of the profiles either when checked using Wilcox rank sum tests.

4.1.2 Concentration of cells over the slide

The concentration of sperm cells across the slide is important for imaging as the autofocus system focuses onto whichever single plane of focus detected that meets the requirements of the autofocus. This results in focus being lost when no cells in the field being imaged resulting in it losing the focal plane of the sample. The solution to this was based around two things; the manner the sperm was pipetted onto the sample and the concentration of cells in the sample. The patterns used to drop slides is shown on figure 20 and they have different strengths and weaknesses. The main point of the dropping pattern is to give the largest square region of cells with an even concentration using the least sample. Patterns 1 and 2 shown in Figures 17 and 20 were standardised on as they were the found to give the most consistent large region of imageable cells. The concentration of the cells was determined using a tester slide, with graticules on and simple estimation as the range of cells per field needed for imaging is very large, it can be anywhere from 1-20 nuclei present. The patterns had very little effect on coverslip or how the fixative evaporates, the main advantages of 1 and 2 over other tested patters were that they resulted in no areas with low cell concentration.



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Figure 20: sample dropping patterns used for sperm imaging.

This was done to determine which was more effective and 1 and 2 were found most effective for preventing loss of focus during automated imaging.

4.1.3 Fluorescence intensity of cells

With a larger coverslip a larger amount of DAPI was needed, to keep the concentration of DAPI consistent for consistent fluorescence of the cells. Fluorescence was also an issue in older samples: as the cells age, damaged fragments of DNA can surround the cell. This extracellular DNA causes a corona or halo of low fluorescence around the cells that is of indistinguishable intensity of fluorescence to regions of the nuclei with low DNA concentration (e.g., the tip of the hook seen

Figure 21). This extracellular DNA was relatively easily removed by washing samples in methanol: acetic acid (MeAc) before making slides.

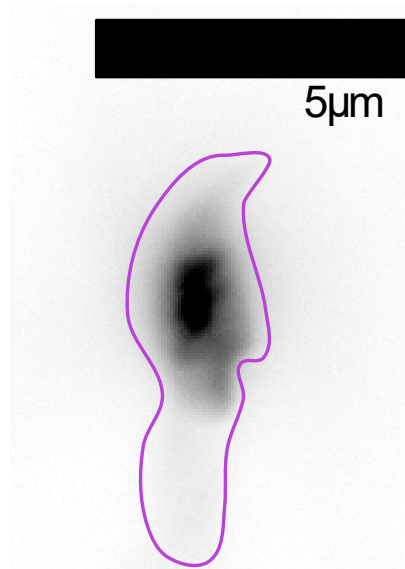


Figure 21 an example mouse sperm cell with extranuclear DNA leakage.

This results in a very faint DNA leakage similar in intensity to the hook. Contrast is increased to make both hook and extracellular DNA visible. Area where intensity is like a real cell highlighted in purple.

4.2 ASSESSING THE QUALITY OF AUTOMATICALLY CAPTURED IMAGES AGAINST MANUALLY CAPTURED IMAGES

The first point of interest was the comparing how similar the data acquired from images acquired manually and automatically. The first major difference is that our automated method images all cells in a region of the slide instead of manually selected fields of cells. To ensure cell imaging quality was comparable to manual imaging, a semi-automated method was performed using an automated stage with manual focusing. Images were analysed with NMA to compare the angle profiles produced. Figure 20 shows the comparison of the same hamster nuclei imaged from each method started at the same position on the same slide, but the automatic method has more nuclei imaged from that slide (the area to image was expanded to allow for the assessing the value of more images). There is little difference between manual and automatic focusing except from some

increased variance around the basal region where the tail attaches caused by the slight increase in artifacts. This occurs due to the focus not being as accurate and since the cells aren't curated by a human, nuclei that are orientated oddly and are difficult to focus on won't be skipped resulting in more cells with lower quality edge detection. Consensus shapes detected were almost identical between manual and automatic imaging as can be seen in figure 22 and 23).

A more variable sample than the hamster sperm of heterozygous *Fbxo7*^{+/-} mouse sperm was also compared, (Figure 22) and confirms that overall measured shapes of are similar between manual and automatic imaging. The increase in data volume also allows us to see rarer phenotypes in samples the het *Fbxo7*^{+/-} samples give a good example of this as they have a rare double hooked phenotype presented in samples from individuals. Differences are subtle and difficult to quantify in any way other than qualitatively, but the increased amount of data makes up for it. The increased data means that although there are proportionally more errors, they are more easily characterised and separated from into their own groups due to their consistency allowing for them dataset as a whole to be used. This can also be seen in the differences of angle profiles of the different imaging methods. It can be seen from these angle profiles in Figure 22 that there are some differences between the average angle profiles of the manual and automatic methods. This is likely because the automated imaging picks different focal planes to an individual for the same cells. These different focusing's results in slight differences in edge detection that cause minor differences however when using the considerably larger data sets is lost in the increased natural variation. This however still is an issue that is solved in section 4.3.

One of the main differences with automatic over manual imaging is an increase in cells slightly out of focal plane for automatically taken images causing more poor edge detection. 8.6% of the of the manual samples had bad edge detection whereas 10.5 % of the semi-automated dataset had bad edge detection and the larger dataset had 28% bad edge detection (this was determined using a simple filter that removed cells with angles that were too sharp to exist in nature which was determined to be 280° by manually filtering errors out of test groups). Despite this, figures 22,23 and 24 show that the increased bad edge detection does not affect the overall angle profiles. Since this bad edge detected data can be filtered out there is relatively little difference and the difference caused by errors is diluted considerably more in larger datasets as stated.

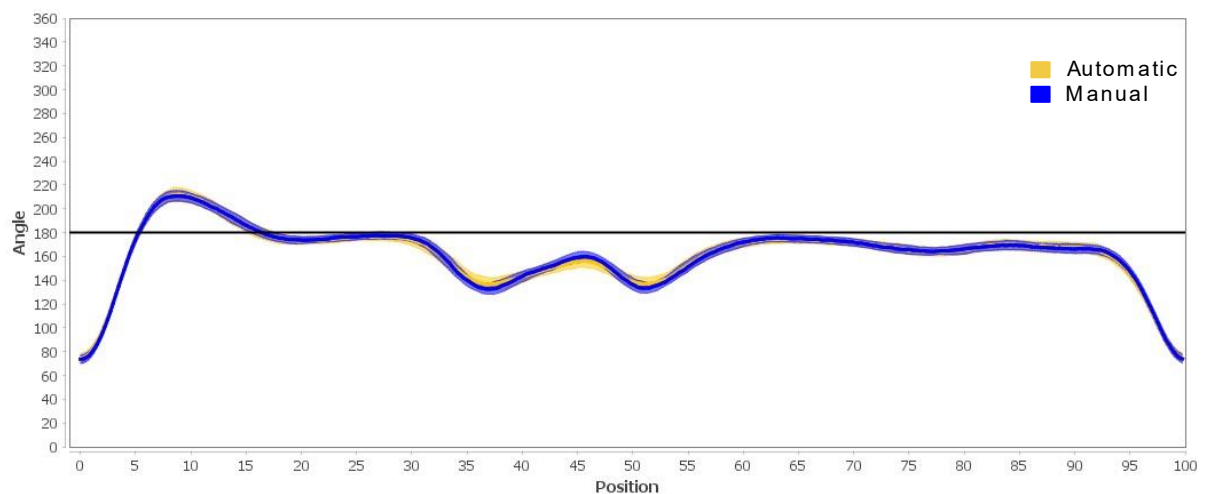


Figure 22: normalised angle profiles of two imaging runs of the same one hundred fields of hamster sperm.

Yellow is automatically acquired, and blue has had each image focused manually. This demonstrates that manual and automatic capture yield very similar results.

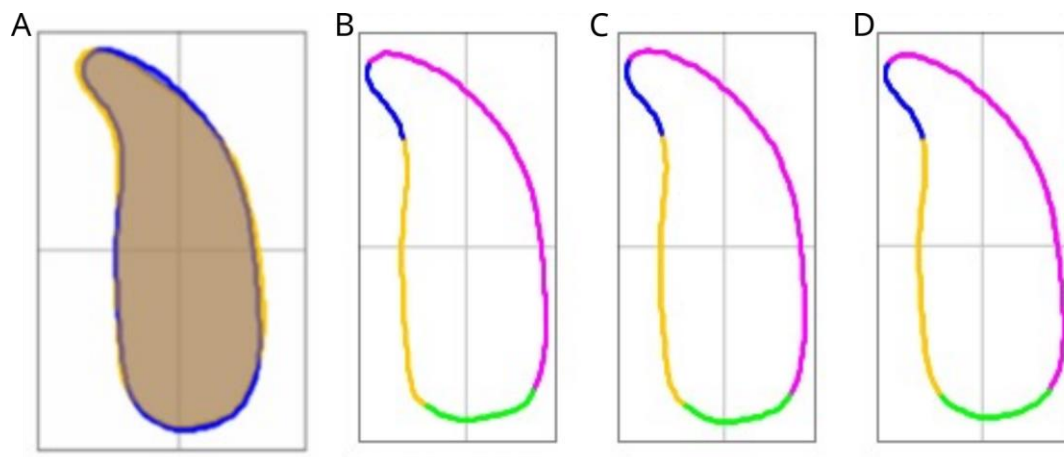


Figure 23: Consensus nuclei of *Phodopus sungorus* hamster sperm.

From left to right an overlap of the consensus (A) of 100 fields-of-view taken manually (B) and the consensus image of the same 100 fields taken automatically (C) and finally the consensus of 7180 cells taken automatically (D) (D is the full imaging run of C).

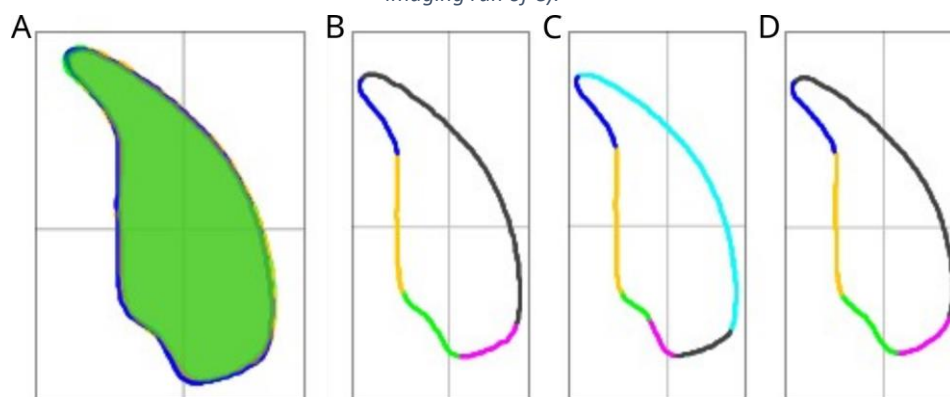


Figure 24: FBXO7 comparison between manual and automated imaging.

A; overlaid consensus images from a comparison of the same 100 fields of cells with the manual in blue (B) and the automatic in yellow (C) and the large dataset in green (D)

4.3 USING PREVIOUSLY IMAGED MOUSE SPERM SAMPLES TO DEVELOP DATA ANALYSIS METHODS FOR IDENTIFYING DIFFERENT MORPHOLOGICAL SUBTYPES

With an automated imaging method established and verified, large image datasets can be rapidly collected. This now opens new avenues of research but to do this the increase in data must be met with an increase in analytical capacity. If the switch is to be made from a low number of high-quality images to a higher number of lower quality images, then there must be a change in the analysis to

facilitate this larger number of images. This larger number of images increases data in respect to the different phenotypes within the samples. This will allow more examples of differing morphologies in variable samples to be captured in image datasets and analysis pipelines to characterise these morphologies will have to be created to take advantage of this. The characterisation of these phenotypes within the sample can then be studied in respect to the environment and genetics of the source of the sample allowing for investigations of their relationships with disease and fertility.

4.3.1 Previous methods used for nuclear morphometric analysis.

The standard method previously used was clustering by angle profiles and then UMAPs on those profiles coloured by the cluster for visualisation. These graphs give a good view based on large morphological differences over the whole sample. The development of a method that can process all the data from NMA and select multimodal regions (regions of profiles that are not unimodal suggesting at least two different phenotypes present in that region) then cluster based on them can give a better view of select differences in phenotypes within the same sample.

Manual curation

This entailed using the built-in functions of NMA to filter the sperm based on visual phenotype. This is very effective at identifying different morphological phenotypes in samples however is time consuming, draining and only effective at smaller datasets.

Simple clustering

This uses the built-in functions of NMA to perform hierarchical clustering using selected parameters from the NMA datasets produced. This allows for intuitive separation of different features that are present and consistently different within a phenotype.

Due to the dilution of morphometric differences when each index valued equally the phenotypes cannot be seen by simple clustering and are hard to characterise in manual curation due to subtle

differences. The dilution of features occurs due to the general similarity of cells in most of the measured features detected by NMA which causes the actual differences to be diluted as these differences may be on only 20 % of a profile and so 80 % of that profile dilutes it as the regions are all weighted the same. This is exacerbated by the fact that samples can have important morphological differences that aren't directly related and therefore can be present on overlapping groups of cells.

4.3.2 Targeted sub profile analysis

An R package was developed (<https://github.com/JosephCliffordPerry/NMAcompanion>) as a companion for NMA that takes exported measurements from NMA and identifies regions of interest within the sperm nucleus. The cell consensuses are built up by NMA using a system of aligning angle profiles with inversion and rotation of the profiles to align them. Figure 25 shows the indexes of an angle profile (or diameter profile) correspond to different features of the sperm outline. The aligned angle profiles give insight into the cells in general as if they are a single discrete phenotype. A shape phenotype could alter an angle profile at any position, while leaving the rest of the profile mostly unchanged. For example, a shape phenotype affecting only the hook of the sperm would change the start and end of the angle profile (as can be seen figure 25 between positions 90-1-10), but the rest of the profile would be unchanged. This causes difficulties in identifying such subtle phenotypes within datasets. Phenotypes such as this can be seen repeatedly in the example analysis (4.3.3), and it is especially evident in phenotypes like the PPLL (PWK X LEWES cross mice) in which the subtle differences between the two hook phenotypes are easily lost by variation and are only detectable in some small sections of the diameter and radius profile. (This is show in section 4.3.5).

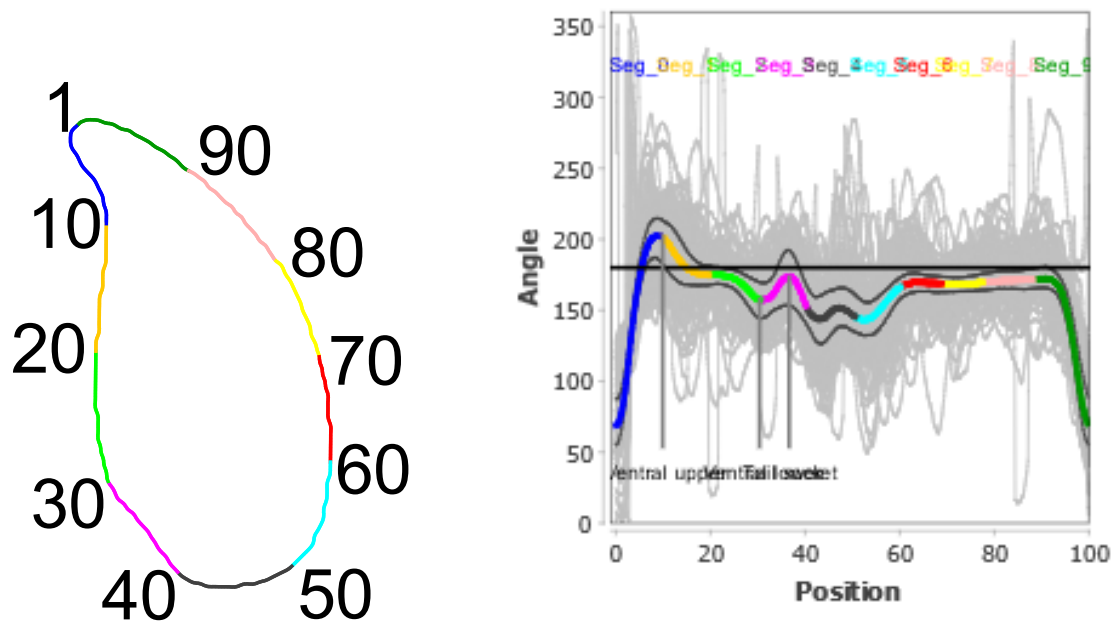


Figure 25 The various regions around the cell and the way they are distributed on an angle profile to explain what the numbered regions correlate to.

This loss of distinction between phenotypes is due to the difference being the sharpness of the tip of the nucleus which is very subtle. When the indexes of the profile are equally weighted in clustering

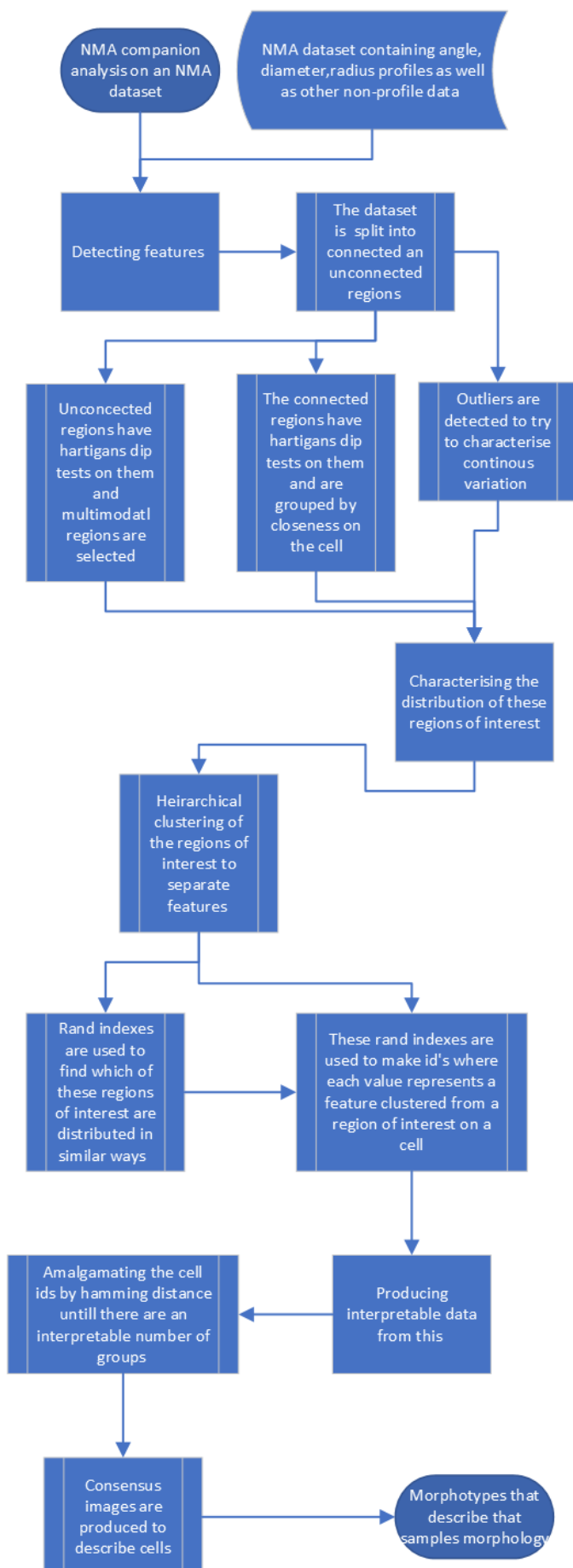


Figure 26 figure showing workflow of a full NMA companion analysis.

This workflow shows the main sections and ideas of the pipeline and the ideas within them. The first process is the detection of features of the nucleus which is done firstly by deciding which sections of the various parameters provided by NMA are connected (can be grouped into sections that represent one morphological feature). The connected regions such as the profiles are then run through an algorithm that reports which sets of indexes are detected as non-unimodal and combines adjacent indexes into a dataset for characterising. The unconnected regions go through the same process without combining the adjacent values. This is also the stage where other ways of picking sections of the data to use for clustering could be implemented such as an outlier system which is in development to try and solve issues with continuous variation.

The output of this feature detection is cluster-able numeric data-frames with a consistent unique id for each nucleus that are then hierarchically clustered to split them across the different modes detected in the earlier step.

The output of the hierarchical clustering is the cluster identity of each for each cell. These clusterings are then tested against each other to see if the grouping of cells are consistent and if they are relatively consistent it suggests that the distribution of features are generally consistent. With this information the regions of interest clusterings that agree via rand index are then combined by joining cluster identities. This produces a series of clusterings of cells by features with many different cluster identities these may be directly useful.

If the rand index based combined clusterings have more than 16 different cell types then there is generally too many clusters to be useful. This is combatted by amalgamating the identifiers by Hamming distance which joins the clusters with the most shared features until there are under 16 groups.

I am using the term morphotype to refer to any grouping separated out from this pipeline that could be of use to describe the sample.

found. To overcome this problem, we have developed a targeted analysis to identify the specific regions of a profile that show differences within a population of nuclei. Each index (an aligned measurement at one of the landmarks of the cell) of the profile is tested using Hartigan's dip test of unimodality to identify regions with a multimodal distribution. These regions are portions of the nuclei with different morphology, allowing for a factor to be assigned providing categorisation of different phenotypes.

These non-unimodal indexes are then clustered using K-means hierarchical clustering with automated calculation of K using a combination of the Silhouette method(compares the point distances within clusters to all other points), gap statistic (log of within cluster dispersion) and the elbow method(determined normally graphically by finding the point where the graph levels off within cluster sum of squares). This is so that the number of clusters is determined in an unbiased way and the use of multiple different validation methods is so that there is a higher confidence as they have variable outputs and so multiple methods make them more likely to provide a useful clustering of the distribution. The results of these clusters are then graphed with two UMAPS (one on just the selected nonunimodal region, and the other on the source of the nonunimodal region to see if the feature is consistent with the rest of the profile), a set of consensus images and a profile graph that displays the different morphologies in the different groups and helps see if what phenotypes the detected features are present in by the similarity of the graphs and the differences between the profiles. The consensus image is made by taking the median coordinates of each index and plotting them as a polygon. It also has a functionality for detecting cells with bad edge detection by filtering out cells that have angles that cannot exist in real mouse nuclei. An example the use of this can be seen in the figure 29. This output is shown in detail in the example analysis.

4.3.3 Example analysis with an artificially diverse testing dataset

To test the ability to identify different regions of interest in a diverse population of nuclei, a pooled dataset was made using *Phodopus sungorus hamster* sperm, sperm from an *Fbxo7*^{LacZ/+} mouse with known shape abnormalities affecting the spermatoproteosomes (which promote the degradation of acetylated histones, allowing them to be removed from DNA and replaced with protamines). This spermatoproteosome abnormality causes issues with nuclear condensation (Rathje et al 2019) and causing structural changes around the junction of the dorsal and basal region of the sperm from perturbed spermiogenesis. The third part of the test dataset is a *Mus musculus musculus* x *Mus musculus domesticus* cross with known abnormalities affecting overall structure of the nucleus giving it a short stubbier shape with a shortened hook (PWK X LEWES (PPLL)). This dataset contains at least five distinct phenotypes that are characterized in Figure 27: A being a relatively normal healthy mouse sperm from the *Fbxo7* sample, B is the deformed phenotype from the *Fbxo7* that is generally very variable and tends to have deformed basal regions and in extreme cases multiple hooks. The C phenotype is hamster sperm and has a lack of tail attachment region as well as a slightly shorter hook. Phenotype D is the short sharp hooked PPLL, and E is the longer dull hooked PPLL.

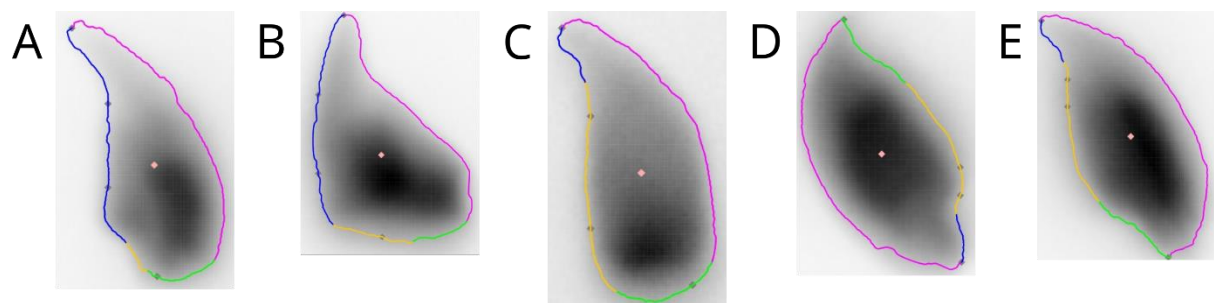


Figure 27: five different phenotypes present in the testing dataset.

The two *Fbxo7*^{+/-} phenotypes A-B, the hamster phenotype C and the two PPLL phenotypes D-E (images are not to actual size scale). The *Fbxo7*^{+/-} phenotype A is a normal mouse sperm with little variation from other mice however phenotype B is massively different being a highly variable deformed phenotype that is difficult to categorise. C is hamster sperm from *Phodopus campbelli* that has highly consistent nuclei that lack a well-defined tail attachment region. The PPLL phenotypes are very similar but are separated by whether they have a sharp or a dull hook.

The analysis detected 11 non-unimodal regions of interest that it has split the dataset into between 2 and 6 clusters based on the variation of features in that region for each region. These 11 sections can all be broken down to explain how the different nuclear morphology led to the different clusters. The regions are made up of four different parts of the dataset, the angle profiles (Figure 28A containing 4 regions of interest: indexes 3:7, 9:15, 27:32 and 34:39) , the diameter profiles (Figure 28B containing 3 regions of interest: indexes 0:3, 52:57 and 95:99), the radius profiles (Figure 28C containing 3 regions of interest: indexes 0:3, 34:43 and 96:99), and hook length (Figure 28D calculated using the difference between the minimum diameter and the width of the bounding box which will be caused by the hook). Profiles reflect the overall structure of the differences between cells in the dataset. These differences are mainly based around the hook's shape and structure as well as the depth and structure of the tail attachment region. The data supports the major differences seen visually between the morphological phenotypes present, there is also the hook length that is relevant to the phenotypes present.

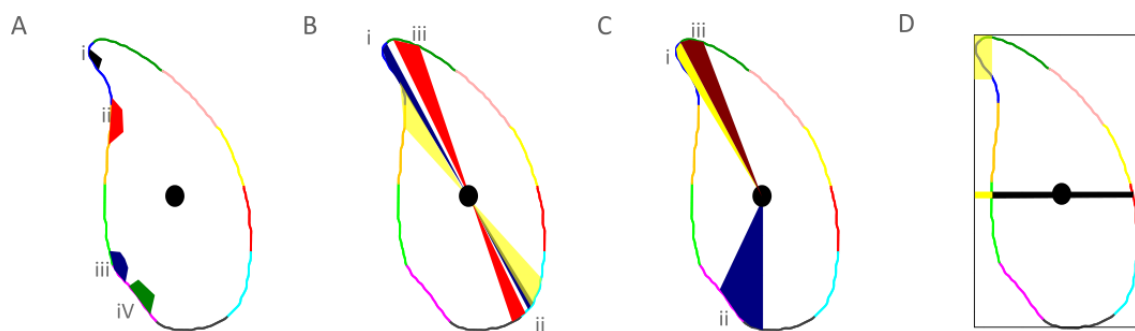


Figure 28 The regions of interest detected by NMA companion.

This uses the overall dataset consensus to base the highlight the regions. A) the angle profiles containing 4 regions of interest: indexes i 3:7, ii 9:15, iii 27:32 and iv 34:39. B) the diameter profiles containing 3 regions of interest: indexes i 0:3, ii 52:57 and iii 95:99. C) the radius profiles containing 3 regions of interest: indexes i 0:3, ii 34:43 and iii 96:99. D) show diagrammatically hook length calculated using the difference between the minimum diameter and the width of the bounding box which will be caused by the hook.

The angle filter rejected 751 cells from the dataset that represent poor edge detection (example in Figure 29) these are removed from the multimodal region detection but are still accessible to allow for reducing detection errors in future. The consensus image shows the very short thin hook that is

caused by it being only partially detected causing a very thin, uneven hook that is not representative of the sample.

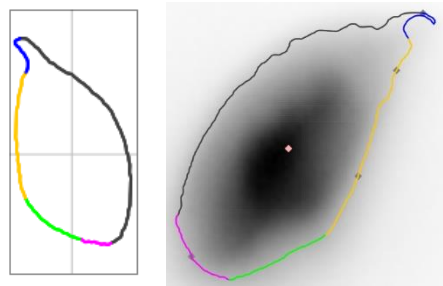


Figure 29: consensus image of the cells filtered from the dataset and a representative DAPI stained example.

The following sections break down the 11 regions of interest:

4.3.3.1 Region 1 of 11: Clustered on Angle Profile indexes 3:7

Angle profile indexes 3-7 are near the tip of the hook of the sperm as can be seen in Figure 28 Ai. This region is relatively consistent between mice and hamsters due to their shared similar falciform shape (Figure 27). The five clusters made by multimodal region clustering over this first set of indexes detected. These clusters do not split the dataset entirely between the three main sample groups that make up the test dataset. However, it does show the actual morphological differences between the angles around the hook. The clusters are numbered arbitrarily to distinguish them. The majority of if hamster sperm is distributed across clusters 1-2 as they possess the general blunt hooks shown by those clusters concentrated mostly in cluster 1. The *Fbxo7*^{+/-} cells are similarly distributed. The PPLL cells make up most of cluster 4 and are also in cluster 1-2 with most on their cells in cluster 2, this reflects their more variable hook point shape. Cluster 4 and 5 are small and shows outliers. The graphs help exhibit these relationships with the small groups of outliers shown in the UMAP of the multimodal regions although it also shows that some other outliers are included in

the other clustering. With the use of the overall angle profile comparison, the cells don't have that much of a difference between their general clusters with a lot of overlap as the small differences between this region is easily diluted by the similarities between the cells. These clusters characterise the hook sharpness with clusters 1 and 2 showing relatively normal hooks, 3 shows very blunt hooks 4 showing very sharp hooks and 5 showing a small group of hooks that are likely detection errors.

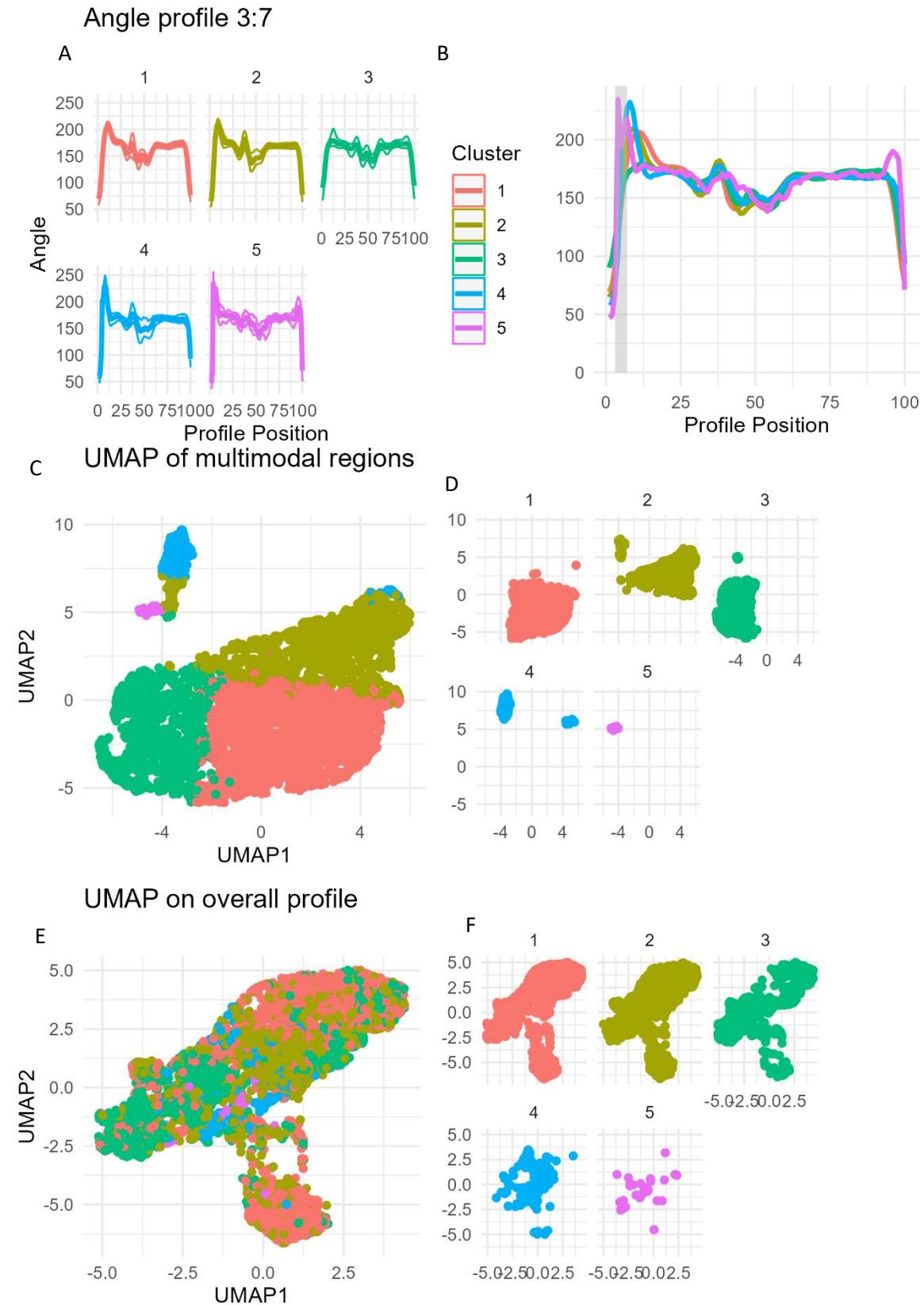


Figure 30 Graphs representing region 1 of 11: Clustered on Angle Profile indexes 3:7.

A) the faceted profile of the region shown and B) is them combined for easy comparison. C) is the combined UMAP of the multimodal region detected with the clusters overlaid, D) is the faceted version of the same graph to combat overplotting and show the shape of the individual clusters. Graphs E) and F) follow the same system however they are on the overall profile region to show how the sample fits into the dataset allowing for cross clustering comparison.

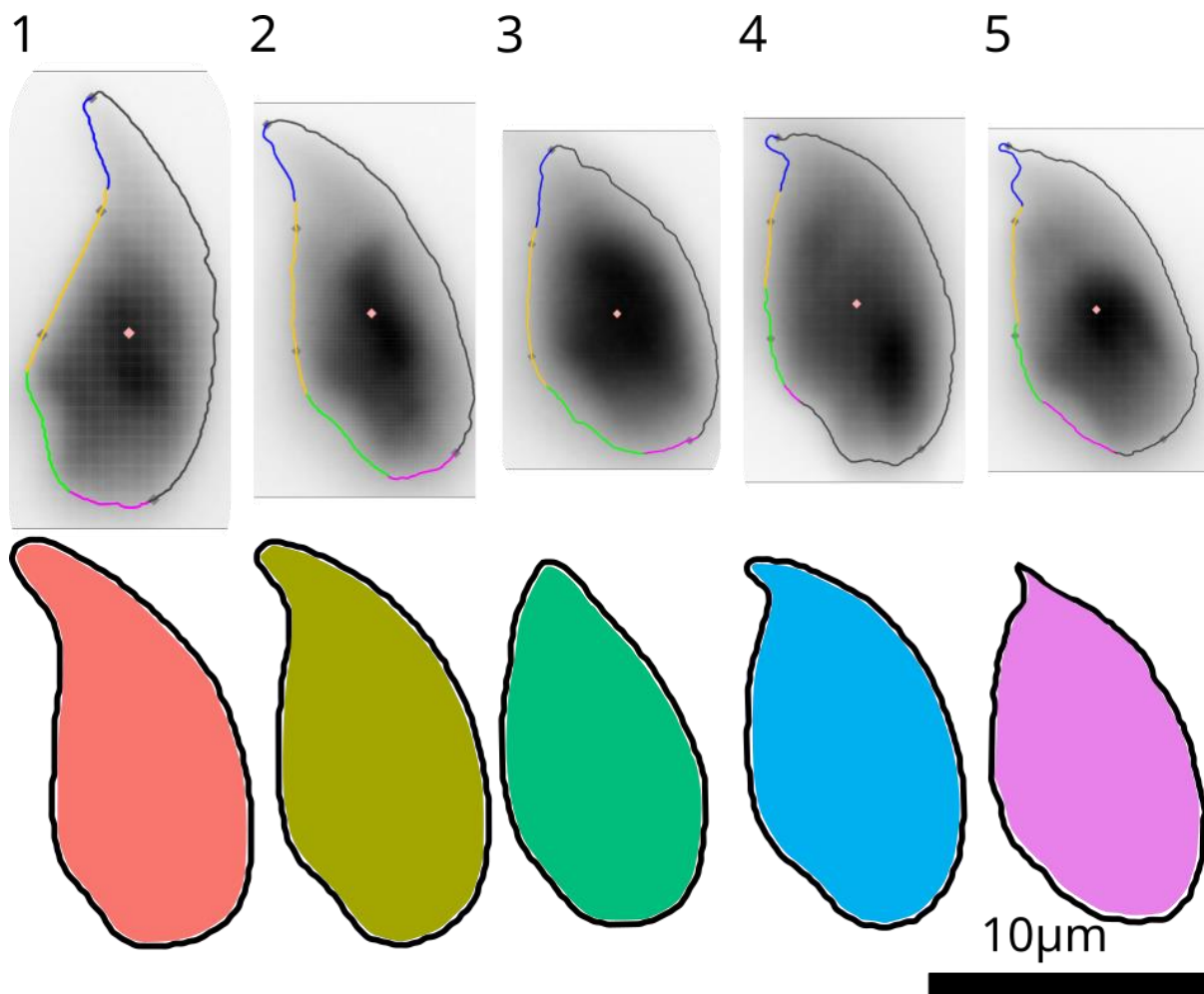


Figure 31: Representative cells of each cluster with their respective consensus images for the cells within the cluster beneath.

These are for angle profile indexes 0:3 and the region they were separated on can be seen in Figure 28 Ai

4.3.3.2 Region 2 of 11: Clustered on Angle profile Indexes 9:15

This region is situated under the tip of the hook and separates the dataset between the sharp hooks and dull hooks as well as how curved the hook is. This region isn't massively relevant to the different phenotypes in the testing dataset and gives a similar story to the previous angle clustering. Clusters 1 and 2 show mostly the Fbxo7^{+/-} and hamster sperm however it differs as it shows the difference in the hook shape of the PLL that shows as the PLL has a relatively even split between cluster 3 and 4 that is due to its two phenotypes one with a short stubby hook and one with a larger sharper hook. This is supported by the filtering of the more deformed Fbxo7^{+/-} cells being filtered into group 4 and the smaller hamster cells being filtered into group 3 exhibiting this morphological difference.

There UMAP graphs show that this feature is clusters in agreement with the general profile clusters which shows the features of the cells are likely different enough to be seen in the overall dataset.

There is a general split between clusters 1-3 and 4-5(Figure 32Figure 33) that show more developed hooks and less developed hooks. This is quite visible in figure 29 D and F which show a separation between the UMAPS. The hooks in group 4-5 (Figure 32Figure 33) could also be missing from detection errors as the hook is one of the regions that stains the worst and is easily lost in detection.

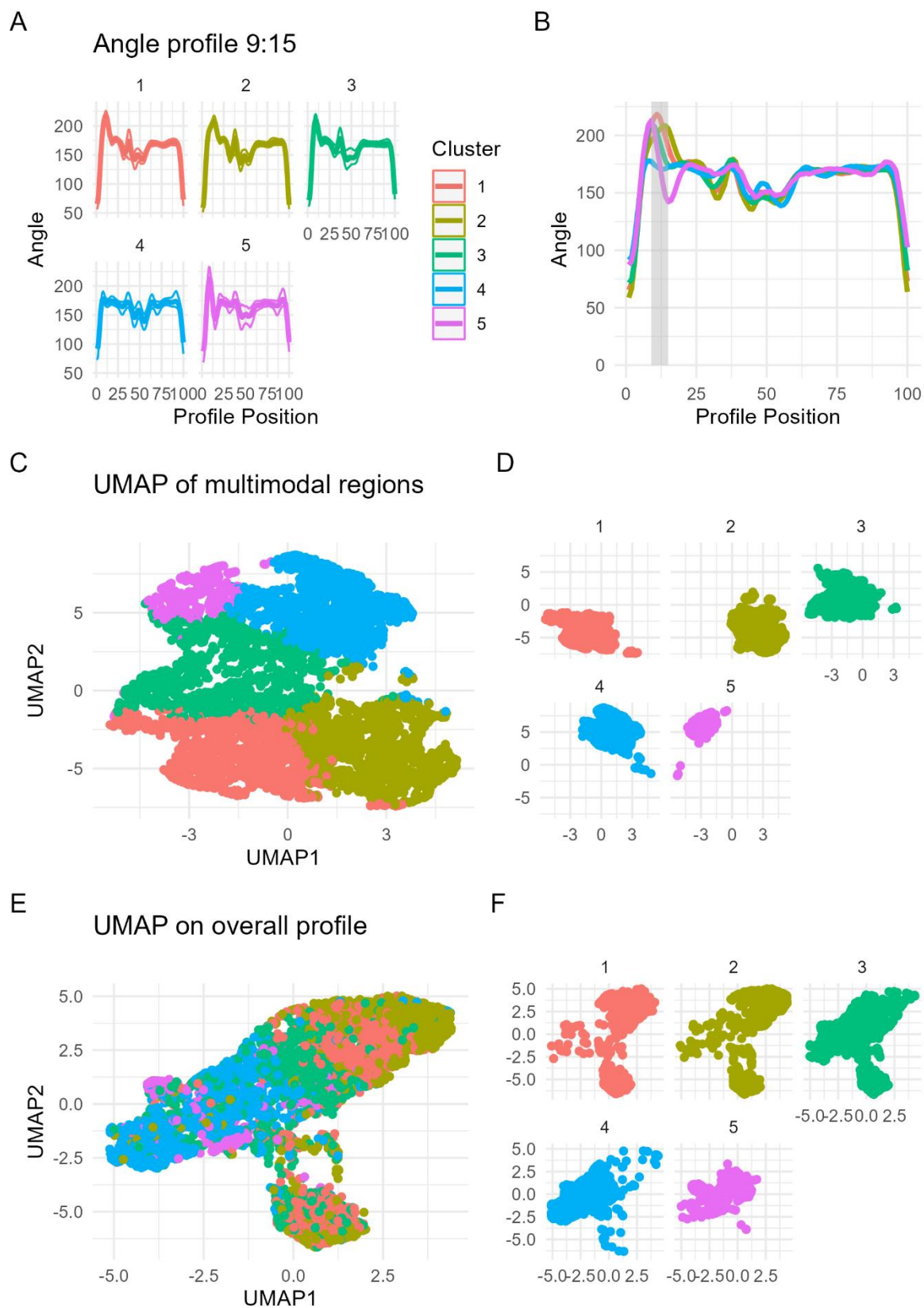


Figure 32 Graphs representing region 2 of 11: Clustered on Angle profile Indexes 9:15.

A) the faceted profile of the region shown and B) is them combined for easy comparison. C) is the combined UMAP of the multimodal region detected with the clusters overlaid, and D) is the faceted version of the same graph to combat overplotting and show the shape of the individual clusters. Graphs E) and F) follow the same system; however, they are on the overall profile region to show how the sample fits into the dataset allowing for cross clustering comparison.

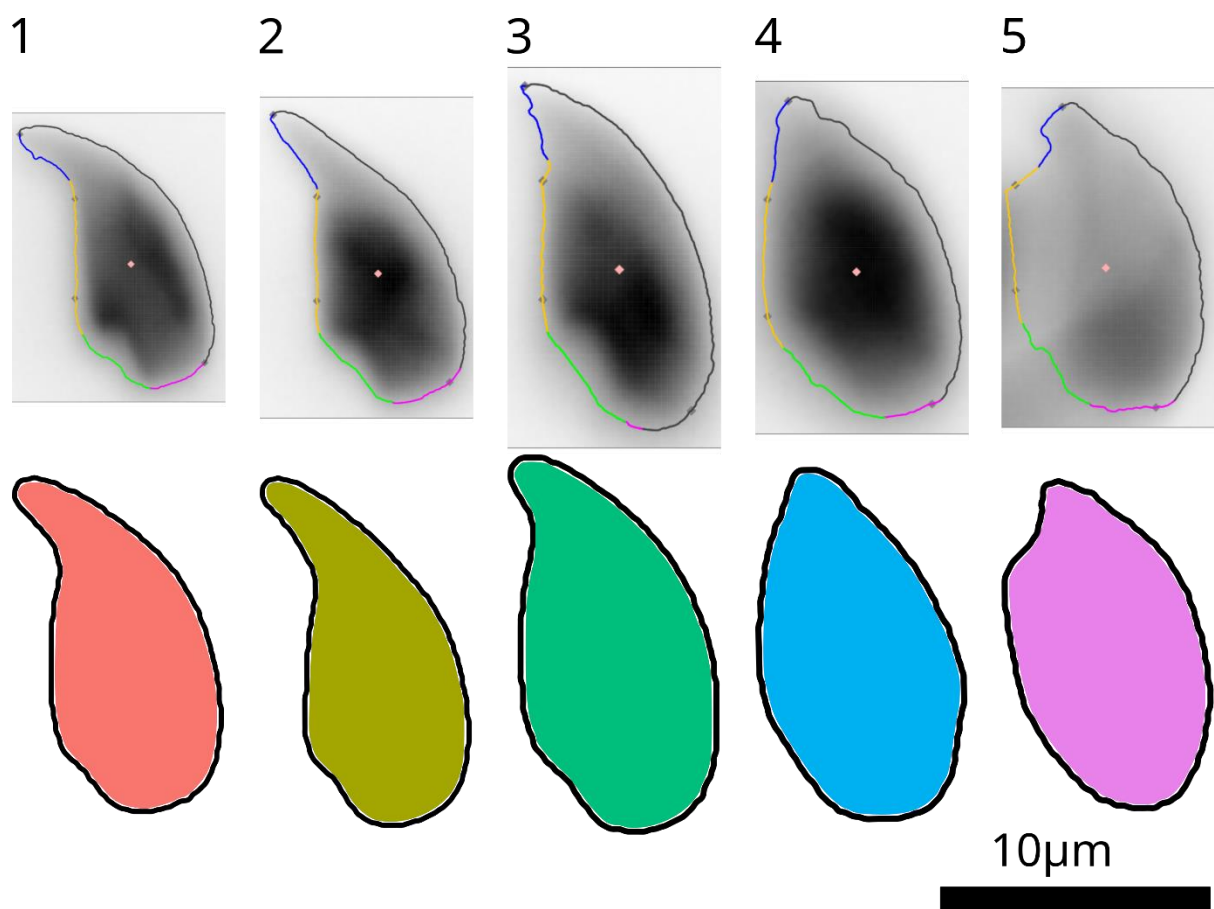


Figure 33 Representative cells of each cluster with their respective consensus images for their region below them. The region they were separated on can be seen in Figure 28 Aii.

4.3.3.3 *Region 3 of 11: Clustered on Angle profile Indexes 27:32*

This section very clearly filters out the sample based on the definition of the tail attachment region shown in section Aiii of Figure 28. Cluster one shows cells with very well-defined tail attachment regions and cluster two shows cells with deformed or completely missing tail attachment regions such as the hamster sperm and some of the more deformed Fbxo7^{+/-} and PPLL. The UMAPS yet again show how the clusters relate individually and to the dataset. There is a split for this kind of generally very continuous data as can be seen in Figure 34 B and C which show a lower density region in-between the two clusters that show that this structure is not made up mostly of slight deformities but a larger more consistent type.

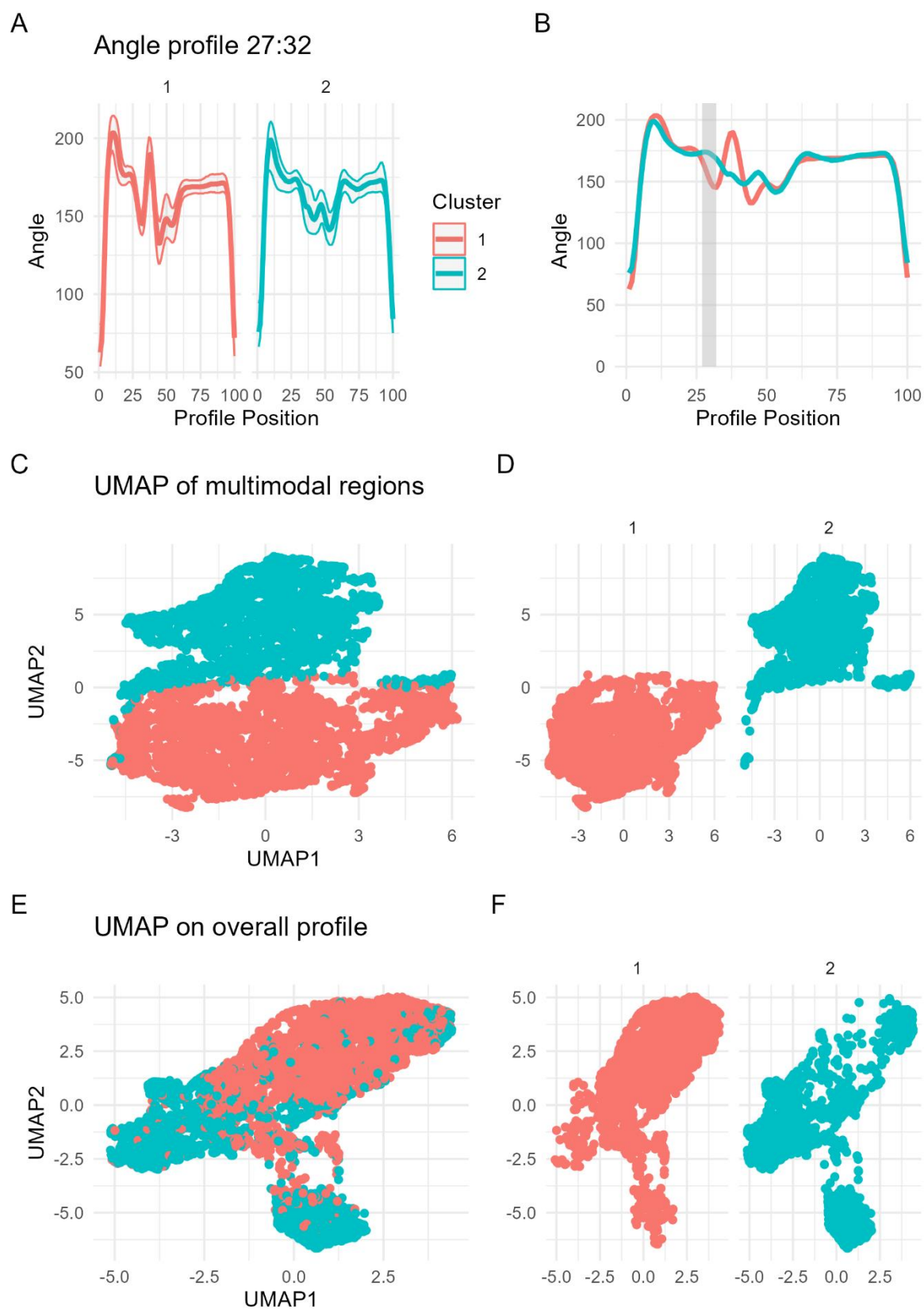


Figure 34 Graphs representing region 3 of 11: Clustered on Angle profile Indexes 27:32.

A) the faceted profile of the region shown and B) is them combined for easy comparison. C) is the combined UMAP of the multimodal region detected with the clusters overlaid, and D) is the faceted version of the same graph to combat overplotting and show the shape of the individual clusters. Graphs E) and F) follow the same system; however, they are on the overall profile region to show how the sample fits into the dataset allowing for cross clustering comparison.

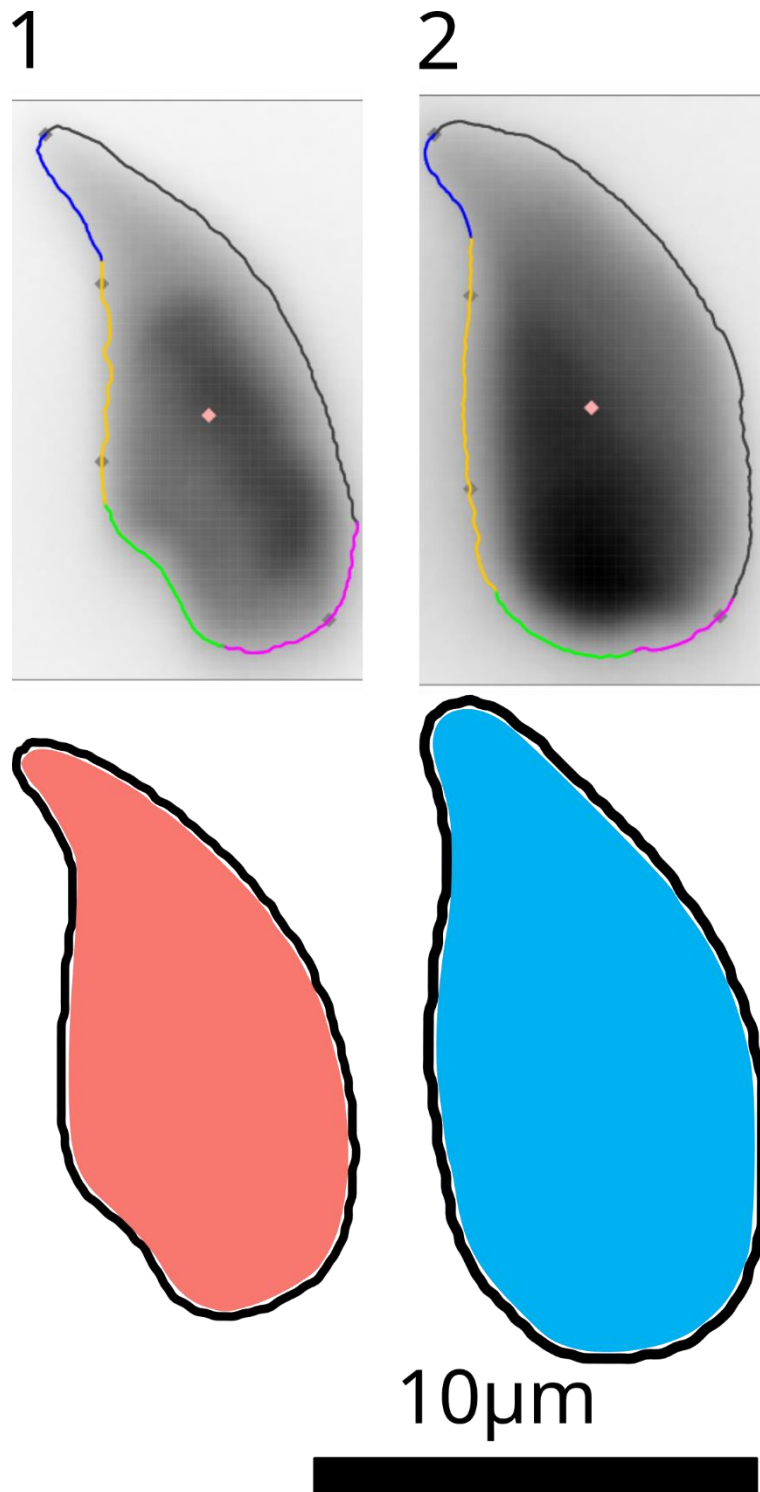


Figure 35 Representative cells of each cluster with their respective consensus images for their region below them. The region they were separated on can be seen in Figure 28 Aiii.

4.3.3.4 *Region 4 of 11: Clustered on Angle profile Indexes 34:39*

This region shows the other side of the tail attachment region shown in section AiV in Figure 28. This clustering filters out those differing tail attachment region phenotypes such as cluster 5 being almost entirely hamster sperm and contains most of the hamster sperm in the sample due to its lack of tail attachment region. The rest of the samples are distributed across clusters 1-4 (Figure 36 and Figure 37) and clusters out the similar cells within the *Fbxo7*^{+/-} samples and PPLL samples. These just show general variation that happens more based on the length of the cells. This is reflected in the UMAP that shows that there are distinct groups with group 5 (Figure 36 and Figure 37) being furthest from the other groups that all border each other.

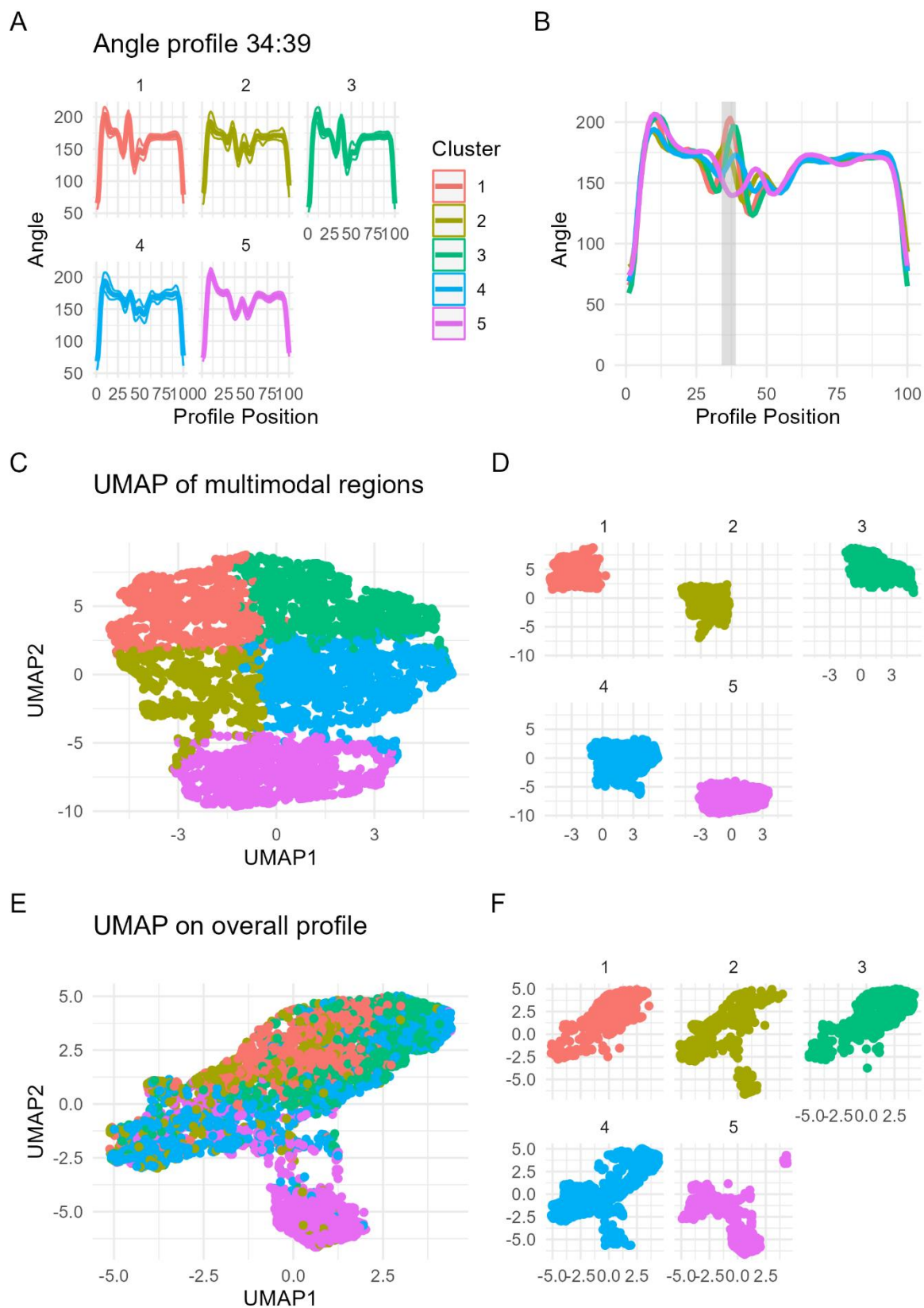


Figure 36 Graphs representing region 4 of 11: Clustered on Angle profile indexes 34:39.

A) the faceted profile of the region shown, and B) is them combined for easy comparison. C) is the combined UMAP of the multimodal region detected with the clusters overlaid, and D) is the faceted version of the same graph to combat overplotting and show the shape of the individual clusters. Graphs E) and F) follow the same system; however, they are on the overall profile region to show how the sample fits into the dataset allowing for cross clustering comparison.

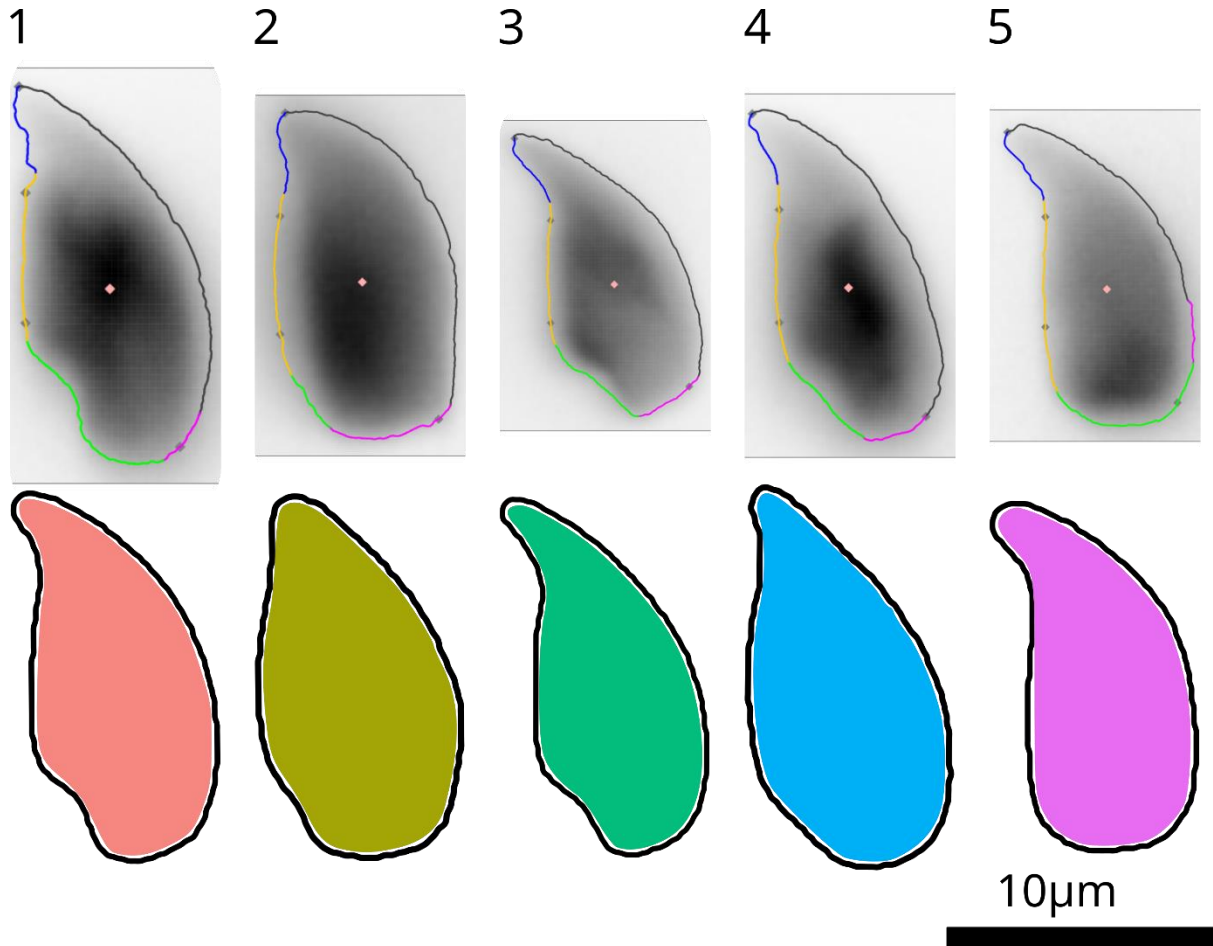


Figure 37 Representative cells of each cluster with their respective consensus images for their region below them. The region they were separated on can be seen in Figure 28 AiV.

4.3.3.5 Region 5 of 11: Clustered on Diameter profile Indexes 0:3

This section is made up of diameter profiles which are the length of the diameter of the cells through the centre of mass as can be seen in Figure 28 Bi showing this region. This gives a more another aspect of features that can be seen other than angle profile and can help with identifying structures with similar angles but different physical shapes. These clusters are based on the overall diameter of

the cells from hook to base with most of the cells being filtered into clusters 1, 2 cluster being mostly the stubbier PPLL phenotype. Figure 38 C and D show that these cells are clustered on their discrete hook morphotypes as can be seen Figure 39 2. Figure 38 E and F also show that there are also some of the morphotype distributed across the rest of the sample sources in the test dataset. This distributed morphotype contains detection errors as can be seen in Figure 39 2.

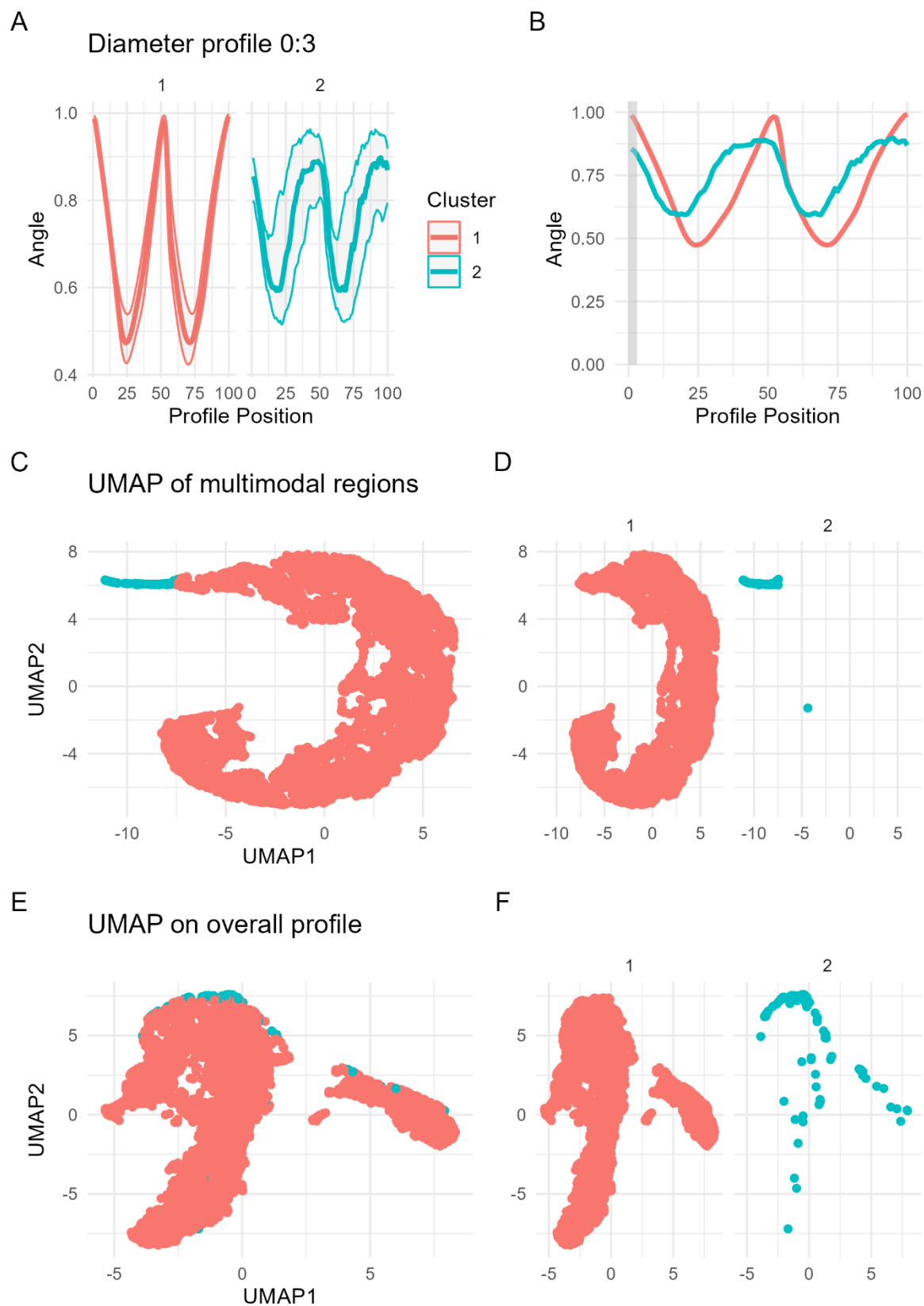


Figure 38 Graphs representing region 5 of 11: Clustered on Diameter profile Indexes 0:3.

A) the faceted profile of the region shown and B) is them combined for easy comparison. C) is the combined UMAP of the multimodal region detected with the clusters overlaid, and D) is the faceted version of the same graph to combat overplotting and show the shape of the individual clusters. Graphs E) and F) follow the same system; however, they are on the overall profile region to show how the sample fits into the dataset allowing for cross clustering comparison.

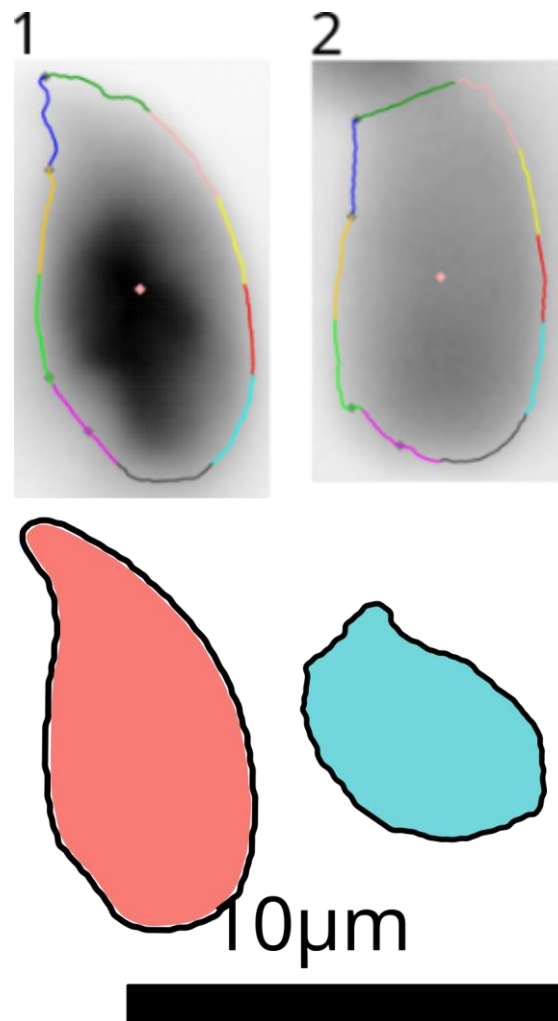


Figure 39 Representative cells of each cluster with their respective consensus images for their region below them. The region they were separated on can be seen in Figure 28 Bi.

4.3.3.6 Region 6 of 11: Clustered on Diameter Profile Indexes 52:57

This diameter profile is across the cell from the other side to the hook and mainly just measures the size of the cells. The groupings (Figure 40 and Figure 41)) are based on the size of the cells, but it is

also affected by the depth of the area around the tail attached zone. The PLL is once again split into its two phenotypes by this clustering in cluster 4 and 5 and hamster sperm is filtered out into mainly cluster 2. Interestingly it very well categorises the *Fbxo7*^{+/-} deformed phenotype into group 5 likely due to its shorter height. Figure 40 B shows an interesting effect of this style of clustering where the morphotypes correlate with large differences in some sections of the diameter profile, such as the widths of the nucleus shown by the differences in the two dips.

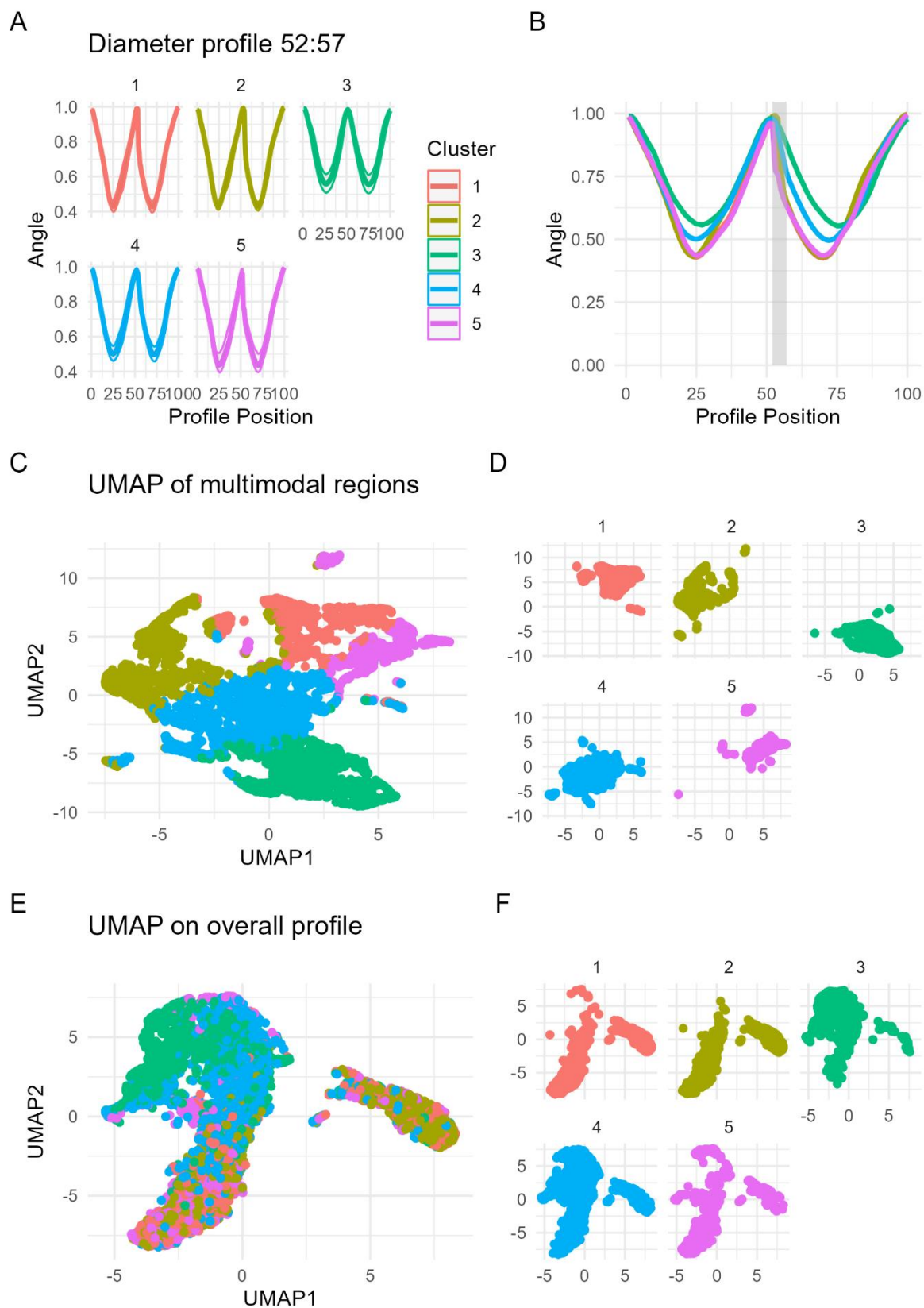


Figure 40 Graphs representing region 6 of 11: Clustered on Diameter Profile Indexes 52:57.

A) the faceted profile of the region shown and B) is them combined for easy comparison. C) is the combined UMAP of the multimodal region detected with the clusters overlaid, and D) is the faceted version of the same graph to combat overplotting and show the shape of the individual clusters. Graphs E) and F) follow the same system; however, they are on the overall profile region to show how the sample fits into the dataset allowing for cross clustering comparison.

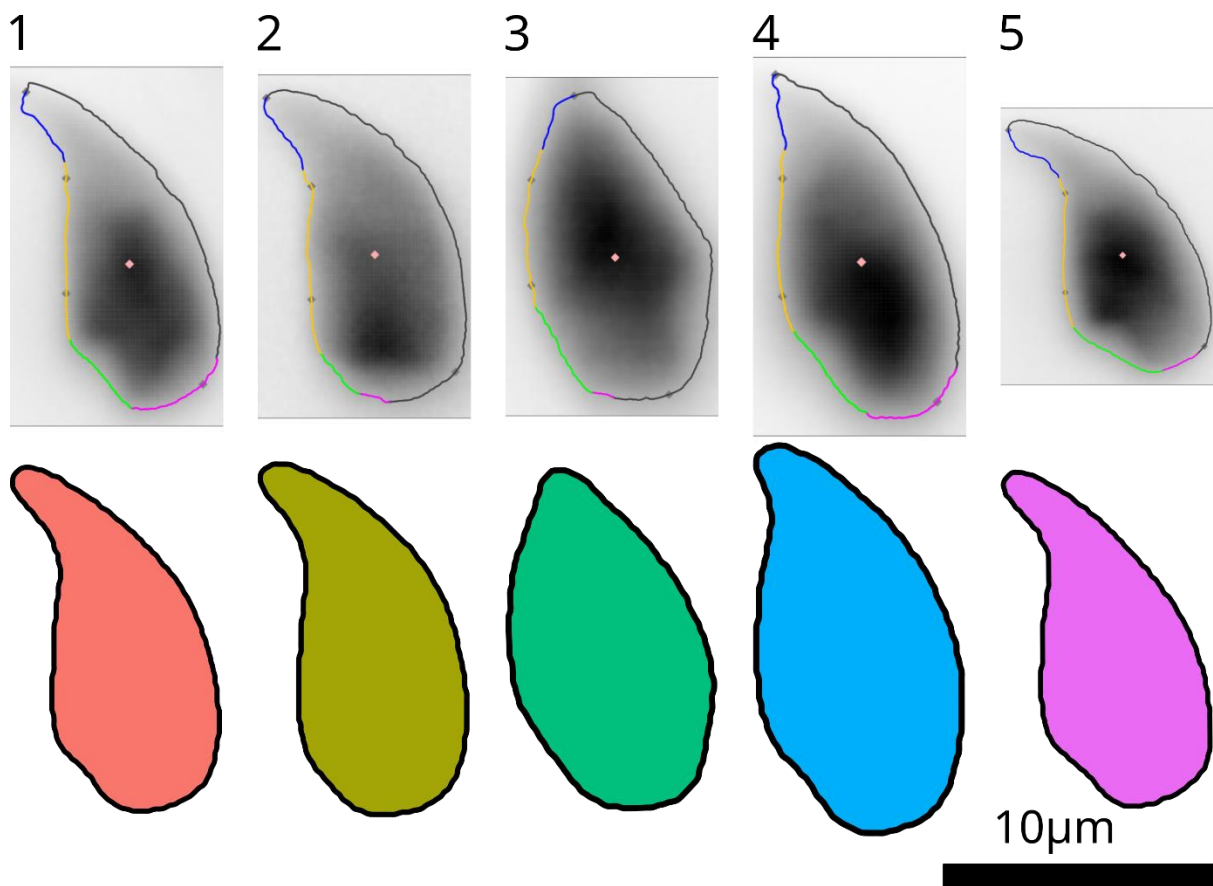


Figure 41 Representative cells of each cluster with their respective consensus images for their region below them. The region they were separated on can be seen in Figure 28 Bii.

4.3.3.7 Region 7 of 11: Clustered on Diameter Profile Indexes 95-99

This region mainly illustrates an issue with the system itself with it being part of the same multimodal region as Diameter profile 0-3 and gives of the same clustering. The detection of multimodal regions is currently done linearly which stops regions that are physically close together being grouped as it cannot be wrapped around top the other end of the dataset as it is circular. It does show some useful groupings of severely malformed sperm and edge detection errors that would be included the rest of the morphology if not filters Figure 42 A and B especially shows this.

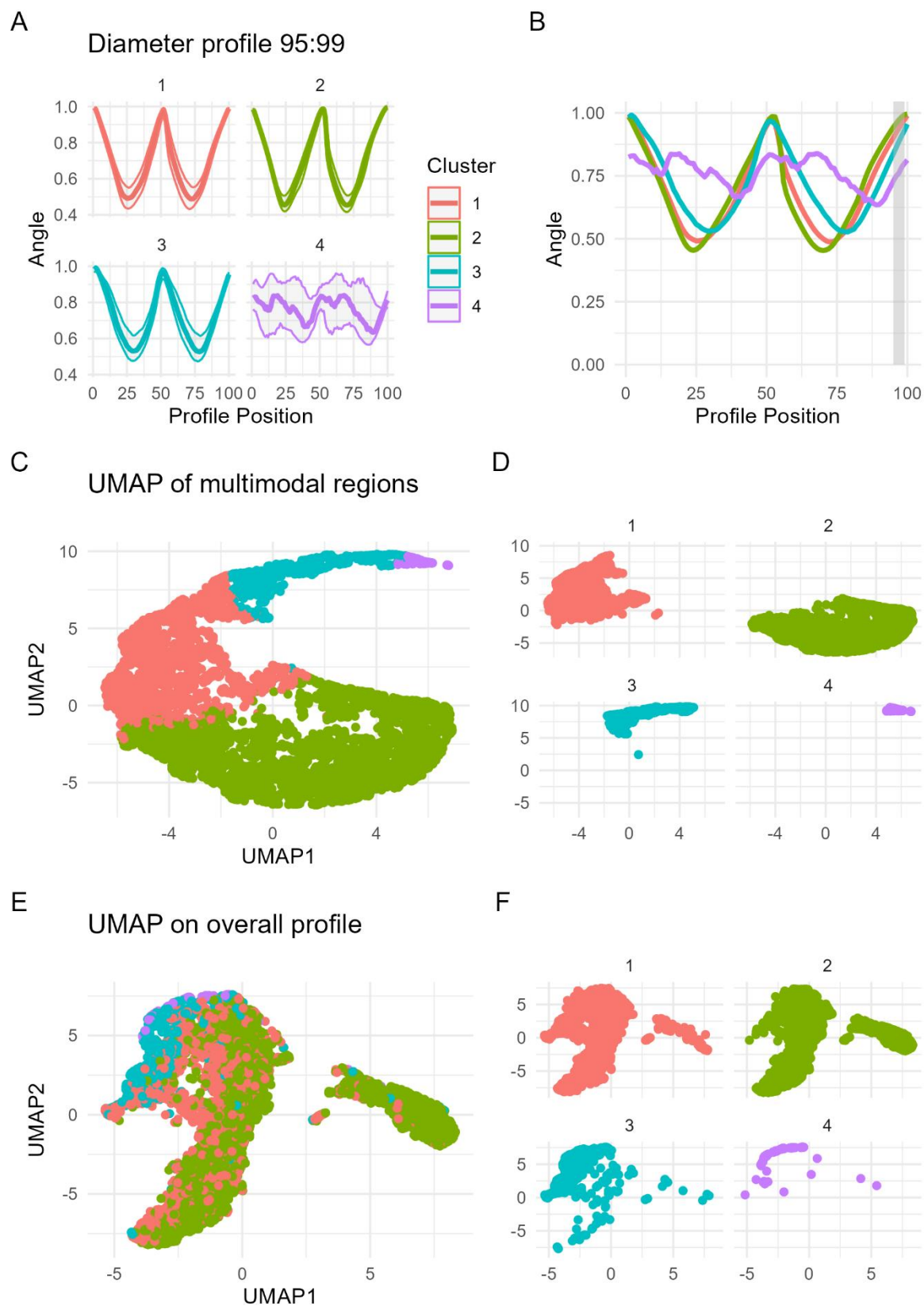


Figure 42 Made up of three graph sets in combined and faceted format for ease of comparison.

A is the faceted profile of the region shown and B is the combined for easy comparison. C is the combined UMAP of the multimodal region detected with the clusters overlaid and D is the faceted version of the same graph to combat overplotting and show the shape of the individual clusters. Graphs E and F follow the same system however they are on the overall profile region to show how the sample fits into the dataset allowing for cross clustering comparison.

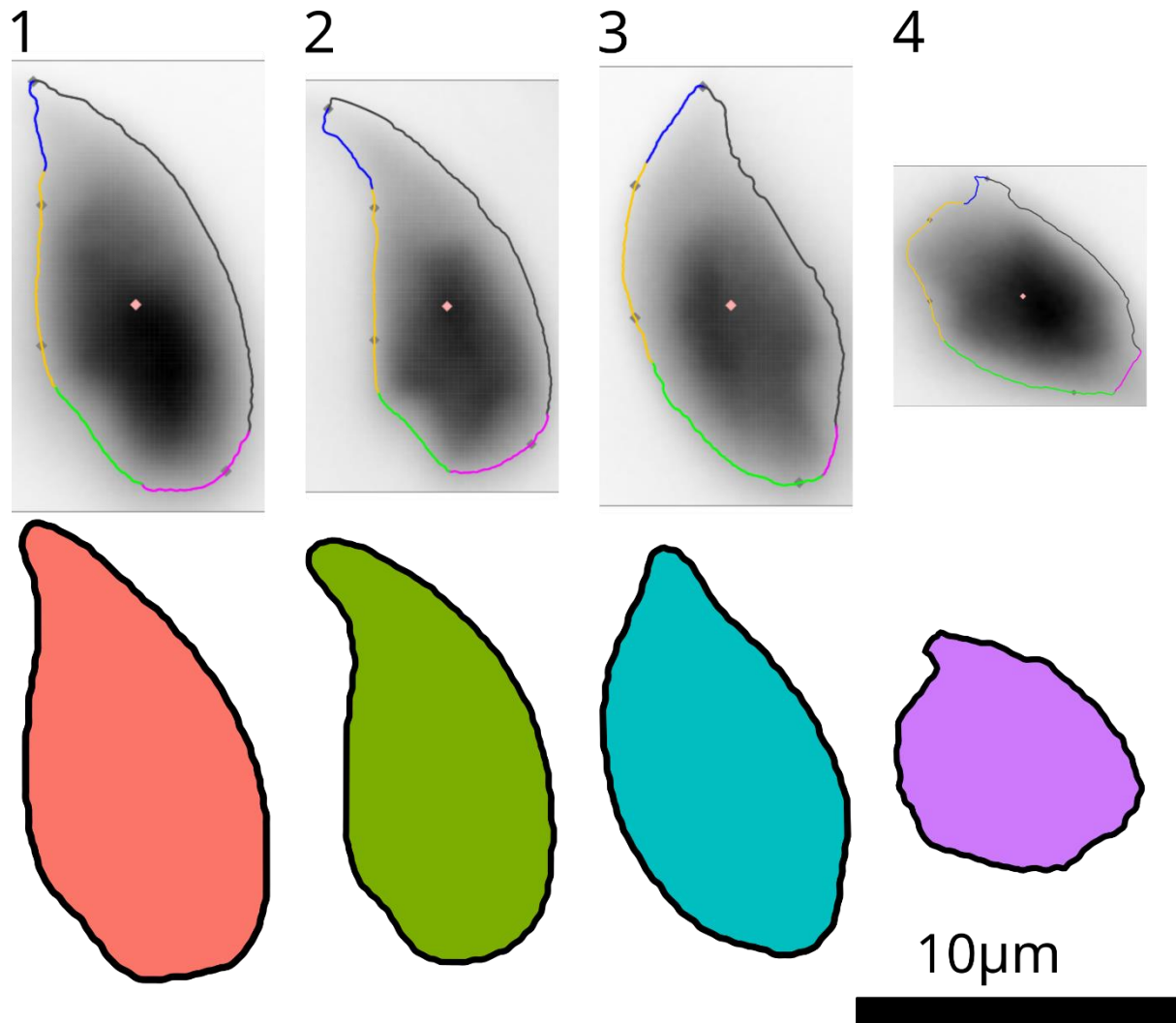


Figure 43 Representative cells of each cluster with their respective consensus images for their region below them. The region they were separated on can be seen in Figure 28 Biii.

4.3.3.8 Region 8 of 11: Clustered on Radius profile Indexes 0-3

This region filters out hook length outliers and is more informative on the nature of the data than the individual phenotypes themselves. They are relatively evenly distributed across all the phenotypes. It gives much the same information as diameter profiles 0-3 as the opposing side in the

profile has little variation this can be seen in Figure 44 C and D being almost identical to Figure 38 C and D. This clustering in combination with the diameter profile shows that the hooks are not also correlated with overall width of the cell.

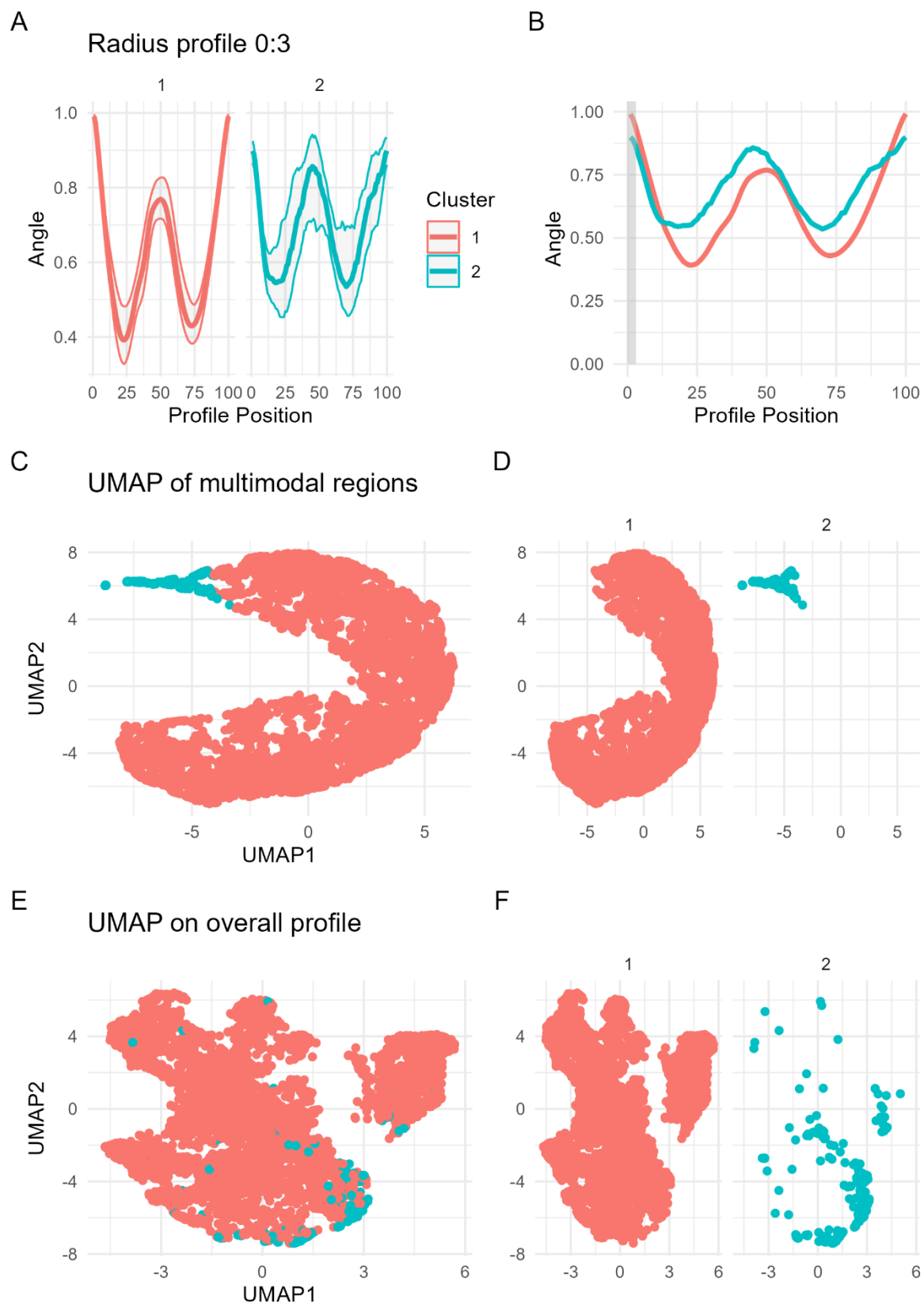


Figure 44 Graphs representing region 8 of 11: Clustered on Radius profile Indexes 0-3.

A) the faceted profile of the region shown and B) is them combined for easy comparison. C) is the combined UMAP of the multimodal region detected with the clusters overlaid, and D) is the faceted version of the same graph to combat overplotting and show the shape of the individual clusters. Graphs E) and F) follow the same system; however, they are on the overall profile region to show how the sample fits into the dataset allowing for cross clustering comparison.

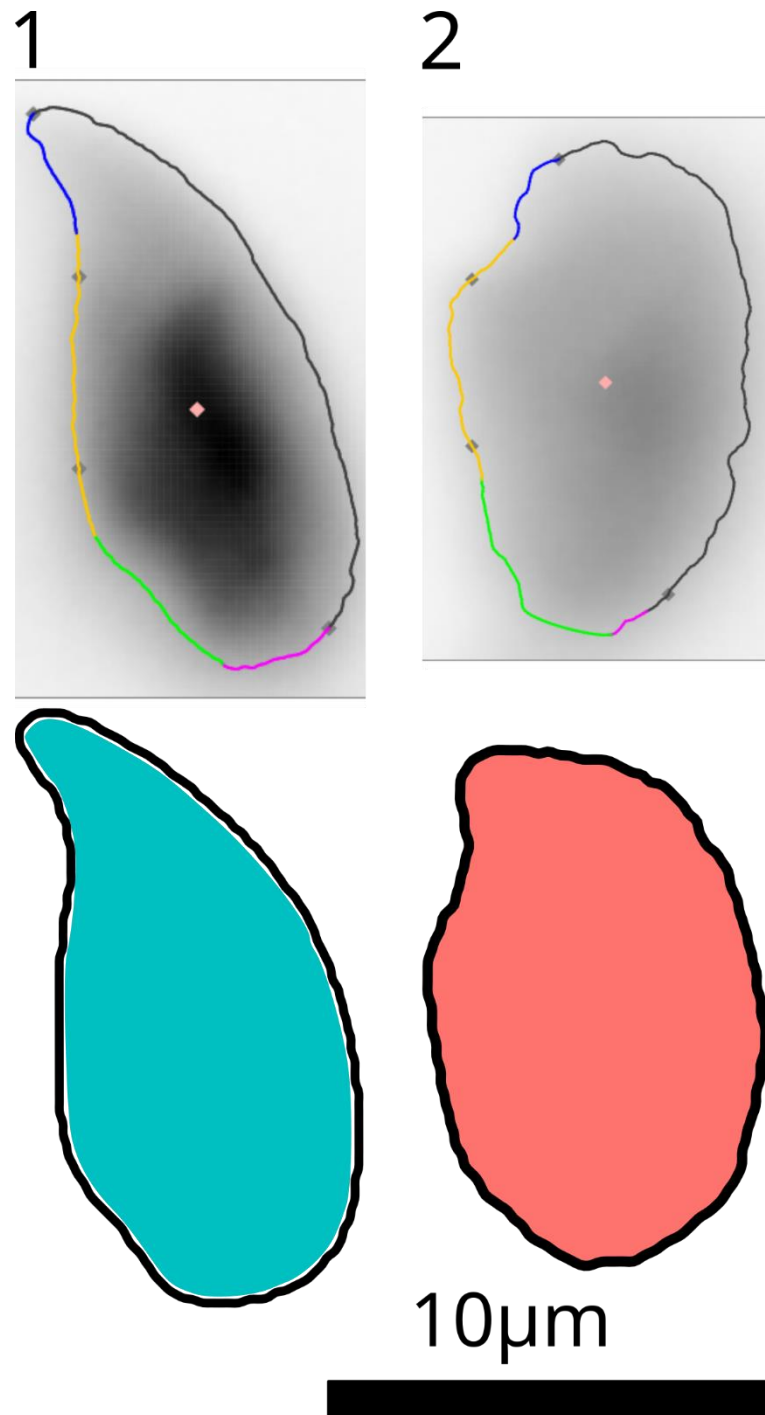


Figure 45 Representative cells of each cluster with their respective consensus images for their region below them. The region they were separated on can be seen in Figure 28 Ci.

4.3.3.9 Region 9 of 11: Clustered on Radius profile Indexes 34-43

This grouping is based on the depth of the tail attachment region (Figure 28 Cii) and filters out the hamster sperm and the cells with less defined tail attachment regions into group 2 (Figure 46 and Figure 47) whereas the cells with well-defined tail attachment regions like the more normal Fbxo7^{+/-} and PPLL are in cluster 1.

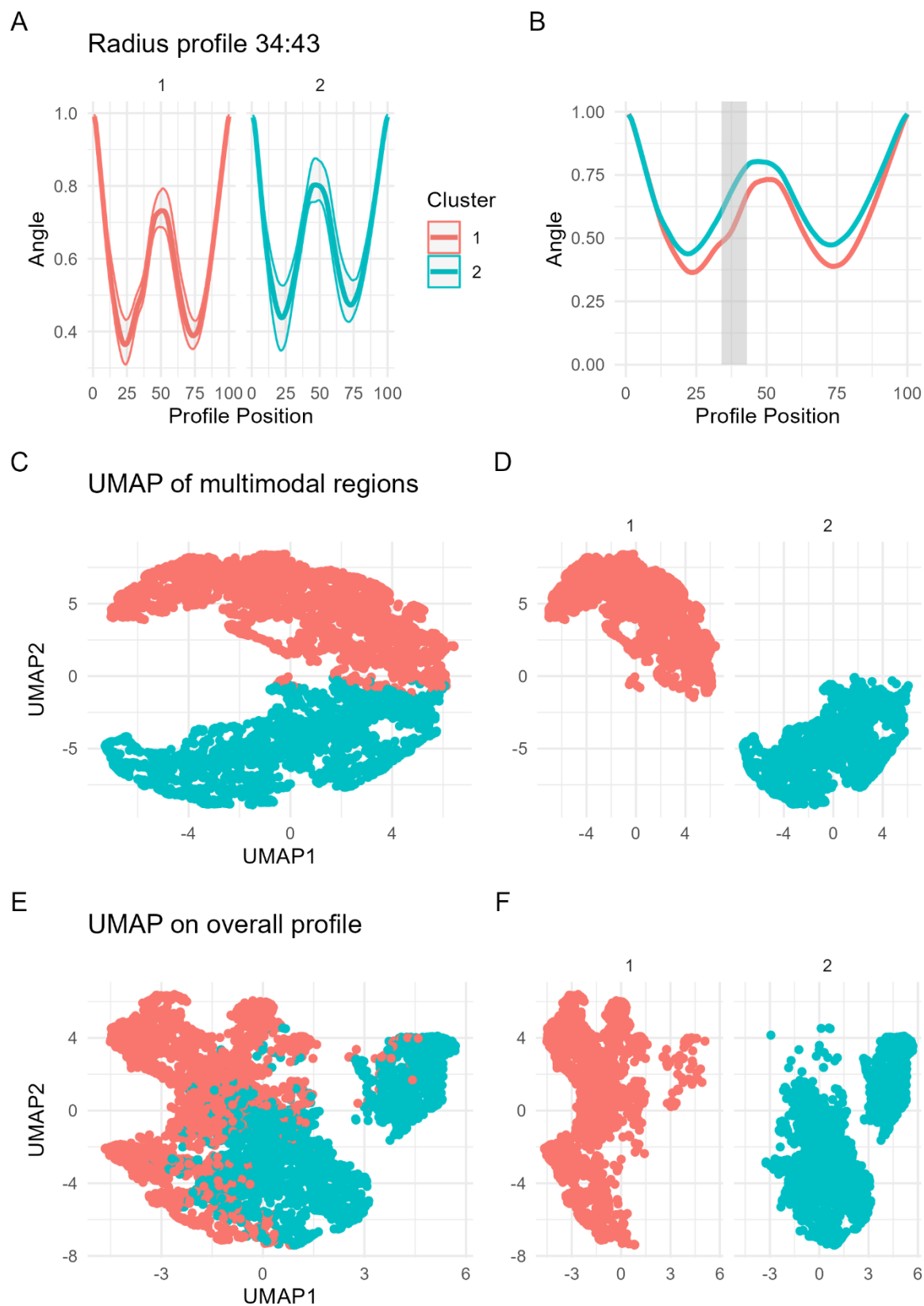


Figure 46 Graphs representing region 9 of 11: Clustered on Radius profile indexes 34-43.

A), the faceted profile of the region shown, and B) combined for easy comparison. C) is the combined UMAP of the multimodal region detected with the clusters overlaid, and D) is the faceted version of the same graph to combat overplotting and show the shape of the individual clusters. Graphs E) and F) follow the same system; however, they are on the overall profile region to show how the sample fits into the dataset allowing for cross clustering comparison.

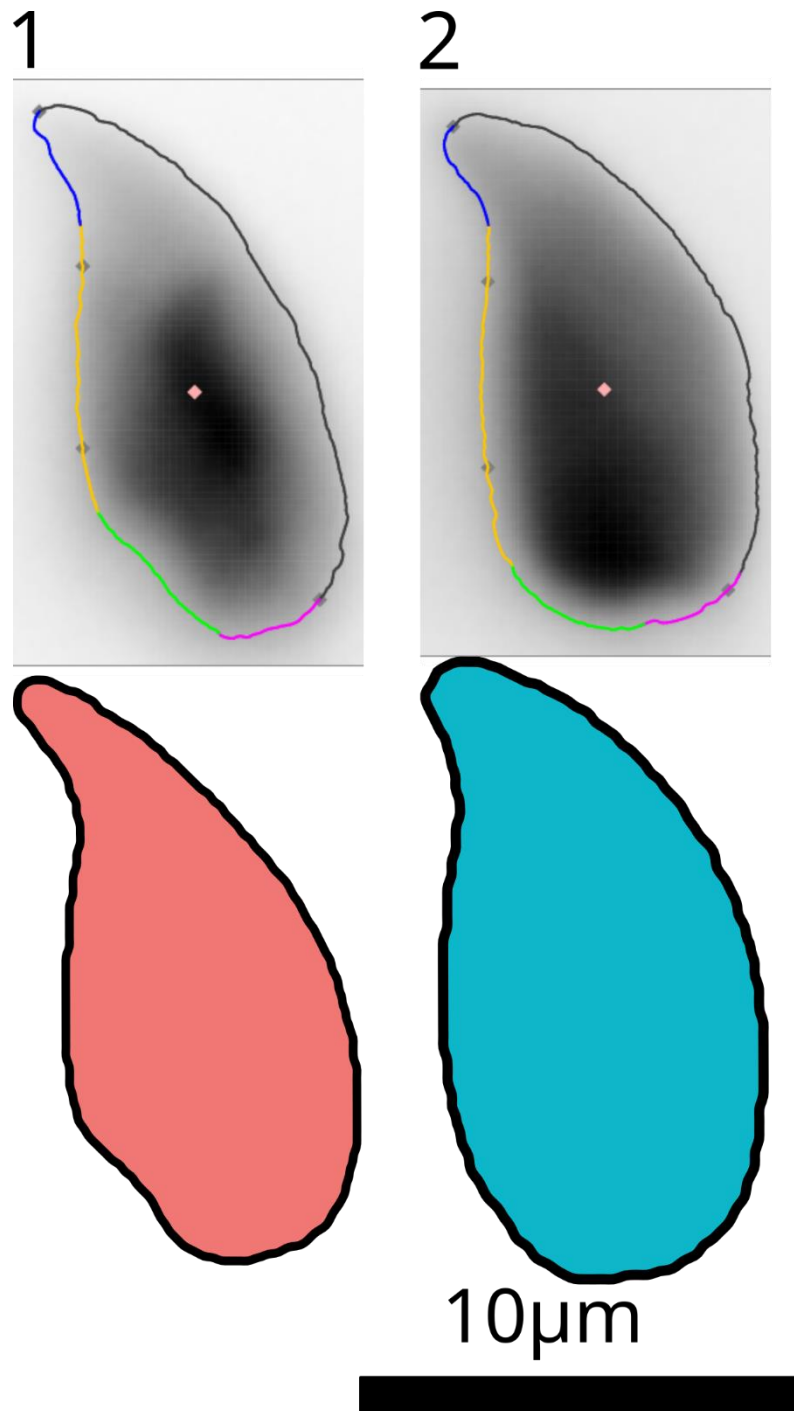


Figure 47 Representative cells of each cluster with their respective consensus images for their region below them. The region they were separated on can be seen in Figure 28 Cii.

4.3.3.10 Region 10 of 11: Clustered on radius profile Indexes 96-99

This Region is a reiteration of the same issues shown in the diameter profile previously and shows something similar but instead of it being such a small outlier group the group is large enough to see the some of the features showing a longer and sharper hook present in low levels across the samples (as can be seen in Figure 48 and Figure 49).

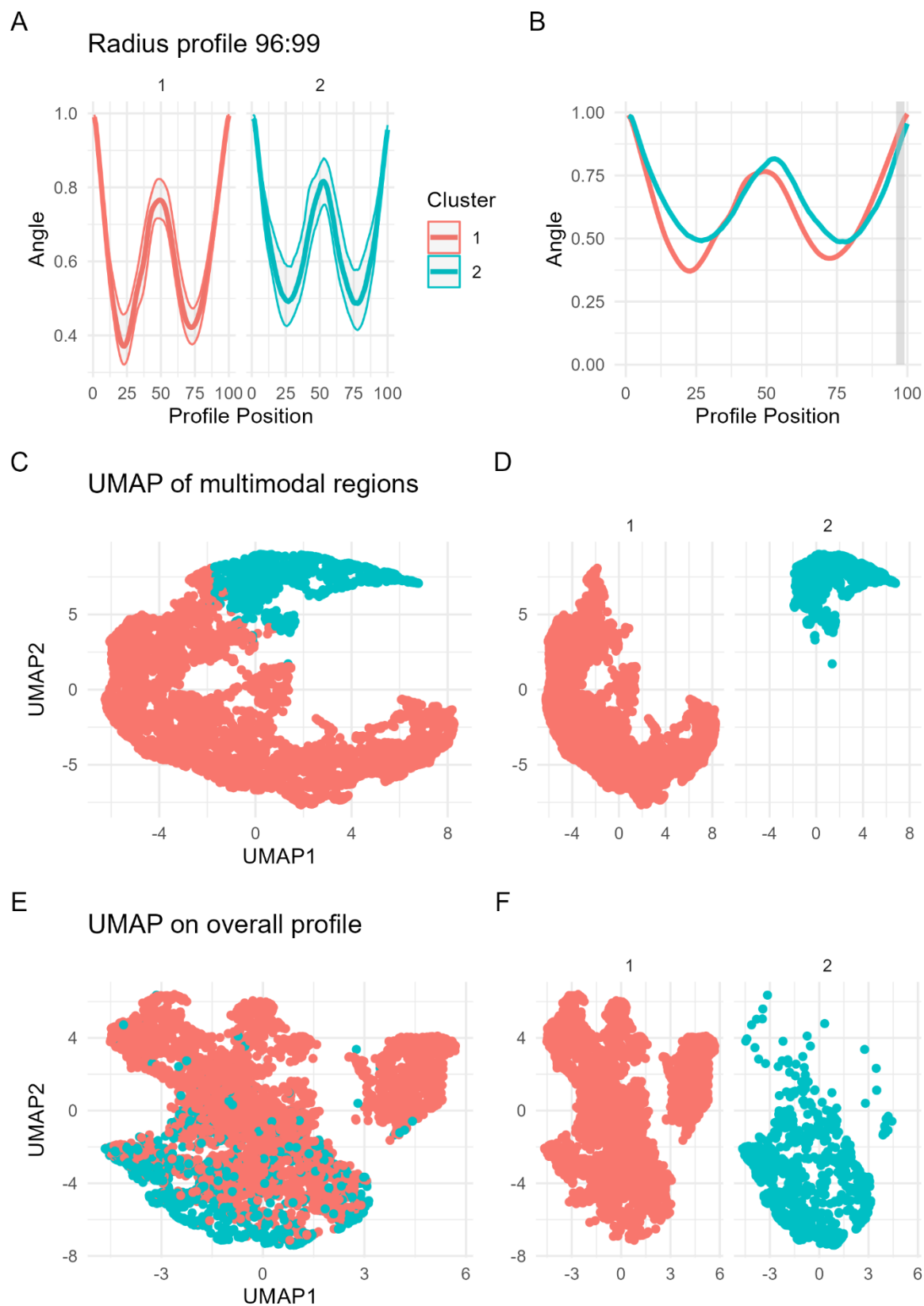


Figure 48 Graphs representing region 10 of 11: Clustered on radius profile Indexes 96-99.

A) the faceted profile of the region shown and B) is them combined for easy comparison. C) is the combined UMAP of the multimodal region detected with the clusters overlaid, and D) is the faceted version of the same graph to combat overplotting and show the shape of the individual clusters. Graphs E) and F) follow the same system; however, they are on the overall profile region to show how the sample fits into the dataset allowing for cross clustering comparison.

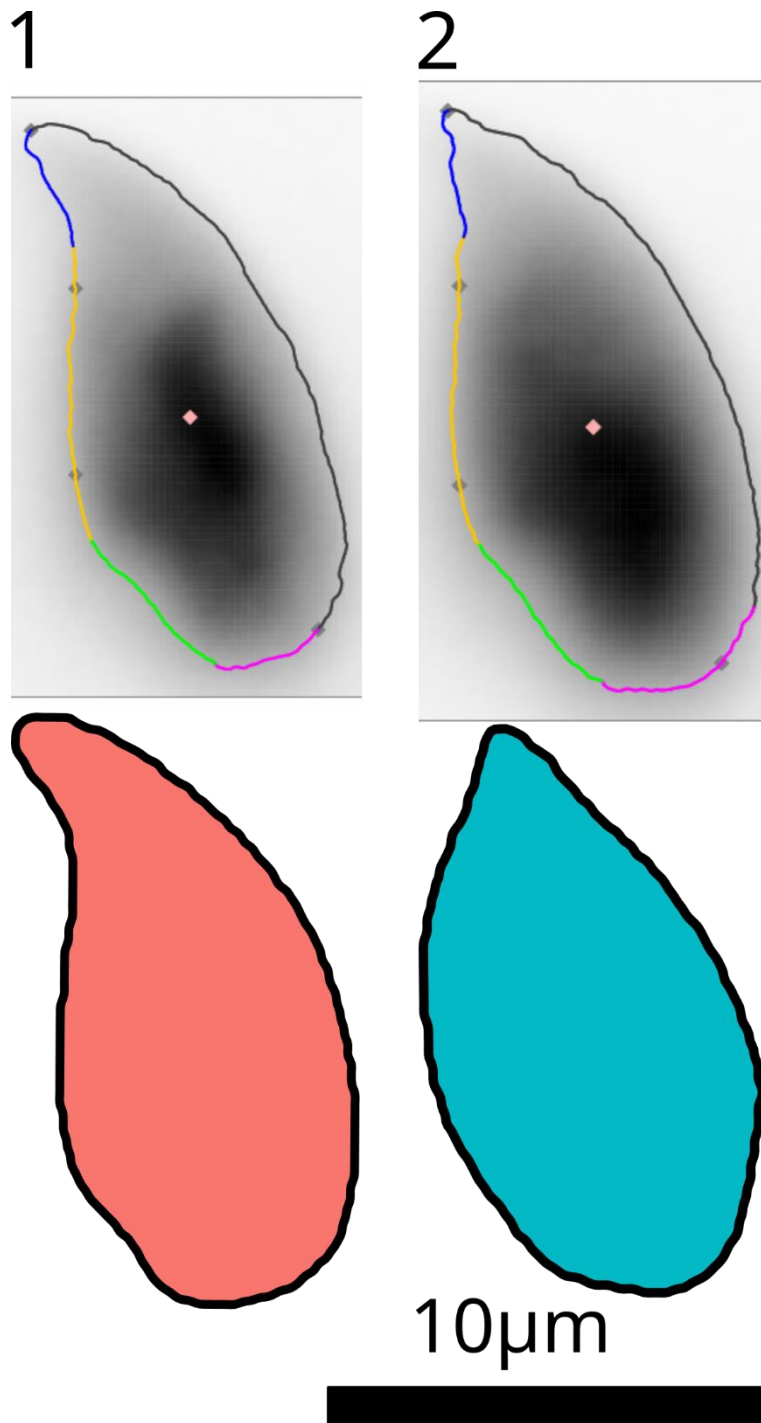


Figure 49 Representative cells of each cluster with their respective consensus images for their region below them. The region they were separated on can be seen in Figure 28 Ciii.

4.3.3.11 Region 11 of 11: Clustered on Length of hook

This clustering is based on hook length and agrees with what was previously thought such as Fbxo7+/- being distributed across long and short hooks the hamster sperm is also distributed across each group as expected mainly being in the longer hook group. The PPLL is almost entirely in the short hook grouping (Figure 50 2).

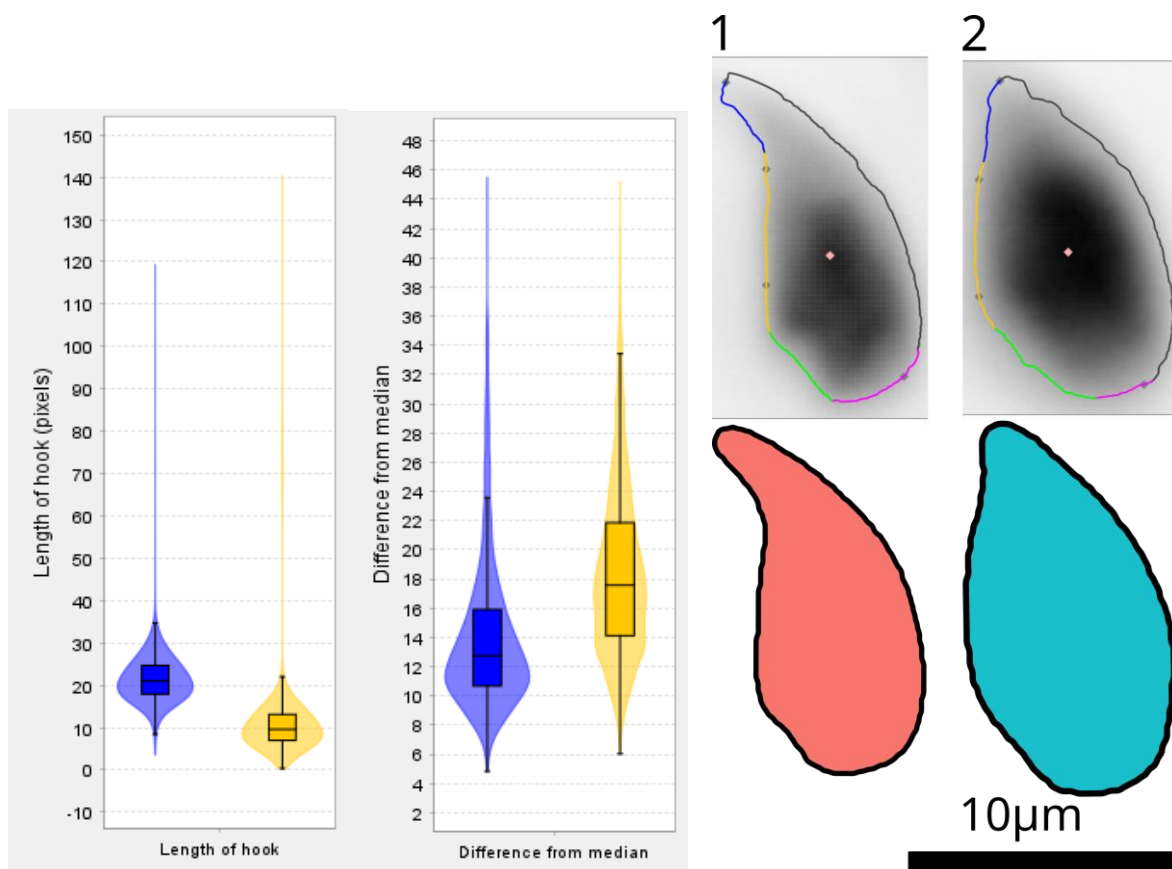


Figure 50 difference in hook length and difference from median are plotted with cluster 1 in blue and cluster 2 in yellow with the representative cell image and consensus image. The region they were separated on can be seen in Figure 28 D.

4.3.4 Using feature-based clusters to characterise morphological phenotypes.

As can be seen from earlier sections there are clear morphological features found within the data and the detection of regions of interest help represent phenotypes in a sample, but the structure of

those phenotypes is difficult to visualise and understand from just the multimodal region clustering. This has caused need for further analysis which is the point of this section. The usage of further analysis allows for the extraction of phenotypes made up of the shared features detected from multimodal regions. This section of the program is not yet automated as it requires a large amount of relatively complex data frame manipulation and so implementation is relatively slow. I have used two main tools to determine the morphotypes of the samples based on features and groupings. The first being using rand indexes (Vavrek, 2020) (this is the calculation of similarity of assignment of clusters used to determine if they agree) to determine the relationship between the sets of clusters created in the multimodal clustering. Thus, determining the relationship between the cells and their region of interest clustering showing which features are related. This was then filtered into three groupings: high, medium, and low confidence groupings low being a rand index between 0.5 and 0.6, medium being between 0.6 and 0.7 and high being over 0.7. This was decided based on the distribution of the clusters and may need to be edited in the future. The high, medium, and low confidence groups give thresholds which are needed to detect phenotypes. Using just these rand indexes-based combinations causes the issue of there being exponentially more combinations of clustering identifiers from rand indexes, and each combination could have at least one cell in it. To combat the multiplicative effect of combining clustering identifiers to start with only the largest groupings were used to make the datasets more processable.

4.3.5 Clustering from purely the rand indexes

4.3.5.1 High confidence groupings

Angle profile 9-15 and Diameter profile 52-57

The first grouping found with high confidence is the region of interest clustering 2 and 6. This detected Angle profile 9-15 and Diameter profile 52-57, the angle region is based around hook structure and the other is the vertical diameter. These clusters are combined by simply joining cluster identifiers into a larger cluster identifier for each cell individually so for example if a cell was in clustering group 1 for the first region of interest and group 2 for the second region of interest it's

new cluster identifier would be 12 (this works as the algorithm used to decide optimal cluster numbers cannot pick more than 8 clusters).

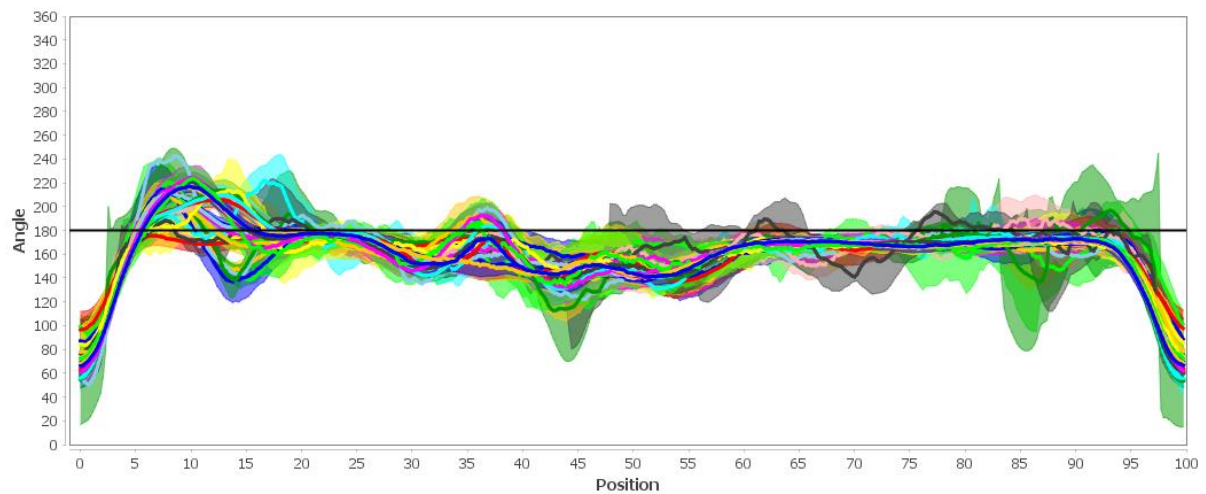


Figure 51 The 25 clusters produced by combining the clusters 2 and 6.

This graph shows that there are too many groupings to be informative and so a way of amalgamating them is needed.

This gives a complex and hard to categorize phenotype grouping as it categorises 25 morphotypes (Figure 51) of the cells however not all categories a reasonable number of cells. The morphological regions have large amounts of variation and as there are 25 possible combinations of the two multimodal region clustering's that are both made up of five clusters. This is relatively useless however shows the need to for some method to condense this data if there is any hope of there being use of it.

Radius cluster 0-3 and 96-99

This grouping Figure 52 potentially shows the need for wrap around (can select indexes from 96-3 through 0 'wrapping around' the start and end of the profile) multimodal clustering as both clusters are based around the hook radius which allows. Both of these regions of interest simply clustered on hook features. It does show there is value to the usage of simple rand index cluster combining where there are few actual clusters, and the features are distributed very similarly. It shows some of the edge detection errors and some misorientation of the cell from NMA in Figure 52.

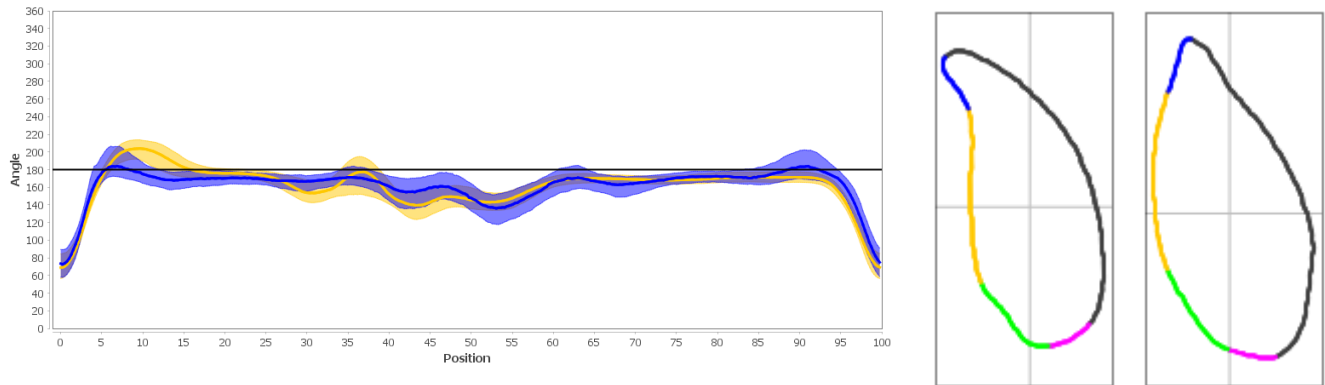


Figure 52 the angle profile and phenotypes produced by combining the two similar radius clusters.

4.3.5.2 Medium confidence grouping

The next step into making sense of the overall dataset was the inclusion of more clustering regions.

The addition of the medium confidence rand index grouping that agree on cluster assignment allow inclusion of regions of interest :1,2,4,5,6,11 (as can be seen in detail in their own respective sections). That includes angle profiles 3-7 ,9-15,34-39, Diameter profiles 0-3, 52-57 And the hook length. This means the clustering is around the hook shape and the tail attachment region as well as central diameter (Figure 53). The inclusion of all these regions it gives a good overall view of the relation of those features to the overall morphology of the sample (shown in Figure 54). However, a much larger set of variables than the previous high confidence groupings for morphotypes gives huge variance of phenotypes detected. This meant a twostep process was used. Firstly, groupings above a threshold for number of cells contained were extracted then they were amalgamated by Hamming distance to their closest group. This resulted in there being 14 morphotypes detected that represented the morphology within the group, they were not necessarily distinct (there is some over or under segmentation of clusters) as the only represent a common morphology. This graph represents these 14 morphotypes.

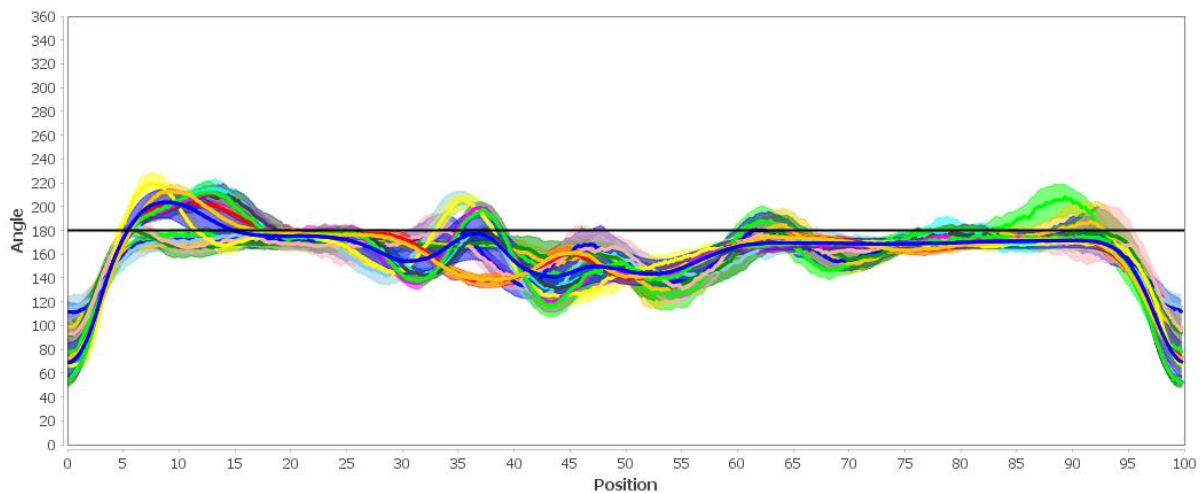


Figure 53 shows the angle profiles of the 14 morphotypes given when performing Hamming amalgamation on the regions :1,2,4,5,6,1.

This is a manageable number of groups to analyse manually in NMA however as can be seen is not easily shown by simple angle profile graphs.

One huge group of cells is made with features very similar to normal rodent morphology that shows the general structural overlap of the whole samples and the error in landmark detection and edge causes a morphological smoothing effect that creates a lot of similarity between cells due to edge detection warping regions around cell that causes less agreement clusters. But this grouping did correctly identify the phenotypes present in the testing dataset that agree with the known phenotypes shown in Figure 27 although requiring some human input. Using some manual amalgamation of the larger groups a Hamster sperm cluster, Fbxo7+/- cluster, PPLL with stubby hook cluster are found (Figure 54). This clustering could not identify the difference between the hook less PPLL and the deformed Fbxo7+/- as the edge detection and landmark placement cannot correctly orient the cells consistently resulting in the cluster segmentation not being able to tell the difference between the phenotypes from edge detection issues. The Fbxo7+/- phenotypes can have multiple hook shapes that result in them looking like cells with bad edge detection that warps the cell shape

to have lumps on it giving a low level of variance across the entire set of profiles making it very difficult to detect. It also showed that bad landmark placement is an issue.

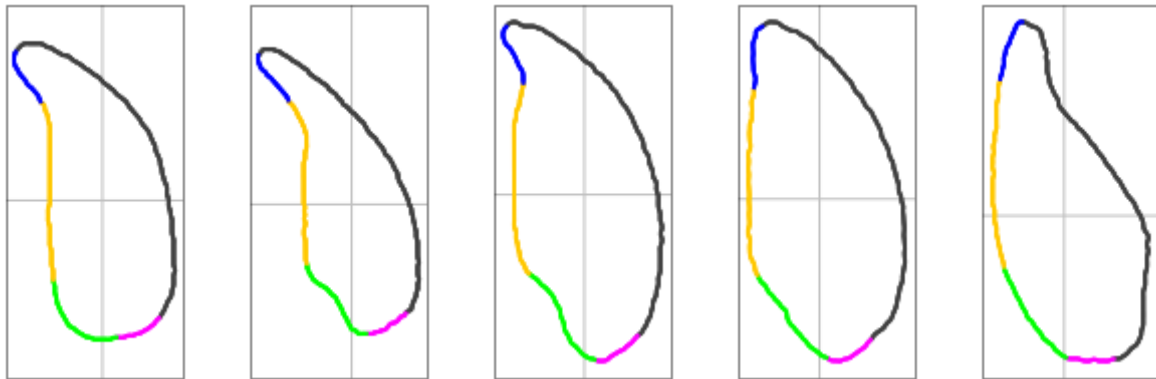


Figure 54 the consensus images of the larger groupings of cells from the clustering regions 1,2,4,5,6 and 11 that have the same cluster identities.

With the inclusion of Hamming clustering the groups become larger and more intuitive (**Error! Reference source not found.**). However, the overall pattern is repeated with the representation of morphological features.

The full process using a Hamming distance to amalgamate the medium confidence rand index groupings clusters the main phenotypes (Figure 55), however, still struggles to characterize phenotypes that are easily swallowed by bad edge detection and landmark placement. This gives an overview of the distinct phenotypes within the sample in detail. It also shows the current limitations of data extracted from NMA and cannot be solved by further data processing in this package.

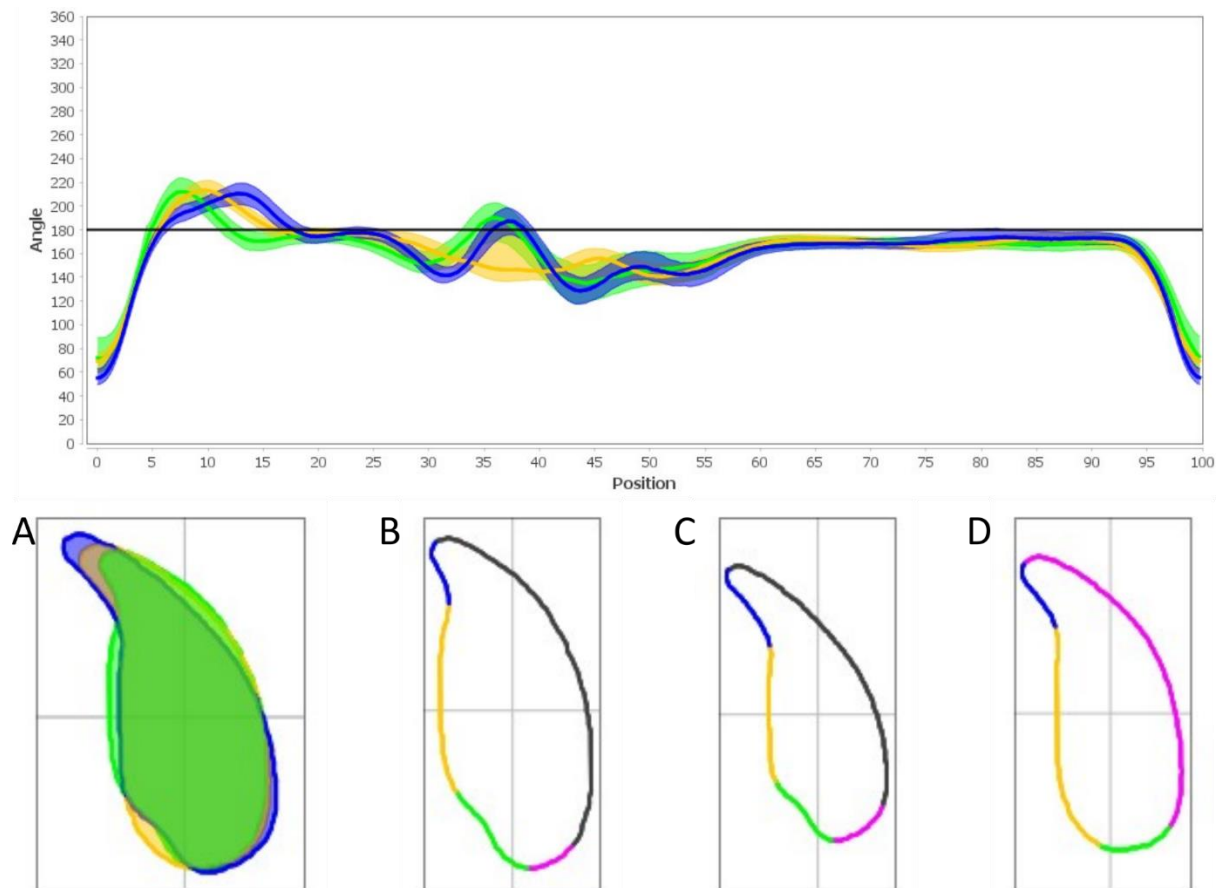


Figure 55 angle profiles and conesus images of the main clusters amalgamated by Hamming distance found of cells from the clustering regions 1,2,4,5,6 and 11. A) is a overlay of the consensus images in the same colours as there angle profiles above. B) is the shorter cells with deformed hooks, C) is the well defined mouse sperm D) is hamster sperm.

4.3.5.3 Low confidence groupings

The low confidence groupings exist for the potential usage with datasets that have loosely connected spectrums of morphological feature distribution that can't be clustered into distinct groups easily. The function of the low confidence grouping would be less of a directly biological useful clustering, but instead as a way to see a continuous morphological phenotype in a cell.

4.3.6 Real Cell grouping tests to detect morphotypes.

The application of analysis with NMA companion with *Musculus X Domesticus* Crosses using a large and a small dataset to show the greater ability to categorise morphological phenotypes. This analysis shows how NMAcompanion can process the data with real samples to prove that it produces

meaningful data on samples other than the artificially diverse dataset made to test it. The two cell genotypes used for this analysis were:

WWLL: This is a highly variable sample as was previously characterized by Watson(2023) in their thesis, a large dataset of 21000 cells was acquired.

PPLL: A more morphologically consistent sample (relative to WWLL) as was previously characterized by Watson(2023) in their thesis and so a smaller dataset of 2000 cells were acquired (due to time constraints). This dataset was thought to be one consistent morphology, however when putting together the testing dataset some preliminary tests using early versions of the NMAcompanion pipeline showed two distinct morphologies. Using NMA companion there were only two real regions of multimodal distributions within the profile data were found.

The usage of NMA companion on two real datasets shows the two different distributions of morphology data that can be created by NMA with this new imaging method and what information that can produce.

4.3.6.1 Analysis of PPLL

The PPLL data is much simpler as it only has two multimodal regions, the radius profile section 0-3, and the diameter profile region 47-56 which when checked with rand indexes do not have great correlation. The reason for this is that the radius profile only categorises the hook whereas the diameter profile shows a larger portion of the cells and detects the various phenotypes Figure 56.

The diameter clusters ran through the full NMAcompanion analysis with no human input shows multiple phenotypes Figure 56.

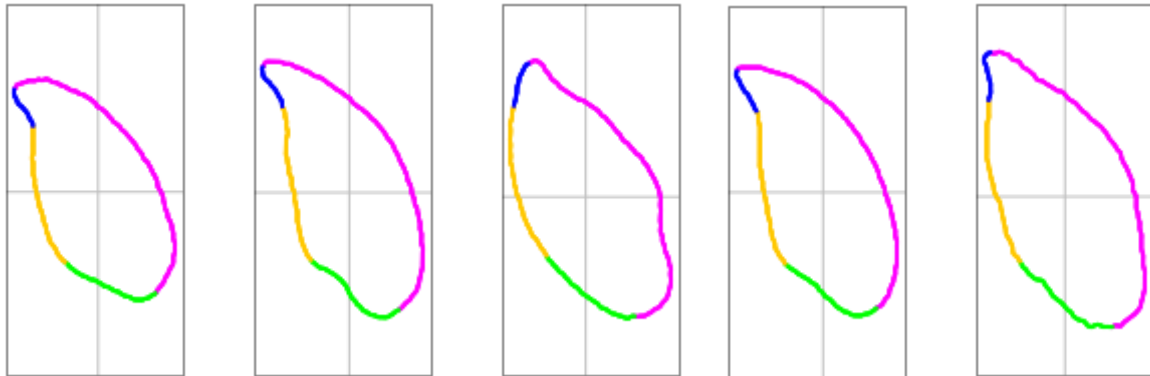


Figure 56 The main phenotypes shown in the diameter clusters.

These clusters are relatively informative but with a simple pass of human editing of the misidentified cells landmarks using the inbuilt NMA interface (this allows for the flipping and rotation of cells that may have been wrongly flipped in the orientation step of edge detection) allows for a new grouping that very clearly shows the two phenotypes of cells (Figure 57 and Figure 58).

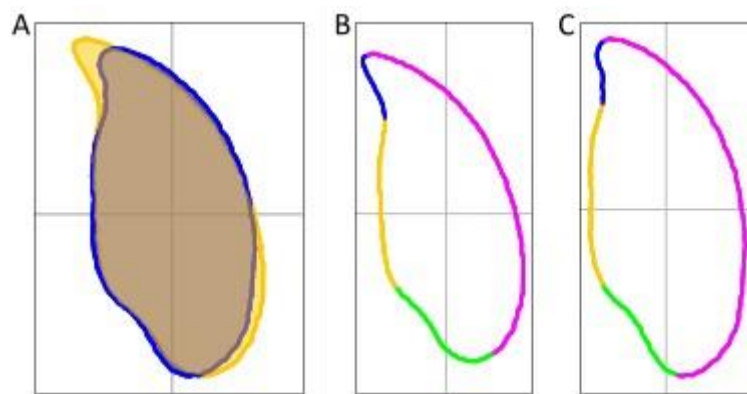


Figure 57 The two phenotypes that are clearly visible when fixing detection errors in the sample. A is an overlay of the two phenotypes. B) is the sharper well formed hook. C) is the dull poorly formed hook.

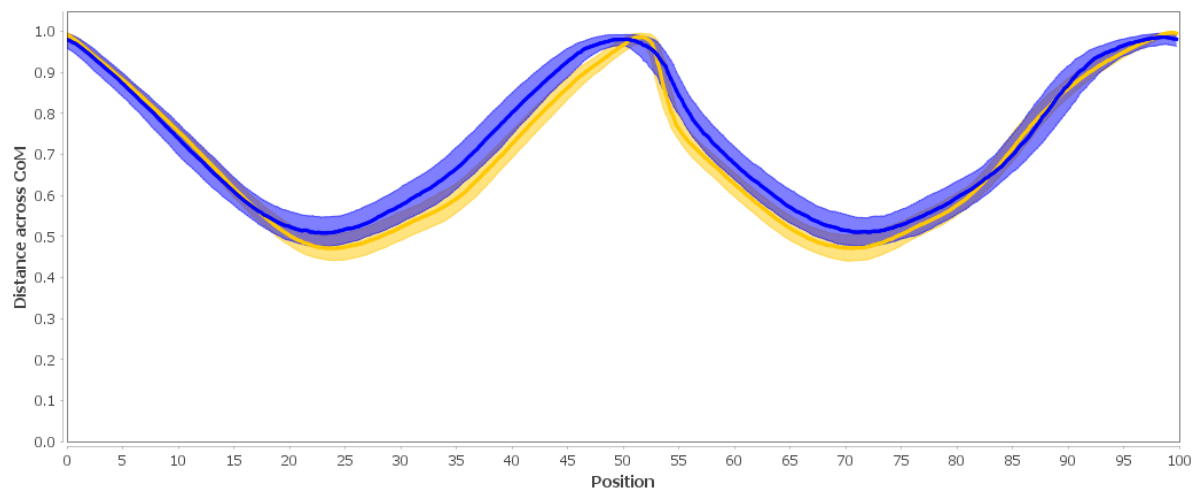


Figure 58 Diameter profile graph of the two phenotypes that are clearly visible when fixing detection errors in the sample for PLL. The yellow shows the sharper phenotype and the blue shows the duller phenotype.

This clear difference as shown in the diameter profile graph which helps characterise the phenotype. The hook is longer, and the lower dorsal region of the cell has a slight bulge in comparison. This detection shows the promise of NMA companion but also some of the issues with landmark placement in NMA.

4.3.6.2 Analysis of WWLL

The WWLL dataset is much larger and has a large amount of variation across the sample. The dataset is too large to run rand indexes on efficiently and therefore to save computing resources needed to be sampled down to 10000. This has reduced some confidence in the groupings, but the output seems to be consistent in the usefulness of the output with other example analyses. When put through NMA companion and Hamming clustered showed a variability but also a consistent general phenotype with 50.17% of the cells in a single cluster the second largest group was the cells with obvious edge detection errors which was 24.93% after that many other cells fit into either smaller group of between 0.4—2% or a single larger group that takes up 8.6%. A considerable portion of the group's 0.4-2% groups are rarer landmark placement errors such as shown in Figure 59 below.

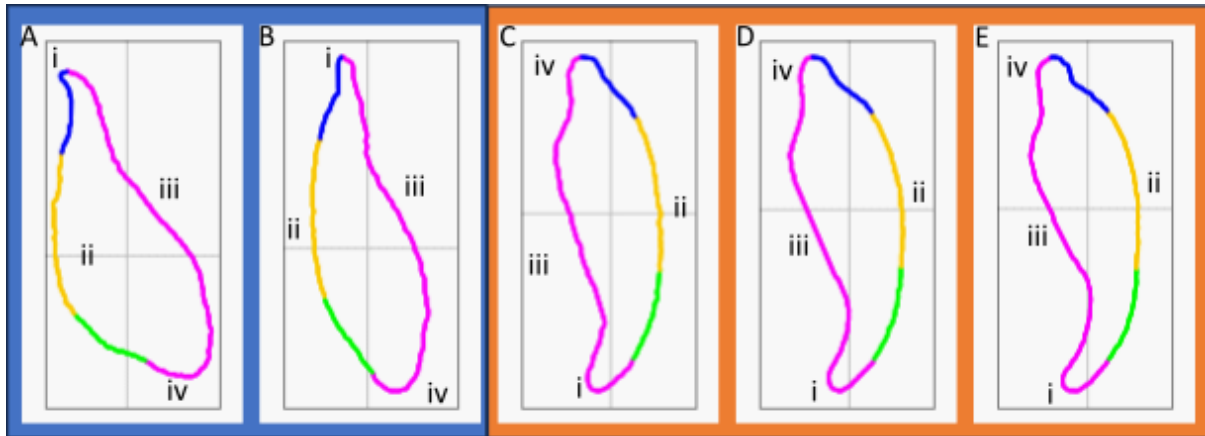


Figure 59 series of the smaller groups and how the detection and land mark placement errors look.

All of the errors have the dorsal and ventral sides misslabeled and the cells in orange also have the tip placed at the basal region instead of at the tip. The coloured regions show sections NMA decided were equivalent and i-iv show the actual parts of the cells. i is the tip, ii is the dorsal side, iii is the ventral side and iv is the basal side.

Some of the cells detected in the first groupings shown of these two figures come from two cells landing on top of each other in such a way that to the landmark placement system they look identical to a deformed cells and so cannot be filtered out without filtering out real cells.

The two main phenotypes visible that the analysis seems to suggest are real are the 50.17% grouping and the 8.6% grouping. The larger grouping encompasses mostly normal mouse sperm whereas the 8.6% cluster shows small very deformed cells that tend to lack hooks and tail attachment regions. Although the 8.6% group definitely contains some artefacts it does suggest that there is a main morphotype with variation that leads to this region of heavily deformed outlier cells which is in almost complete agreement with what was found by Watson, (2023) previously with the morphology ranging from mostly normal (Figure 60 B) with variation moving towards a morphotype similar to PPLL (Figure 60 A and C).

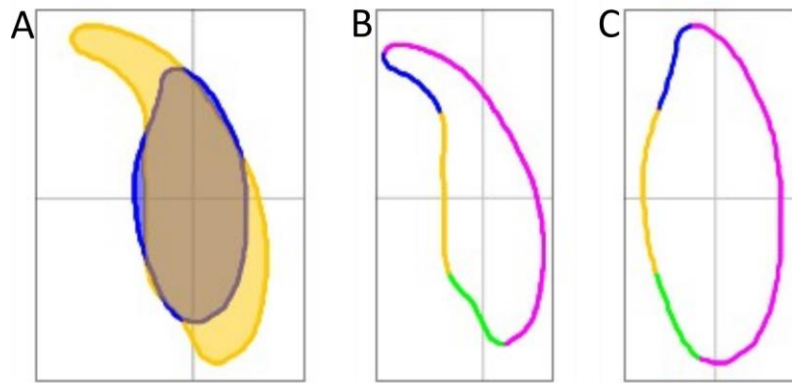


Figure 60 shows the two main WWLL phenotypes

There is however more that can be found due to the application of NMA companion as it shows that the deformed morphotype is more like morphotype of PLL that has a stubby hook (Figure 61 B) than the more normal hook phenotype however it also shows that this phenotype is small (WWLL deformed phenotype is $2\mu\text{m}$ smaller than regular WWLL and $0.5\mu\text{m}$ smaller than PLL) even relative to the PLL phenotype (Figure 61 C) as can be seen in the consensus image (Figure 61 A).

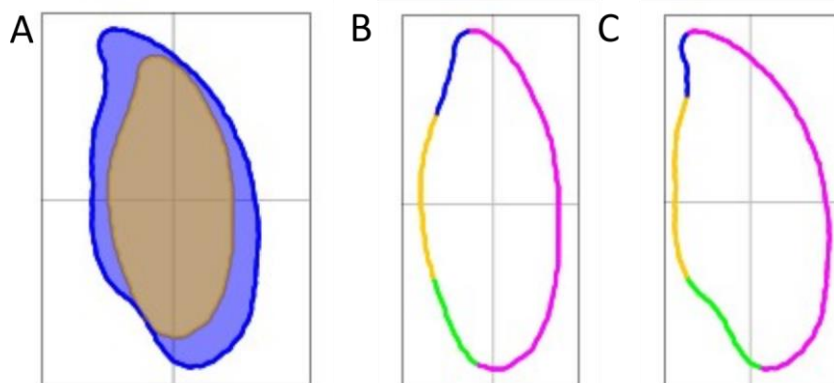


Figure 61 compares PLL main phenotype and WWLL deformed phenotype

4.3.7 The Pipeline as a whole

This example analysis has shown the features of this pipeline and the advantages it has over previous manual analyses, especially the speed of data acquisition, speed of analysis and relative ease of use. The time taken to image enough nuclei for analysis going from 6 hours to 1 hour, and the automated analysis now taking less time, however it is difficult to show tangibly how the time is

saved in gaining understanding of datasets and so analysis time may vary depending on the individual processing the data. The number of individual cells acquired in single imaging sessions has increased to the thousands and with access to better microscopes that number increases to tens of thousands while the work needed to acquire the images decreases. The analysis is reaching a level of automation that removes a lot of the microscopy skill requirements and allows for nuclear morphometric analysis with a much lower investment of individual time and training. The phenotype detection of NMAcompanion as shown in table 2 has some useful features, it is relatively useful at segmenting out discrete phenotypes and although there is some over-segmentation it is still of considerable use as the over-segmentation is relatively easily resolved by remerging groups. Where it really comes into use is in the usage in sample feature diagnostics and phenotype distribution within the sample. The morphotypes given by NMAcompanion are useful for preliminary investigations of samples. The morphotypes as a whole can give useful information on a NMA dataset from as sample as well as the sample, it detects artifacts relatively well although not completely automatically this final step of interpretation of the morphotypes are still human in the loop. Segregating these morphotypes is considerably easier visually as they are generally more

consistent and allow for the determination of whether that morphotype is a distinct phenotype, and amalgamation of a continuous phenotype or an artifact from poor edge detection.

Table 2 three main test runs of NMAcompanion analysis. This table outlines important qualitative information for the usage of NMAcompanion,

Analysis run through	Artifacts detected (%)	Over-segmentation	Under-segmentation	Known phenotypes found
Training analysis	11.0	Some over-segmentation of the largest discrete phenotype on small size differences	Under-segmentation of continuous phenotypes	All known phenotypes found however the continuous Fbxo7+/- phenotype was poorly segmented
PPLL	25.2	Many artifacts over segmented, real phenotypes unaffected	Little to non	Main phenotype represented well; dataset quality shown to be relatively low
WWLL	~27 (depends on the level of landmark error)	Large amount of over-segmentation due to artifacts	Potential under-segmentation of continuous phenotypes	Main phenotype represented well, and sub-phenotype found however the nature of it was not quantified

4.4 USING THE NEW IMAGING PIPELINE TO PERFORM BIOLOGICAL COMPARISONS OF HAMSTER AND MOUSE SPERM TO UNDERSTAND THE DEVELOPMENT OF THE RODENT SPERM HEAD SHAPE ACROSS 30 MILLION YEARS OF DIVERGENT EVOLUTION

Given the availability of hamster sperm from two species, we wanted to assess the variation in phenotypes to compare against the very diverse mouse phenotypes already described.

Two main large datasets were made of the hamster sperm; one of each of our selected species (Phodopus sungorus and Phodopus campbelli). These data sets are orders of magnitude larger (20000 nuclei can be imaged with a similar level of time investment for an individual a 500 used to take without automation) than the old standard for data sets and gives a

good overview of the morphology of the hamster sperm. The two closely related species of hamsters have indistinguishable gross morphology as can clearly be seen in

Figure 62. An interesting feature of the hamster sperm is that they have no dent in their basal region of their nucleus where the tail attaches unlike mice.

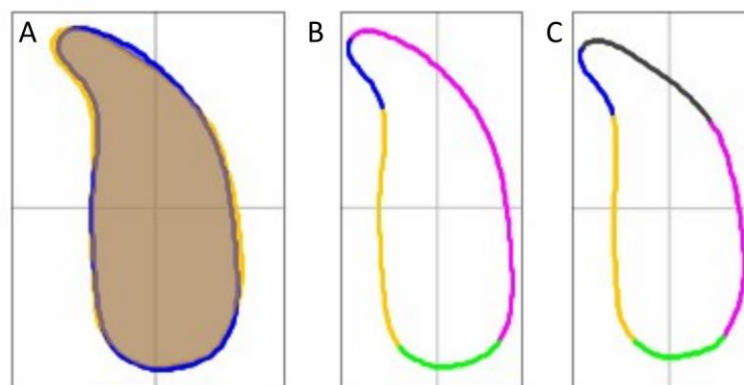


Figure 62 comparison of the hamster species and are identical in terms of gross morphology.

A) is overlay of Phodopus sungorus and Phodopus campbelli B) Phodopus sungorus and C) Phodopus campbelli)

Much of the is comparison between mouse and hamster sperm can be seen already in section 4.3.3 and repeating it airs on the side of redundancy however there are clear regions that exhibit different features to mouse sperm. This is supported mainly by the analysis with the test dataset in detail and shows these differences such as in angle profile region 27-32 which shows a structure of the lower ventral region that is distinct from in the mice, section 4.3.3.3 shows this in detail and the difference is shown in Figure 36 and Figure 37 and 4.3.3.4 Figure 38 and Figure 39 that characterise the tail attachment region of hamsters.

A direct comparison between a consensus of mouse nucleus and a hamster nucleus as shown in Figure 64 from healthy rodents with low levels of variation within the samples. The main differences between the nuclear morphology of the hamster and mouse sperm are very clearly shown in this

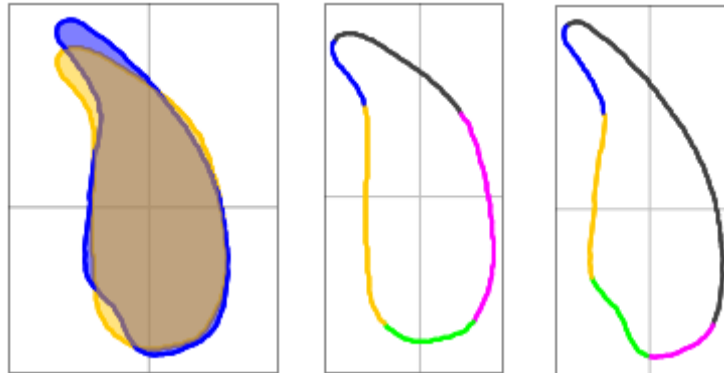


Figure 63 overlaid hamster and mouse sperm (The hamster sperm is in yellow, and the mouse sperm is in blue.) with hamster and then mouse sperm after.

This exhibits the morphological differences between the two species especially the hook curvature and tail attachment region.

diagram. The main differences are at the tail attachment region and the curvature of the hook. This hook curvature is not massively different from some of that found in many more variable rodent sperm types as can be seen in section 4.3.3 throughout as there was no clear separations on the zones relevant to this hook curvature, it is much more variable in mouse sperm than in hamster sperm. The hamster sperm in general is very consistent as can be seen in Figure 64 which shows the difference from median of a mouse sperm

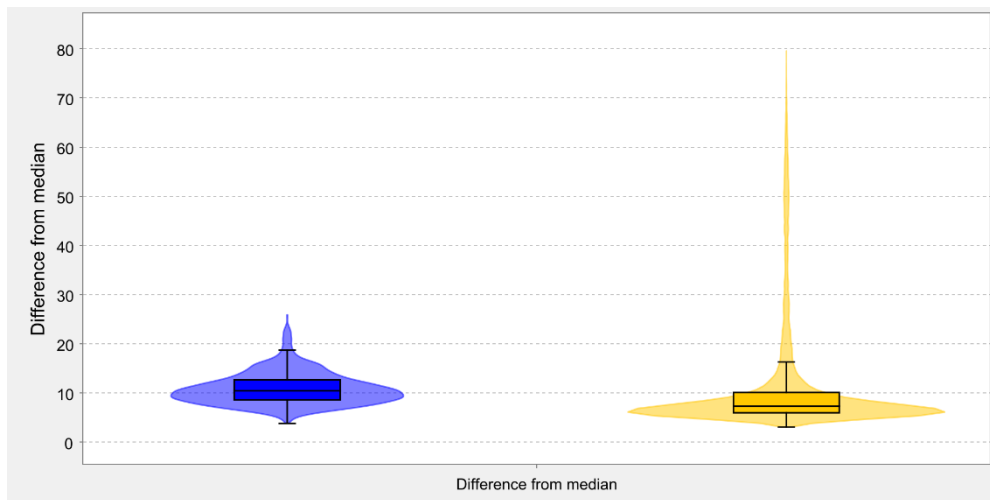


Figure 64 shows the difference from median angle profile (produced by taking the median value at each index giving an angle profile that all other angle profiles can be compared to but generally won't match perfectly) of the hamster and mouse sperm from sample images taken on the same microscope.

The hamster sperm is in yellow, and the mouse sperm is in blue.

Sample and a hamster sperm sample to show the level of variation the samples have. The hamster sperm has a larger range of the distribution because the dataset is considerably larger.

5 DISCUSSION

The goals of this project have been met with an unbiased automated image acquisition system produced that has allowed months of data acquisition to be streamlined to hours with 20000 nuclei able to be imaged unsupervised over a weekend. That datasets were found to be robust enough for analysis and a bioinformatics pipeline in R was developed for the characterization of morphotypes within a sample. The mouse and hamster sperm studied has been well characterized into distinct feature-based clusters, and their differences and similarities have been clearly shown with these clusters that allow for comparison of morphological feature. Rarer phenotypes of samples have been detected and to a characterised either discretely or by showing the continuous variation.

5.1 THE PIPELINE AS A WHOLE AND ITS PLACE AS A NUCLEAR MORPHOMETRIC ANALYTICAL METHOD

This method has some insights to morphology that are not present in currently used morphometric analysis methods allowing for the distribution of morphological features in a sample to be studied. The usage of the angle, radius and diameter profiles and the dissection of them based on their multimodal regions are directly building from the findings in Skinner *et al.*(2019) and the advancements it allows in segmenting the different features of nuclei. The method has one main advantage over some of the simpler methods which is that it gives insight into feature distribution within samples. Further improvements to methods such as this are likely vital to the continued usage of nuclear morphometric analysis. The current standard is sperm images in the thousands and tens of thousands of nuclei such as in Skinner *et al.*, 2019; Skinner *et al.*, 2019; Kopania *et al.*, 2022 where each study had less than 20000 nuclei studied. This study however had over 50000 with considerably less time investment, and most of the microscopy done an individual with considerably less experience, which is a simple improvement over the current literature methods.

The context put into the morphological differences by being able to characterise the differences between phenotypes within a single sample is another direct improvement on other methods in the literature. There are considerably more parameters of morphology than used as standard in morphometry provided by NMA, and with the assistance of NMA companion, they are put into context. The pipeline has room for improvement especially in landmark placement, as either methods of orientation independent feature extraction in NMAcompanion or increasing the quality of landmark placement in NMA. This pipeline is also lacking a common feature which is 3D analysis. Tango (Ollion *et al.*, 2013) is good example of an the implementation of 3D using composite Z-stack images which would give much greater opportunity for analysis. NMAcompanion although lacking refinement provides many clear differences between morphotypes of cells that are not present in the literature methods. This method allows for much more sophisticated comparisons between cells

than previous studies using Nuclear Morphology Analysis, as it combats some of the issues previously found with clustering systems and the variation of samples. Previously the method used was manual clustering and some hierarchical clustering based on angle profiles and then UMAPS for visualisation. This previous pipeline is effective for categorising large differences between samples, however when there are subtle continuous phenotypes the pipeline can only show that they are continuous and struggles to characterise the specific features in this kind of sample. This is due to the dilution of morphometric differences when each index valued equally the phenotypes cannot be seen by simple clustering and are hard to characterise in manual curation due to subtle differences. The dilution of features occurs due to the general similarity of cells in most of the measured features detected by NMA which causes the actual differences to be diluted as these differences may be on only 20 % of a profile and so 80 % of that profile dilutes it as the regions are all weighted the same. This is exacerbated by the fact that samples can have important morphological differences that aren't directly related and therefore can be present on overlapping groups of cells.

This problem is solved detecting features of the cells from multimodal regions of the data and clustering the cells based on only these regions, which prevents the features being diluted. The solution to the overlap of phenotypes is the usage of rand indexes on these features to find out which morphological features are related to which morphological groupings. This allows for the multiple different features that make up groupings to be compared in the same dataset, this allows for complex analysis of samples that may contain two independent sets of morphotypes. These can be seen in the test dataset in the results section that exhibits a morphotype based on just hook length(Radius clusters 0-3 and 96-99), which is highly weighted due to an issue with the way the program reads the profiles however there is more support for the clusters given by angle profile 9-15 and diameter profile 52-57 rand indexes this shows that the regions can independently identified.

5.2 THE USAGE OF THE PIPELINE FOR ANALYSIS OF RODENT SPERM IN GENERAL AND HOW IT HELPS IDENTIFY STRUCTURE

This pipeline from acquisition to NMAcompanion was built using analysis of rodent sperm for testing. Due to this, it is very efficient at processing of rodent sperm data. This pipeline allows for a much greater ability to compare the features of rodent sperm. It also allows for a much higher speed analysis of rodent sperm phenotypes and opened plans for multiple new analyses to be made in rodent sperm. This includes a plan to study the sperm of mice from a gut microbiome study to see if it affects the morphology of the sperm. In this study the pipeline has been used on many different rodent samples already, that have shown some insights that suggest that it likely may be useful to perform it on all samples at the lab at some point as there have been some new morphological subtypes detected on some of the samples that were not detected in previous studies. The morphological features in samples with large amounts of variation may have more order than previously thought. This can be seen in the new morphotypes found in the PPLL *musculus X domesticus* cross data that has two distinct morphotypes based on their hook structure that were not visible from clustering angle profiles that were manually validated.

This calls into question that there may be considerably more distinct morphologies (with specific distributions of morphological features) in the samples studied previously. This however is not certain, as highly variable samples have been studied in this study and the results weren't as useful as desired with the general result being a main morphotype detected and a minor morphotype detected then a series of different intermediaries in between that share features between both. This structural difference is likely because mice naturally are promiscuous (Ramm *et al.*, 2014) and in nature have consistent sperm similar to the wild type hamsters studied in this study however the mice we are studying are inbred strains and crosses selected for their interesting sperm and therefore are likely to have odd morphologies caused by few mutations unlike a sample from a

monogamous species such as humans that may have many different mutations that have built up over time that effect morphology negatively.

A potential future study for developing the usage of NMAcompanion against sperm of a real sample that has multiple distinct morphologies instead of an artificially diverse dataset with known composition. A good target of study for this would be the sperm of Balb/C mice or a similar strain as they have been inbred for a long period of time (Nakamura, 2013) which has artificially made them comparable to a monogamous species with low sperm quality as their breeding has been artificially monitored by humans for over 230 generations. Their sperm is well documented in previous studies to have obvious morphotypes (Skinner *et al.*, 2019) however the exact differences between these morphologies are not completely categorised region by region as is now possible with NMA companion. This can potentially help give greater insight into the factors causing these morphological differences and give the structural changes more biological insight as with access to samples from multiple individuals and access to their environmental and genetic information. The environmental and genetic information can then be correlated with the changes in distribution of morphological features between individuals.

5.3 INFERENCE ON THE STRUCTURE OF RODENT SPERM BASED ON NUCLEAR STRUCTURAL

FINDINGS OF CLOSE RELATIVES

The ability to actively observe different regions of the cells in detail and compare their features allows for greater insights towards the structure of the nuclei studied and allows a better comparison between their morphology. This allows for theories on why they possess that morphology such as developmental issues caused by various processes during spermatogenesis or other causes of differing morphology such as nuclear damage from environmental factors such as oxidative damage (leaving a slide on the lab bench overnight and DAPI leaching out) or damage from other similar sources.

Hamsters and mice are evolutionarily different, living in different environments with mice ranging most of the world but hamsters being more isolated in the middle East and central Asia, but their sperm is generally quite similar in size and shape sharing the same falciform hook. There is 30 million years of evolution between them and yet their nuclei are almost indistinguishable in the upper

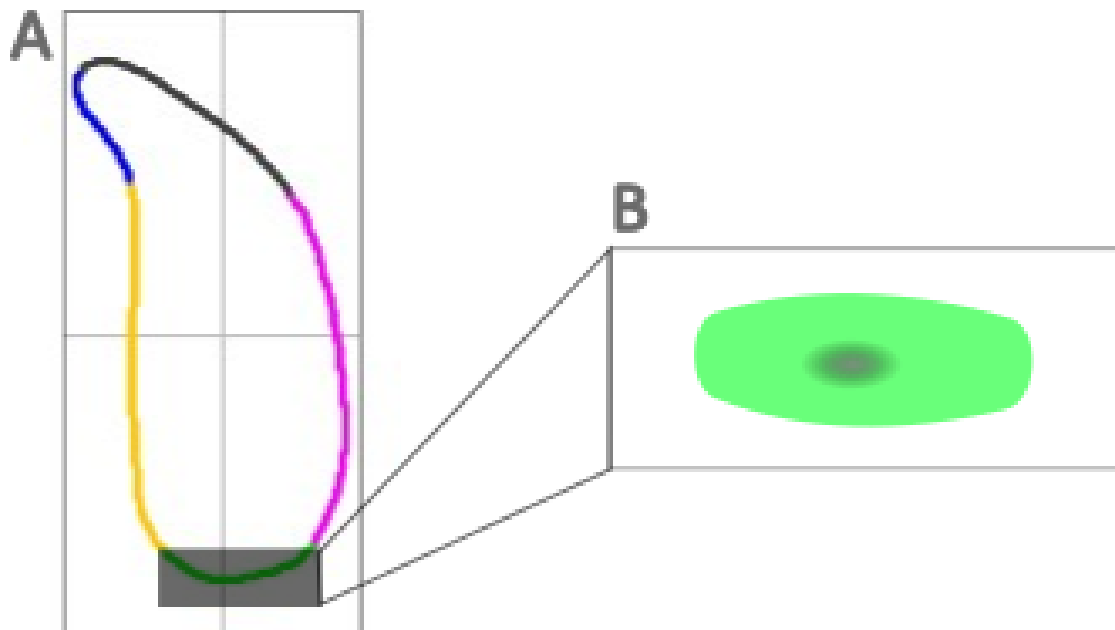


Figure 65 Diagrammatic representation of a basal view of a hamster sperm.

A) a consensus image of the hamster sperm. B) basal view showing a potential tail attachment region of the hamster sperm.

dorsal and ventral region. From the analysis completed there is a major difference between the hamster sperm and the mouse sperm tail attachment region as it is in a completely different place and structure (Figure 64). The tail attachment region in hamster sperm is smooth in consensus (Figure 66A) there may be a dimple in the bottom due to a more three-dimensional tail attachment of the sperm which was shown in some preliminary three-dimensional images. A diagrammatic representation of this tail attachment region can be seen in Figure 66B which shows the dimple being surrounded by the rest of the cell making it out of perspective from 2D imaging.

The tail attachment region of the mouse nucleus is much more defined with an indent that is not present on the hamsters with that region being relatively symmetrical in hamsters. The two hamster species diverged from a common ancestor between 0.1 and 1.7 million years ago in comparison to

their divergence from *Mus musculus* 24.2 - 33.0 million years ago. This divergence time puts them as close enough to be interesting as they are similar morphologically in sperm but have many other relatively large morphological differences with the rest of their overall morphology such as mice having tails and hamsters only have little nubs. This contrasts from the findings in mice in studies such as in Skinner *et al.* (2019) where two similar species of mice: *Mus musculus* and *Mus spretus* were studied that were of a similar difference evolutionarily (2 million years) but had vastly different sperm morphologically. The hamster species are so close that there has been some debate over them being the same species, but suffer from hybrid unviability (Brekke and Good, 2014) that has significantly different mechanisms of unviability to that of *musculus* X *domesticus* crosses (The hamsters have a postzygotic barrier (Brekke and Good, 2014) whereas the mice have a prezygotic barrier (Watson, 2023)). The differences between the mice and the hamsters suggest that either mouse sperm is highly morphologically variable, or hamster sperm is highly consistent. A way to find out the variability in rodent sperm morphology would be the categorisation of more rodent types, a likely candidate would be the similarly closely related member of the Muroidea superfamily Nesomyidae, performing a morphometric analysis on a few species from this family of rodents would categorise well how mice and hamsters' sperm morphology fits into Muroidea.

The structure and formation of the nucleus in mammalian sperm is relatively well categorised and with the work done in this project in combination with the work done by Kierszenbaum and Tres, (2004); Wei and Yang, (2018); Zakrzewski *et al.* (2020) it is possible to make some inferences on the reasons for the structural difference between hamster sperm and other similar rodents. These previously mentioned studies focus on the acrosome-acroplaxome-manchette complex mentioned them earlier in the introduction. The acroplaxome-manchette complex is likely the culprit for this difference between the basal regions of the sperm morphology as this complex is required for the attachment of the tail of sperm. The mechanism of this however cannot be determined by just fluorescence imaging however it does tell us where and how to look. The reasons for these morphological differences can likely be found genetically and in the spermatogenesis of the animals.

A further experiment to find the reason for this differing tail attachment region could be to perform imaging of testicular sections of the hamsters with additional actin stains such as phalloidin. Imaging with a combination of stains would allow observations the structure of the manchette as well as the processes occurring during spermatogenesis to be characterized in a manner that allows comparisons.

There is also likely a mechanism that affects the condensation of the acrosome-acroplaxome-manchette complex in the PPLL sample that makes it have a stubbier overall shape and deformation around the hook. Since the acrosome-acroplaxome-manchette mediates spermiogenesis and is important for morphology and is condensed by the ectoplasmic specialisation of the Sertoli cell (Kierszenbaum, Rivkin and Tres, 2003). This abnormal shape and hook structure is potentially due to over condensation around the acrosome-acroplaxome portion of the complex during spermatogenesis preventing normal elongation. The reason for this would be able to be studied in an experiment imaging the PPLL cells during spermatogenesis, within the Sertoli cells with stains of the structures like dynins, kinesins or nectins to see the interactions between connecting complexes and molecular motors within the Sertoli cells and determine if or how they were causing this morphological difference. There is also another potential morphological difference detected in one of the samples that can be explained by structures present in spermatogenesis such as in the deformed *Fbxo7*^{+/-} sperm studied in Rathje *et al.*(2019). In Rathje's study it is said that the sperm provides a new model for late spermiogenic failure, and the research done in this study supports that and with the categorisation of the morphology of the *Fbxo7*^{+/-} giving new structural insight into again a potential reason for some of these deformed morphotypes in the *Fbxo7*^{+/-}. There are a few different *Fbxo7*^{+/-} deformed morphotypes that are characterized by double hooks, deformed hooks, and warped tail attachment regions (shown in Figure 27 B but not fully shown in the consensus images due to their continuous nature). The double hooks potentially occur due to LINC complex errors causing the acroplaxome to form in both directions, this could be tested by staining the acrosome to see if the double hooked cells have two acrosomes, the warped tail attachment region

is potentially due to issues with the manchette. The deformed hooks are potentially due to these same acroplaxome issues resulting in the improper elongation of the hook. All of the protein complexes such as the LINC complex in the Sertoli cells having issues in Fbxo7+/- knock outs makes a lot of sense as it supports the findings of Rathje *et al.*(2019) completely with the proteosomes dysfunction causing these series of issues with the sperm.

5.4 CLINICAL POTENTIAL OF NUCLEAR MORPHOMETRICS

There are many applications opened with this advancement that can make nuclear morphometrics a much more useful field of study especially in human fertility as well as animal husbandry and conservation. A first pass of 2D morphometric analysis of human sperm has already been done and has already started to suggest there is likely valuable clinical insight to found (Turner *et al.*, 2020; K. A. Turner *et al.*, 2021; K. J. Turner *et al.*, 2021). This pipeline with the imaging bottleneck removed and a way to characterise the morphotype within a sample, all at relatively low running cost and training requirement, give potential for clinical classification of the nuclear morphology of human sperm. To make this viable the development of a 3D system for edge detection and landmark placement would need to be implemented into NMA as well developing the NMA data acquisition to work with 3D datasets. The reason a 3D system would be needed for human sperm is that its 3D shape has phenotypes that can't be captured in 2D. One of the avenues of interest for the 3D morphometric analysis of human sperm is the usage of AI (Artificial intelligence) based identification of the edges and landmarks of the cells using a segmentation model (the yolo model is a good example of this (Redmon *et al.*, 2016)).

The main benefit of using a system like this theoretical Ai assisted 3D version of the pipeline over commercially available systems is the increase in capacity to characterize the sperm with the more in depth feature analysis, this system would not need to necessarily replace systems like CASA ('Proiser Computer Aided Sperm Analysis (CASA) Systems – ISAS and Sundries | Cryolab Limited', 2017). This pipeline instead could provide a different form of analysis to give in depth morphological

analysis for more complex fertility issues that might go undetected due to the sperm only having visually subtle nuclear morphological abnormalities that are connected to other fertility parameters like motility or oxidative damage (Aziz *et al.*, 2004) and otherwise looking like a fertile sperm. The method of imaging used in this pipeline is undoubtedly worse than currently used analysis systems such as CASA ('Proiser Computer Aided Sperm Analysis (CASA) Systems – ISAS and Sundries | Cryolab Limited', 2017) for the highspeed 2D categorisation of simple morphology however this pipeline does hold its own in morphological feature categorisation and does have the capacity to provide valuable information not currently categorised (such as the distributions of different morphological features within individuals) if the adaptation to 3D can be made.

This should then allow for the categorisation of different morphological features of human sperm and their association with specific conditions allowing parameters to be set for disease phenotypes and increasing the quality of human semen analysis. Aberrant sperm morphology is a signifier of male infertility but the root cause of this aberrant morphology is not directly characterised (Sharma *et al.*, 2020). This characterisation of infertility morphotypes of sperm could allow for a greater understanding of the diseases involved and the differences between the causes of infertilities associated with aberrant sperm. Since human sperm tends to have an ovoid spatulate symmetrical shape the nature of the morphological differences characterised and so the type of comparisons made will have to be to find things like bulges and indents.

This system could have value outside of just human fertility such as in animal breeding. Preliminary testing with pig sperm has shown some evidence for variant morphological groups that may be relevant to the current ideas of the distributions of morphology found in samples found. This was however held back by the morphology of the pigs varying in such a way that the system for orientating the sperm for comparison doesn't work effectively resulting in consensus errors like can be seen in Figure 67 ii, iv, vi and ix and xi which all don't show the morphology of the cells they

represent. It may be possible to characterize and test for infertility morphotypes in many animals that are valuable in commercial animal husbandry.

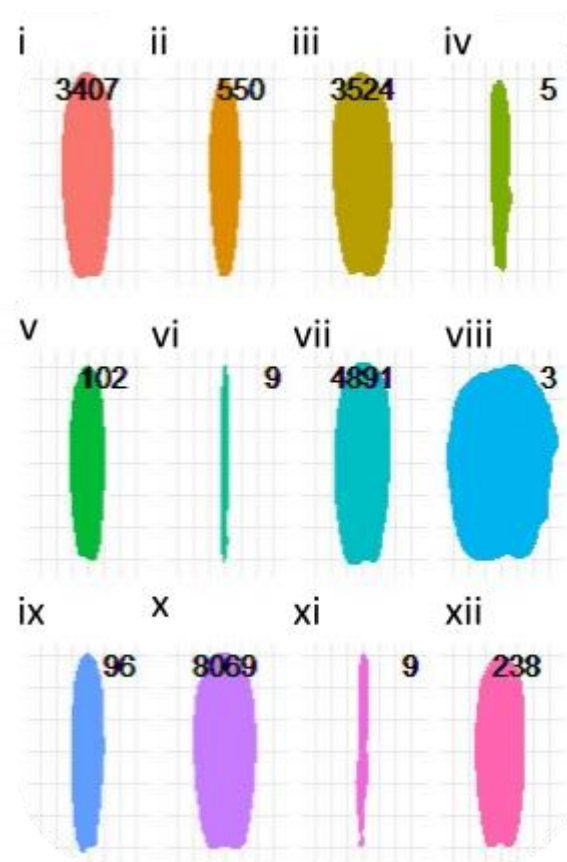


Figure 66 Morphotypes detected with NMAcompanion in pig sperm.

The morphotypes have the number of cells detected in the analysis overlaid on them and are number i – xii to distinguish them.

3D morphometric analysis and Ai based automatic landmark placement, provide the potential for a much more widespread use of nuclear morphometric analysis for cell classification and characterisation of all kinds. Although there is a large amount of other software that is effective more cell classification in fields like immunology, histology, and oncology there may still be novel uses for the morphometric break down of cell features assuming the automated landmark placement is implemented. There are many morphological features in these fields that categorising could provide insight into however this has not been investigated in detail.

6 CONCLUSION

This project has been successful in the expansion of the methodology of nuclear morphometrics. It has opened many new avenues of research and increased the potential for morphological analysis of nuclei. The reduction of time and effort for performing research has made future projects require much less investment and the increase in analytical capacity has allowed for better characterization of the nuclei allowing for new morphological questions to be asked. This project has laid the foundation for the analysis of more complex 3D morphology and shown the potential for male fertility categorisation.

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8 APPENDICES

This is the code used with NMA companion functions to build the figures to explain NMA companion. All other code is available on Github at:

<https://github.com/JosephCliffordPerry/NMAcompanion>

```
# building dissertation graphs
library (dplyr)

makeDissetation_graphs<-function
(graphnum,graphtype,testgraphlist2,minumapgraphs,selected_datasets){
gromblo <-testgraphlist2[[graphnum]][["graph2"]]
ganb<-miniumapgraphs[[graphnum]]

#####
#Make profile graphs
title <- paste (names (selected_datasets[[graphnum]]), collapse = " ")

words <- str_extract_all (title, "\\b\\w+\\b")[[1]]
# Extract words and numbers
extracted2 <- gsub ("_\\d+", "", words[1])
words2 <- unique (gsub ("_", " ",extracted2 ))

numbers <- gsub ("^[0-9]+", " ", title)

# Split the numbers by space
number_parts <- as.numeric (unlist (strsplit (numbers, " ")))
```

```

# Determine the range of numbers
number_range <- paste (min (number_parts, na.rm = TRUE), max (number_parts,
na.rm = TRUE), sep = ":")
#if (any (grepl ("\\d+:\\d+", number_range))) {

# Create new title
title <- paste0 (words2, sep = " ", number_range, recycle0 = TRUE)
#else {
# title <- paste0 (words2, sep = " ", recycle0 = TRUE)}

# Initialize a list to store the matrices
a <- list ()
clusterinos <- cbind (radius_data, clusters[[graphnum]][["Clustering_file"]])
# Loop through clusters
for (j in 1:max (clusters[[graphnum]][["Clustering_file"]])) {
  A1 <- clusterinos %>% filter (clusters[[graphnum]][["Clustering_file"]] ==
j)
  a1 <- apply (A1[1:100], 2, median)
  a2 <- apply (A1[1:100], 2, quantile, probs = 0.25)
  a3 <- apply (A1[1:100], 2, quantile, probs = 0.75)

  # Combine the matrices for each cluster into a list
  a[[j]] <- list (median = a1, Q25 = a2, Q75 = a3)
}

# Create a long-format data frame for plotting
a_long <- lapply (a, function (cluster) {
  data.frame (Position = 1:100, Angle = cluster$median, Q25 = cluster$Q25, Q75
= cluster$Q75)
}) %>%
  bind_rows (.id = "Cluster")

# Create the ggplot plot
x51 <- ggplot (a_long, aes (x = Position, y = Angle, group = Cluster, color =
Cluster)) +
  geom_line (linewidth = 1.2) +
  annotate ("rect", xmin = min (number_parts, na.rm = TRUE), xmax = max
(number_parts, na.rm = TRUE), ymin = 0, ymax = Inf, fill = "gray", alpha =
0.5)+
  #geom_ribbon (aes (ymin = Q25, ymax = Q75), alpha = 0.05) +
  labs (title = NULL ,x = "Profile Position", y = graphtype, color =
"Cluster") +
  #facet_wrap (~Cluster)+
  theme_minimal ()+
  theme (legend.position = "none")+
  theme (title = )
  scale_color_discrete ()

```

```

# Create the ggplot plot
x5 <- ggplot (a_long, aes (x = Position, y = Angle, group = Cluster, color =
Cluster)) +
  geom_line (linewidth = 1.2) +
  geom_ribbon (aes (ymin = Q25, ymax = Q75), alpha = 0.05) +
  labs (title = title ,x = "Profile Position", y = graphtype, color =
"Cluster") +
  facet_wrap (~Cluster)+
  theme_minimal ()+
  scale_color_discrete ()

plotoutput1<- (x5+x51)/ganb[[1]]/gromblo
plotoutput1}

grampth<-makeDissetation_graphs (graphnum = 10,graphtype =
"Angle",testgraphlist2 = testgraphlist2,minumapgraphs =
miniumapgraphs,selected_datasets = selected_datasets)

grampthlabel<-grampth+plot_annotation (tag_levels = "A")
grampthlabel

ggsave (plot = grampthlabel,filename = "grampt10.png",path =
"C:/Users/User/Documents/0stuff/Masters project/Write up/Figures/Results
figures", dpi=300, units="mm", width = 170, height = 239)

```

This is the code used to generate the fixative bar chart.

```

library (ggplot2)
comparison <- c ("PFA" = 46,"Methanol:acetic acid" = 117)
ggplot (comparison)+geom_bar ()
# Load the ggplot2 library
library (ggplot2)
# Load the ggplot2 library
library (ggplot2)

# Data
comparison <- data.frame (Method = c ("PFA", "Methanol:acetic acid"),
  Value = c (46, 117))

# Create the plot with color-blind friendly theme and color palette
ggplot (comparison, aes (x = Method, y = Value, fill = Method)) +
  geom_bar (stat = "identity") +
  scale_fill_viridis_d () + # Use the viridis color palette

```

```
theme_minimal () +      # Use a minimal theme
labs (title = "Comparison of Fixation Methods",
      x = "Fixative used",
      y = "Cells detected")
```