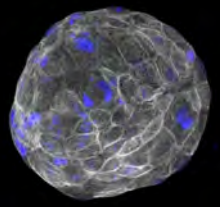
Biomarkers of Sperm Quality in Livestock:

G2P & AI^2

Peter Sutovsky
University of Missouri
ANSCI & OBGYN

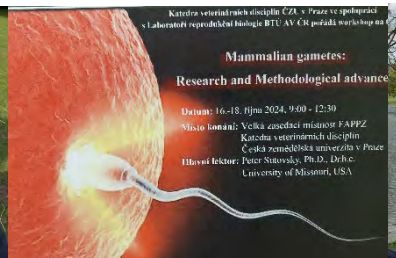


Genome to Phenome Initiative
Putting AI in AI

Thank You

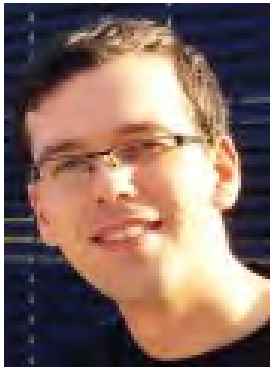
- Prof. Filipp Georgijevič Savvulidi
- Prof. Martin Ptáček
- Organizing & support teams
- Czech University of Life Sciences faculty & staff
- Audience
- All my Czech friends & collaborators




Czech Trainees & Visiting Scholars




Michal Zigo **Veronika Benešová** **Lucie Tůmová** **Barbora Klusáčková**

Jan Nevoral **Miriam Štiavnická** **Natálie Zelenková** **Kateřina Grygarová**

Overview

- Sperm structure & fertility
- Biomarker discovery & validation - putting artificial intelligence in the artificial insemination (AI^2)
- Genome to phenotype (G2P) approach
- Applications, conclusions & impacts
- *Fertility as a low heritability, polygenic trait*

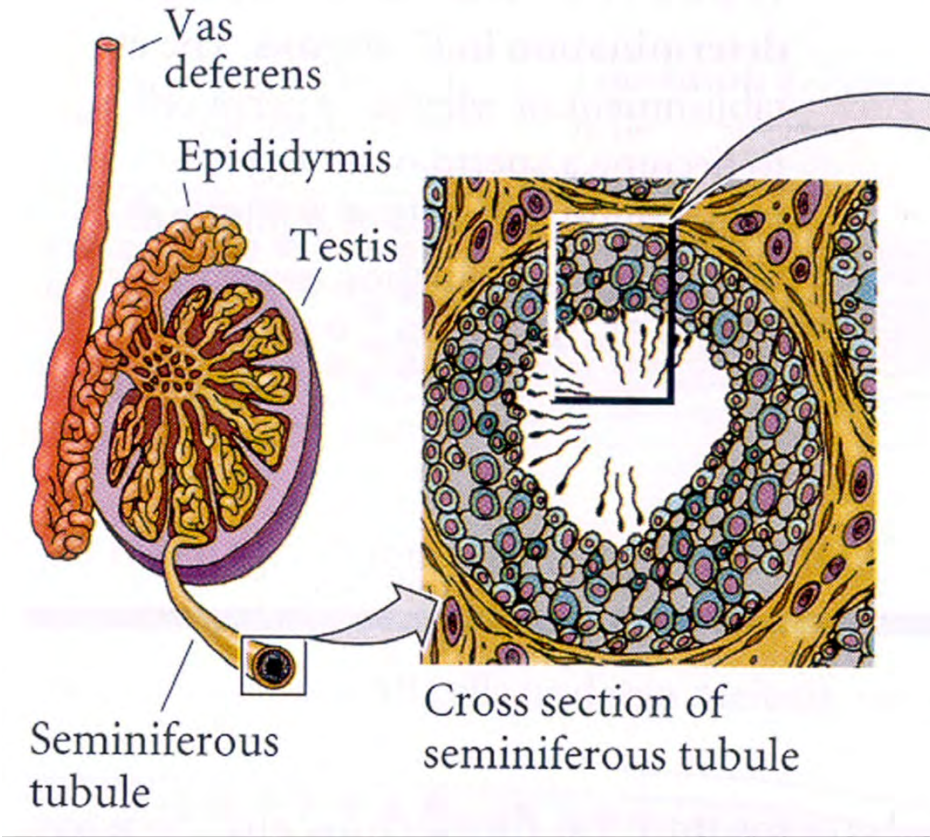
Spermatogenesis & Sperm Maturation

Daily sperm output:

- Bulls: ~5 billion/day
- Boars: 15-20 billion/day
- Men: ~120-180 million/day

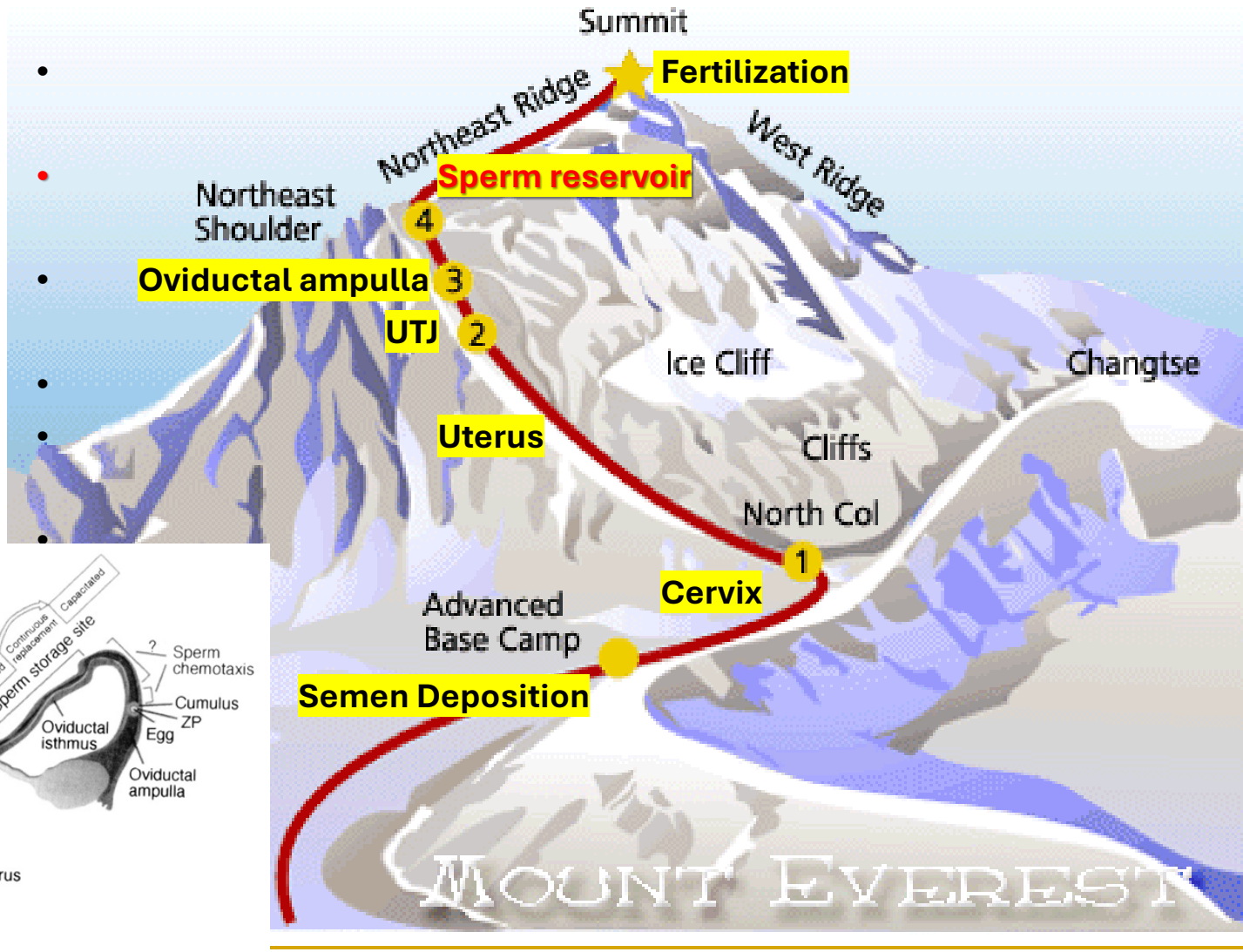
Total seminiferous tubule length in an individual is up to 400 meters (1/4 mile)

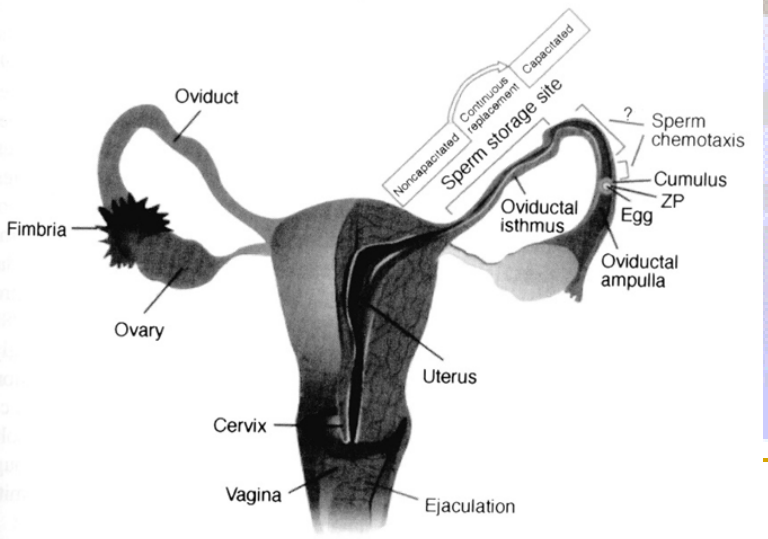
Defects happen



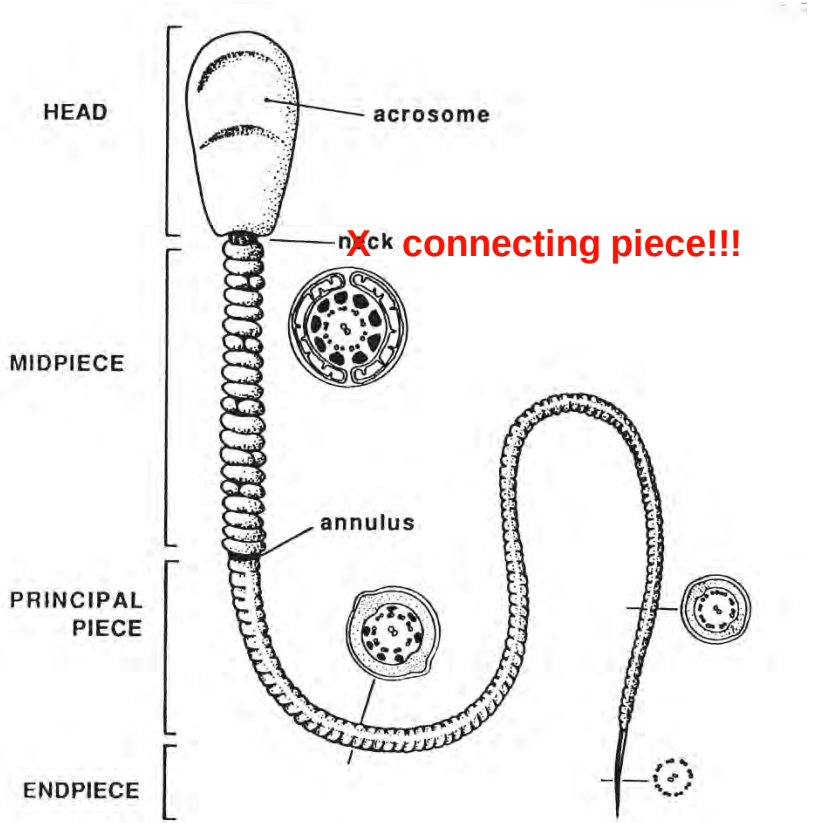
Sperm Transport: **The Conquest of Mount Everest**

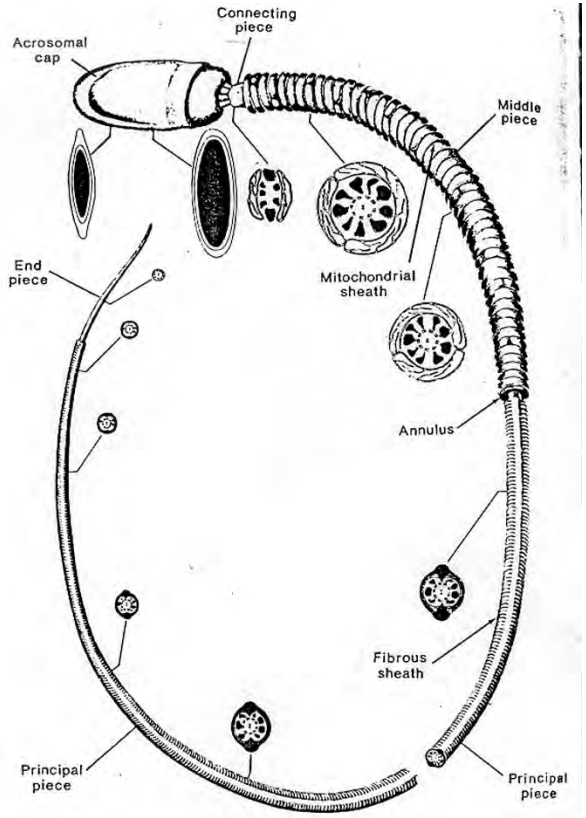
Edmund Hilary & Tenzing Norgay
1953

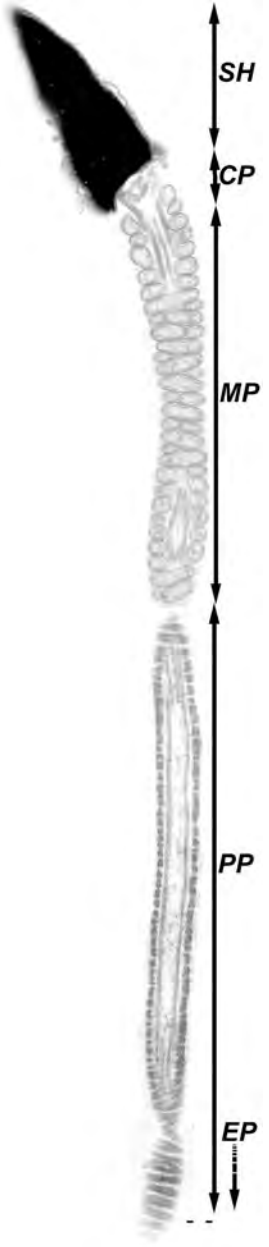


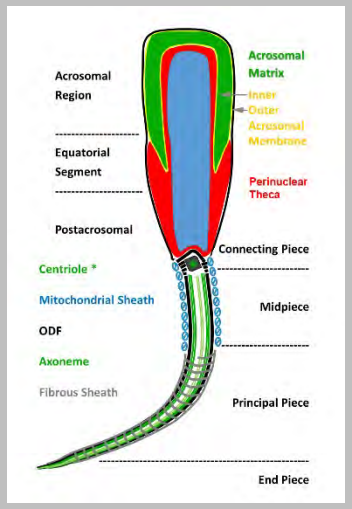


Sperm Structure 101





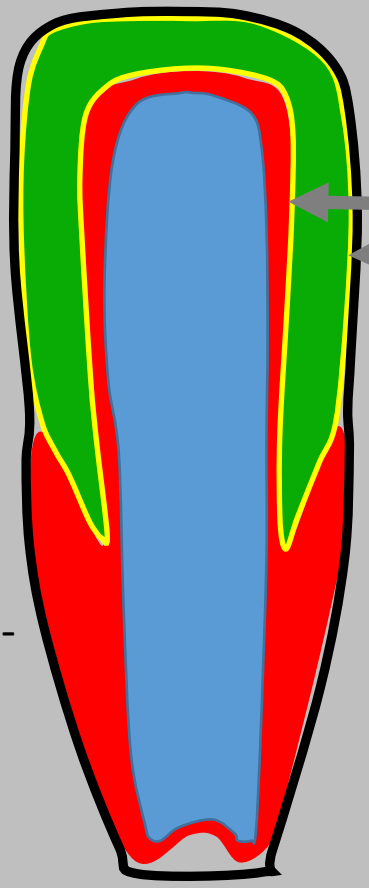




Acrosomal Region

Equatorial Segment

Postacrosomal Sheath



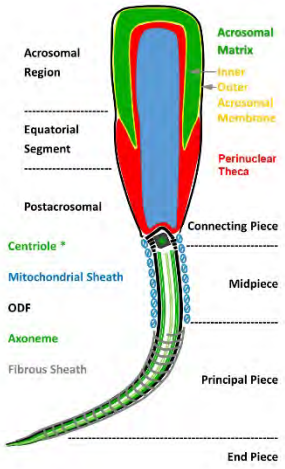
Acrosomal Matrix

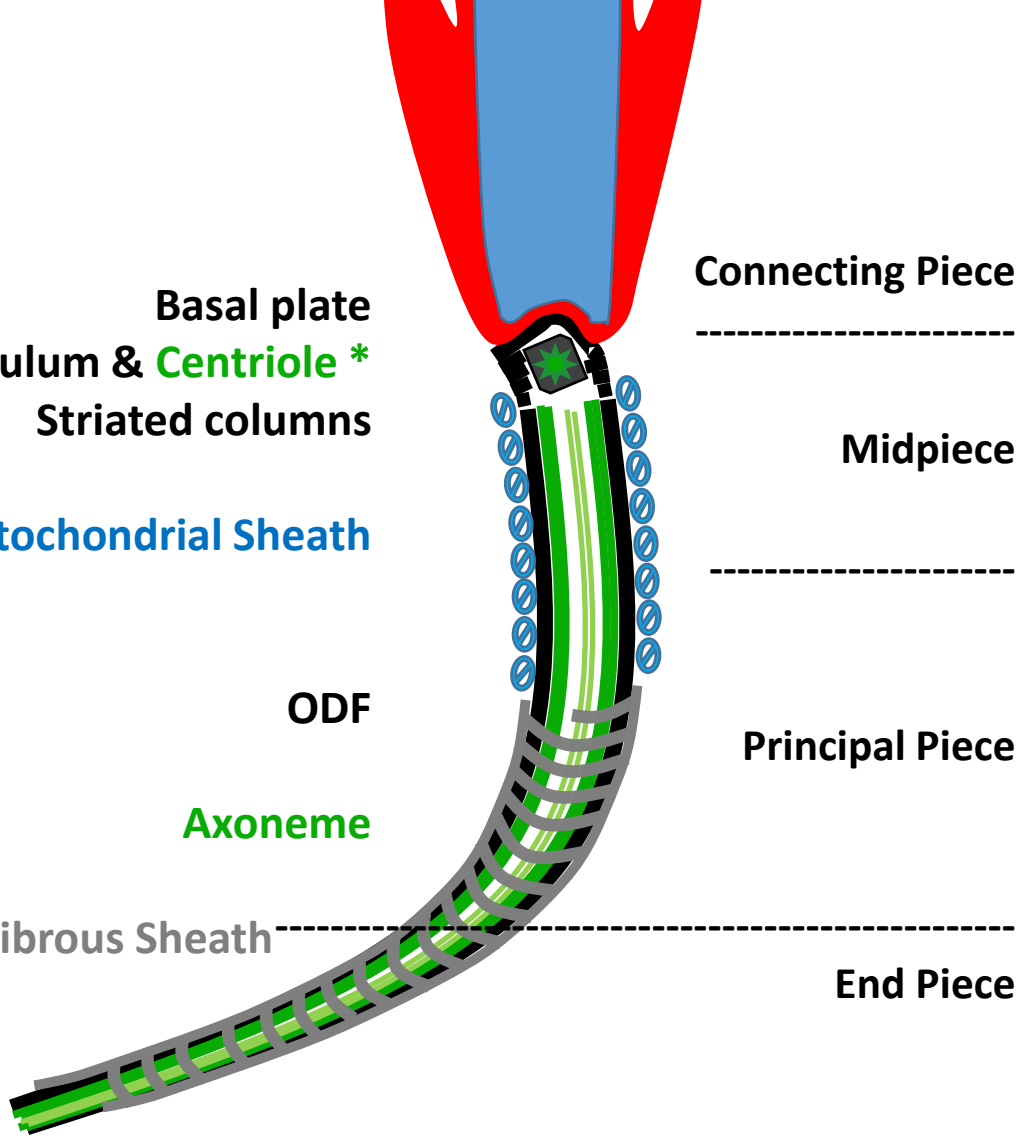
Inner

Outer

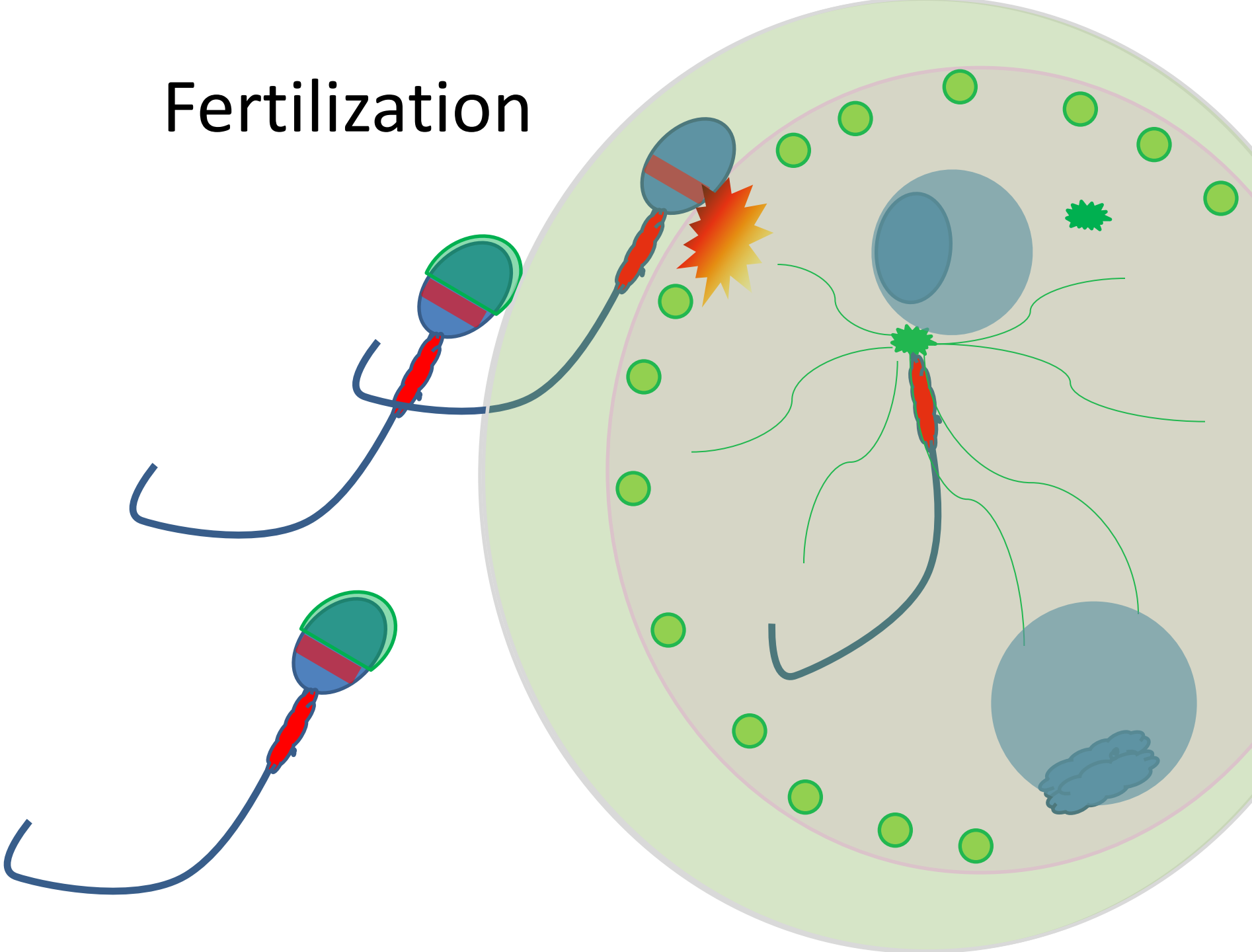
Acrosomal Membrane

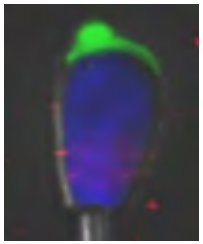
Perinuclear theca

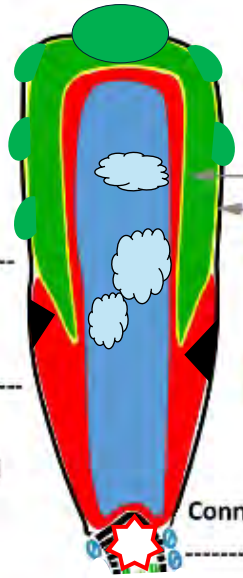

Acrosomal Region
Acrosomal Matrix
Inner
Outer
Acrosomal Membrane
Equatorial Segment
Perinuclear Theca
Postacrosomal
Connecting Piece
Centriole *
Mitochondrial Sheath
Midpiece
ODF
Axoneme
Fibrous Sheath
Principal Piece
End Piece


Basal plate
Capitulum & Centriole *
Striated columns
Mitochondrial Sheath
ODF
Axoneme
Fibrous Sheath
Connecting Piece
Midpiece
Principal Piece
End Piece

Fertilization







Acrosomal Region

Equatorial Segment

Postacrosomal

Acrosomal Matrix

Inner

Outer

Acrosomal Membrane

Perinuclear Theca

Connecting Piece

Centriole *

Mitochondrial Sheath

Midpiece

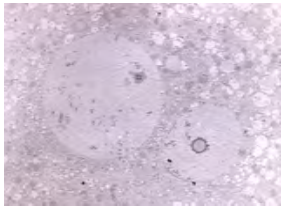
ODF

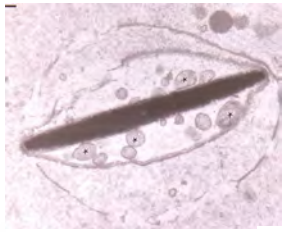
Axoneme

Fibrous Sheath

Principal Piece

End Piece





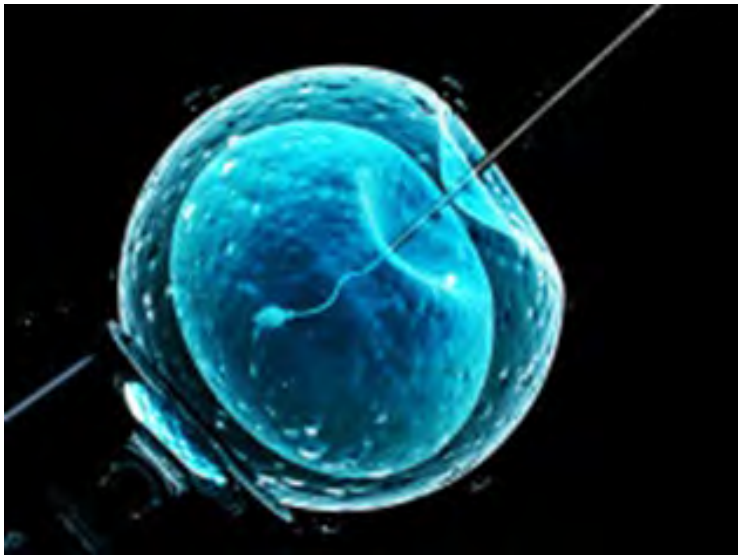
Infertility

LIVESTOCK: Diminished or absent capacity to produce viable offspring.

CATTLE: The goal should be to have 90-95% of cows bred in a 65 day breeding season.

HUMANS: Failure to get pregnant despite having frequent, unprotected sex for at least a year for most couples.

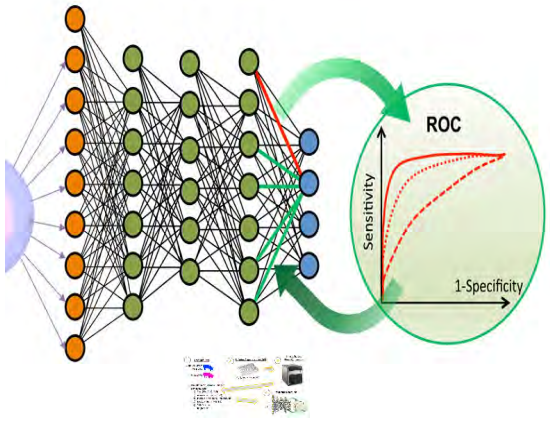
Livestock animal reproductive efficiency, and the success rate and safety of human ART* can be improved by new technologies for semen analysis, handling and purification



*Assisted Reproductive Therapy

Andrology in the Age of Precision Medicine and Agriculture

Looking Back While Moving Forward



JANUS – Two faced god who looked forward and backward.
Month of January was named after him by ancient Romans.

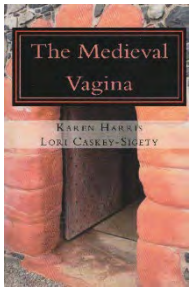
Andrology

Andrology (from Ancient Greek: ἀνήρ, anēr, genitive ἀνδρός, andros, "man"; and -λογία, -logia) is the medical specialty that deals with male health, particularly relating to the problems of the male reproductive system and urological problems that are unique to men.

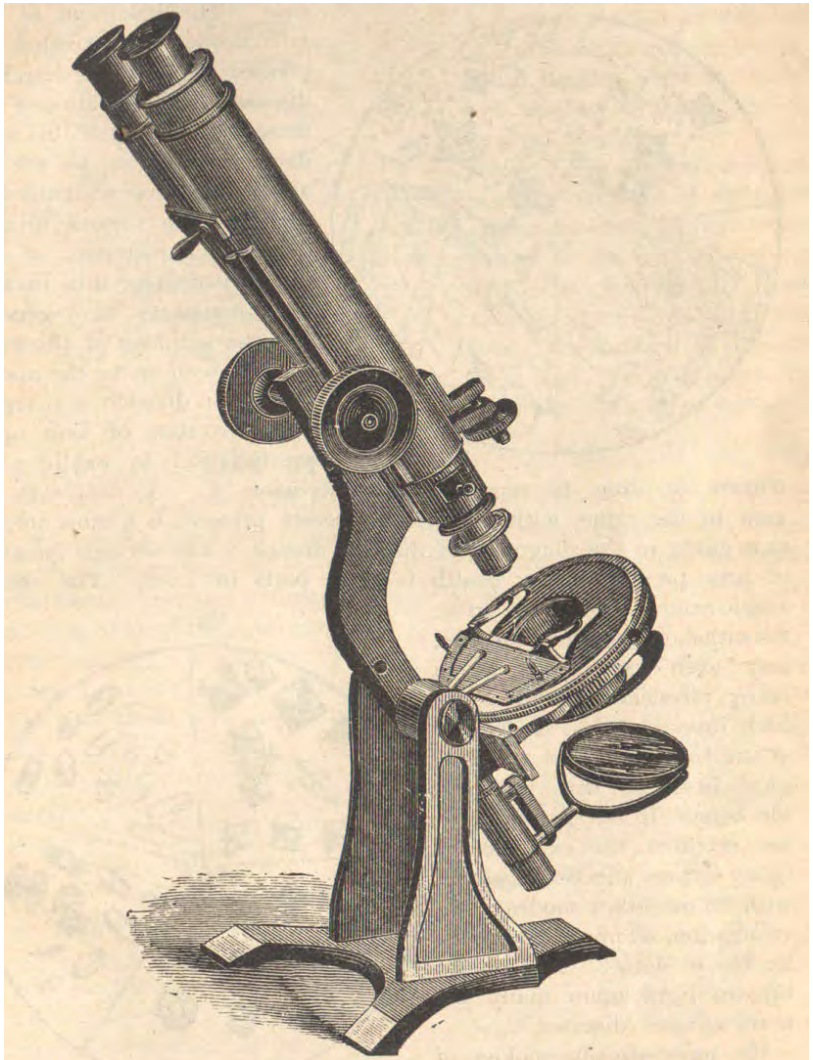
First Fertility Test

Take two new earthen pots, each by itself; and let the woman make water in the one, and the man in the other; and put in each of them a quantity of wheat bran, and not too much, that it be not thick, but be liquid or running; and mark well the pots for identification, and let them stand for ten days and ten nights, and thou shalt see in the water that it is in default small live worms; and if there appear no worms in either water, then they be likely to have children in process of time when God will.

~Common medieval fertility test, often attributed to the female physician, Trota of Salerno (Trocta/Trotula; 1050-1097)

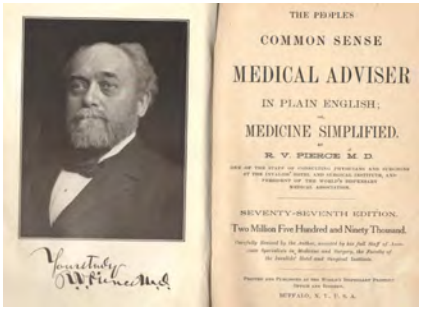


De curis mulierum (On Treatments for Women)
Practica secundum Trotam (Practical Medicine According to Trota)



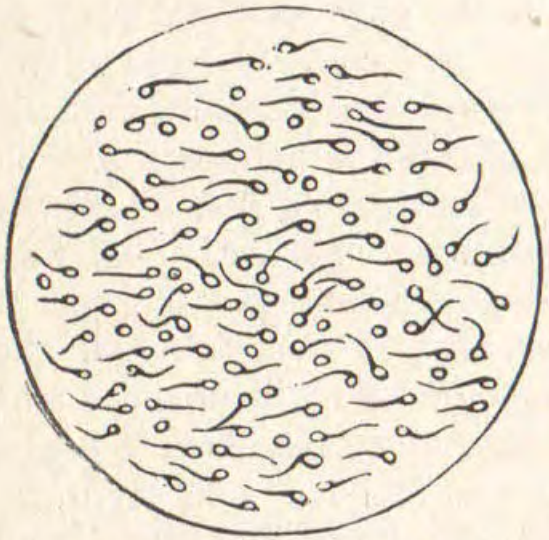
Binocular Microscope used at the Invalids' Hotel and Surgical Institute.

1909: A CAREFUL MICROSCOPIC EXAMINATION IS A VALUABLE AID IN DETERMINING THE NATURE OF CHRONICAL DISEASES OF GENERATIVE ORGANS



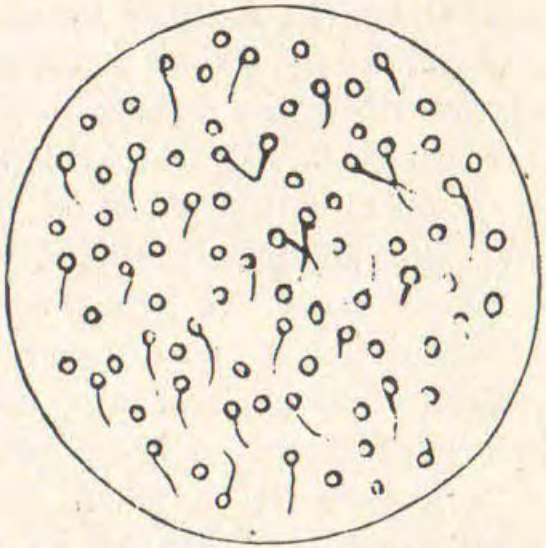
SPERMATORRHEA – SEMINAL WEAKNESS

Fig. 3.



Microscopic appearance of healthy semen.

Fig. 4.



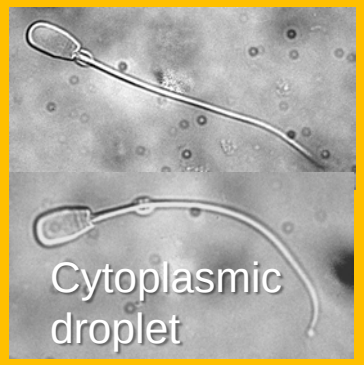
Microscopic appearance of semen which will not fecundate.

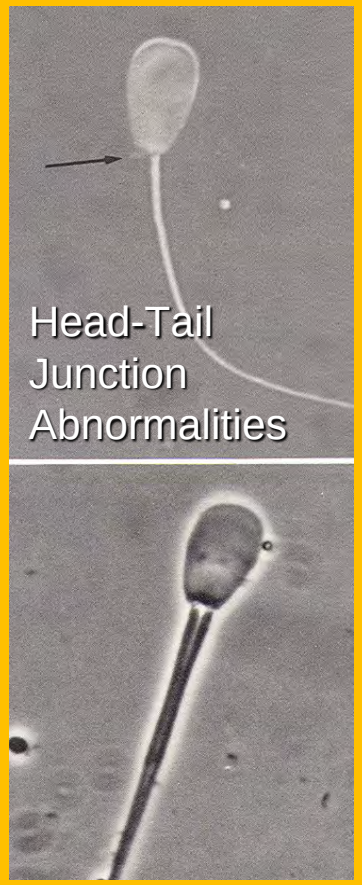
"MAY BE A RESULT OF MARITAL EXCESS"

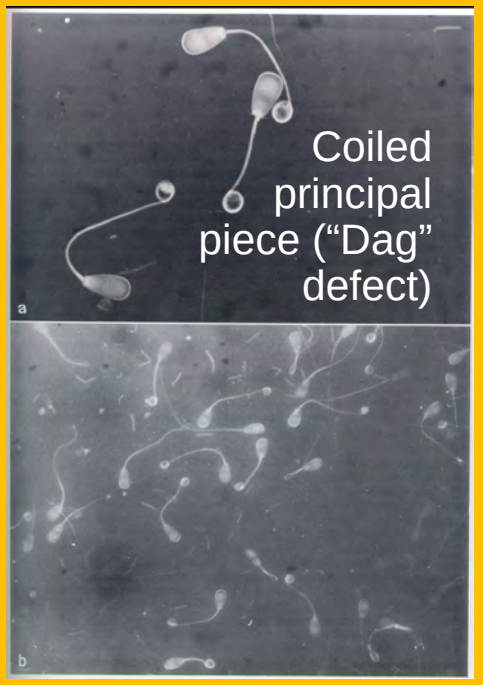
Conventional Semen Analysis 2024

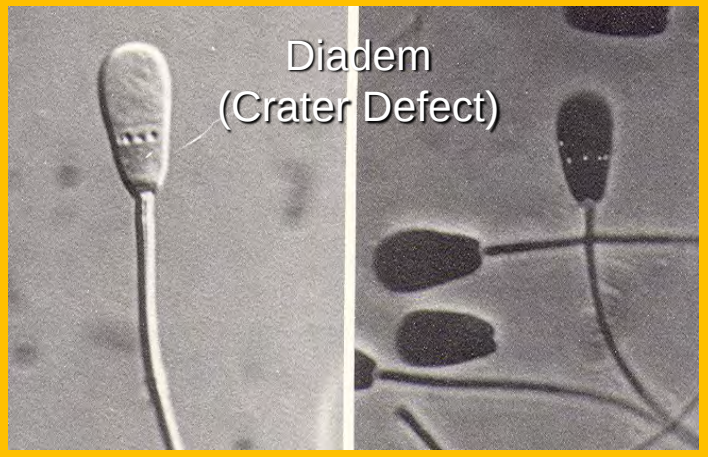
- Front line semen assessment (volume, density, color, swirl)
- Sperm Count
- Motility
- Appearance/Morphology
- Contaminants (Leukocytes, spermatids, epithelial cells, residual bodies, cellular debris)

Livestock Animal Sperm Defects



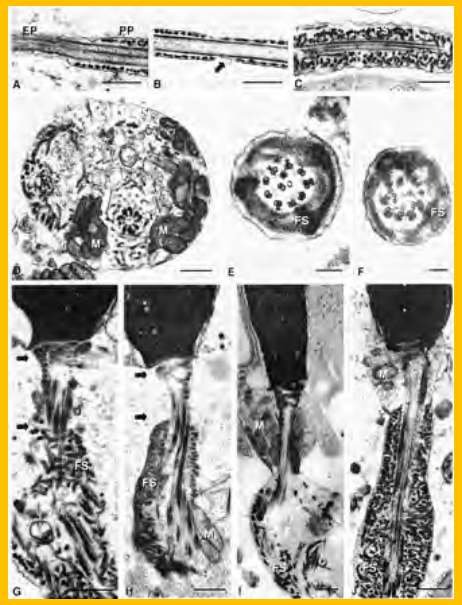




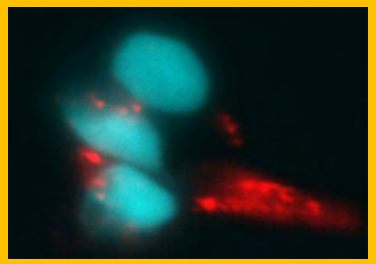


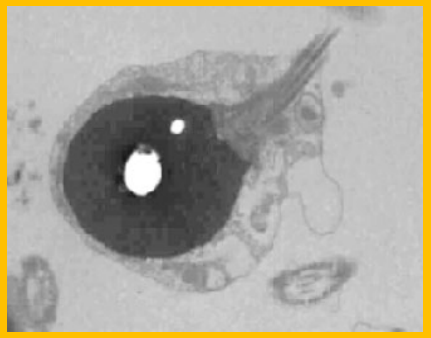
Genetic Defects

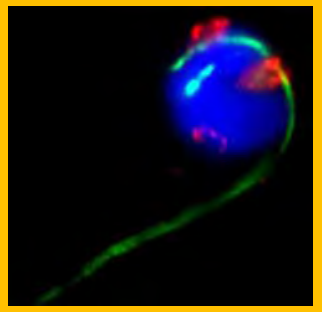
Repro-Toxic Insult



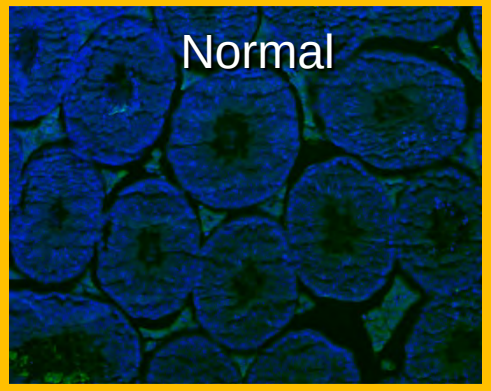
Primary Ciliary Diskinesia & Dysplasia of The Fibrous Sheath (DFS/Stump Tails Syndrome)

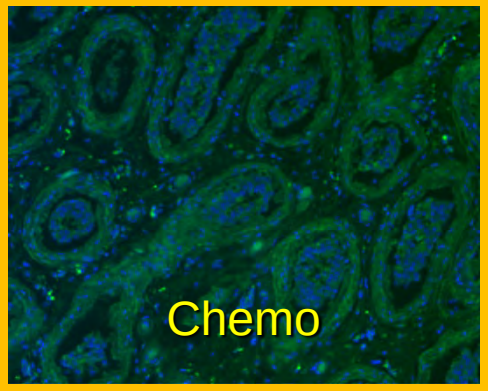






Globozoospermia (Round Headed Sperm Syndrome)





Semen Parameters - Livestock

	Bull (desired)	Boar (desired)
Volume mL	5 mL (range 1-15 mL)	100-500 mL
Concentration/mL	>500 million (range 300-2500 million)	100-200 million
% Motile	>60% (>30% BSE)	Min. 80% (prog. mot.)
% Normal	>70%	80-90%
Single AI dose	20 million	1-3 billion

- Past: Sires with poor semen quality eliminated during breeding soundness evaluation
- Present: Emphasis on genetic value, not fertility

Do Conventional Semen Parameters Reflect Fertility?

- Yes, but to a limited extent…
- Why? Because…

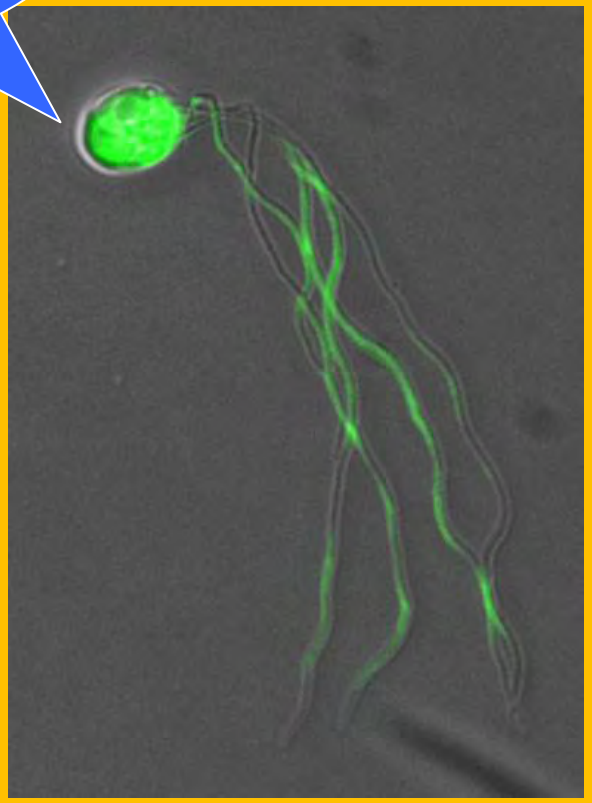
SPERMATOZOA- THE GOOD, THE BAD AND THE UGLY

STARRING PETER SUTOVSKY, PHD
AS
BLONDIE "THE GOOD" (SPERM GUY)

PRODUCTION: DIVISION OF ANIMAL SCIENCES AND THE DEPARTMENTS OF OBSTETRICS, GYNECOLOGY AND WOMEN'S HEALTH, UNIVERSITY OF MISSOURI, COLUMBIA, MO, USA

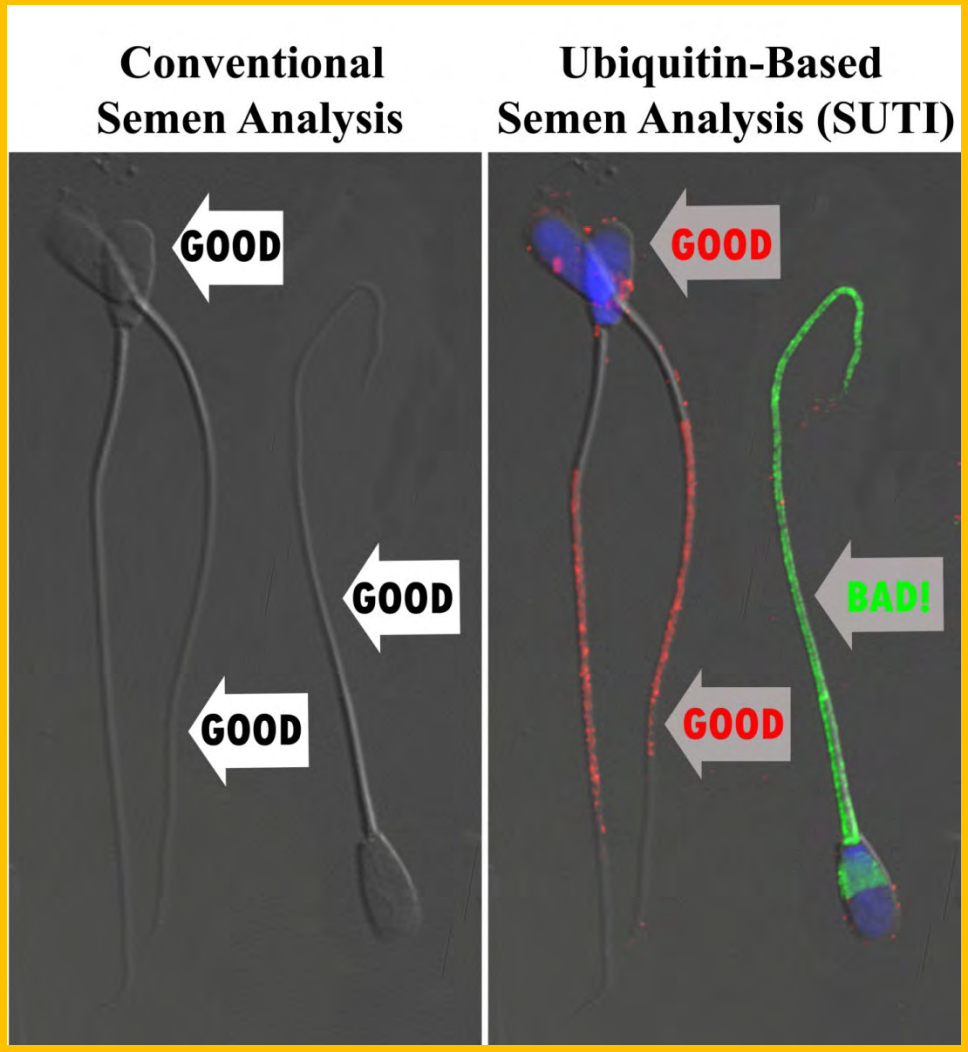
BAD & UGLY

Do You Feel Lucky, Punk?



Multinuclear – multiflagellar sperm defect in bull

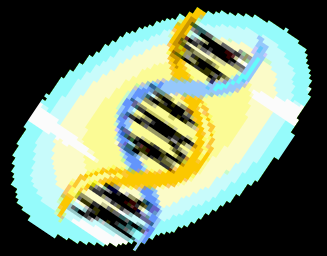
ALL GOOD?


Conventional
Semen Analysis
Ubiquitin-Based
Semen Analysis (SUTI)
GOOD
GOOD
GOOD
GOOD
GOOD
BAD!

Improved Semen Quality Assays: What to Consider:

- Objective - evaluation is not based on subjective judgment
- Universal - recognizes multiple types of semen abnormalities
- Detects hidden sperm abnormalities
- Correlates with fertility
- High throughput 50-500 samples /day/technician
- Representative of a sperm sample - measures 10-20,000 cells in each semen sample as opposed to 100-200 cells/sample capacity of microscopic evaluation
- Measurements are not distorted by sperm damage during collection, storage and thawing
- Low cost: ready to use technology; probes & reagents are commercially available or easy to produce on a large scale
- Combined/multiplex tests with other related assays possible

OMICS



- Sperm transcriptomics/mRNA & miRNA analysis in semen: Ejaculated spermatozoa contain a complex repertoire of mRNAs that could be used as a non-invasive proxy for investigations of testis-specific infertility. (Ostermeyer et al., Lancet. 2002; 360:772-7) Also includes sperm epigenome.

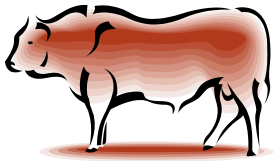
Spermatozoa carry small non-coding RNAs that could affect female reproductive system & zygote

- Proteomics: Sperm proteomes differ between fertile and infertile males

- Metabolomics: Sperm and seminal plasma metabolite profile reflects fertility

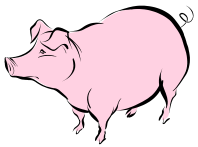
Sperm Proteome: “Negative” Biomarkers of Male Fertility

- **Ubiquitin (UBB) & 26S proteasome**
  - -polyubiquitinated proteins
  - -ubiquitin-rich aggresomes
  - -amyloid and polyglutamine tract proteins
  - -sperm proteasome activity
  - -deubiqutinases
- **15-lipoxygenase (ALOX15)**
- **Spermatid-Specific Thioredoxin 3 (TXNDC8)**
- **Perinuclear theca WBP2NL (PAWP)**
- **Seminal plasma spermadhesins & BSPs**
- **Nuclear DNA-binding proteins**
  - -protamines
  - -testis/sperm-specific histones
  - -acetylated histones
- **Platelet activating factor-receptor (PAFR)**



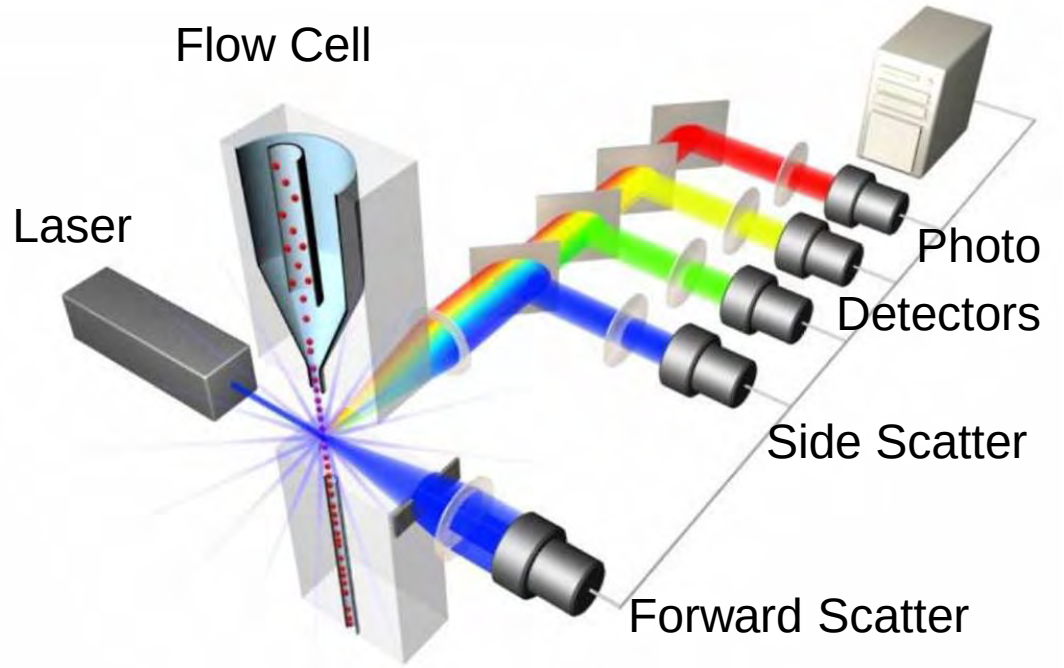




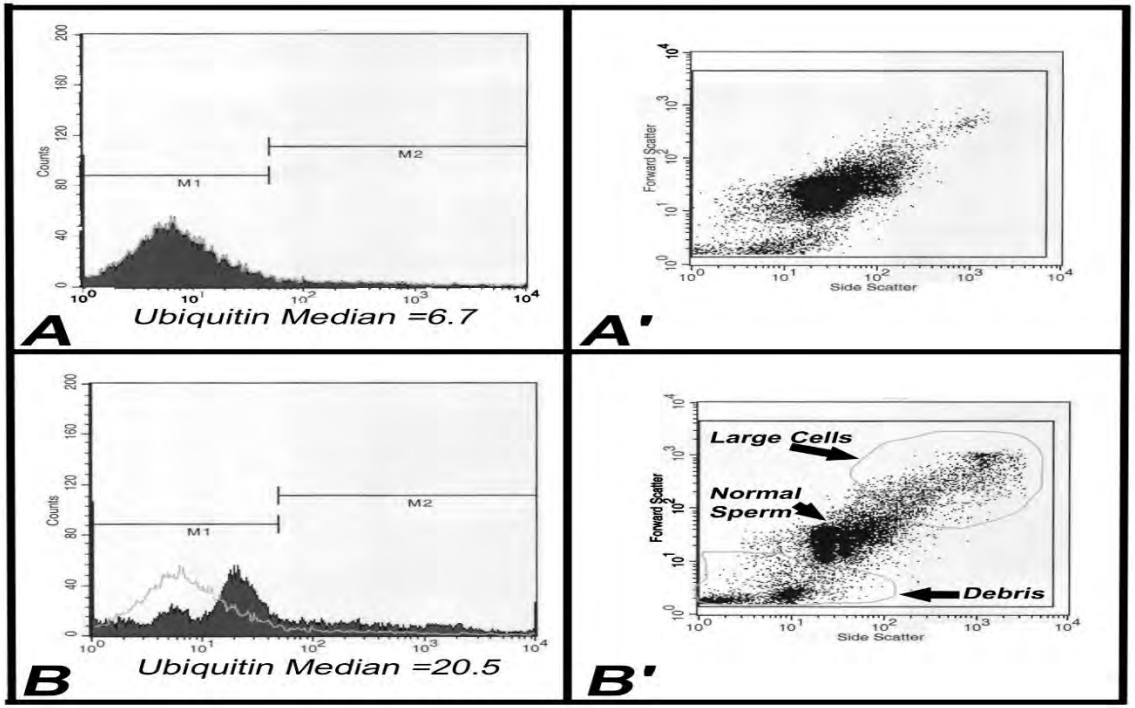

Sutovsky et al., (2024) Biomarker-based human and animal sperm phenotyping: The good, the bad and the ugly. ***Biol. Reprod.,*** Special Issue on Imaging, https://doi.org/10.1093/biolre/ioae061

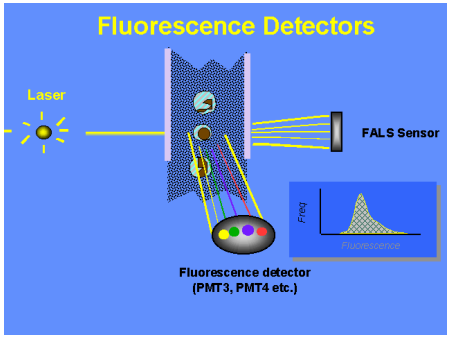
Flow Cytometry*

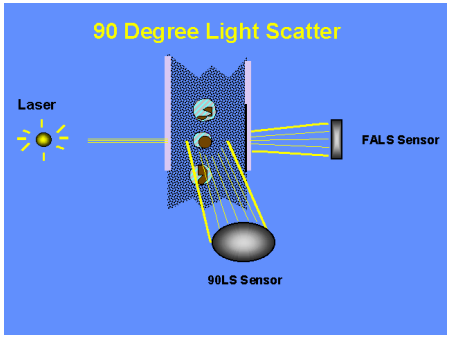


*High throughput (5-10K cells measured in seconds)

Histogram & Scatter Diagram

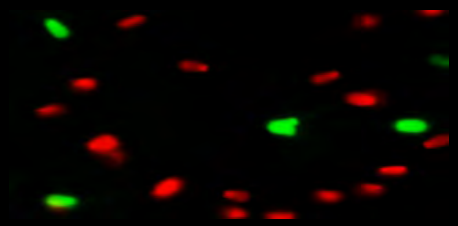


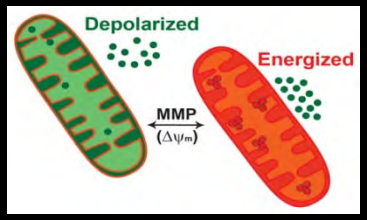




Vital Stains and DNA Stains

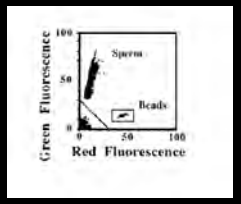
Mito Potential

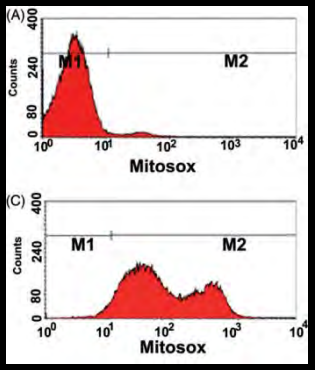




Viability - Live/Dead

ROS – Reactive Oxygen Species



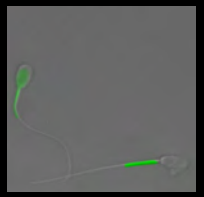


SCSA – Chromatin Structure

TUNEL – DNA Damage

Acrosomal Integrity – PNA lectin

Zinc signature of sperm capacitation – FluoZin3



Calcium fluxes – Fluo3/Fluo4

Antioxidants of Commercial Black Wattle (*Acacia mearnsii*) Tannin Extract Analysed by UPLC-QTOF MS.

Fractionation & mass spectrometry identified four major compounds:

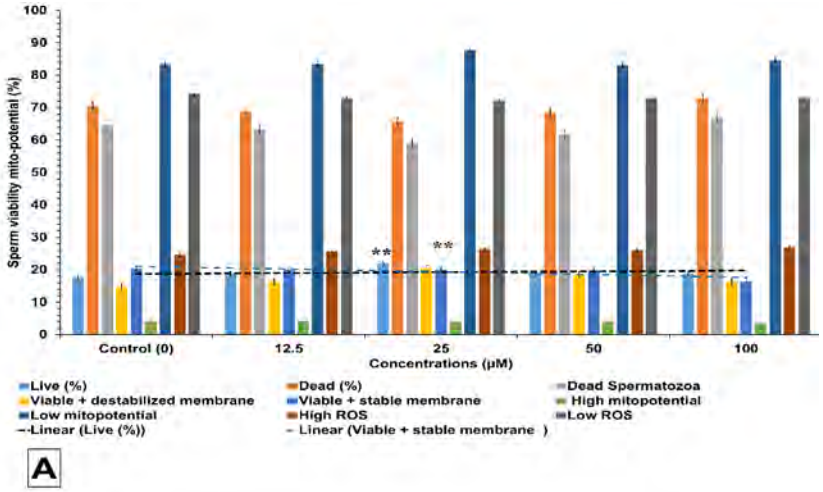
- Gallocatechin (305.06)
- Catechin (289.07)
- Kaempferol-3-Glucoside-2''-p-coumaroyl (593.12)
- (*epi*)-Catechin-(*epi*)-catechin (577.13)

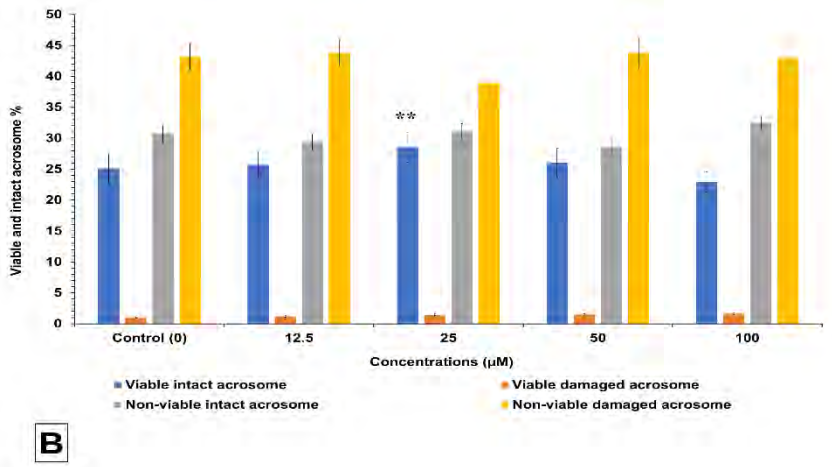
Mohammed Liman & Dietmar Holm

University of Pretoria Faculty of Veterinary Science

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Post-Thaw Mitochondrial Activity and Acrosomal Integrity in Merino Ram Semen Cryopreserved with Gallocatechin





Liman MS, Hassen A, Smuts M, Biraima ADA, Sutovsky P, McGaw LP, Holm DE (2025) Influence of a tannin-rich extract of commercial *Acacia mearnsii* and a flavonoid compound on ovine chilled and cryopreserved semen viability. ***Sys. Biol. Reprod. Med.,*** 71:1, 90-101, DOI:10.1080/19396368.2025.2465260

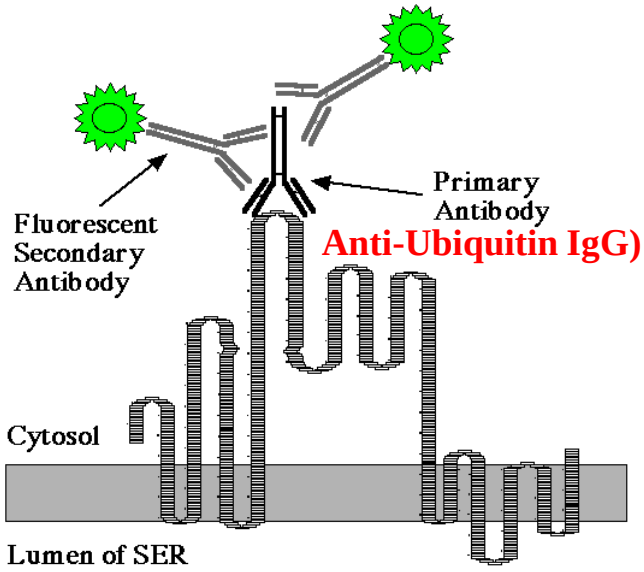
Antibodies - Immunofluorescence Protocol for Flow Cytometry

•Fixation (OPTIONAL)

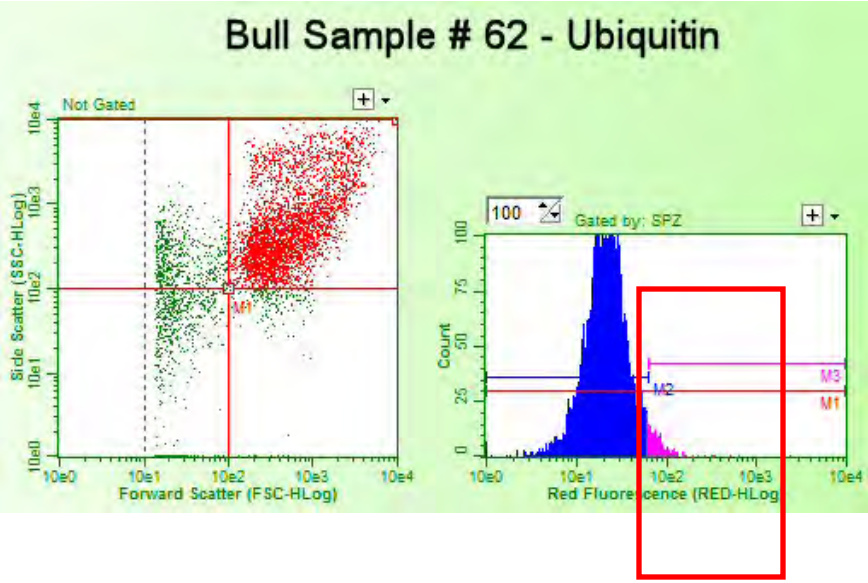
•Primary antibody; monospecific IgG developed in mouse/rabbit/other species

•Secondary antibody; Anti-mouse/rabbit/other IgG coupled to a fluorescent dye (FITC)

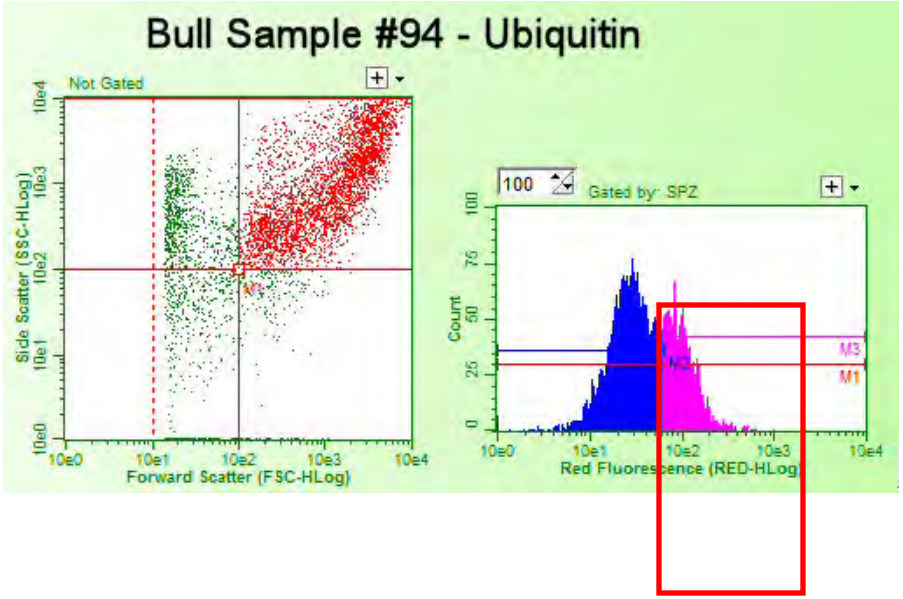
•Evaluation by epifluorescence microscopy and/or flow cytometry



Ubiquitin – Too Much Bad Protein

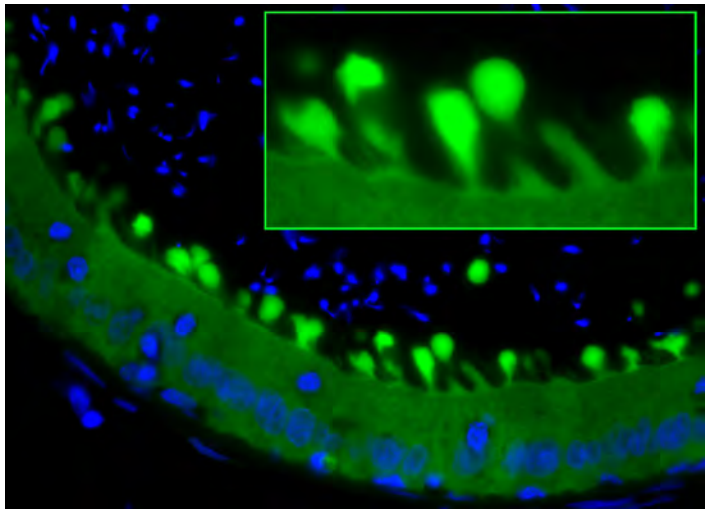


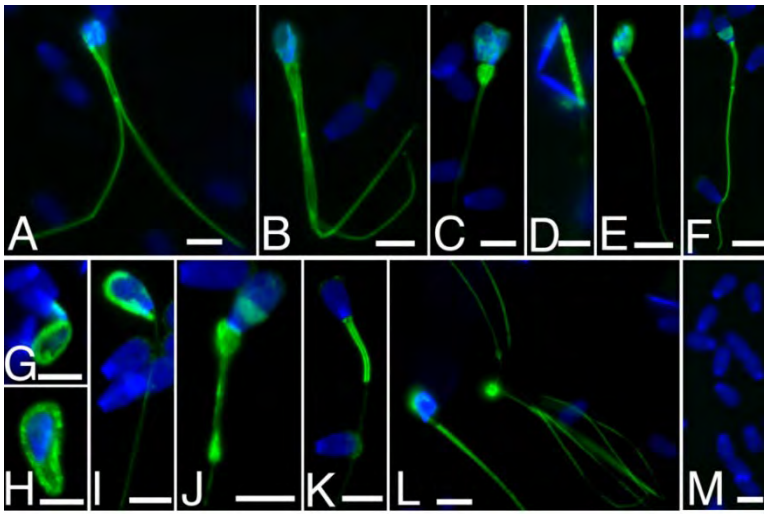
Normal Histogram

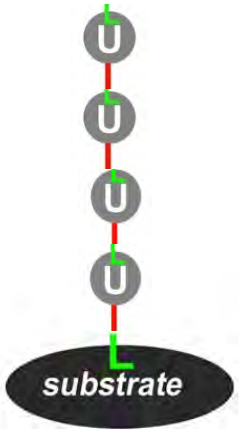


Abnormal Histogram

Epididymal Sperm Quality Control by Sperm Surface Ubiquitination







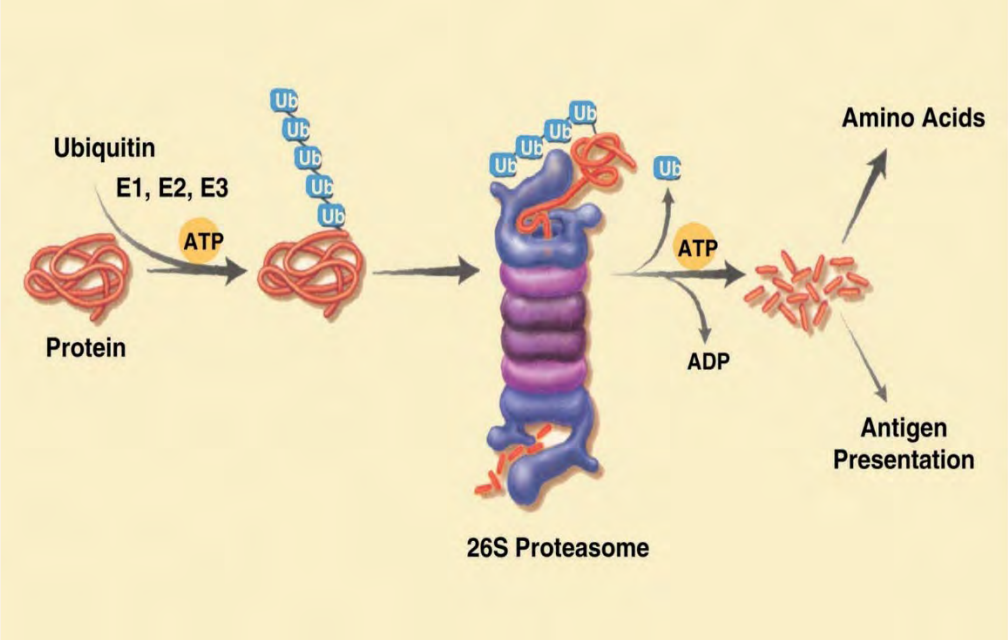
Possible Roles of Defective Sperm Coating:

- Clustering of defective spermatozoa
- Prevention of autoimmune infertility
- Degradation of defective spermatozoa

Sutovsky *et al.*, 1998 *J. Cell Sci*

Ubiquitin–Proteasome System (UPS)

- A major cellular protein degradation pathway
- Substrate-specific protein recycling
- 26S Proteasome
- Autophagosome



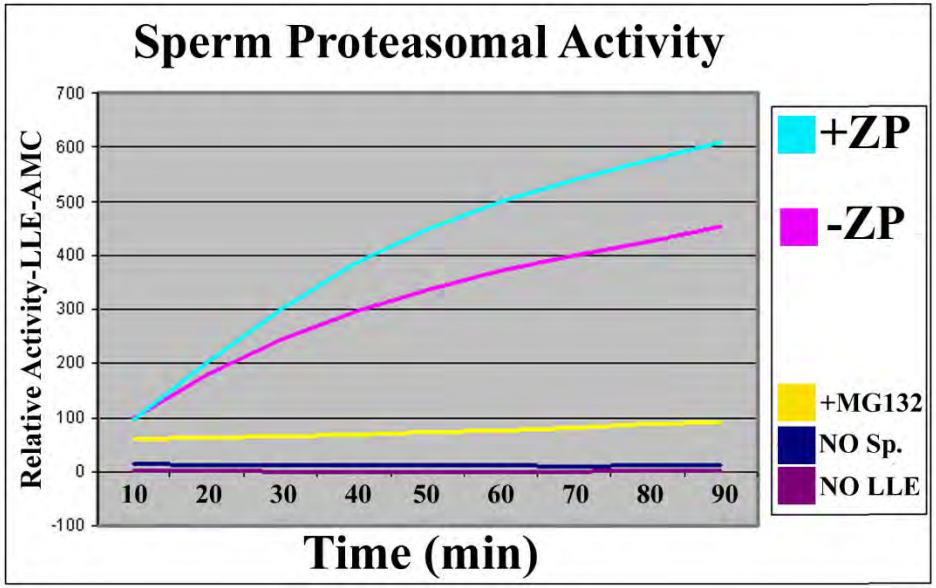
Kisselevy and Goldberg 2001

Proteasomal Activity in Motile Boar Spermatozoa Exposed to Soluble Zona Pellucida Protein

20S

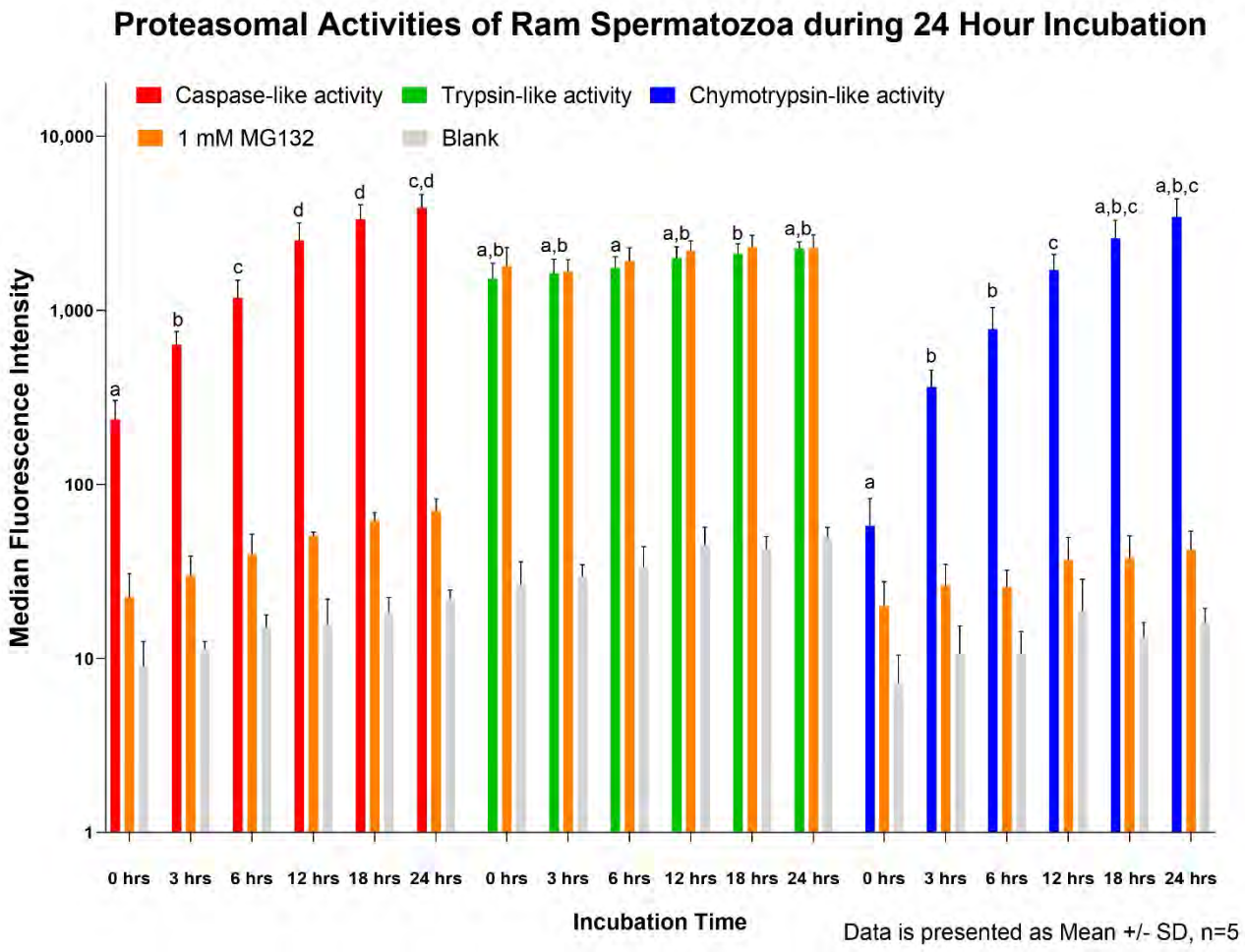
↓

Substrate-LLE **-AMC**



+ZP=Human α-Acid Sialylated Protein

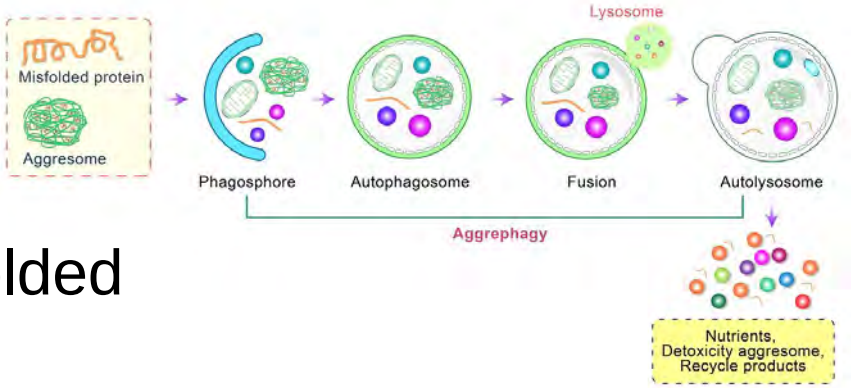
Proteasomal Activity in Ram Spermatozoa Incubated for 24 Hours



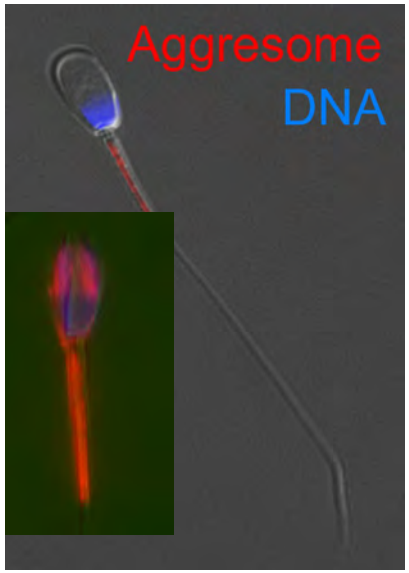
- Collaboration with Dr. Taylor Pini, University of Queensland and Dr. Jessica Epple-Farmer, Lincoln University, MO USA

Aggresomes

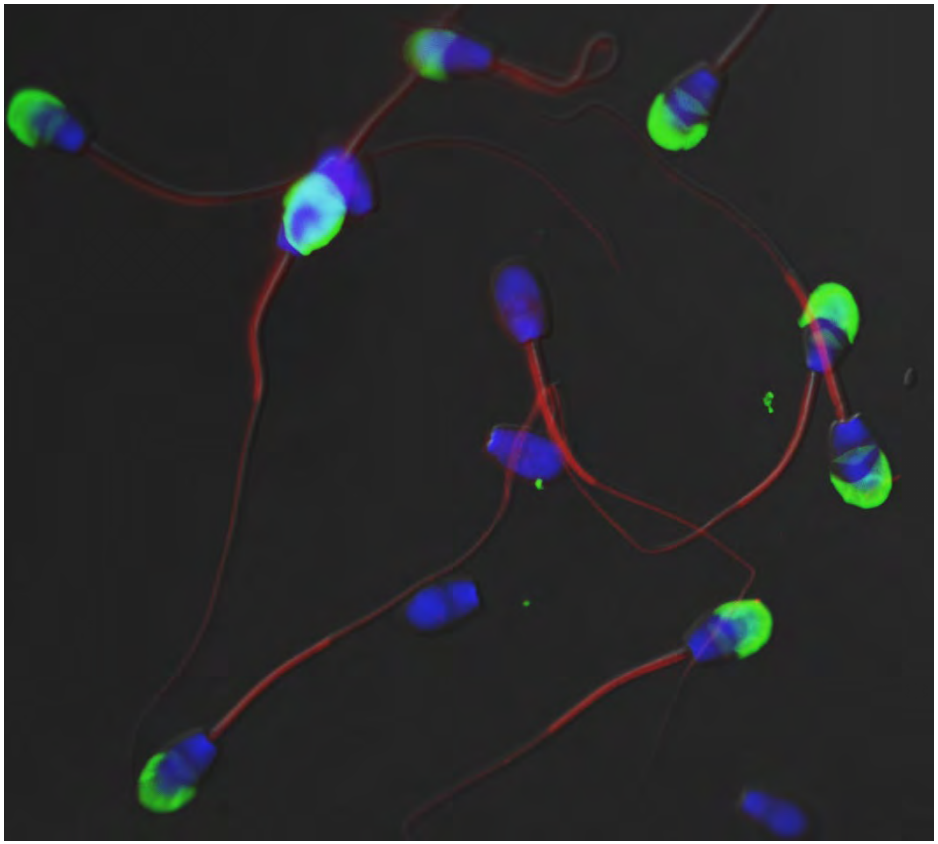
- Recycled by autophagy →

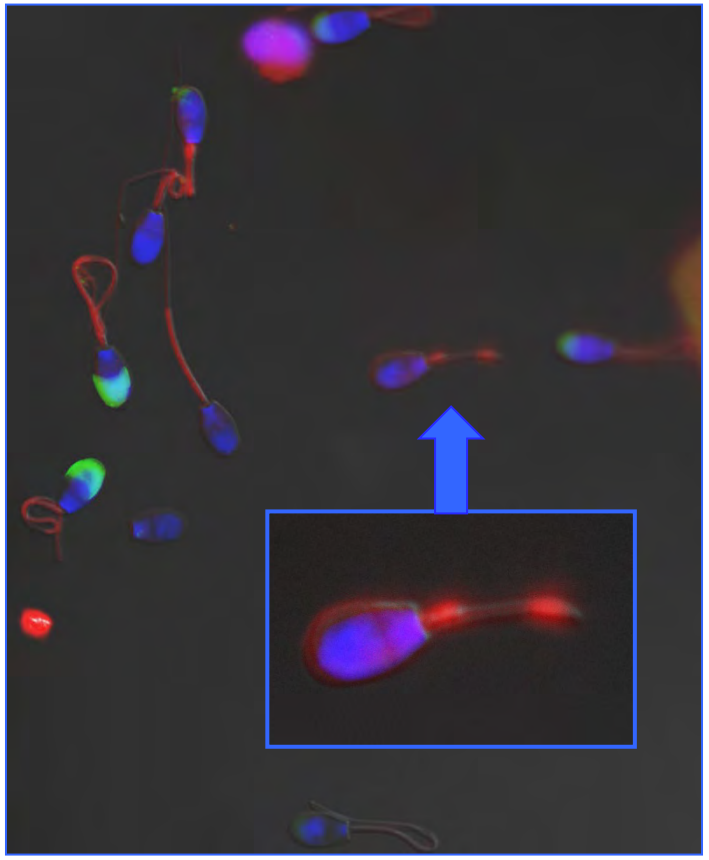


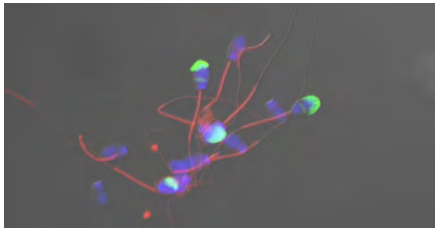
- Cellular stress induced, misfolded protein aggregates.
- Ubiquitinated, reflective of altered proteostasis/UPS saturation, when not cleared by autophagy.
- Measured by fluorescent probes
- Associated with neurodegenerative brain disorders.
- Present in normal sperm midpiece and defective sperm heads.

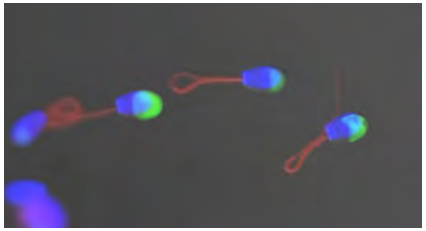


Ram Sperm Aggresome

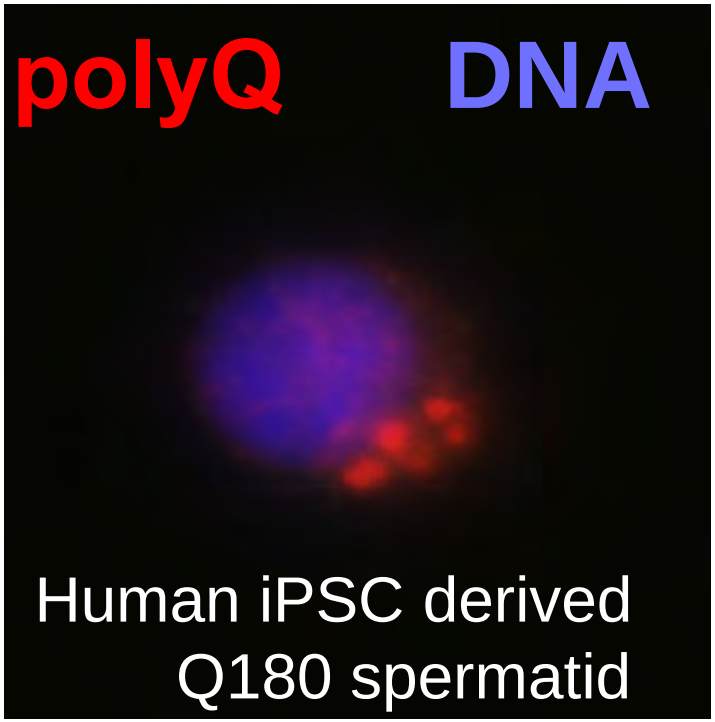


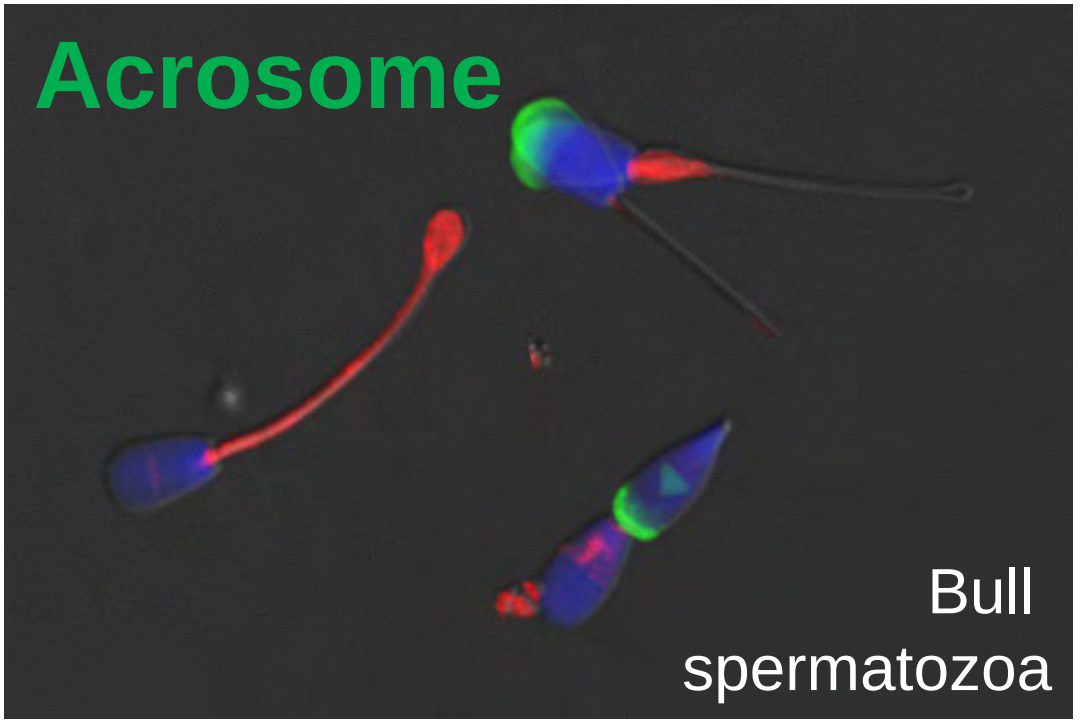






Aggregation of Glutamine Rich & Polyglutamine Tract Sperm Proteins





International Journal of Molecular Sciences

Lawlor et al., 2022

Polyglutamine Tract Disease

- Associated with Huntington, Alzheimer and other progressive neurodegenerative, neurological & neuromuscular diseases.
- Repeat instability mutations: Abnormally elongated CAG repeats translate into abnormal poly-glutamine tracts in the protein sequence.
- About 40 different diseases result from polyQ elongation.
- 178 polyQ genes identified in bovine genome (most are testis expressed & polymorphic).

Healthy gene:

cagcagcagc agcag

QQQQQ

Diseased gene:

cagcagcagc agcagcagca gcagcagcag

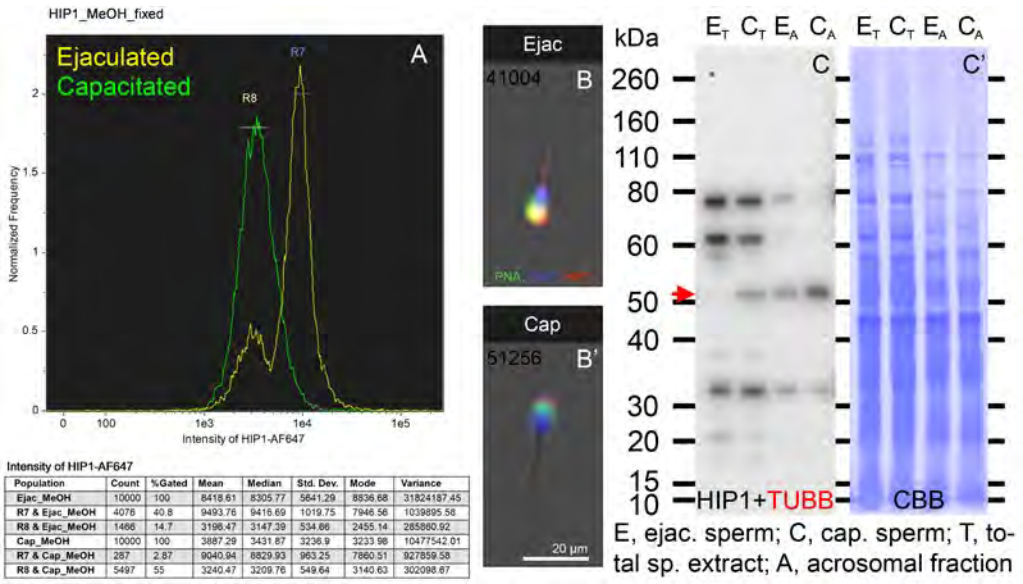
QQQQQ QQQQQ

Whan et al., *BMC Genomics* **11,** 654 (2010). https://doi.org/10.1186/1471-2164-11-654

Neurons With Tails

- Huntington, Alzheimer and other neurodegenerative disease pathways are involved in sperm function and sperm pathology

Huntingtin Interacting Protein 1 (HIP1)



Population	Count	%Gated	Mean	Median	Std. Dev.	Mode	Variance
Ejac_MeOH	10000	100	8418.61	8305.77	5841.29	8836.68	31824187.45
R7 & Ejac_MeOH	4076	40.8	9493.76	9416.69	1019.75	7946.56	1039895.58
R8 & Ejac_MeOH	1466	14.7	3198.47	3147.39	534.66	2455.14	285860.92
Cap_MeOH	10000	100	3887.29	3431.87	3236.9	3233.98	10477542.01
R7 & Cap_MeOH	287	2.87	9040.94	8829.93	963.25	7860.51	927859.58
R8 & Cap_MeOH	5497	55	3240.47	3209.76	549.64	3140.63	302098.67

Zigo et al., 2022 Commun. Biol. (Nature)

Emerging Concept: Proteostasis as An Endpoint Biomarker of Sperm Quality

CAUSE/INSULT — Mutation/deletion, somatic-reproductive comorbidity, environmental pollution, nutrition, lifestyle, occupational exposure.

DAMAGE TO — Plasma membrane, DNA/nucleus, acrosome, perinuclear theca, mitochondria, axoneme.

PROTEOSTATIC ENDPOINT

UPS
Ubiquitination

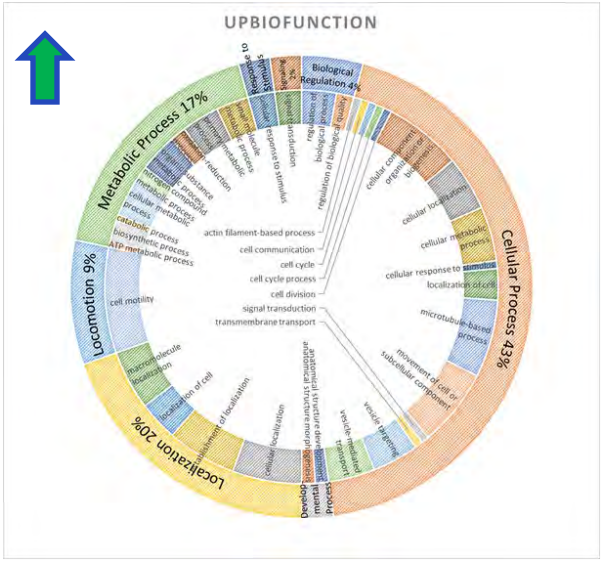
Autophagy
Protein Aggregation

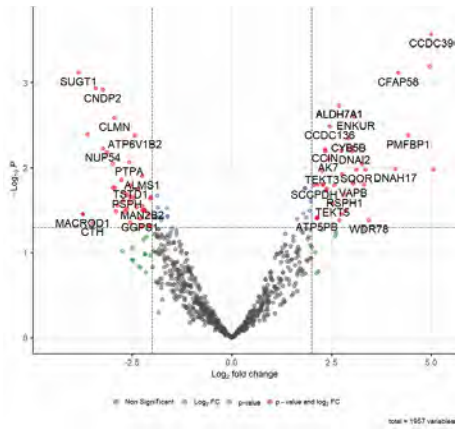
PolyQ Repeat
Disease

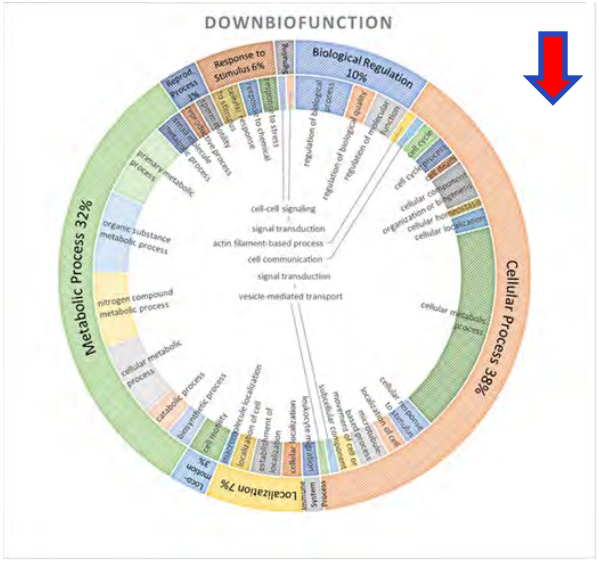
Common denominator: Saturation of spermatogenic cell/post-testicular sperm proteostasis mechanisms.

Related posttranslational modifications: Sperm surface glycosylation, carryover histone methylation and acetylation, protein sulfhydrylation, amyloid formation, SUMOylation, ISGylation, lipid peroxidation, other PTM.

Zincoproteome of Mammalian Sperm Capacitation





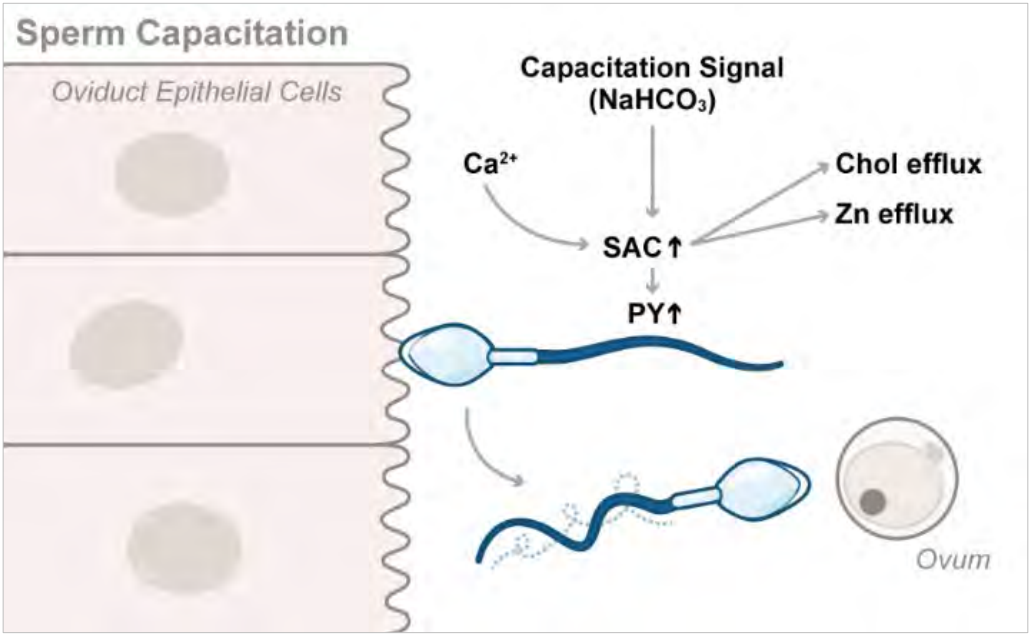


Increased

Decreased

Affinity of zinc-containing/interacting proteins to zinc-binding protein purification matrices before vs. after sperm capacitation

Zigo et al., 2022 Commun. Biol. (Nature)

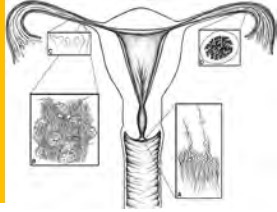


Sperm Capacitation

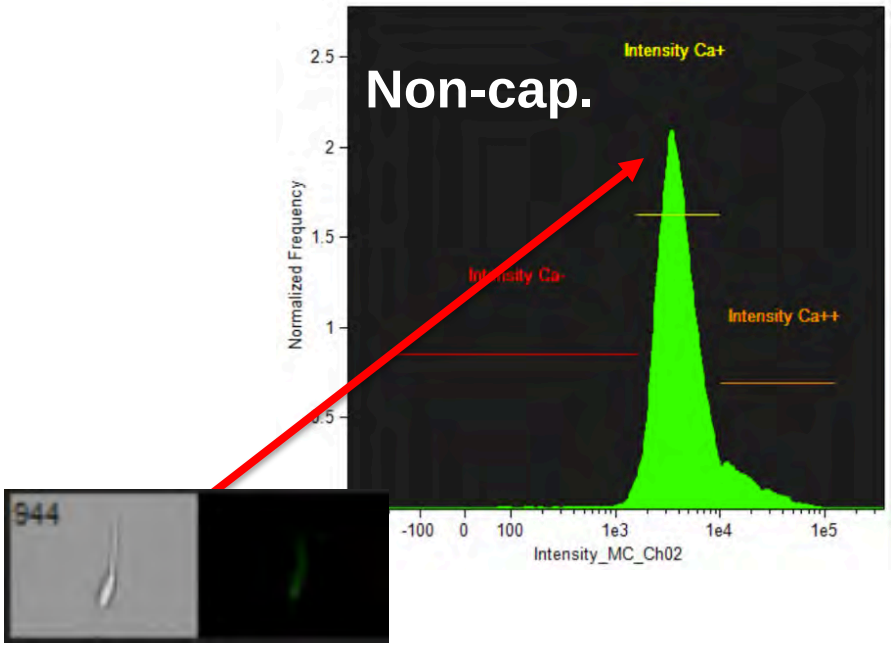
Set of changes in the sperm plasma membrane that enables a cell to undergo acrosome reaction and fertilize an egg (fertilizing ability)

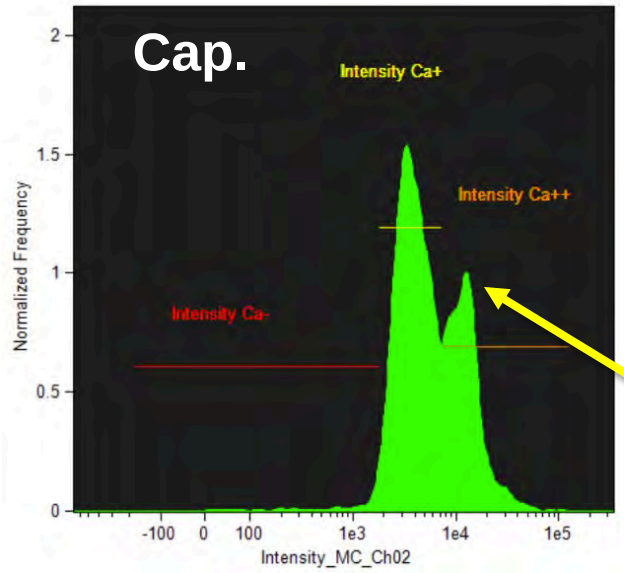
- Terminal maturation event
- Not fully reversible
- Post-capacitated spermatozoa die
- Inducible by semen processing (incl. sexing & cryostorage)

Sperm Capacitation Status

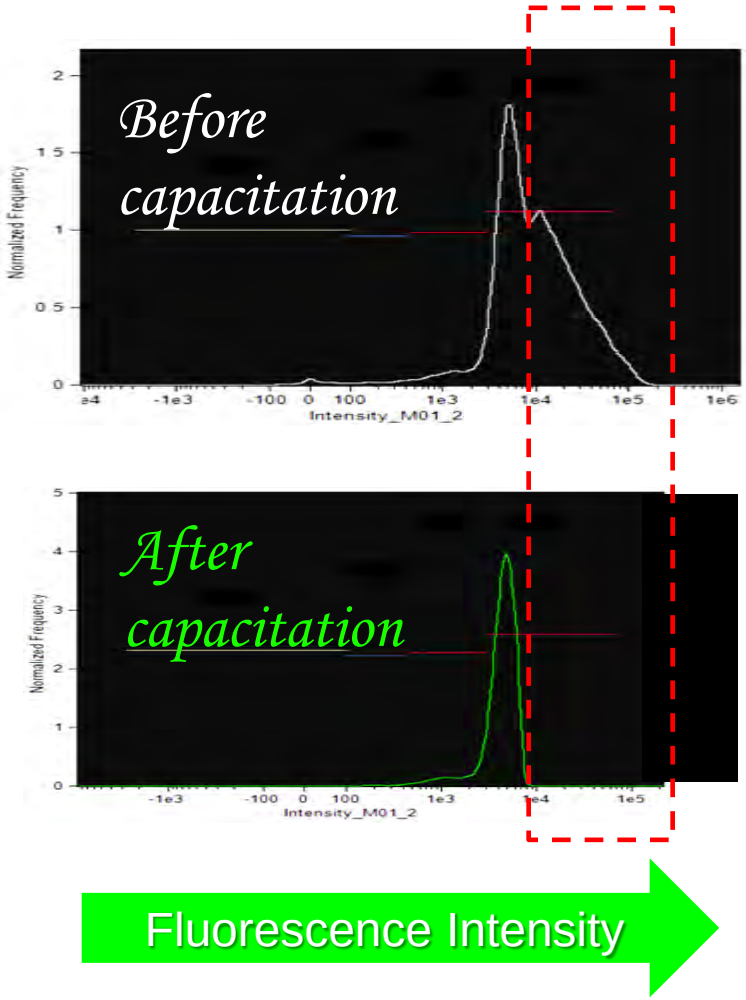


- Endows spermatozoa with FERTILIZING ABILITY
- Spermatozoa do not capacitate naturally until they bind to oviductal sperm reservoir epithelium
- Premature capacitation kills spermatozoa
- Associated with Ca-influx in sperm
- Measured by fluorescent Ca-dyes Fluo-3/Fluo-4 (flow cytometry) or by chlorotetracycline (epifluorescence microscopy)

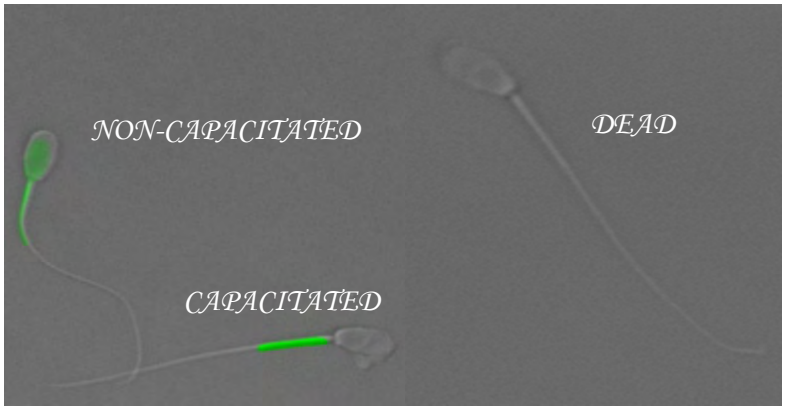




Zinc Signature of Capacitation

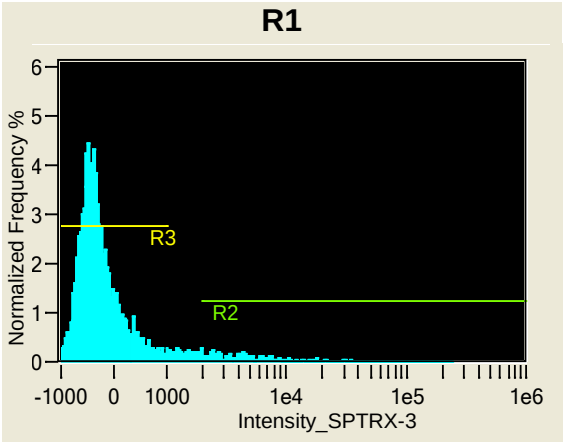


- Quick one step live sperm staining, detects & quantifies premature capacitation
- Also detects & quantifies death spermatozoa
- Indicates sperm ability or readiness to undergo timely capacitation



Kerns E et al 2018 Nature Communications

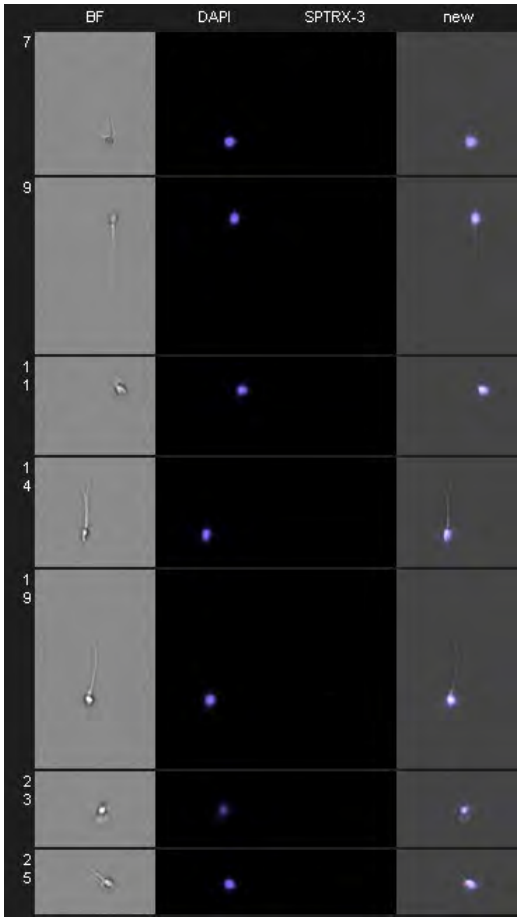
Image-Based Flow Cytometry: Cytometer & Microscope in One Box



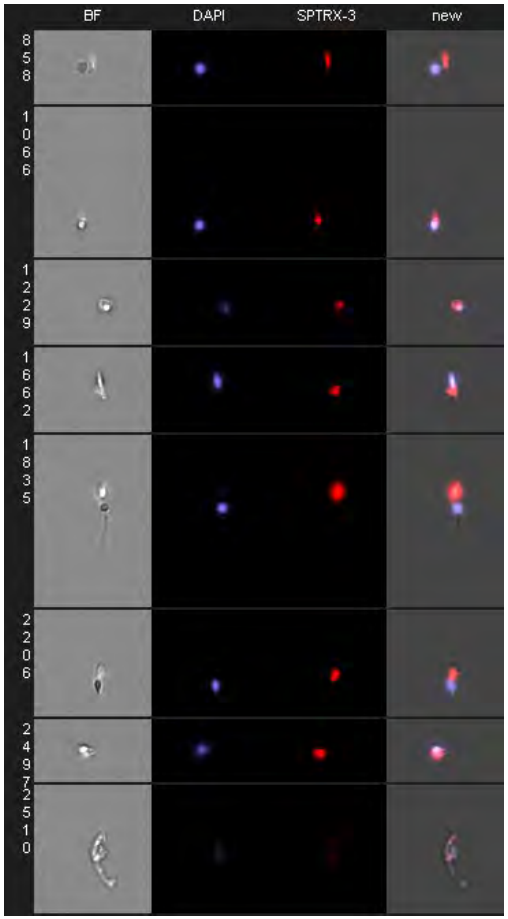
Population Statistics

Population	Count	%Gated
R1	5099	100
R3 & R1	4567	89.6
R2 & R1	319	6.26

Normal Sperm (R3)

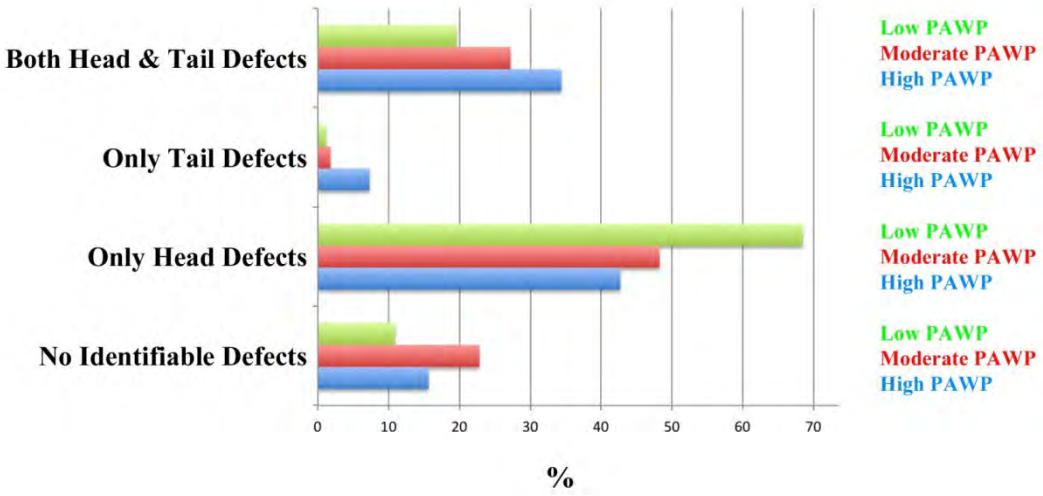


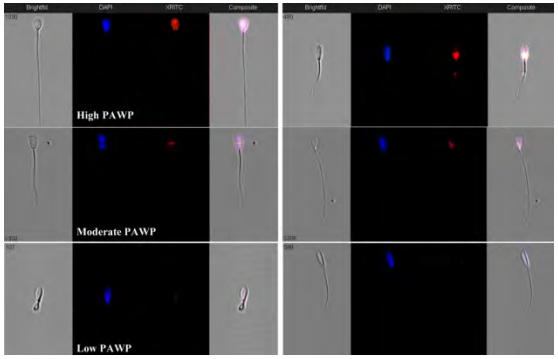
Defective Sperm (R2)



- Amnis Corp., Seattle WA [Buckman *et al.,* 2009, *Systems Biol. Reprod. Med.,* 55(5-6):244-251]

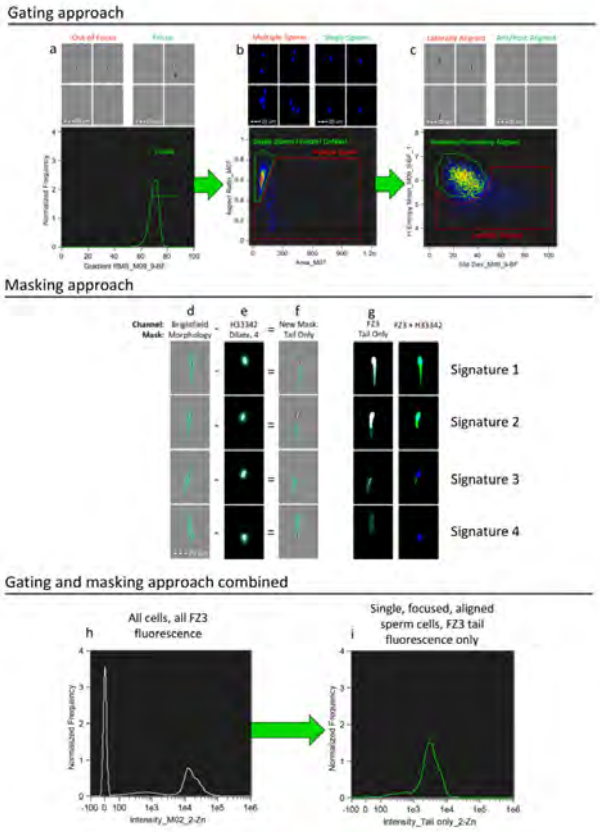
Sperm Phenotyping by Image Based Flow Cytometry





Kennedy *et al.,* 2014, Mol Reprod. Dev. 81:436-449 .

Gates & Masks


Gating approach
Masking approach
Signature 1
Signature 2
Signature 3
Signature 4
Gating and masking approach combined
All cells, all FZ3 fluorescence
Single, focused, aligned sperm cells, FZ3 tail fluorescence only

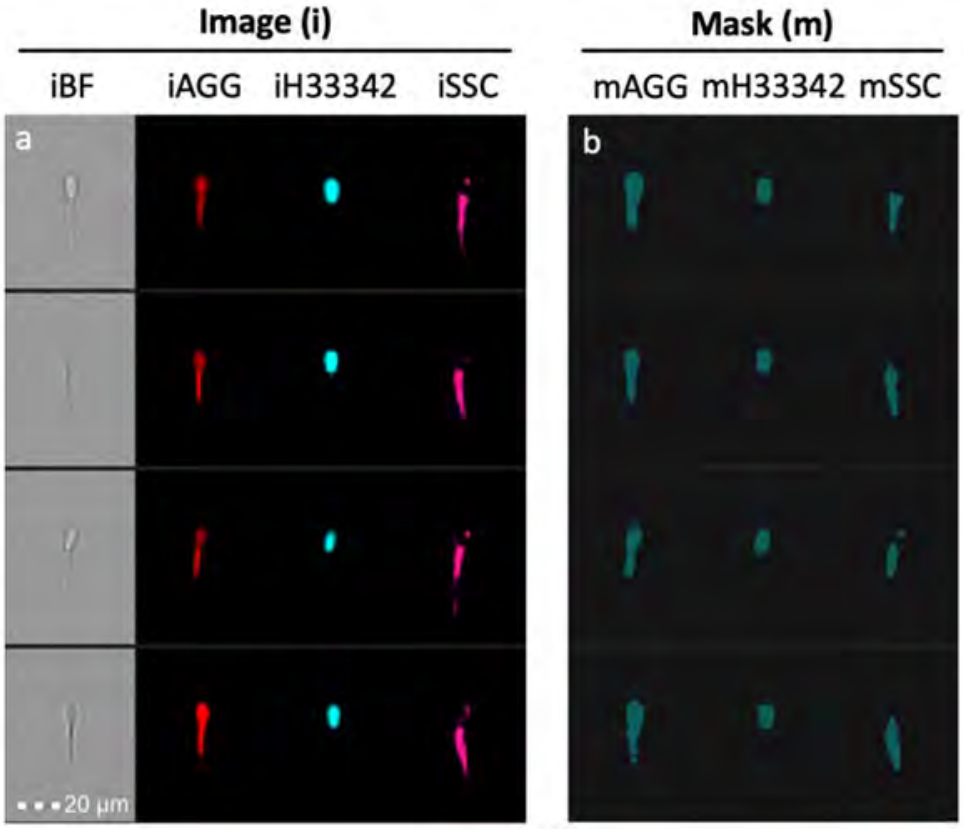
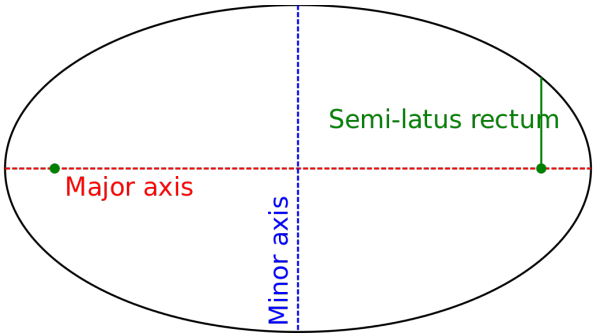
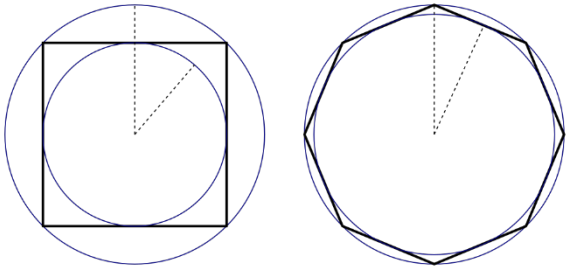


Image (i)
Mask (m)
iBF
iAGG
iH33342
iSSC
mAGG
mH33342
mSSC
20 µm

Feature Finder



Aspect ratio



Circularity
(Roundness)

Image Feature	Definition
Angle Intensity	Angle is the angle of the major axis from a horizontal plane in radians.
Area	The number of microns squared in a mask is equal to the Area. 1 pixel = 1.0 μm².
Aspect Ratio	Aspect Ratio is the Minor Axis divided by the Major Axis and describes how round or oblong an object is.
Aspect Ratio Intensity	Aspect Ratio Intensity is the Minor Axis Intensity divided by the Major Axis Intensity.
Bright Detail Intensity R3	The Bright Detail Intensity R3 feature computes the intensity of localized bright spots within the masked area in the image. The intensity of bright spots that are 3 pixels in radius or less are included and the local background around the spots is removed before the intensity computation.
Centriod Y	Centroid Y is the number of pixels in the vertical axis from the upper, left corner of the image to the center of the mask.
Circularity	Circularity measures the degree of the mask's deviation from a circle. Its measurement is based on the average distance of the object boundary from its center divided by the variation of this distance. Thus, the closer the object to a circle, the smaller the variation and therefore the feature value will be high. Vice versa, the more the shape deviates from a circle, the higher the variation and therefore the Circularity value will be low.
Diameter	The Diameter feature provides the diameter of the circle that has the same area as the object.
Gradient RMS	The Gradient RMS feature measures the sharpness of an image by detecting large changes of pixel values in the image. It is computed using the average gradient of a pixel normalized for variations in intensity levels.
H Correlation	The Haralick (H) texture features are a set of texture features based on the second order statistics computed from the joint 2-D probability distribution of pixel intensities in the image. This distribution is referred to as the Gray Level Co-occurrence Matrix (GLCM). Correlation measures how similar pixel pairs are, and is the opposite of contrast. Images with high correlation are very uniform and lack variant texture.
H Energy	Energy is a measure of intensity concentration in the cell. At one extreme is the case of a uniform distribution with all probabilities being equal. This image has several intensity variations with no noticeable concentration of high intensity and thus has low energy. At the other extreme is the case of a very narrow distribution with a few elements having high values. This image will have notable intensity concentrations and thus, has high energy.
H Entropy	Entropy is also a measure of high intensity concentration in the cell. However, this feature relates to the randomness of the intensities in the image. Images that have distinct areas of intensity concentration are less random and thus, have low entropy. Images that have a range of equally likely intensity pairings have less distinct intensity concentrations, and correspondingly, have higher entropy. Entropy is the opposite of energy.
Height	Using the bounding rectangle, Height is the number of microns of the longer side.
Length	Length measures the longest part of an object. Unlike the Major Axis feature, Length can measure the object's length even if it folds to form a cashew, banana, or doughnut shape, where in many of these cases the major or minor axis features would not be able to differentiate these with true circular shaped objects with no hole.
Symmetry 3	The Symmetry 3 feature measures the tendency of the object to have a three-fold axis of symmetry.

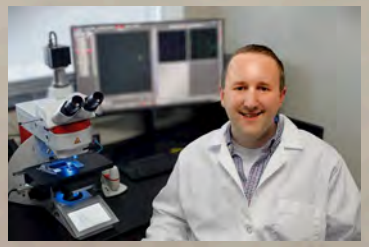
Table: Image Feature definitions from IDEAS ® Software Version 6.2 (Luminex Corp., Seattle, WA)

Correlation of IBFC Cell Features with Sperm Parameters and Fertility Outcomes in 45 AI Boars

PARAMETERS	r	P-value
Fertility Related		
Bright Detail Intensity R3_mH33342_iAGG, Median Absolute Deviation (MAD), Head High AGG: **Total Born**	**-0.423**	**0.004**
Sperm Trait Related		
H Energy Mean_mSSC_iSSC_5, Median: **% Viable with Intact Acrosome**	**0.621**	**< 0.00001**
Centroid Y Intensity_mSSC_iSSC, MAD: **% Viable with Intact Acrosome**	-0.607	< 0.00001
Bright Detail Intensity R3_mSSC_iSSC, Median: **% Viable with Intact Acrosome**	0.531	0.0002
Bright Detail Intensity R3_mH33342_iAGG, Median, Head High AGG: **CompDNA**	0.496	0.0005
Bright Detail Intensity R3_mH33342_iAGG, Mean, Head: Low AGG: **Mean Length MS**	0.442	0.002
Symmetry 3_mSSC_iSSC, Median: **% Normal Morphology**	0.441	0.002
Length_mH33342, Mean: **% Viable with Intact Acrosome**	0.420	0.004
Height_mH33342, Mean: **% Viable with Intact Acrosome**	0.413	0.005
% Head No AGG [gated]: **% Viable with Intact Acrosome**	-0.377	0.011
% Head: Mid AGG [gated]: **% Viable with Intact Acrosome**	0.351	0.018
% Head Low AGG [gated]: **% Viable with Intact Acrosome**	0.339	0.023
Mitochondria Related		
Symmetry 3_mSSC_iSSC, Std. Dev.: **% Depolarized Mitochondria**	**0.650**	**0.000002**
Aspect Ratio_mSSC, MAD: **% Depolarized Mitochondria**	0.516	0.0003
Angle Intensity_mSSC_iSSC, Mean: **% Depolarized Mitochondria**	-0.515	0.0003
Bright Detail Intensity R3_mSSC_iSSC, Median: **% Depolarized Mitochondria**	-0.477	0.001
Symmetry 3_mSSC_iSSC, Mean: **Oxidation (Basal:Induced Ratio)**	-0.440	0.002
Area_mSSC, Median: **% Depolarized Mitochondria**	-0.406	0.006
Aspect Ratio Intensity_mSSC_iSSC, Mean: **% Depolarized Mitochondria**	0.403	0.006
% Head: No AGG [gated]: **Oxidation (Basal:Induced Ratio)**	0.321	0.032
% Head Low AGG [gated]: **Oxidation (Basal:Induced Ratio)**	-0.334	0.025
% Head: Mid AGG [gated]: **Oxidation (Basal:Induced Ratio)**	-0.315	0.035

Kerns et al., 2020 *Antioxidants*

AI^2 - Putting AI in the AI



Prof. Karl Kerns

HEADLINE: AI helped make a song on 'the last Beatles record'

"[Jackson] was able to extricate John's voice from a ropey little bit of cassette — it had John's voice and a piano. He could separate them with AI, they could tell the machine 'that's a voice, this is a guitar, lose the guitar'

Sir Paul McCartney describing AI use by documentary film director Peter Jackson

BBC - Homepage

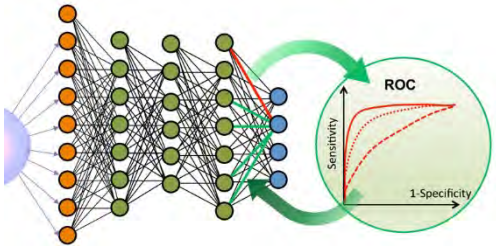
Machine Learning – Face ID For Sperm Cell

Genotyped sire cohort characterization based on prevailing sperm defect

Brightfield and epifluorescence light microscopy

Identification of cellular features associated with specific sperm morphologies and biomarker phenotypes

Image based flow cytometry and *Feature Finder* data processing



Machine learning, training the computer to recognize defective sperm phenotypes without biomarker-based labeling

Validation of cellular features associated with specific sperm phenotype

“Face ID” for spermatozoa

Label-free flow cytometry

Artificial Intelligence Analysis of the Mammalian Sperm Zinc Signature Predicts Male-factor Subfertility

Biomarker Development Workflow

Step 1 - BIOMARKER DISCOVERY

Cell biology, proteomics, genomics, polymorphism identification, infertility screening, fertility analysis in animal models propagated by artificial insemination, knock-out animals.

Step 2 - BIOMARKER VALIDATION

Cell biology, immunocytochemistry, biochemistry, CASA, flow cytometry, infertile couple screening, low-fertile sire screening in livestock models. Antibodies & fluorescent probes are required.

Step 3 - MACHINE LEARNING

IBFC – association of infertile biomarker phenotypes with advanced multifactorial sperm morphometry patterns.

Step 4 - APPLICATION

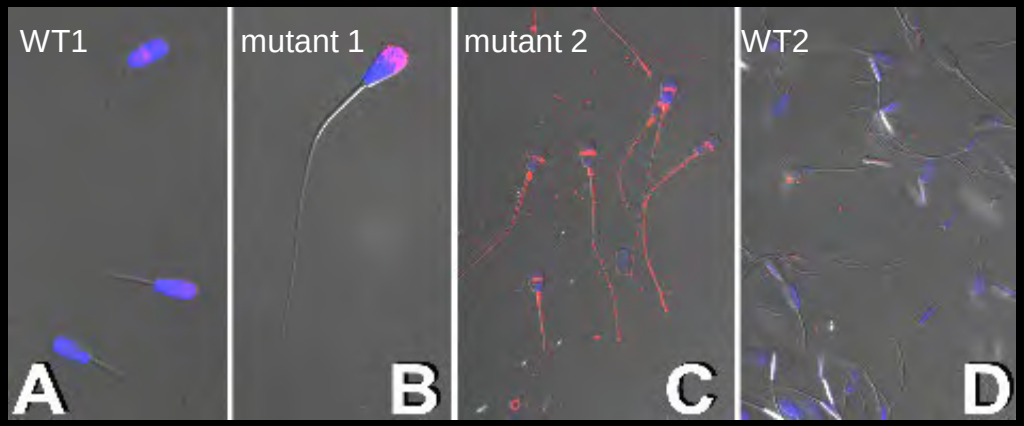
Limited/no probes required (“label-free”)

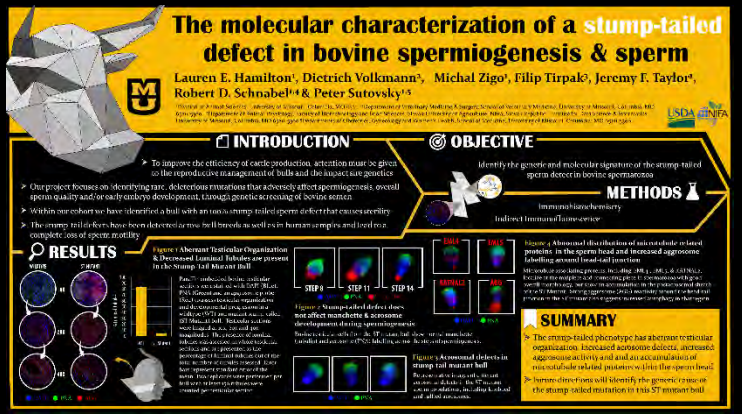
Male infertility diagnostics, sire selection

Ejaculate assessment, AI dose quality control


G2P

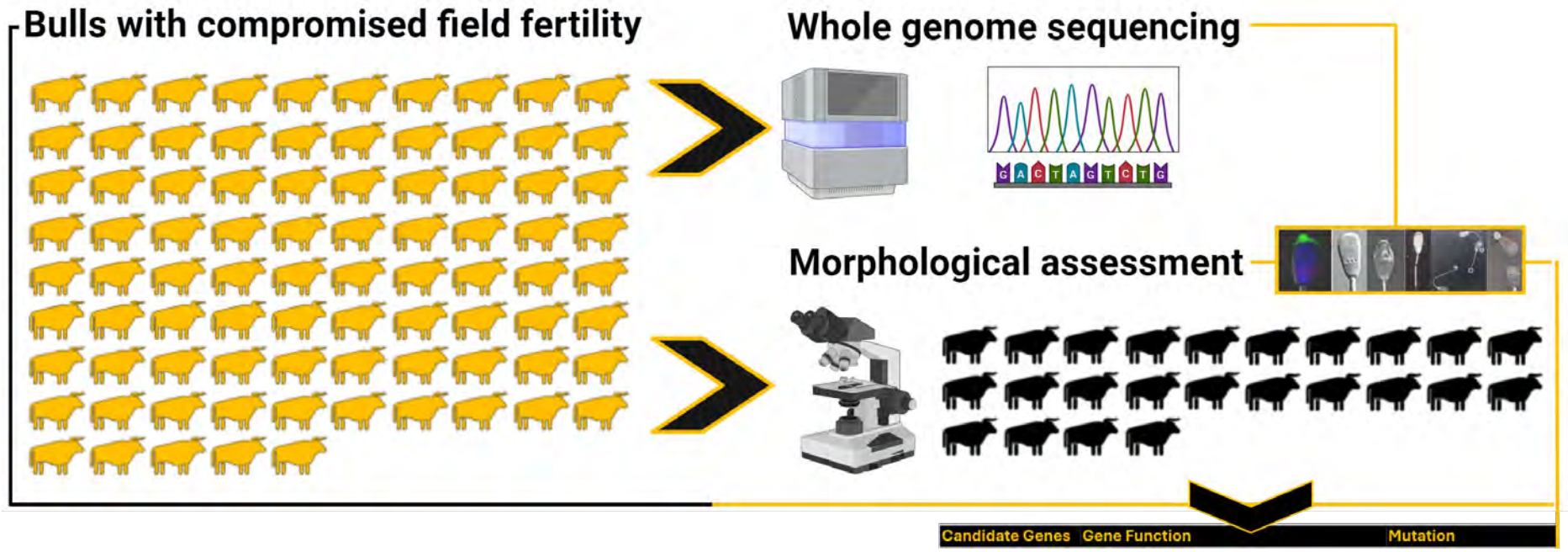
Sperm Genomics – SNPs, INDELS Affecting Spermatogenesis, Sperm Structure and Sperm Function





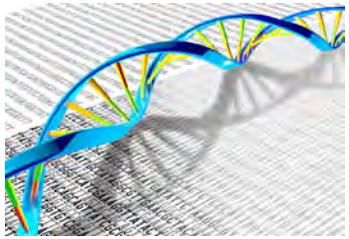
Unique sperm phenotypes associated with predicted loss of function (LOF) mutations in bulls used for AI service

Field Fertility Phenotype → Genome → Sperm Phenotype



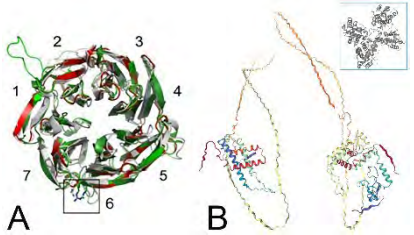
Genome to Phenome Approach

Sperm Phenotype-based Gene Variant Identification & Validation



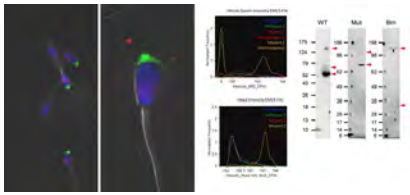
- Genome sequenced bulls (n=85) with high prevalence of a single morphological defect (>20% of cells affected) compared to the fertile cattle population of Run9 of the 1000 Bull Genomes project.
- Genetic mutations considered candidates based on: (1) homozygosity in all affected individuals, (2) rare within the fertile population (<0.01), (3) deleterious and/or high impact based on SIFT score (missense) and/or affected protein coding region.

Protein Modeling



- Amino acid sequence of the affected protein region run through HHpred analysis to identify existing and reliable templates.
- Identified templates input into MODELLER to create an overall visualization of the affected region.
- Model validation by TrRosetta, with molecular dynamics refinement in YASARA and AlphaFold2.
- Effect of each mutation modelled by mutagenesis wizard of PyMOL.

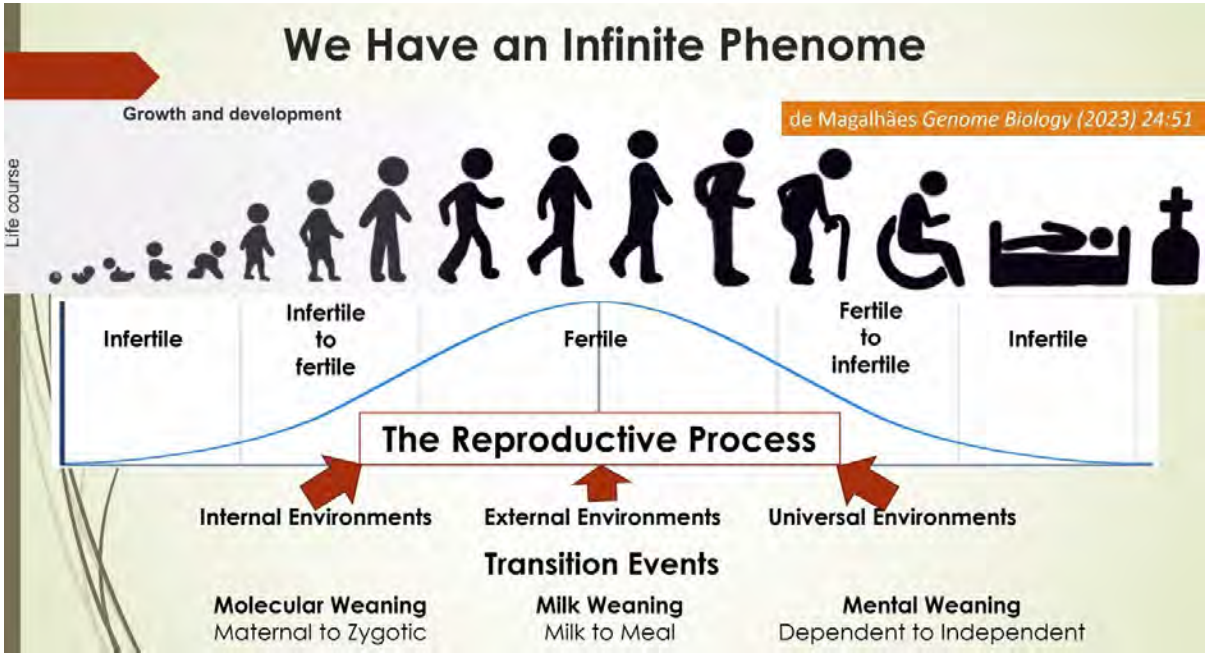
Sperm Phenotyping (+/+ +/- -/- sires)



- Immunofluorescence on spermatozoa, isolated testicular cells and testicular tissue section
- Image-based flow cytometry (IBFC) on carrier and wild type spermatozoa
- Western blotting
- Immuno-electron microscopy
- Omics
- Functional phenotypes – ability to undergo capacitation, fertilize eggs in vitro, produce embryos

Finite Genomes, Infinite Phenotypes

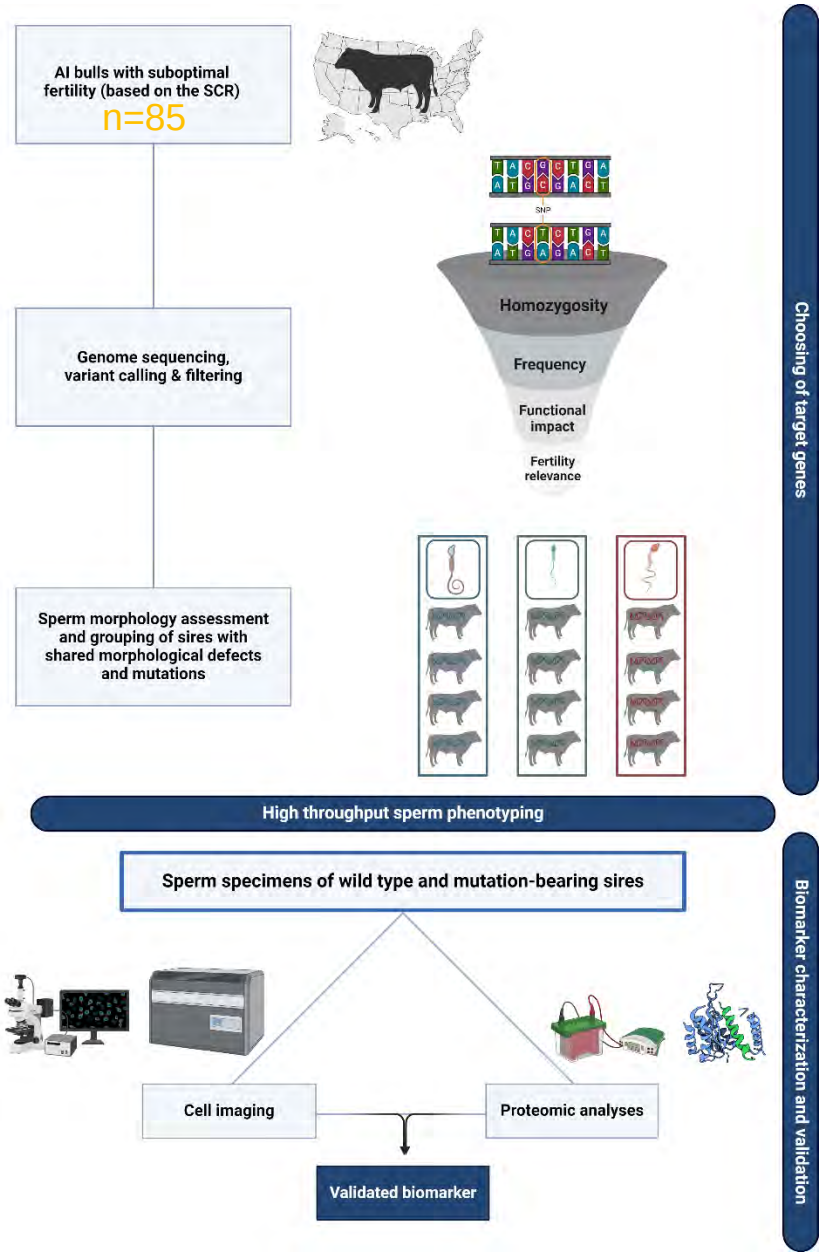
- Meiotic recombination
- Imprinting & small regulatory RNAs
- Alternative transcription and mRNA splicing
- In utero influences
- Environmental & postnatal influences
- Aging



$$P = G_{enome} + R_{na} + E_{nvironment} + G \times R \times E$$

High Throughput Phenotyping of Polygenic Heritable Sperm Defects

USDA NIFA Animal Genomics & Phenomics Foundational Project



Abnormal Sperm Morphology

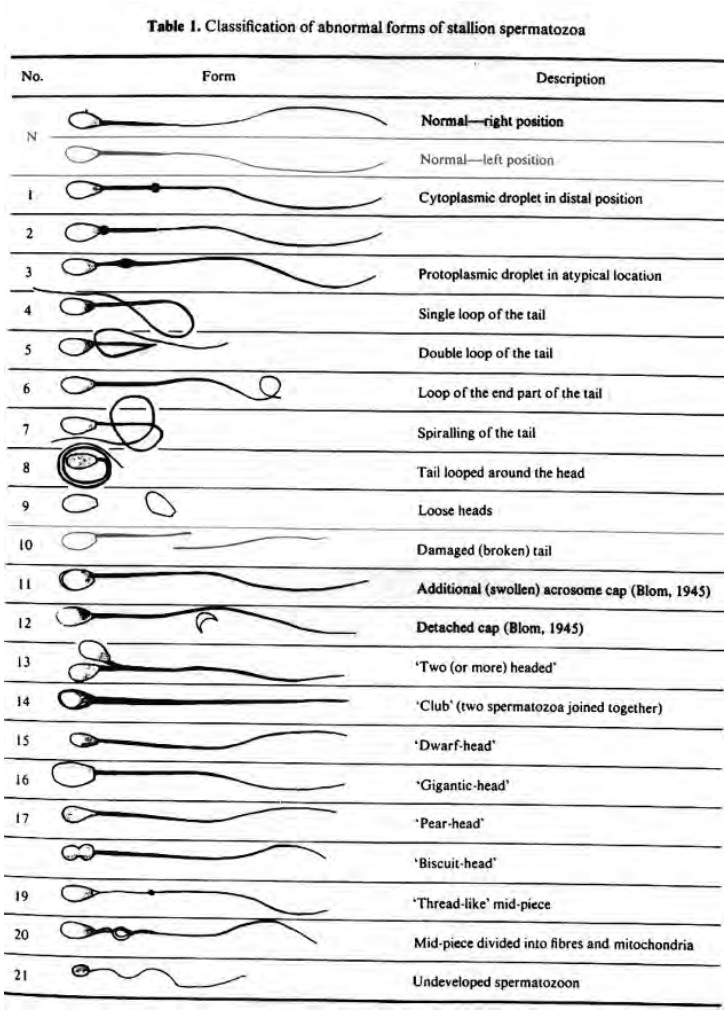


Table 1. Classification of abnormal forms of stallion spermatozoa

No.	Form	Description
N		Normal—right position
		Normal—left position
1		Cytoplasmic droplet in distal position
2		
3		Protoplasmic droplet in atypical location
4		Single loop of the tail
5		Double loop of the tail
6		Loop of the end part of the tail
7		Spiralling of the tail
8		Tail looped around the head
9		Loose heads
10		Damaged (broken) tail
11		Additional (swollen) acrosome cap (Blom, 1945)
12		Detached cap (Blom, 1945)
13		'Two (or more) headed'
14		'Club' (two spermatozoa joined together)
15		'Dwarf-head'
16		'Gigantic-head'
17		'Pear-head'
		'Biscuit-head'
19		'Thread-like' mid-piece
20		Mid-piece divided into fibres and mitochondria
21		Undeveloped spermatozoon

Expectations

Breeding Bulls: >70-75% normal morphology acceptable; 20 million spermatozoa per AI dose.

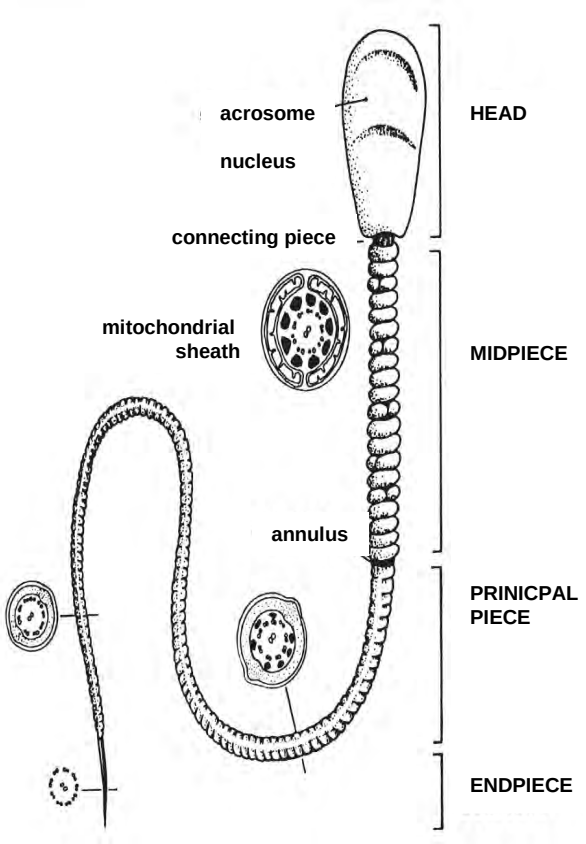
Non-compensable Sperm Abnormalities

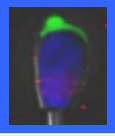
•Interfere with sperm function/fertilization process.
•Cannot be compensated for by increasing sperm number per AI dose.
•Heritable.
•Diadem defect/nuclear craters; nuclear vacuoles; “Dag” defect (coiled tails), knobbed acrosome, primary ciliary dyskinesia (asthenozoospermia).

Preliminary Data (collaboration with Schnabel Lab at MU)

•85 genome sequenced AI sires with varied but acceptable fertility.
•5 prevalent defect groups (non compensable defects).
•22 group-specific, rare, homozygous recessive gene variants identified, predicted to be deleterious.

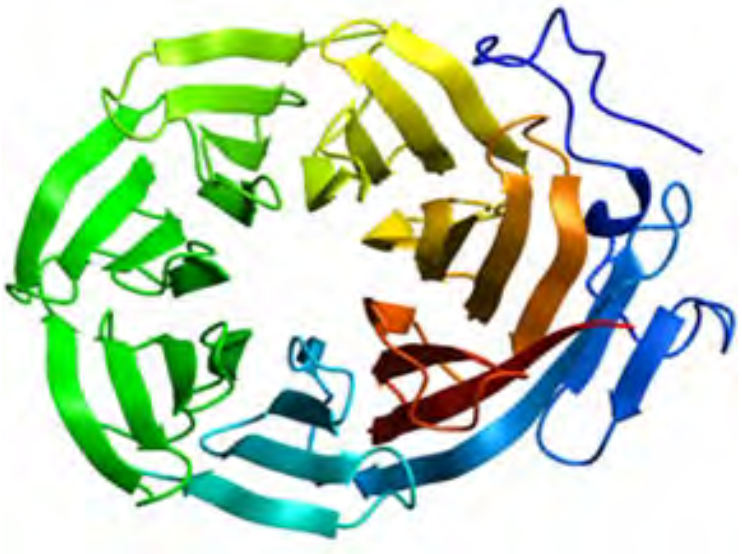
Non-Compensable Sperm Defects and Genes of Interest



Morphological Defect	Candidate Genes	Gene Function
Knobbed Acrosomes 	EML5	Vesicular trafficking and protein complex scaffold protein
	PSD3	Regulation of ARF protein signal transduction and guanyl-nucleotide exchange factor
	KLHL14	Vesicle trafficking and scaffolding protein, locates to ER/Golgi network
	ADGRA2	Regulator of the WNT pathway, development of blood-testis barrier and membrane organization
Nuclear Vacuoles 	EP400	Part of the Snf2-related CBP-activator protein chromatin remodelling complex (SRCAP)
	PEG3	DNA Damage & Repair Pathways, Imprinted gene
	MSL3	Chromatin remodelling, transcription regulation and X-inactivation factor
	PIR	Transcription cofactor, replication and gene transcription regulator at the level of DNA complexes
	TDRD9	Helicase implicated in spermatogenesis, involved in the silencing of potential transposable elements, and protecting the integrity of the male germline
Mitochondrial Aplasia 	SUPVSL1	Major helicase regulating mitochondrial metabolism
	RXFP2	Functions in Golgi apparatus and flagellar organization
Asthenozoo-spermia 	CNTLN	Functions in centrosome adhesion
	PHIP	Cell morphology and cytoskeletal organization
	FGD1	Actin cytoskeleton and cell shape regulation
	FGD5	Directional movement of actin, cytoskeletal and cell shape regulation
	MYH1	Actin-based motor protein
	BRCA1	DNA repair and E3 ubiquitin ligase activity
Stump Tail 	POC1A	Functions in ciliogenesis
	DCDC2C	Critical component of the human sperm microtubulome
	KIAA0586	Functions in ciliogenesis
	CEP152	A major microtubule-organizing center that influences cell shape, polarity, and motility
	MICAL1	Cytoskeletal regulator that interacts with intermediate filaments

From Sire Genome to Sperm Phenotype: Phenotyping of a Rare, Fertility Affecting Mutation in *Eml5* Gene

- Echinoderm microtubule-associated protein-like 5 isoform X5
- Rare mutation in WD40/TAPE domain
- Repetitive, circular solenoid protein domain for multi-protein complex assembly



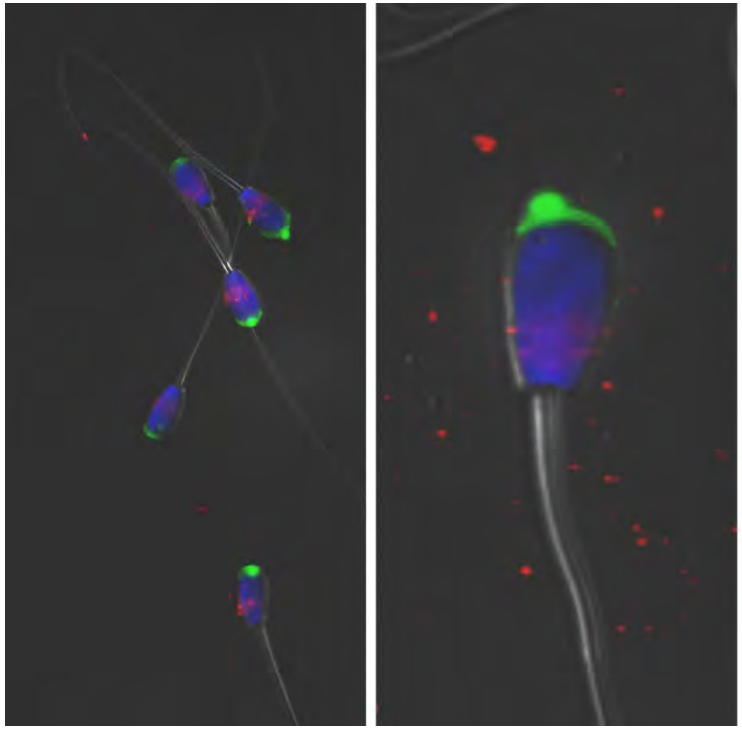
Nogueira et al., 2022

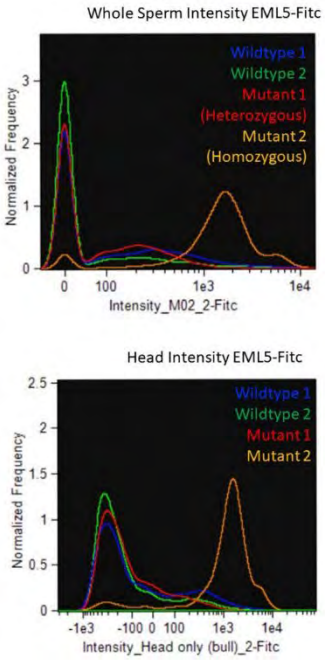
frontiers

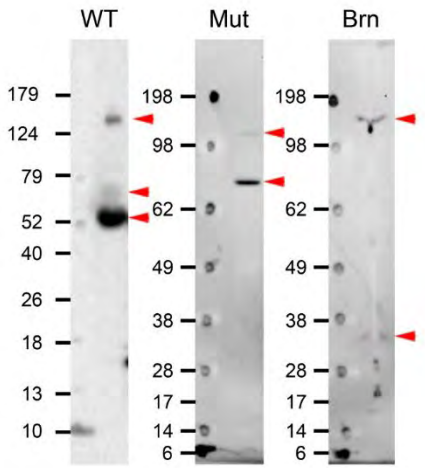
Karolinska Institutet

Embrapa

Protruding Knobbed Acrosome Phenotype in Homozygous *Eml5*$^{wd40+/+}$ Mutant Bull

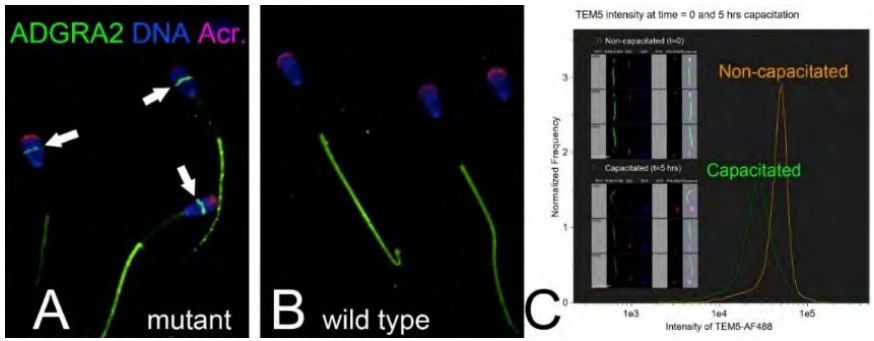


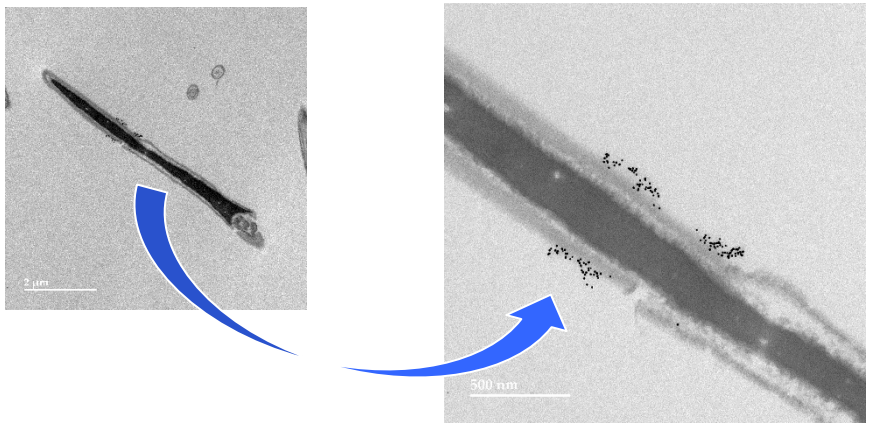




ADGRA2/TEM5 – Adhesion G Protein-Coupled Receptor A2

- Rare recessive-homozygous deleterious mutation present in sires with high % knobbed acrosome phenotype.
- Protein restricted to sperm tail principal piece in WT sires.
- Accumulates in sperm head acrosome/equatorial segment in mutation carriers.
- Involved in WNT pathway signaling.
- Collaboration with Mayra E. Ortiz D'Avila Assumpção, University of São Paulo

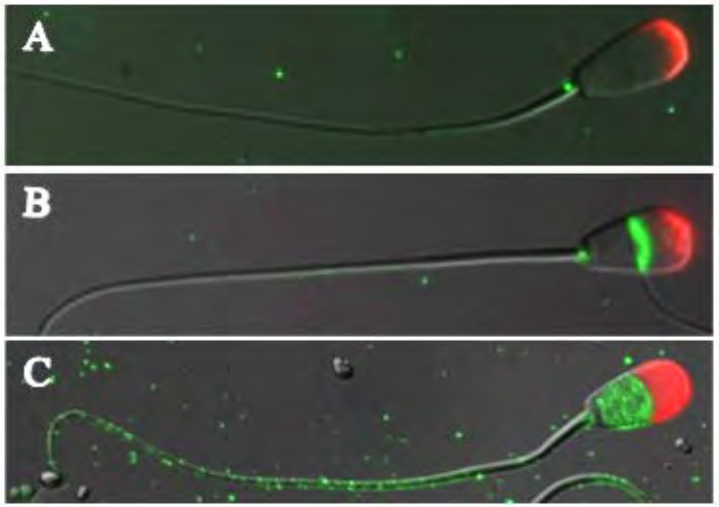




Immunofluorescence, IBFC and nanogold immunoelectron microscopy of ADGRA2 in WT & carrier bull spermatozoa

KLHL14 - Kelch-Like Family Member 14

KLHL14
Acrosome (lectin PNA)
DNA (DAPI)



morphological normal

knobbed acrosome

normal after sexing

- Torsin A ATPase-interacting, adaptor protein for ubiquitin ligase enzymes
- Implicated in cancer cell proliferation
- Localized in endoplasmatic reticulum (somatic cells) and Golgi (spermatocytes)
- Rare, deleterious, homozygous-recessive mutation discovered in our genome-sequenced AI bulls with inward-knobbed acrosomes.

Phenotyping of Nuclear Vacuole Defect Associated Gene Variants

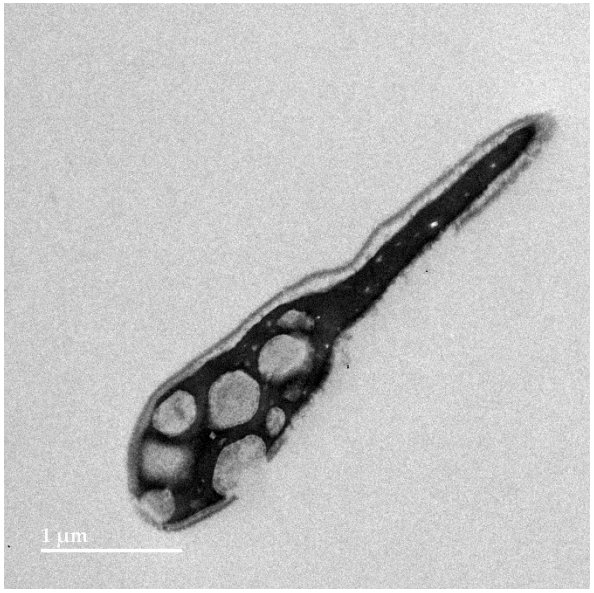


TABLE: Candidate genes harboring rare deleterious mutations associated with sperm nuclear vacuole phenotype. All candidate gene were identified in all three out of 85 genotyped sires with highest NV frequency through our established genomics pipeline.

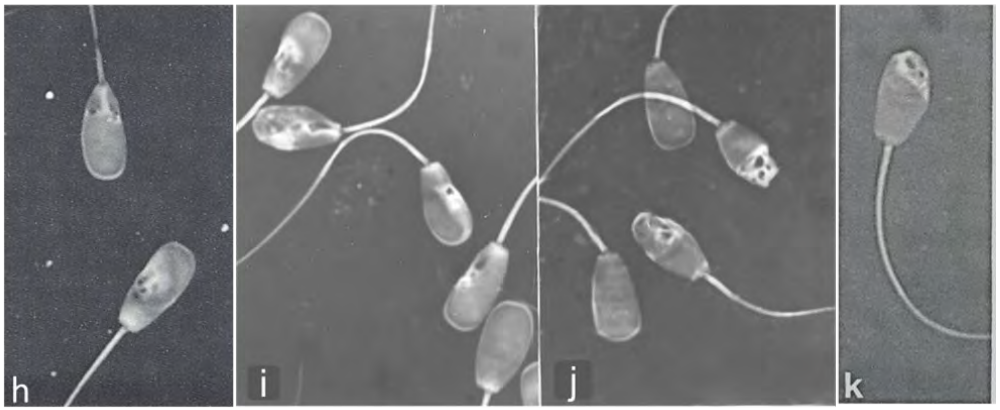
Gene Name, Function, Mutant Phenotype & Reference	Mutation in NV Bulls
EP400 – E1A Binding Protein 400. Part of the Snf2-related CBP-activator protein chromatin remodelling complex (SRCAP). Mice homozygous for a knock-out allele die at E11.5 and display severe defects in yolk sac erythropoiesis, anemia, and a neural tube deformity [14] .	Missense mutation (amino acid change)
PEG3 – Paternally Expressed 3. Regulates DNA damage & repair pathways; imprinted gene. Embryo lethal homozygous knockout; heterozygous mutants have postnatal lethality, decreased oocyte numbers, small testis, abnormal imprinting and decreased body weight [15].	Missense mutation (amino acid change)
MSL3 - Male Specific Lethal Ribonucleoprotein. Chromatin remodelling, transcription regulation and X-inactivation factor Male embryo-lethal KO in Drosophila [16]. Mutant mouse [17] has neurological and behavioural defects/hyperactivity. Human gene is linked to Basilicata-Akhtar syndrome; syndromic X-linked intellectual disability characterized by global developmental delay apparent from infancy, feeding difficulties, hypotonia, and poor or absent speech that has material basis in hemizygous or heterozygous mutation in MSL3 on chromosome Xp22.2 [18].	Splice region variant
PIR – Pirin/Iron Binding Nuclear Protein. Transcription cofactor, replication, and gene transcription regulator at the level of DNA complexes. One-fifth of male chimeras hemizygous for a gene trapped allele exhibit a developmental delay. Overall, the effect was growth retardation [19].	5'UTR modifier, alters translation.
TDRD9 – Tudor Domain Containing 9. DDX3 type DNA helicase implicated in spermatogenesis, involved in silencing of transposable elements, and protecting the integrity of the male germline. Male homozygous mice are sterile, with small testis, arrest of male meiosis and abnormal spermatocyte morphology [20]. Females are fertile. Also associated with spermatogenic failure (nonobstructive azoospermia or cryptozoospermia) caused by homozygous or compound heterozygous mutation on human chromosome 14q32.33.	Missense mutation (amino acid change)

- Mutations shared by sires with high % sperm nuclear vacuole phenotype.
- All are expressed in testis and during preimplantation embryo development.
- Cold be paternal contributors to post-fertilization developmental failure and/or early pregnancy loss.

Nuclear Vacuole Associated Gene Variants Ctd’

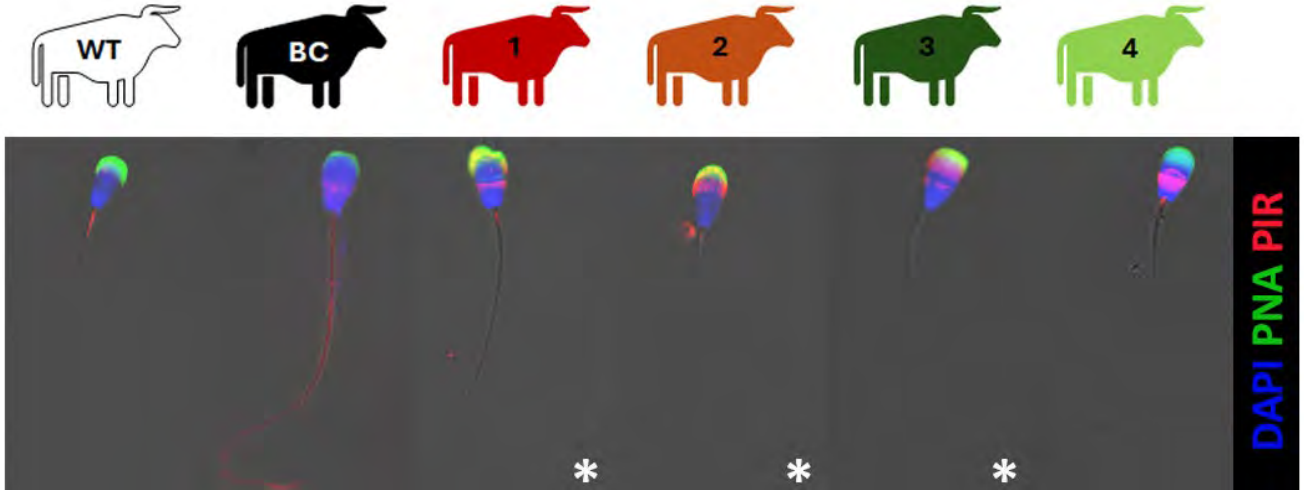
Gene/Sire	PIR	MSL3	PEG3	TDRD9	EP400
1RNK	+	+		+	+
2DJO	+	+			
3RIO	+	+			
4CRO	+		+		
5MOE			+		

Photo credit:
Barth A, Perry V, Hamilton L, Sutovsky P & Oko R
Abnormal Morphology of Bovine Spermatozoa
Second Edition, 2025
Springer-Nature



Estimated Breeding Values of Bulls Harboring Gene Mutations Related to NV Phenotype

Bull	DAUS	HERDS	NM$	DPR (%)	HCR (%)	CCR (%)
A	479	293	-361	-0.4	-0.6	-1.8
B	513	78	447	-3.0	-1.4	-3.6
C	3243	772	-2	-2.0	1.8	-1.3
D	203	48	212	0.3	0.5	1.0
E	6626	1843	47	-1.5	0.4	-2.0

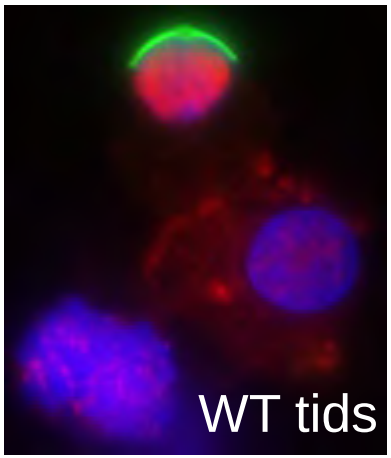


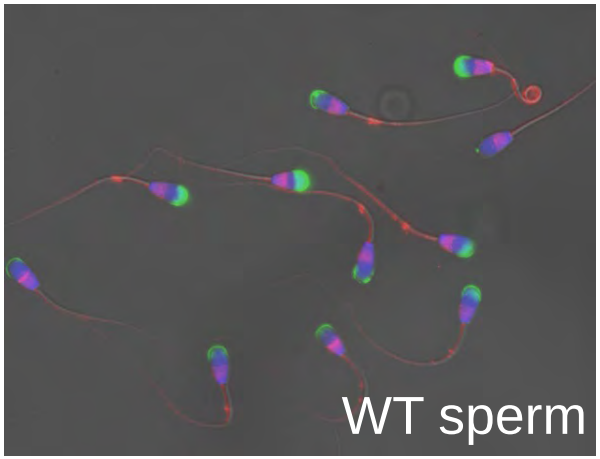
WT &1-3: genome sequenced **BC:** unknown genotype *****recessive homozygous variant allele carriers***** **4:** half brother of 3

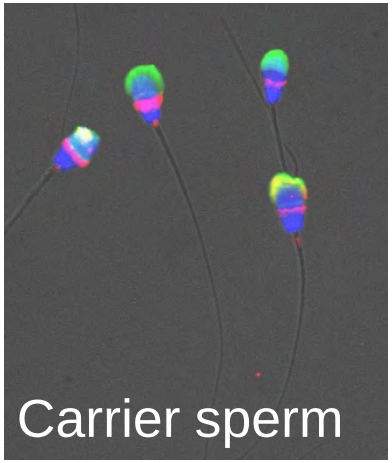
- Number of daughters in evaluation (DAUS).
- Number of herds in evaluation (HERDS). Lifetime net merit (NM$) index (ranks dairy animals based on their combined genetic merit for economically important traits).
- Daughter Pregnancy Rate (DPR; predicts the percentage of non-pregnant cows that will become pregnant during each 21-day period compared to the breed base).
- Heifer Conception Rate (HCR; predicts maiden heifer's ability to conceive, defined as expected percentage to become pregnant at each insemination in comparison to the breed base).
- Cow Conception Rate (CCR; predicts the lactating cow's ability to conceive, defined as expected percentage to become pregnant at each insemination in comparison to the breed base).
- Data (originally published in 04/2024) retrieved from the Council on Dairy Cattle Breeding (CDCB) database.

PIR - Pirin/Iron Binding Nuclear Protein

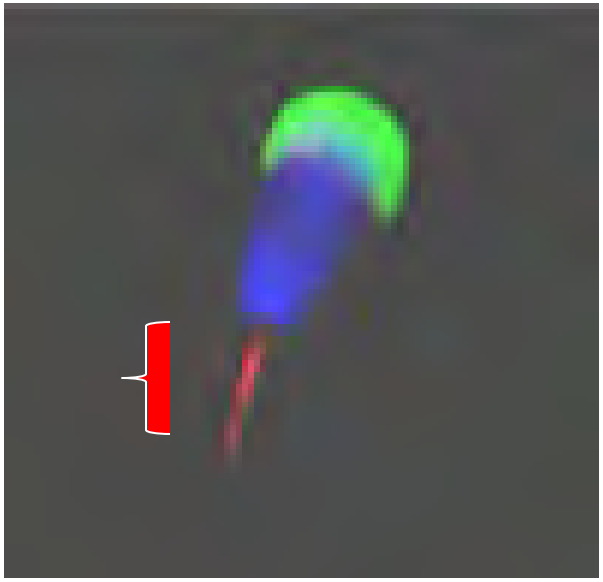
- Nuclear vacuole associated in mutation carriers (4/5 NV sires in present set).
- Transcription cofactor, replication, and gene transcription regulator at the level of DNA complexes.
- One-fifth of male mouse chimeras hemizygous for a gene trapped allele (multiple mutations) exhibit a developmental delay, causing growth retardation.



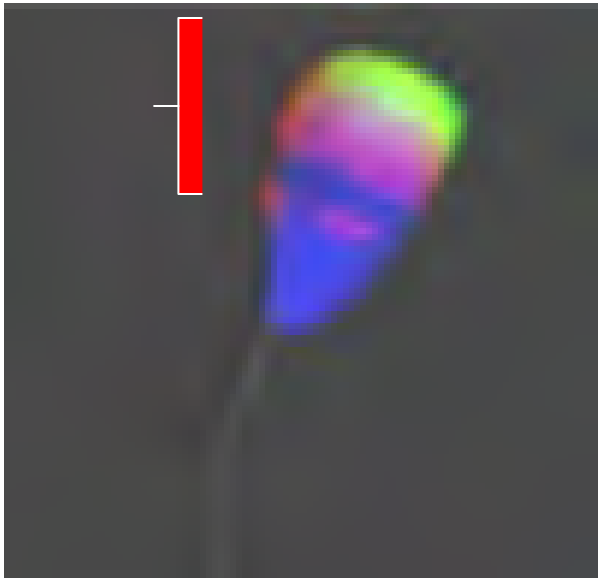




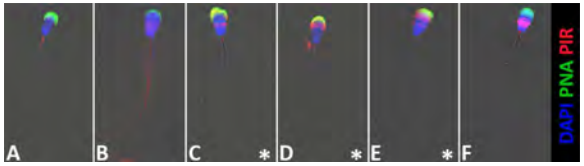
Typical, Representative PIR Phenotypes



Wild Type

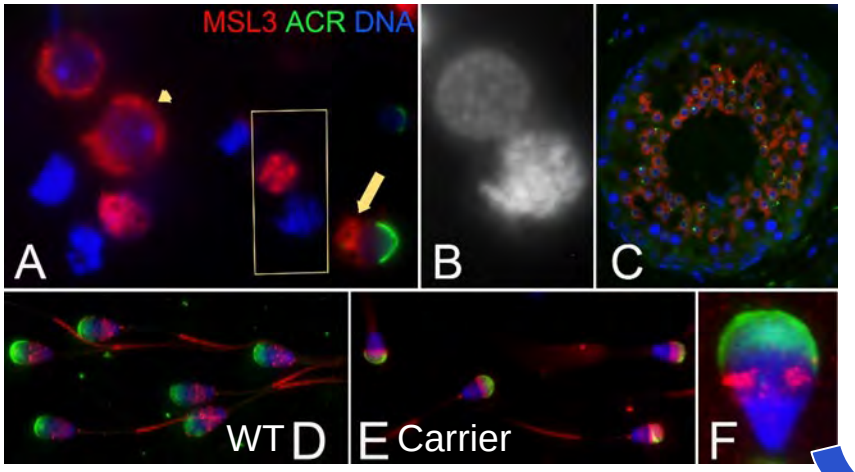


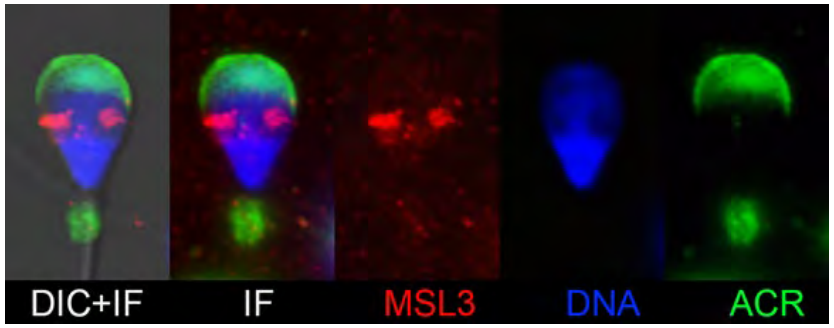
Deleterious Variant Carrier



MSL3 - Male Specific Lethal Ribonucleoprotein

- Chromatin remodeling, transcription regulation and X-inactivation factor
- Nuclear vacuole associated in mutation carriers (3/5 in present cohort).
- Equatorial in carriers, postacrosomal in WT sperm.
- Present only in acrosome and sperm tail principal piece in WT sires.
- Imprinted gene expressed from paternal copy in cattle
- Mutation kills male *Drosophila* embryos.





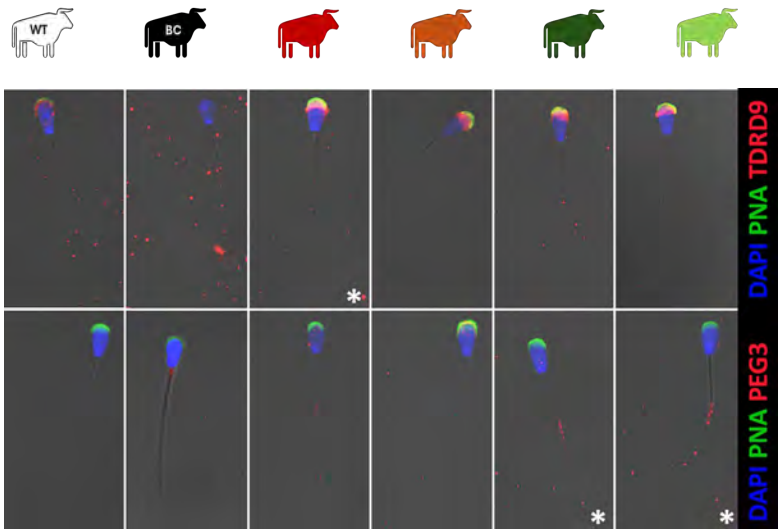
DNA Binding Proteins TDRD9 & PEG3

TDRD9 - Tudor Domain Containing 9
DNA helicase

- Spermatogenesis & protection of male germline integrity
- Silencing of potential transposable elements in the nucleus
- Associated with spermatogenic failure & azoospermia

PEG3 - Paternally Expressed 3

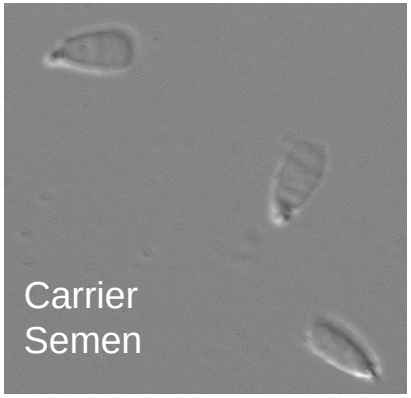
- DNA Damage & Repair
- Imprinted gene



TDRD9

PEG3

Ongoing – Stump Tail Mutations



GENE	FUNCTION	MUTATION
POC1A*	Ciliogenesis	Missense variant (AA change)
DCDC2C*	Major sperm microtubulome component in humans	Missense variant (AA change)
KIAA0586*	Cilliogenesis	Missense variant & loss of stop codon
CEP152	MTOC protein; cell polarity, shape & motility	Splice region variant
MICAL1	Cytoskeletal regulator, interacts with IMF	Missense variant (AA change)

*Posses alternative polyadenylation sites (collaboration with Dr. Zhihua Jiang, WSU)

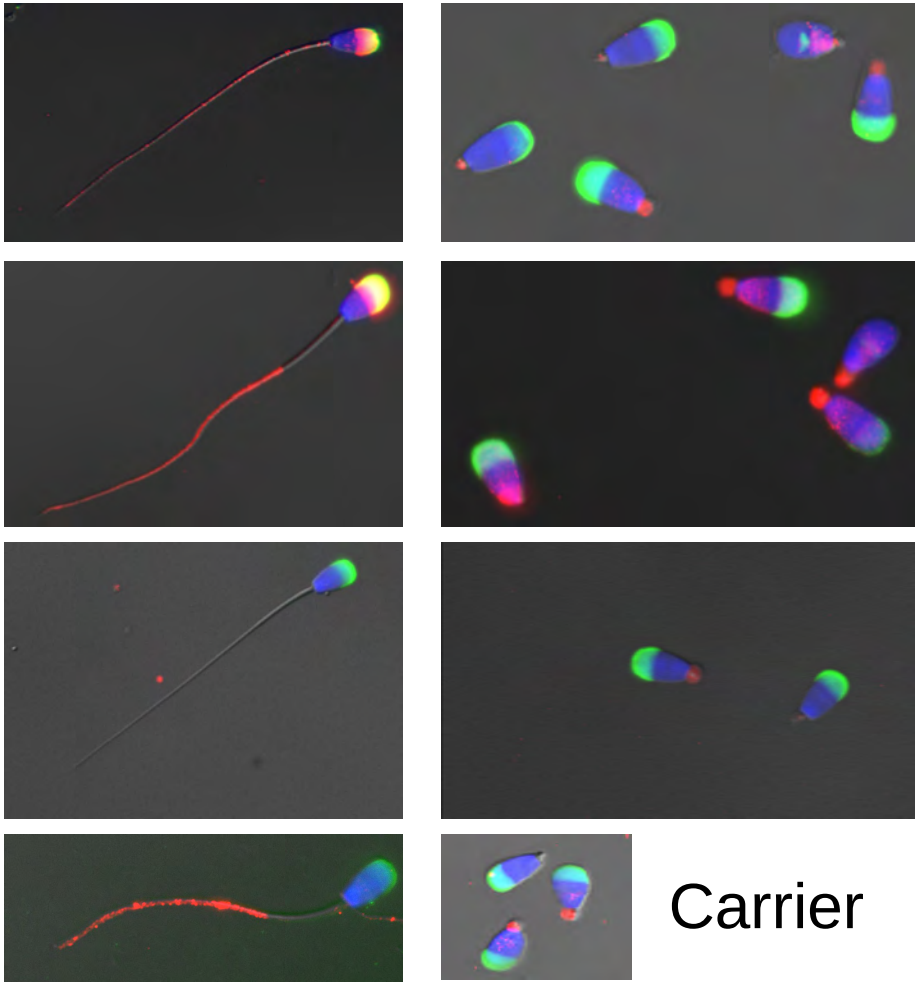
Stump Tail Mutation Phenotypes

CEP152

MICAL1

POC1A

KIAA0586

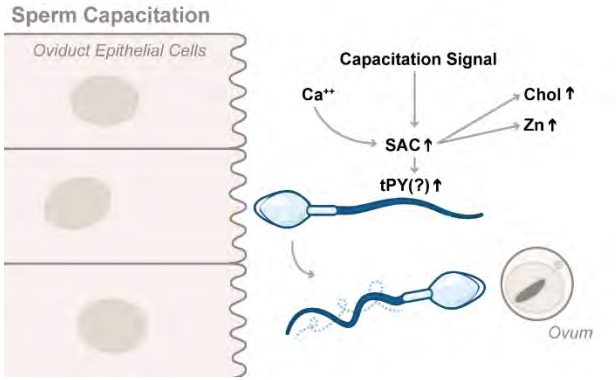


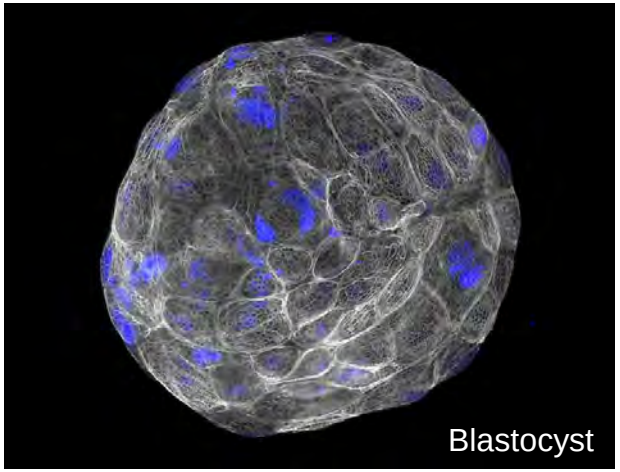
Wild Type

Carrier

Functional Sperm Phenotypes

- *In vitro* sperm capacitation (IVC) competency (acquisition of fertilizing ability, zinc signature change)
- Fertilization *in vitro*
- Development to blastocyst *in vitro*
- Development to term after embryo transfer



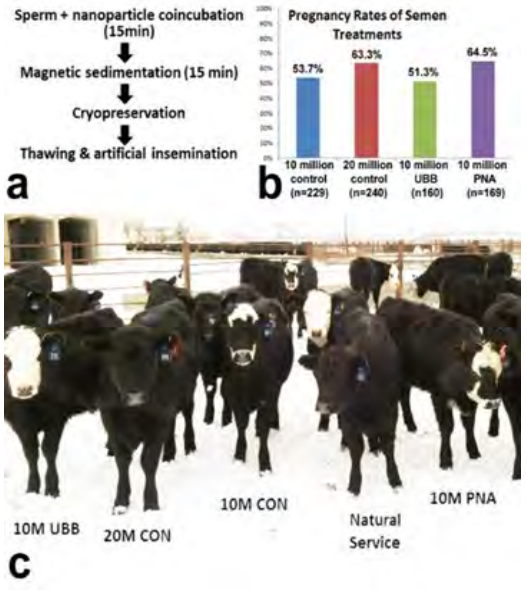


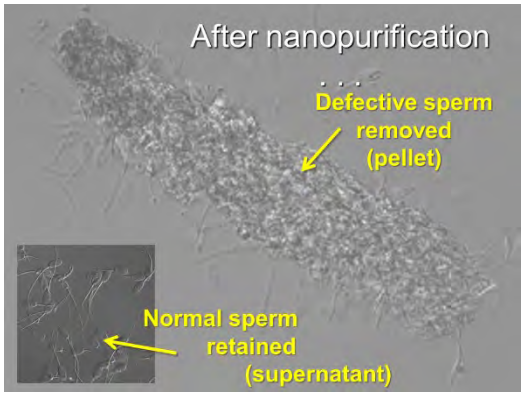
SUMMARY

- Biomarker-based sperm quality assays reveal molecular sperm defects undetectable by conventional semen analysis.
- Livestock models propagated by artificial insemination are ideal for biomarker development.
- Omics and biomarker-based machine learning will enable label-free, fully automated andrological analysis, *in livestock species.*

APPLICATIONS

- Veterinary diagnostics
- Breeding soundness evaluation
- Semen nanopurification (getting rid of the bad and the ugly)
- Semen extender improvement
- Semen sexing improvement
- Fertility-neutral genomic selection





Fertility Redefined

Low heritability, polygenic trait

Complex phenotype ready to be explored and exploited for the advancement of animal agriculture

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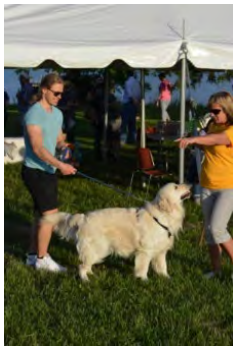

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Current Funding

USDA-NIFA Animal Genomics
USDA NIFA Animal Reproduction
NIH/NIEHS
Seed Funding MU CAFNR

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Sutovsky et al., (2024) Biomarker-based human and animal sperm phenotyping: **The good, the bad and the ugly**. ***Biol. Reprod.,*** Special Issue on Imaging, https://doi.org/10.1093/biolre/ioae061

Sutovsky et al., (2024) **Proteostasis as a sentry for sperm quality and male fertility**. In: *Molecular Male Reproductive Medicine: Biology and Clinical Perspectives*. C. Yan Cheng, Editor; Springer Nature, ***Adv Exp Med Biol,*** 2025

Photo: Ostrich spermatids, SSR 2024 Photo Contest

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