

Alternative techniques for preservation of small ruminant spermatozoa without liquid nitrogen

Sheep endangered species in Mediterranean area

Country	LOCAL			REGIONAL			INTERNATIONAL			TOTAL
	At risk	Not at risk	Un-known	At risk	Not at risk	Un-known	At risk	Not at risk	Un-known	
Morocco	0	14	27	0	3	1	0	30	3	78
Algeria	0	4	12	0	7	1	0	9	0	33
Tunisia	0	3	5	0	1	0	0	13	10	32
Lybian A.J.	0	1	2	0	1	0	0	2	0	6
Egypt	3	33	40	0	1	0	0	29	5	111
Israel	0	0	3	0	0	0	1	6	2	12
Lebanon	0	1	0	0	0	1	0	3	0	5
Syrian A.R.	1	4	3	0	0	1	0	7	3	19
Turkey	11	16	45	1	1	2	0	20	4	100
Greece	13	17	5	0	1	0	0	19	0	55
Albania	7	29	6	0	2	1	0	23	1	69
Montenegro	0	0	0	0	0	0	0	0	0	0
Bosnia &H.	1	3	15	0	2	0	0	3	0	24
Croatia	8	27	2	3	2	0	0	13	0	55
Slovenia	45	10	4	18	11	0	12	53	2	156
Italy	100	67	15	0	6	0	2	25	0	215
France	91	55	152	10	24	0	5	72	6	399
Spain	91	62	20	1	2	0	1	10	1	188
Portugal	4	28	0	2	2	0	0	9	0	45
Cyprus	3	10	4	0	1	0	0	11	0	29
Malta	1	1	0	0	1	0	0	4	0	7
TOTAL	379	385	340	35	68	7	21	361	38	1634

Conservation Methods

Conservation methods and their potential to contribute to various objectives

Objective	Type of conservation (if implemented as a stand-alone measure)		
	<i>In situ</i>	<i>Ex situ in vivo</i>	Cryoconservation
Maintaining flexibility for the future			
Insuring against changes in production conditions	Yes	Yes	Yes
Safeguarding against diseases, disasters, etc.*	No	No	Yes
Providing opportunities for research	Yes	Yes	Yes
Genetic factors			
Allowing continued evolution/genetic adaptation	Yes	Limited	No
Increasing knowledge of breed characteristics	Yes	Limited	Limited
Limiting exposure to genetic drift**	Yes	No	Yes
Sustainable management of rural areas			
Providing opportunities for rural development	Yes	Limited	No
Maintaining agro-ecosystem diversity	Yes	Limited	No
Maintaining rural cultural diversity	Yes	Limited	No

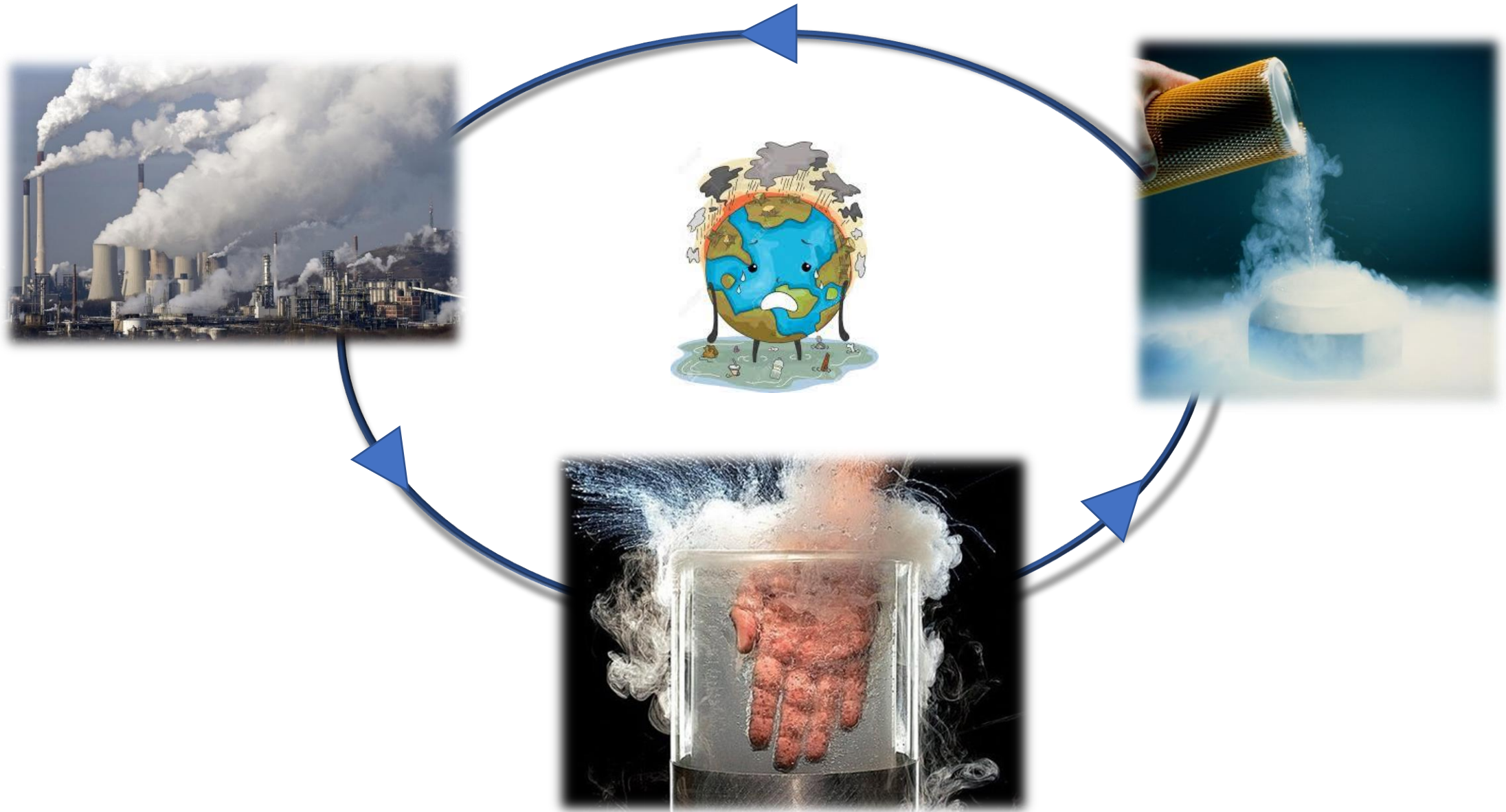
Note: *Risk from disease in *in vivo* programmes can be decreased by maintaining animals in geographically dispersed locations.

**The extent of genetic drift will depend on the population size *in situ* and the number of animals sampled for cryoconservation.

Genetic drift cannot be eliminated in *in vivo* populations, but proper management can limit drift to an acceptable level.

Source: FAO, 2013.

«Liquid Nitrogen Cycle»



Freeze-dry



Is it possible to freeze-dry spermatozoa?



What has been done?

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RESEARCH

1998
Mouse

Development of normal mice from oocytes injected with freeze-dried spermatozoa

Teruhiko Wakayama^{1,2} and Ryuzo Yanagimachi^{1*}

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Received 16 April 1998; accepted 3 June 1998

MOLECULAR REPRODUCTION AND DEVELOPMENT 75:890–894 (2008)

2008
Rat

Live Rats Resulting From Injection of Oocytes With Spermatozoa Freeze-Dried and Stored for One Year

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³The Graduate University of Advanced Studies, Okazaki, Aichi, Japan

2017
Mice from
the space

PNAS

Healthy offspring from freeze-dried mouse spermatozoa held on the International Space Station for 9 months

Sayaka Wakayama^{a,1}, Yuko Kamada^b, Kaori Yamanaka^c, Takashi Kohda^d, Hiromi Suzuki^e, Toru Shimazu^e, Motoki N. Tada^f, Ikuko Osada^f, Aiko Nagamatsu^g, Satoshi Kamimura^g, Hiroaki Nagatomo^{a,h}, Eiji Mizutani^b, Fumitoshi Ishino^g, Sachiko Yano^g, and Teruhiko Wakayama^{a,b,1}

2004
Rabbit

BIOLOGY OF REPRODUCTION 70, 1776–1781 (2004)
Published online before print 11 February 2004.
DOI 10.1095/biolreprod.103.025957

Freeze-Dried Sperm Fertilization Leads to Full-Term Development in Rabbits¹

Ji-Long Liu,^{3,5} Hirokazu Kusakabe,^{4,6} Ching-Chien Chang,⁵ Hiroyuki Suzuki,⁷ David W. Schmidt,⁵ Marina Julian,⁵ Robert Pfeffer,⁵ Charles L. Bormann,⁵ X. Cindy Tian,⁵ Ryuzo Yanagimachi,⁶ and Xiangzhong Yang^{2,5}

¹Department of Animal Science/Center for Regenerative Biology,⁵ University of Connecticut, Storrs, Connecticut 06269
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³Faculty of Agriculture and Life Sciences,⁷ Hirosaki University, Hirosaki 036-8561, Japan

REPRODUCTION
RESEARCH

«2011
Horse»

Production of live foals via intracytoplasmic injection of lyophilized sperm and sperm extract in the horse

Y H Choi¹, D D Varner², C C Love², D L Hartman³ and K Hinrichs^{1,2}

Departments of ¹Veterinary Physiology and Pharmacology and ²Large Animal Clinical Sciences, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, Texas 77843-4466, USA and ³Hartman Equine Reproduction Center, Whitesboro, Texas 76273, USA



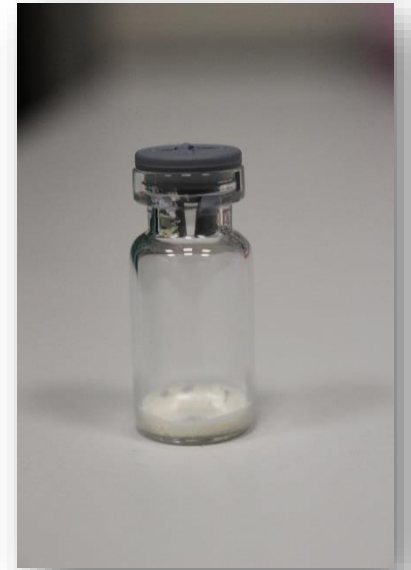
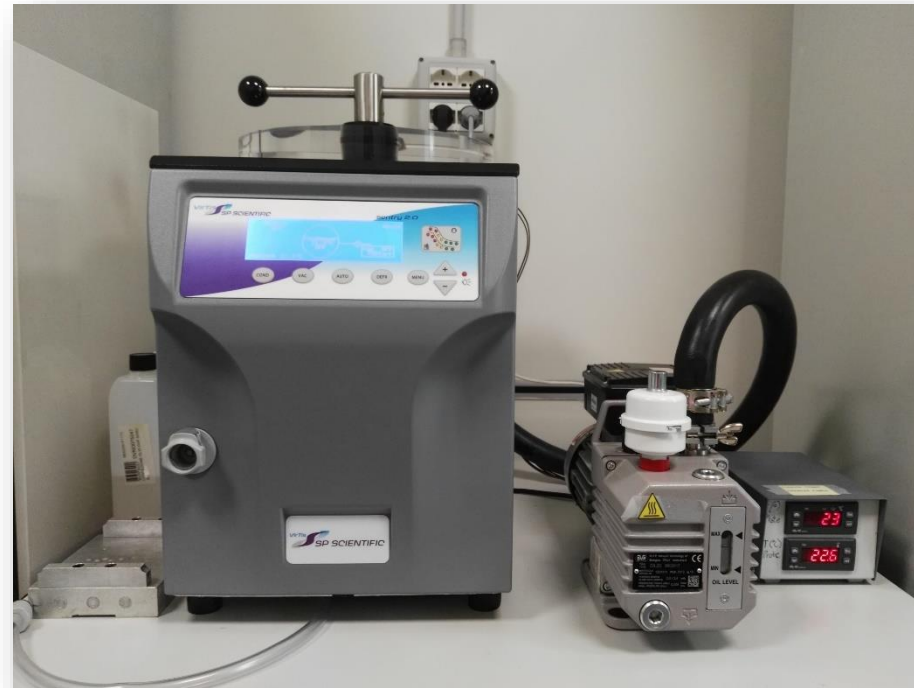
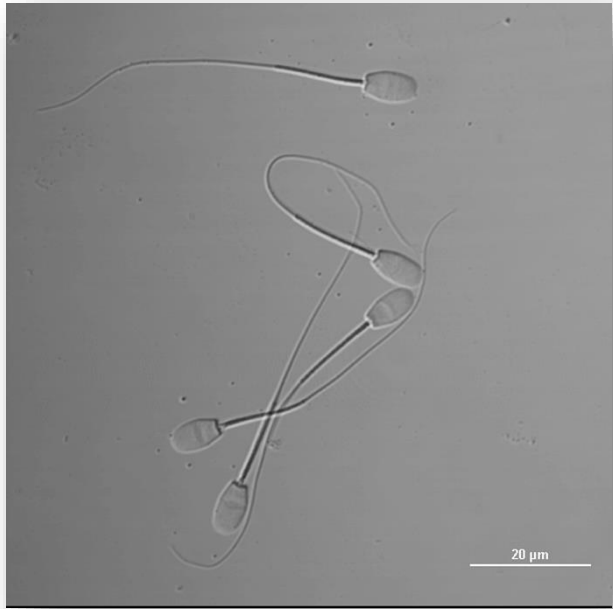
Pagliarola breed

“...il contadino le somministrava quanto vi ha più di scadente fra le materie diverse che si usano e servono come foraggio: le fronde di potatura dell’olivo, le foglie verdi o secche, gli sterpi, le radici, l’erbe raccolte nei fossi o lungo le prode dei campi, la gramigna, lo strame, la paglia...”

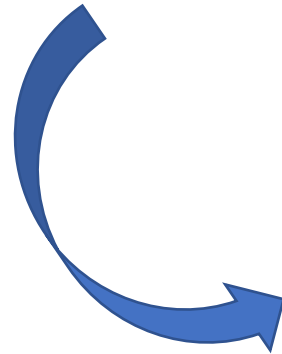
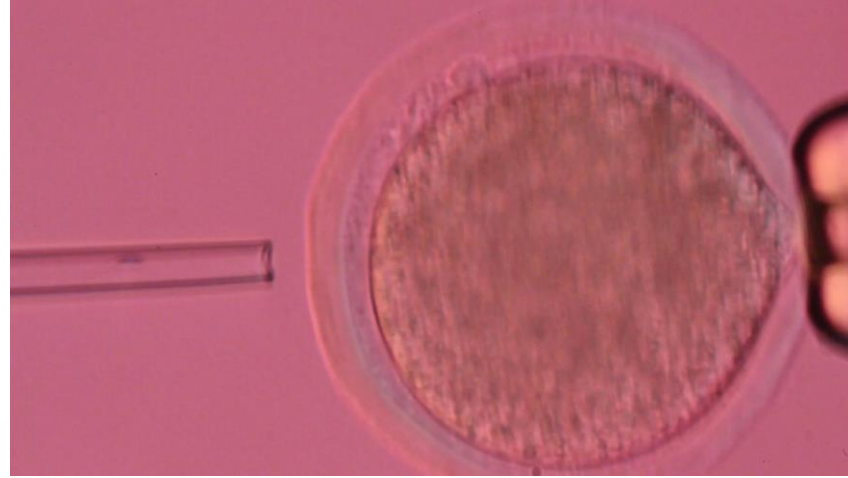
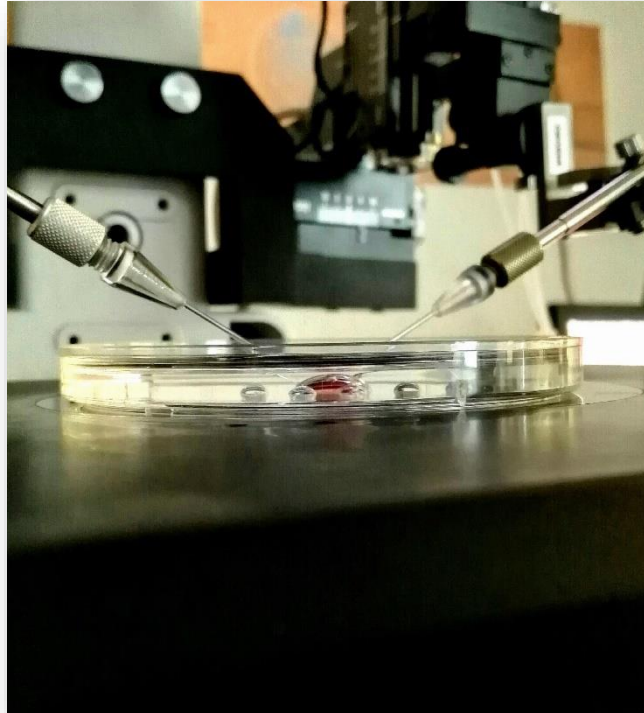
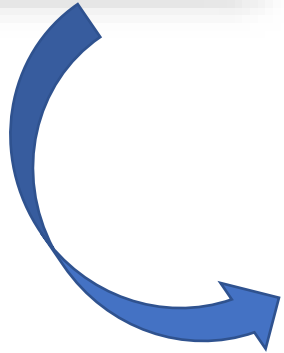
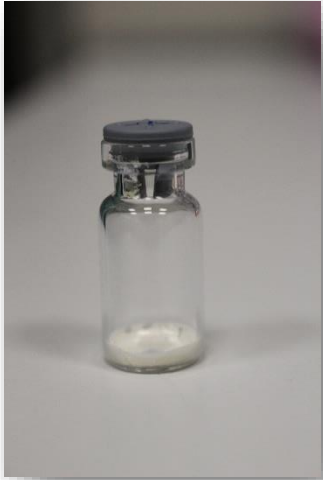
Giovanni Savazzini (1910) *Le Pagliarole*



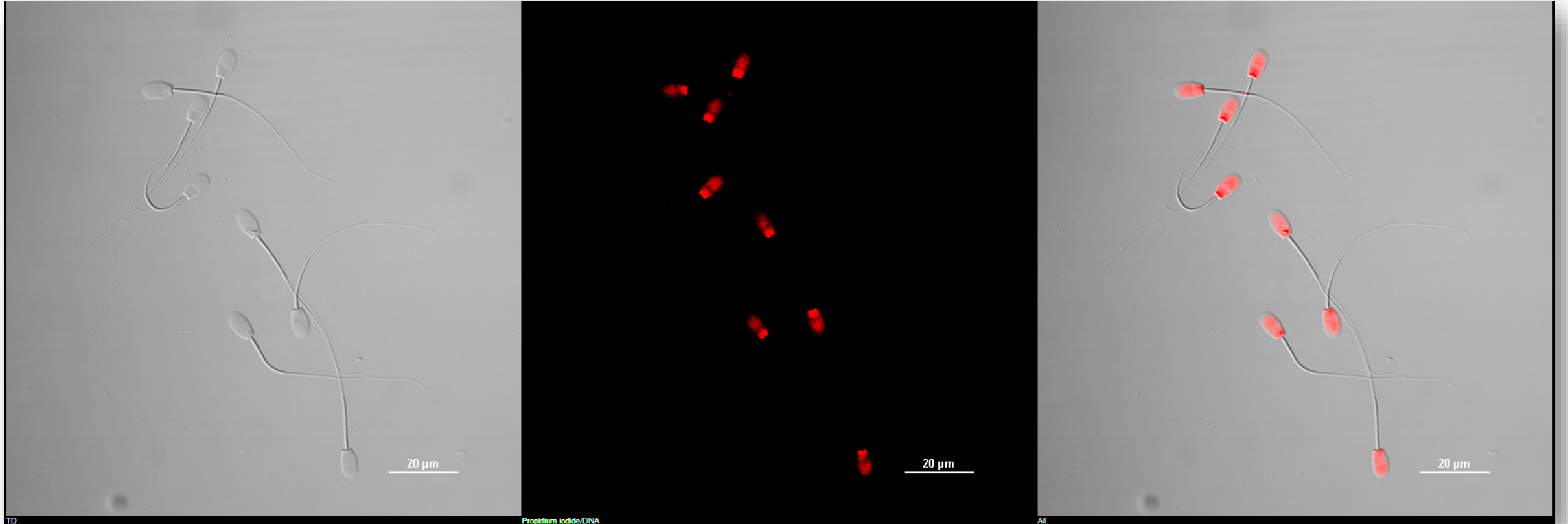
Freeze-dry Ram Spermatozoa



ICSI with freeze-dried semen

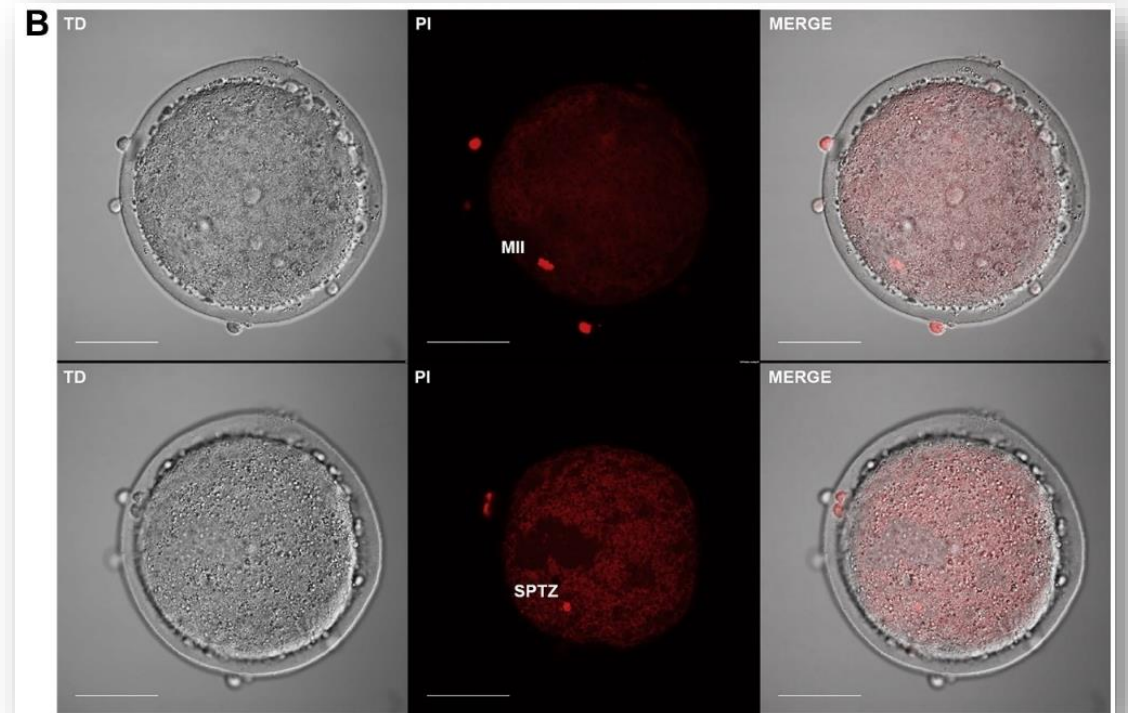
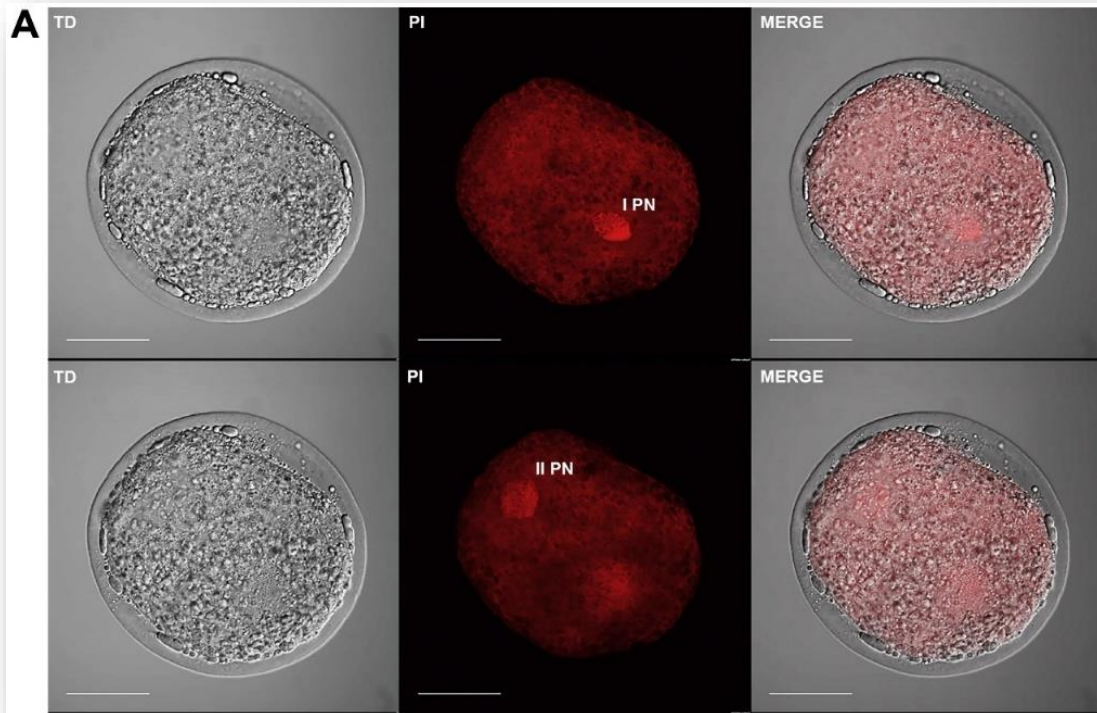


The lyophilized spz preserves the morphology post-rehydration

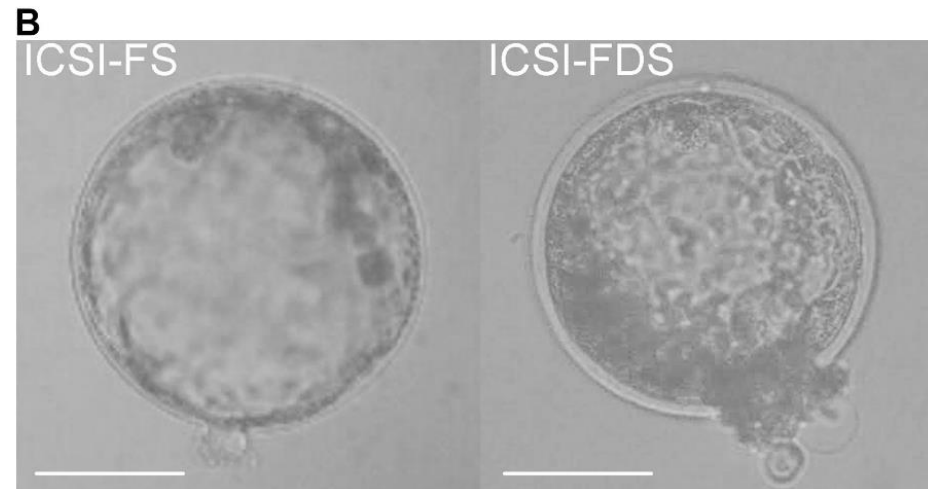
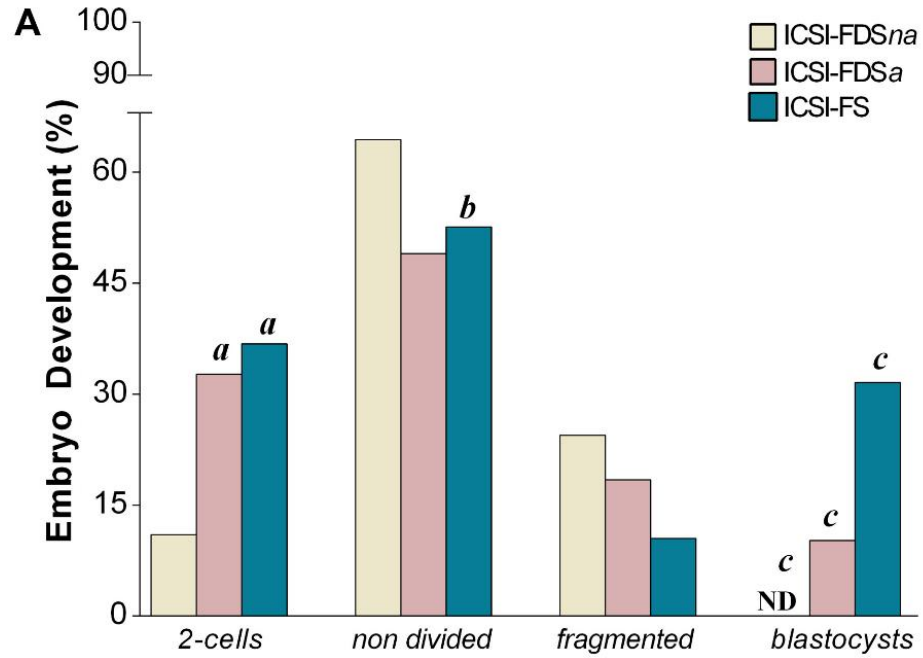


ICSI with FD spz requires chemical activation

Groups	Nr. Oocytes	Lysed (%)	2-Cells (%)	Non divided (%)	Fragmented (%)	Blastocyst (%)
ICSI Frozen	22	3/22 (13,6)	7/19 (36,8)	10/19 (52,6)	2/19 (10,5) ^c	6/19 (31,57)
ICSI- FDSna	52	7/52 (13,5)	5/45 (11) ^a	29/45 (64,4)	11/45 (24,4)	0/45 (0)
ICSI-FDSa	42	4/42 (9,5)	12/38 (31,6)	15/38 (39,5) ^b	11/38 (29)	2/38 (5,2) ^d



Freeze-dried spermatozoa: an alternative biobank for endangered species



Lyophilization of the epididymal sperm

npj © 1998 Nature Publishing Group <http://www.nature.com/naturebiotechnology>

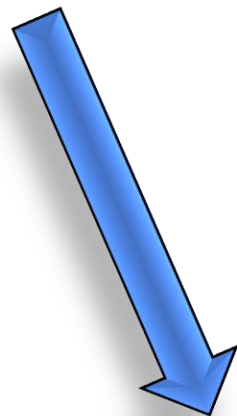
RESEARCH

Development of normal mice from oocytes injected with freeze-dried spermatozoa

Teruhiko Wakayama^{1,2} and Ryuzo Yanagimachi^{1*}

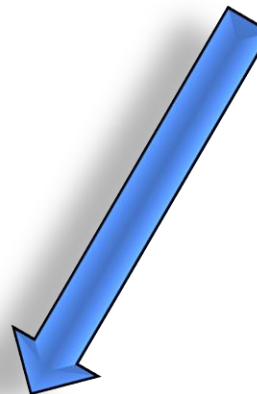
¹Department of Anatomy and Reproductive Biology, University of Hawaii School of Medicine, Honolulu, HI. ²Department of Veterinary Anatomy, Faculty of Agriculture, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan. *Corresponding author (e-mail: yana@hawaii.edu).

Received 16 April 1998; accepted 3 June 1998

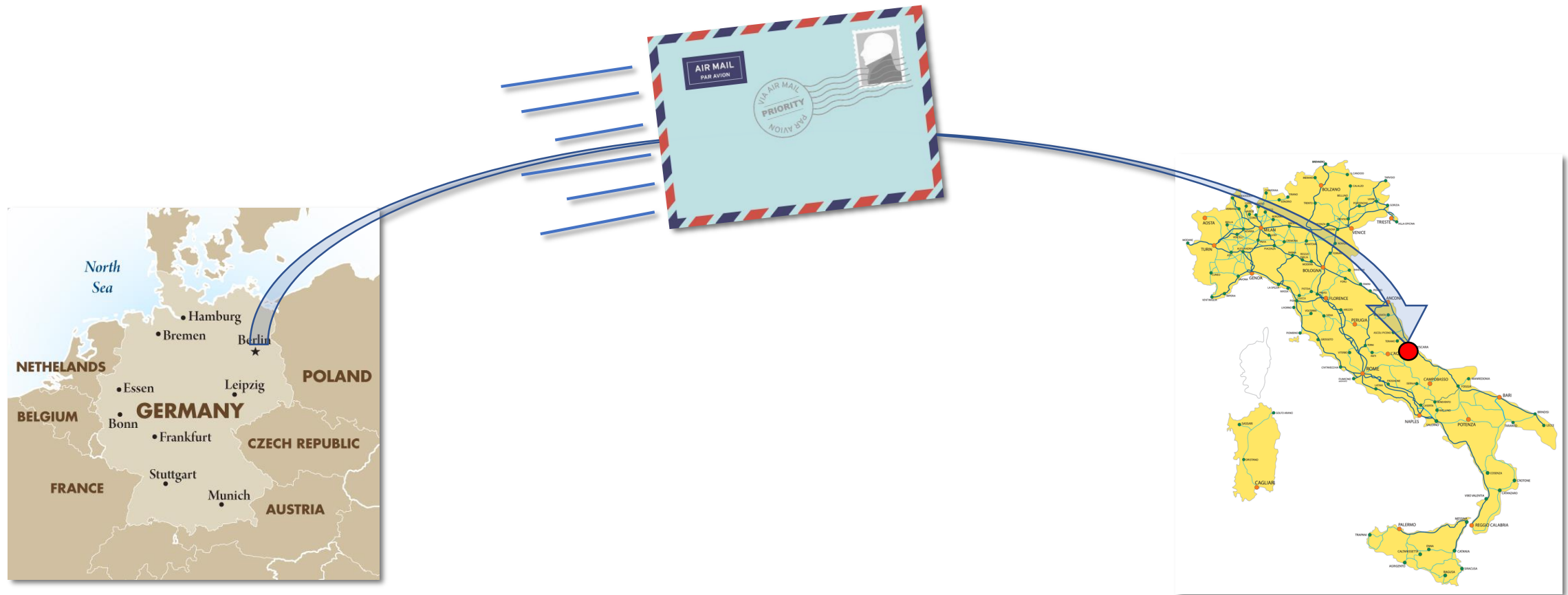


Epididymal spermatozoa

Cases of male infertility (eg Azoospermia)

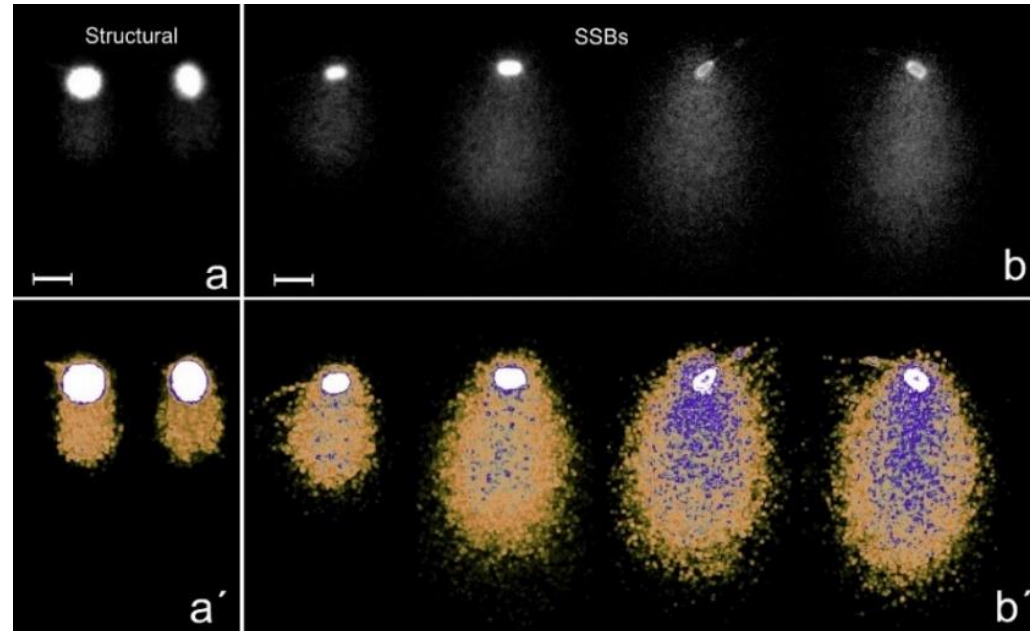
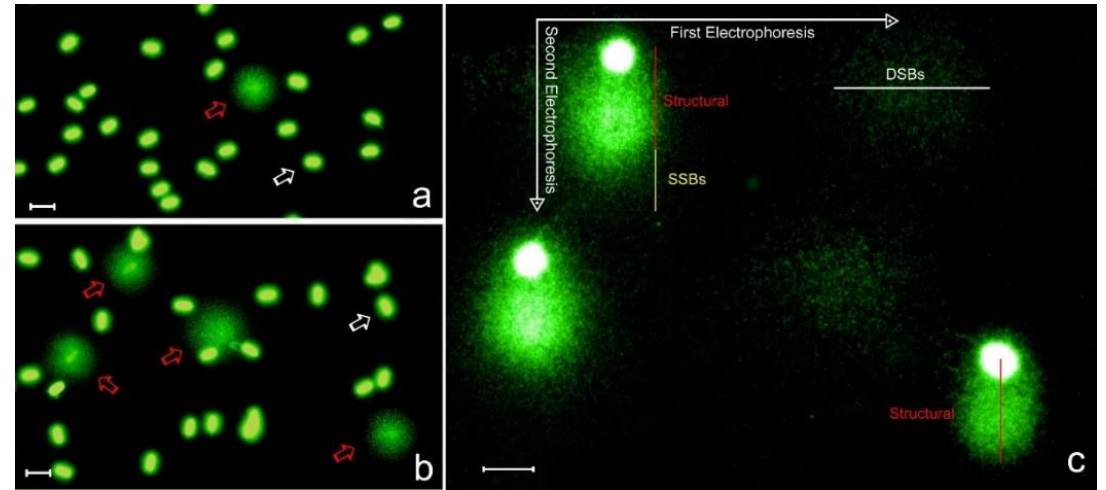


Lyophilization of the epididymal sperm



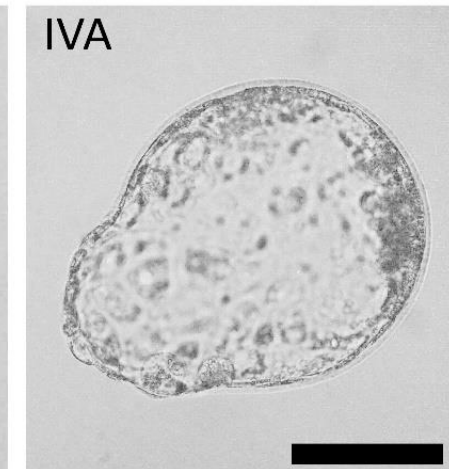
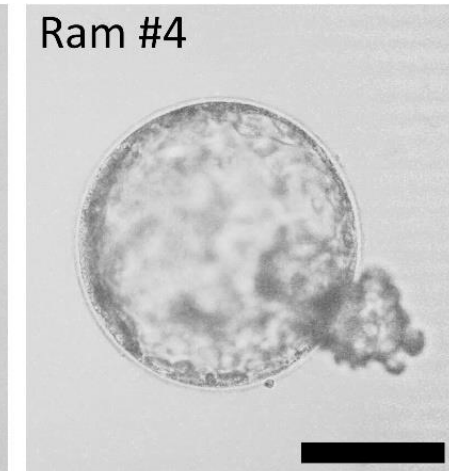
Lyophilization of the epididymal sperm

	Spermatozoa with Normal DNA (%)	Spermatozoa with Fragmented DNA (%)	
		SSBs	DSBs
Ram #1	3.8	95.9	0.3
Ram #2	28.0	70.0	2.0
Ram #3	2.8	92.6	4.6
Ram #4	5.0	93.0	2.0



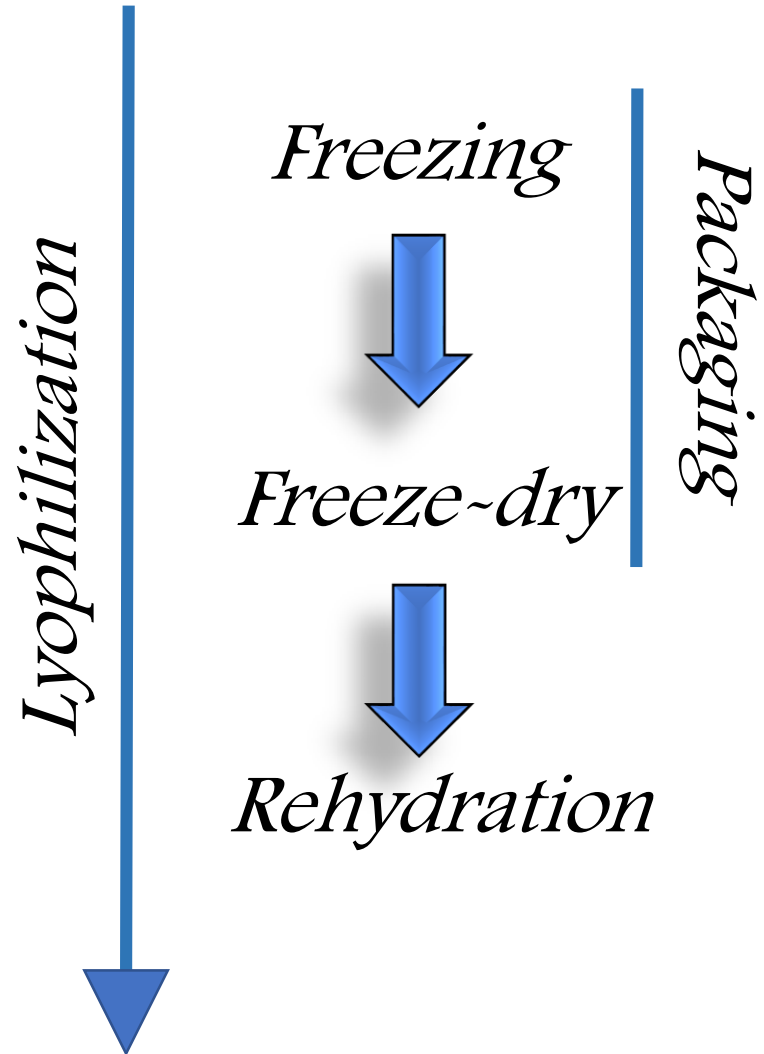
The DNA fragmentation of the epididymal ram spz affects embryonic development

	N. oocytes	Fragmented (%)	Not-Divided (%)	2-Cells (%)	Expanded Blastocyst (%)
Ram #1	72	6 (8.3) ^a	57 (79.2) ^c	9 (12.5)	0 (0)
Ram #2	83	22 (26.5)	38 (45.8)	23 (27.7)	5 (6.0) ^g
Ram #3	58	12 (20.7)	29 (50.0)	17 (29.3)	0 (0)
Ram #4	64	16 (25.0)	36 (56.3)	12 (18.8)	4 (6.3) ^h
EJ-sperm	242	57 (23.6) ^b	107 (44.2) ^d	78 (32.2)	23 (9.5) ⁱ
IVA	210	52 (24.8)	44 (21.0) ^e	114 (54.3) ^f	42 (20.0) ^j



*How to improve
the freeze drying protocol?*

Understanding the lyophilization Step by step



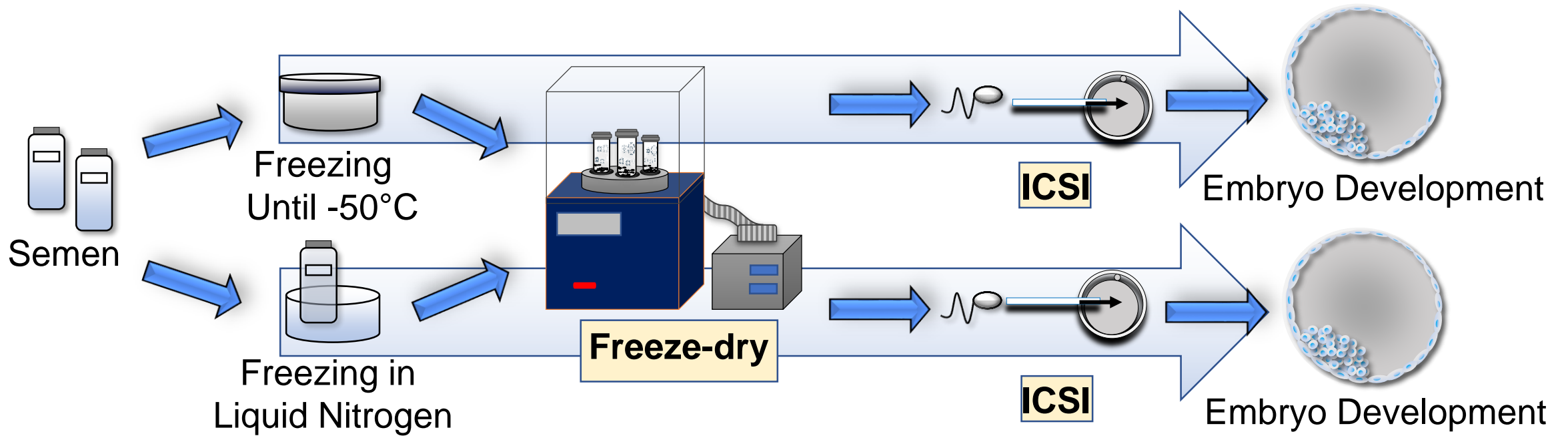
Elimination of liquid nitrogen



Economical, safety and environmental issues?

Can we use milder sub-zero temperature before sublimation?

Experimental Design

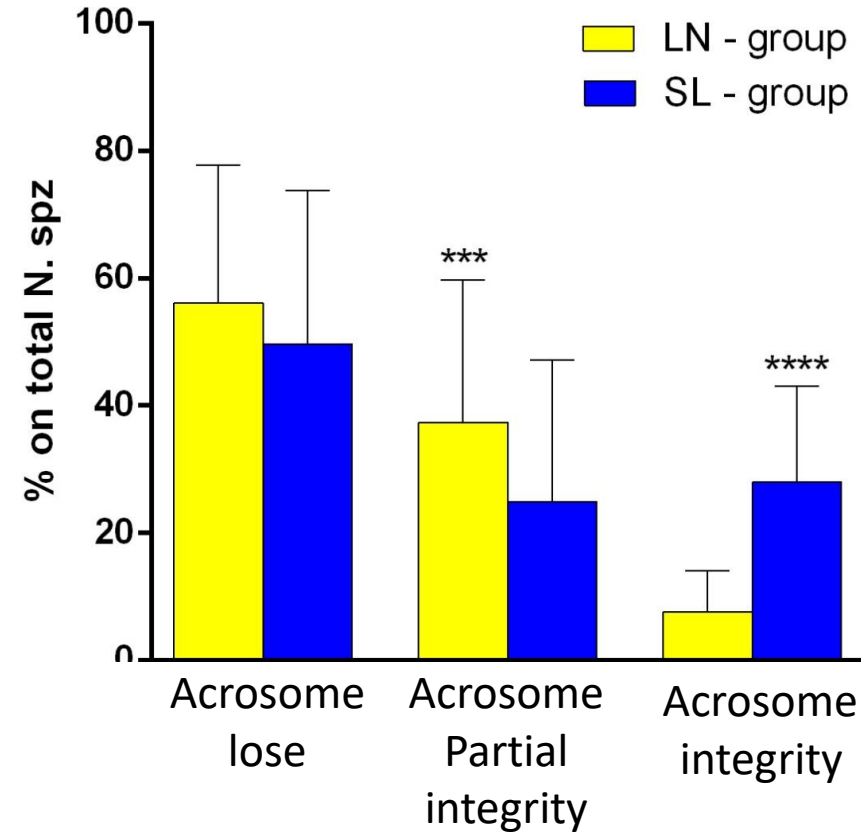
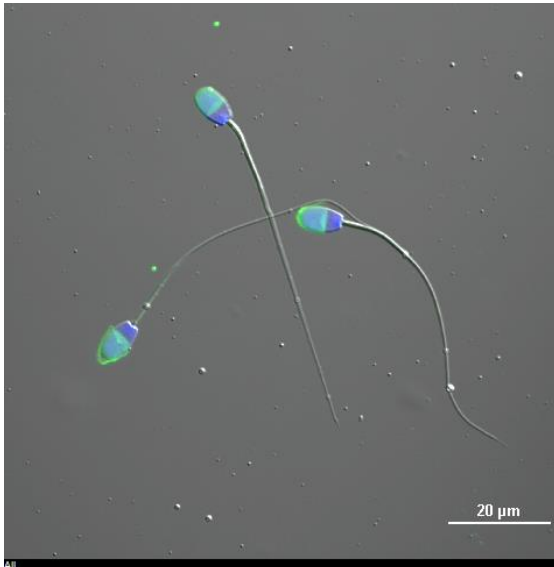


Slow freezing better preserve acrosome integrity

LN-group



SF-group



Sperm Chromatin Structure stability in LN and SF group

Assessed by Sperm Chromatin Structure Assay (SCSA)

	LN~Group	SF~Group
ALPHAT	0.4105±0.0023	0.4085±0.0022
ATSD	0.0206±0.0015	0.0194±0.0014
DFI	9.203±1.465	5.385±1.403
HG	1.475±0.214	1.314±0.205

- No significant difference between the two methods but more DNA fragmentation in LF-group*

ALPHA-T=the degree of abnormal chromatin structure with an increased susceptibility to acid-induced denaturation

ATSD = ALPHA-T standard deviation, that shows the extent of abnormality in chromatin structure within a population

%DFI = percentage of sperm with fragmented DNA

%HG = percentage of sperm with high green fluorescence that is representative of the % of immature cells with reduced nuclear condensation

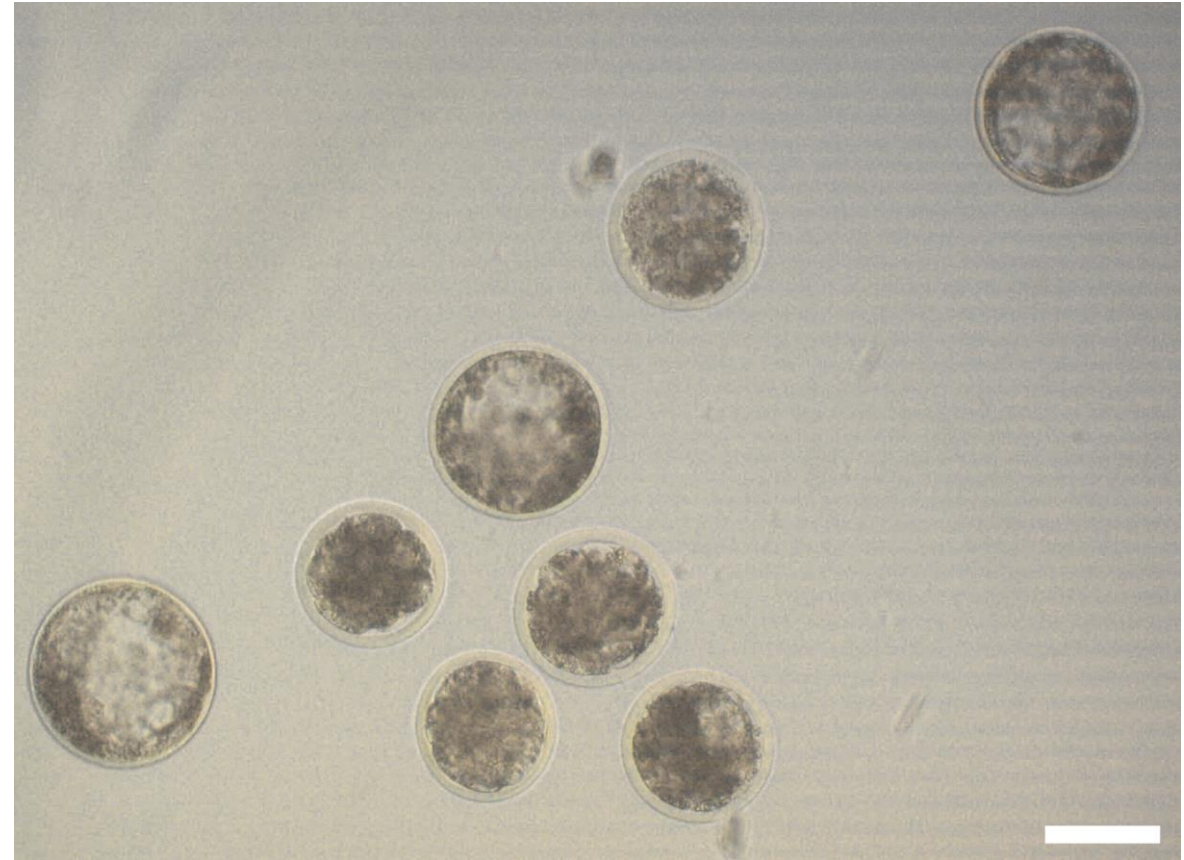
ICSI outcome

Short-medium storage (1-3 months)

	N. oocytes	2-Cells (%)	Expanded Blastocyst (%)
LN-group	75	19 (25.3)	2 (2.7)
SF-group	100	42 (42)*	7 (7)*

Long storage

	N. oocytes	2-Cells (%)	Expanded Blastocyst (%)
1 Month	283	89 (31.4)*	25 (8.8)*
1.5 Year	101	16 (15.8)	2 (2)





OPEN

Whole genome integrity and enhanced developmental potential in ram freeze-dried spermatozoa at mild sub-zero temperature

Luca Palazzese^{1,6}, Debora Agata Anzalone^{1,6}, Federica Turri², Marco Faieta³, Anna Donnadio⁴, Flavia Pizzi², Paola Pittia³, Kazutsugu Matsukawa⁵ & Pasqualino Loi¹✉

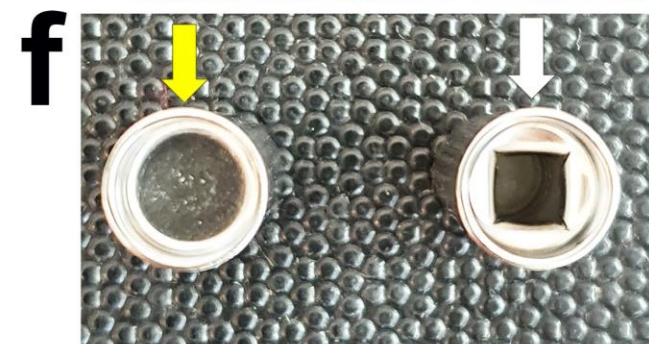
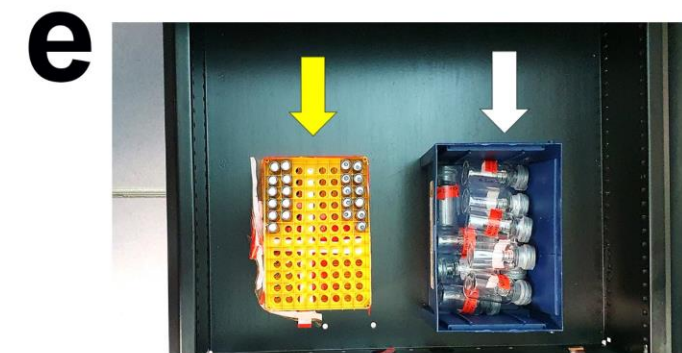
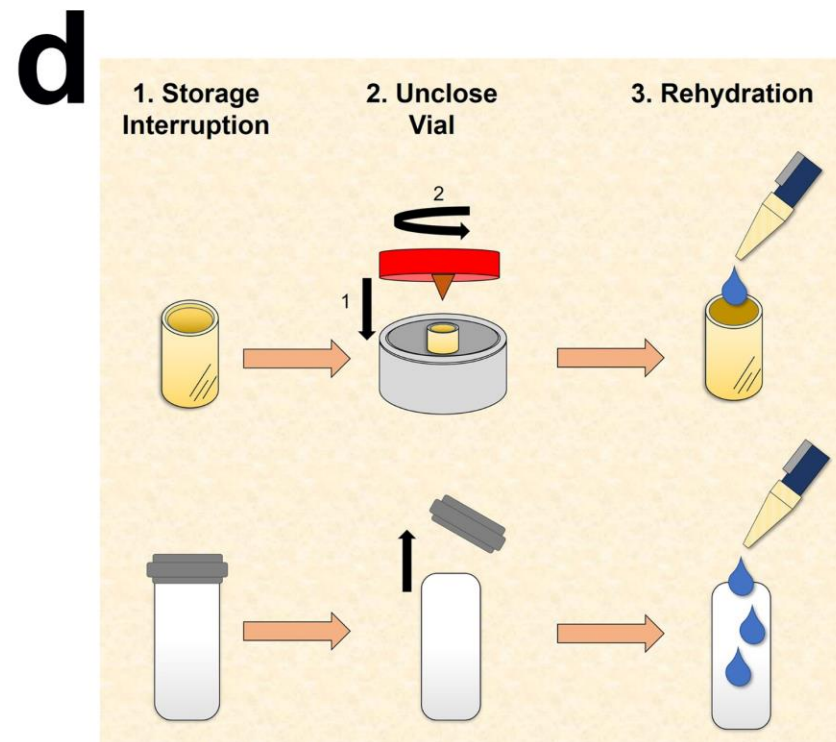
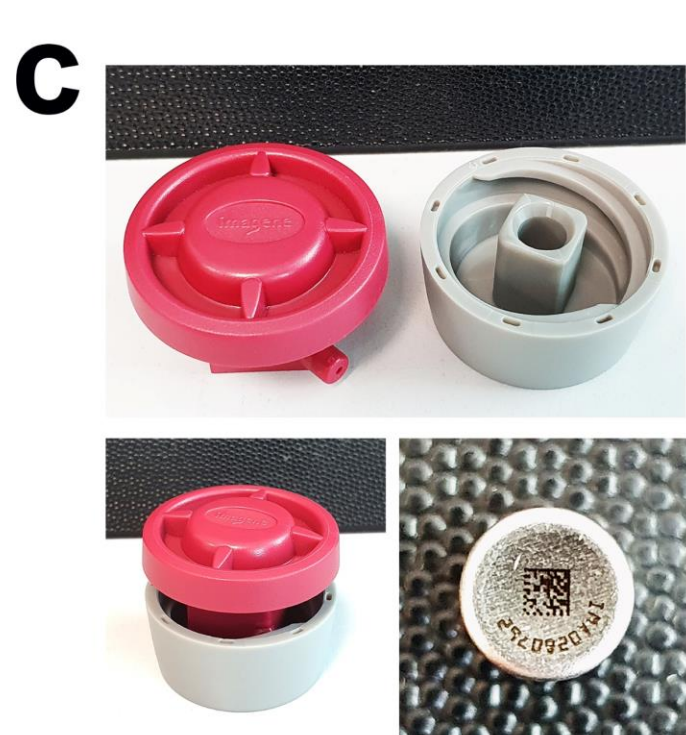
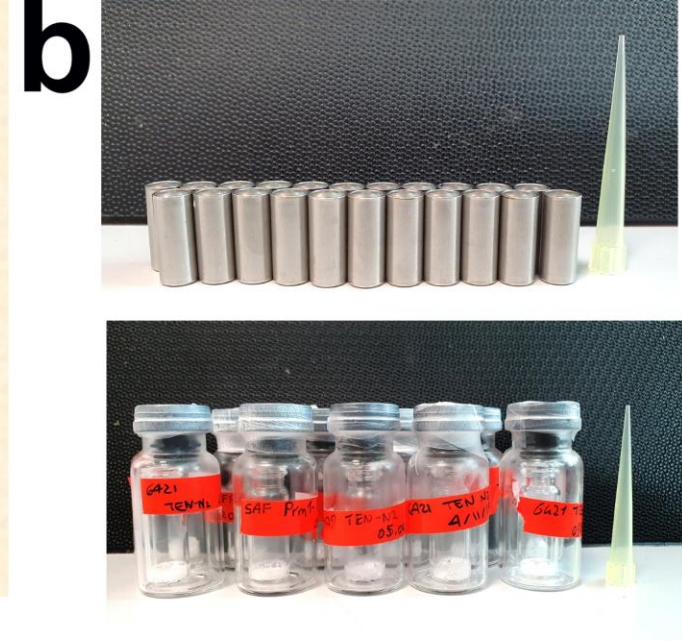
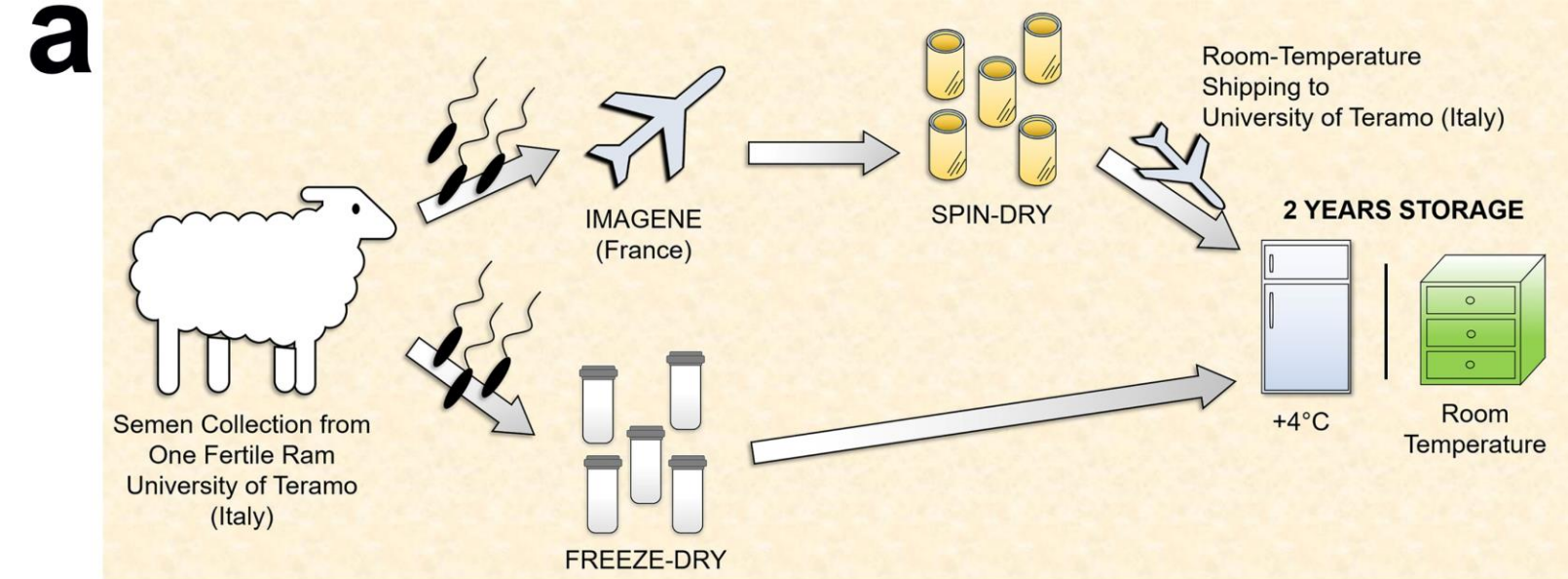
Journal of Reproduction and Development, Vol. 57, No. 2, 2011

SRD Young Investigator Award

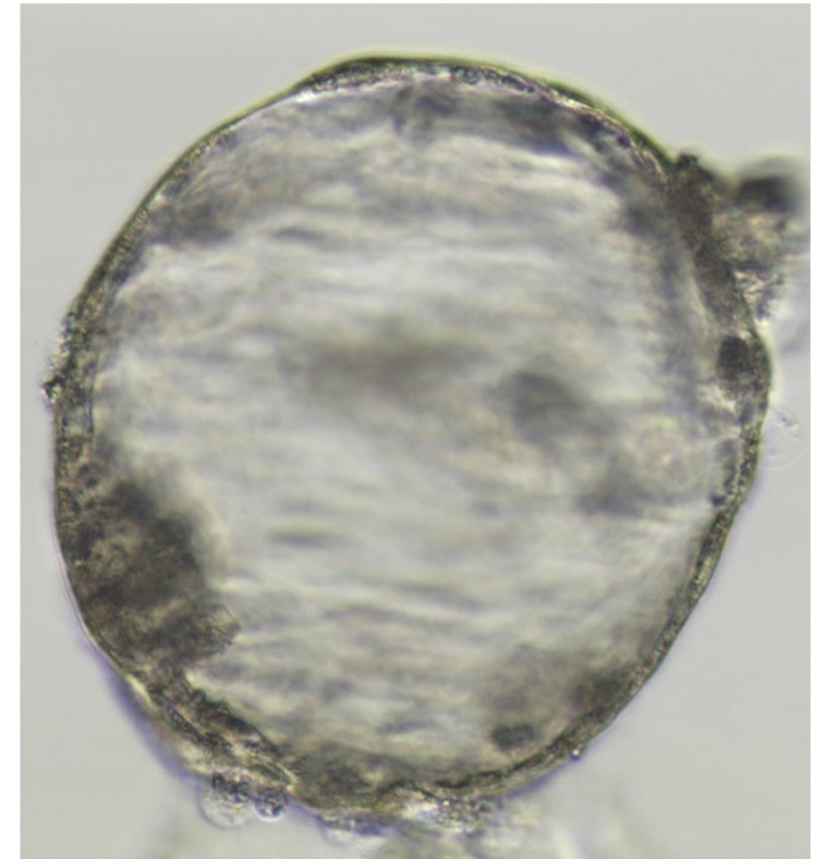
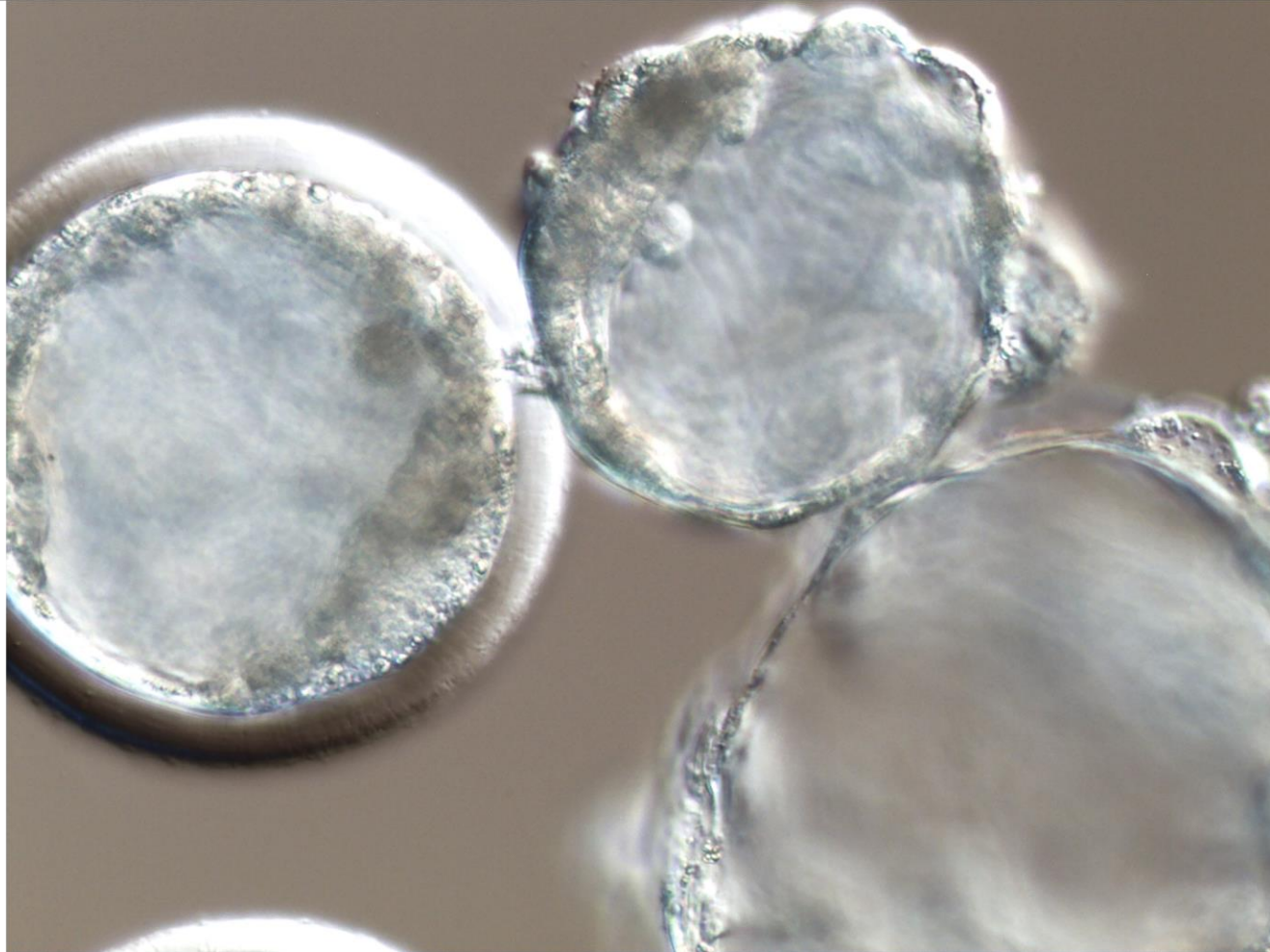
A Study on Freeze-Drying as a Method of Preserving Mouse Sperm

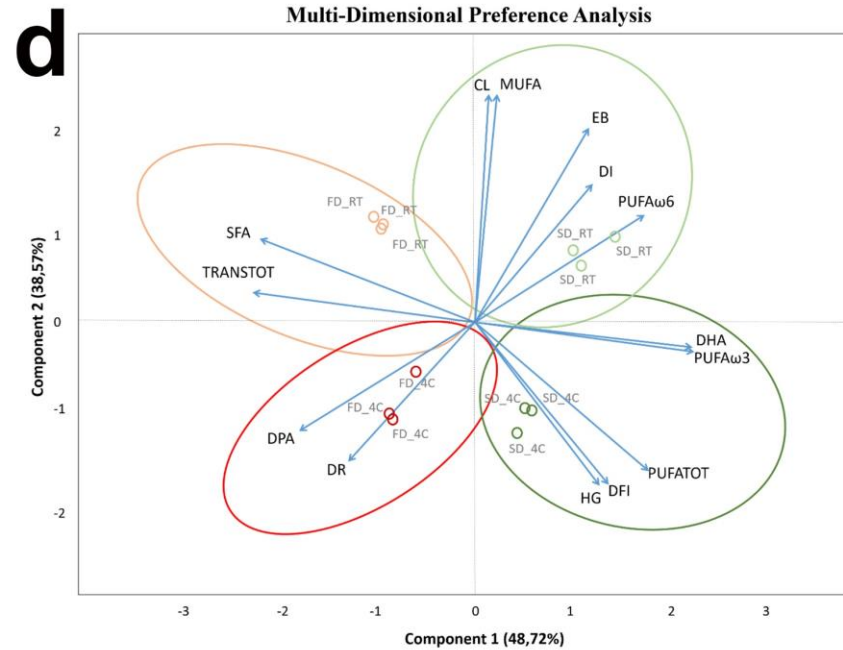
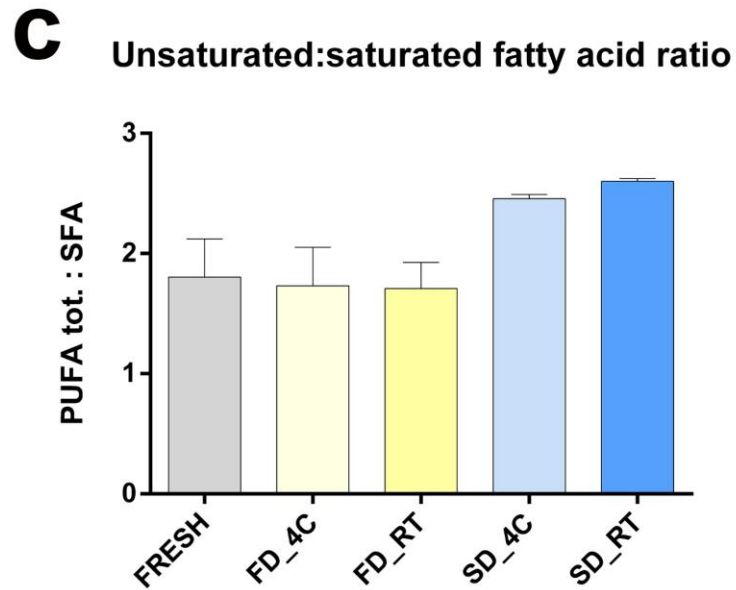
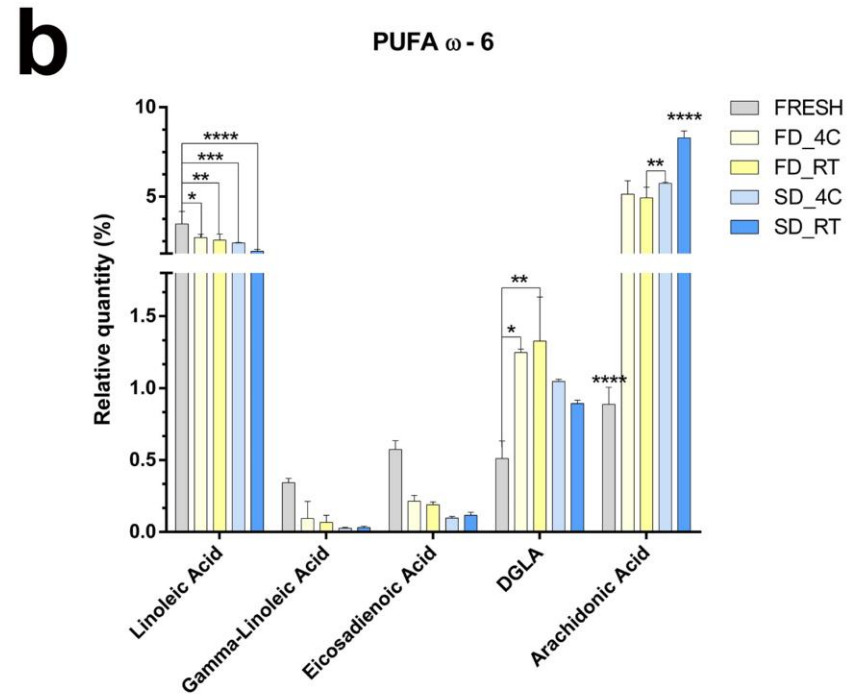
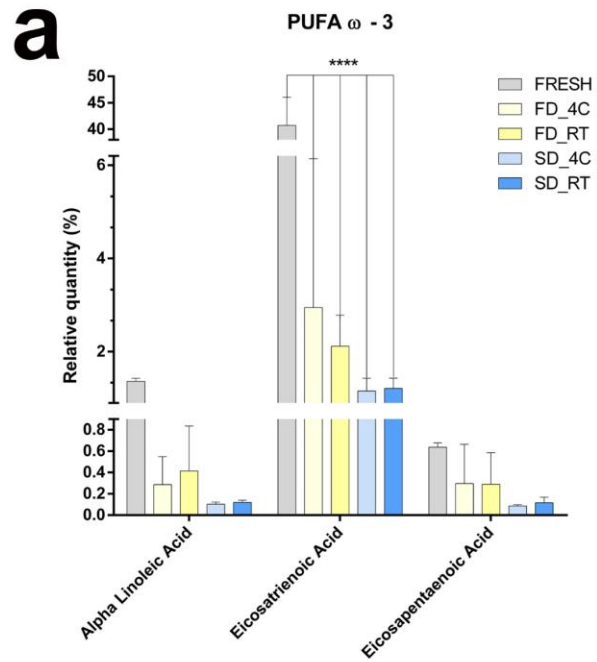
Yosuke KAWASE¹⁾ and Hiroshi SUZUKI²⁾

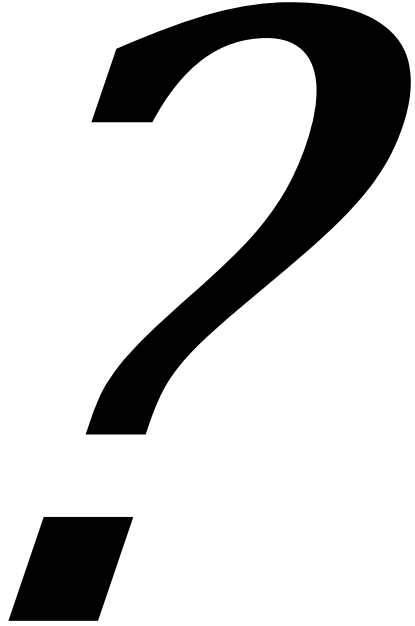
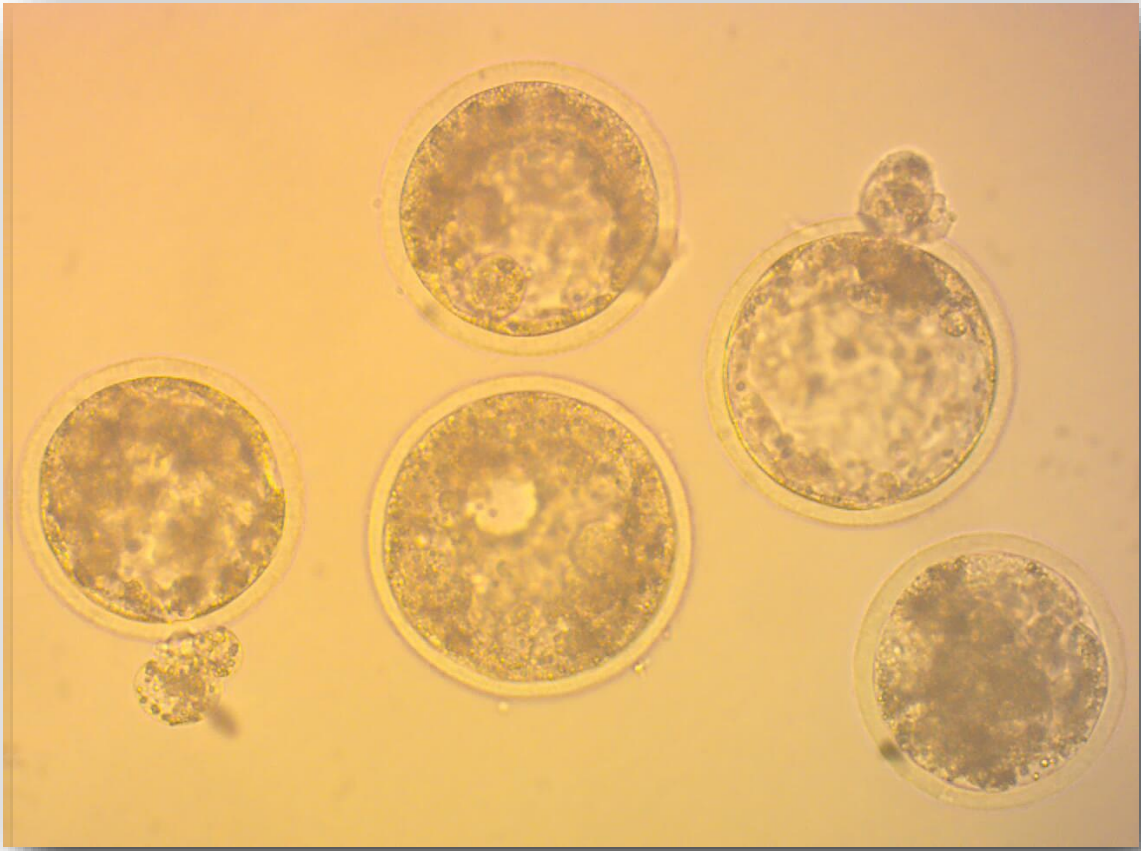
¹⁾Chugai Research Institute for Medical Science, Inc., Gotemba 412-8513 and ²⁾Research Unit for Functional Genomics, National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro 080-8555, Japan



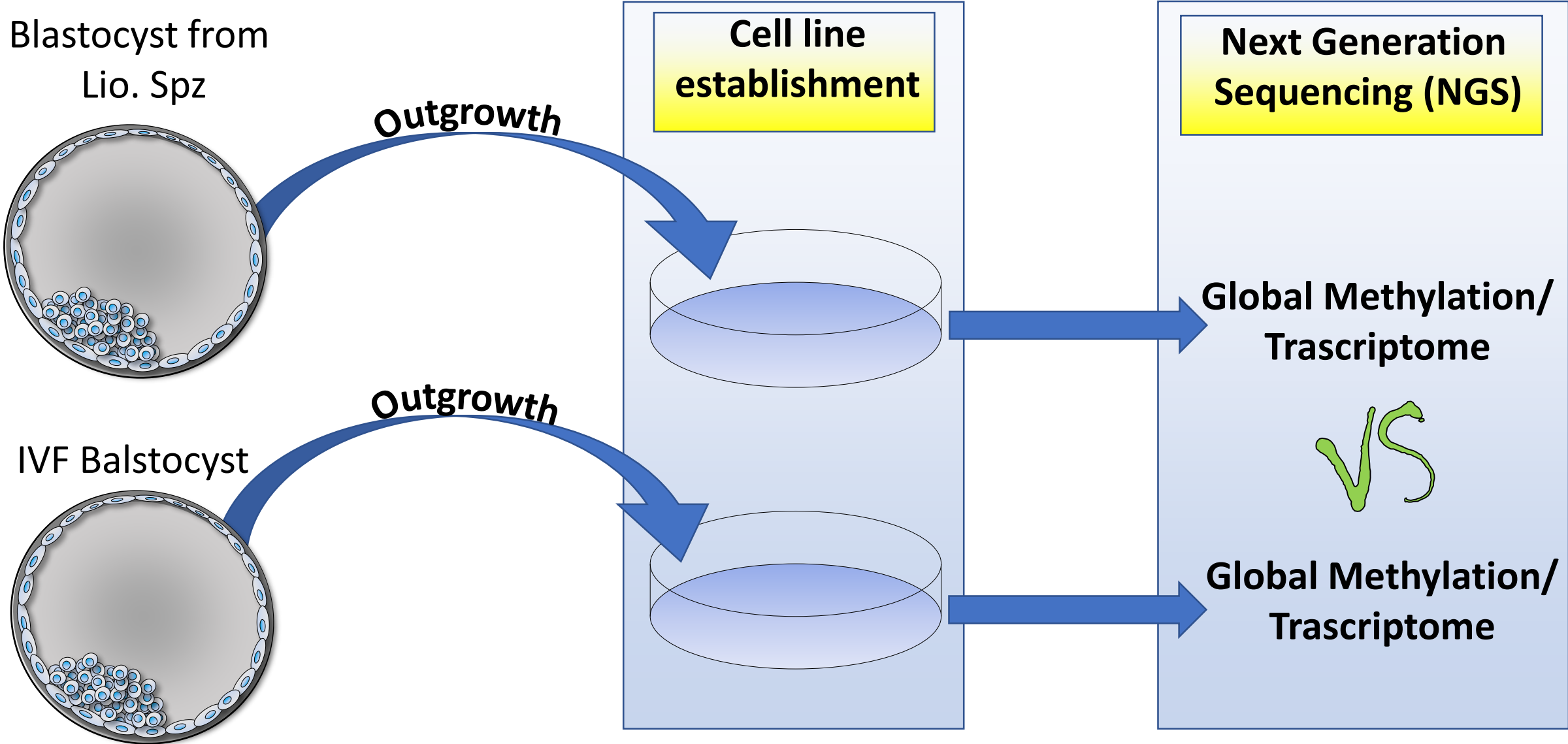
Method	Storage Condition	Cleavage Rate (%)	Expandend Blastocyst
SPIN-DRY	RT	25/53 (47.17)a	7/53 (13.21)a
	4°C	23/70 (32,86)a, b, c	4/70 (5.71)a
FREEZE-DRY	RT	31/62 (50.0)b	5/62 (8.06)a, b
	4°C	15/83 (18.07)a, b,c	2/83 (2,41)a, b



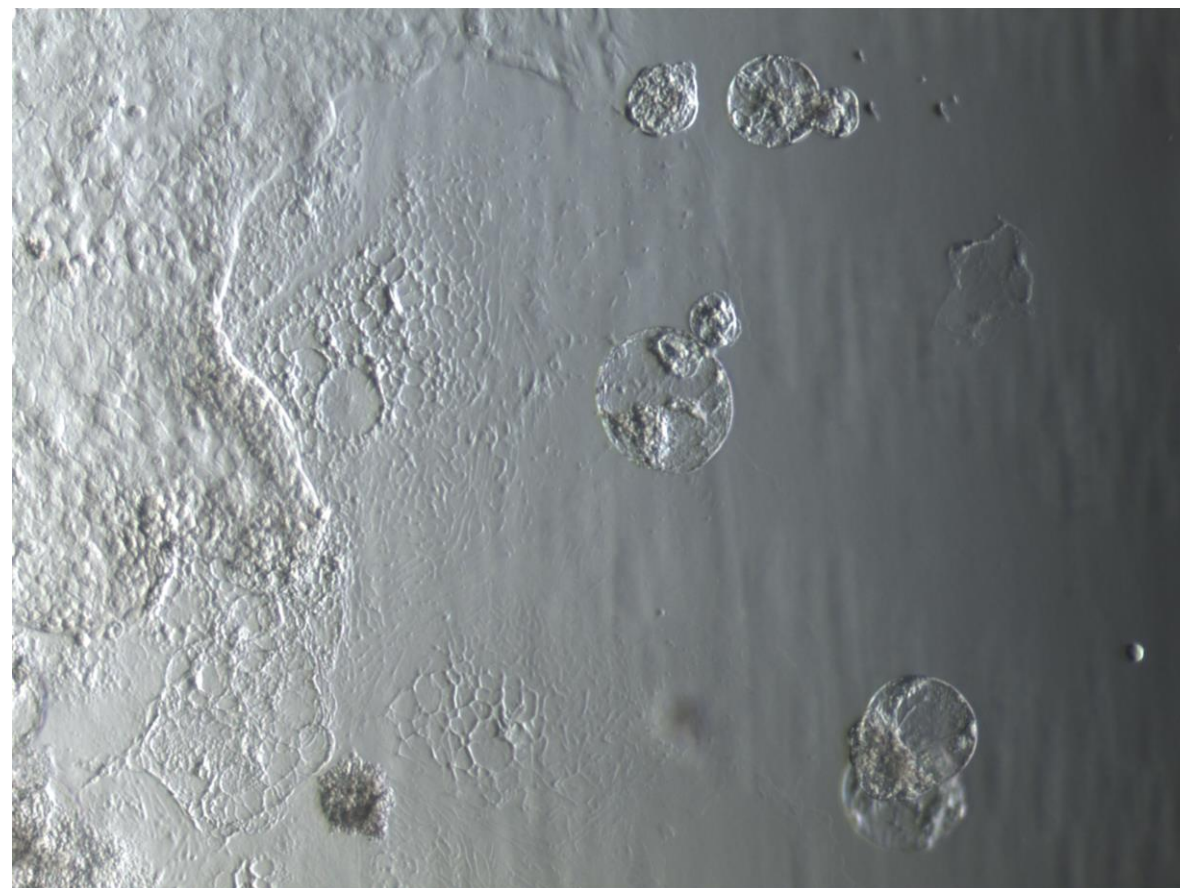
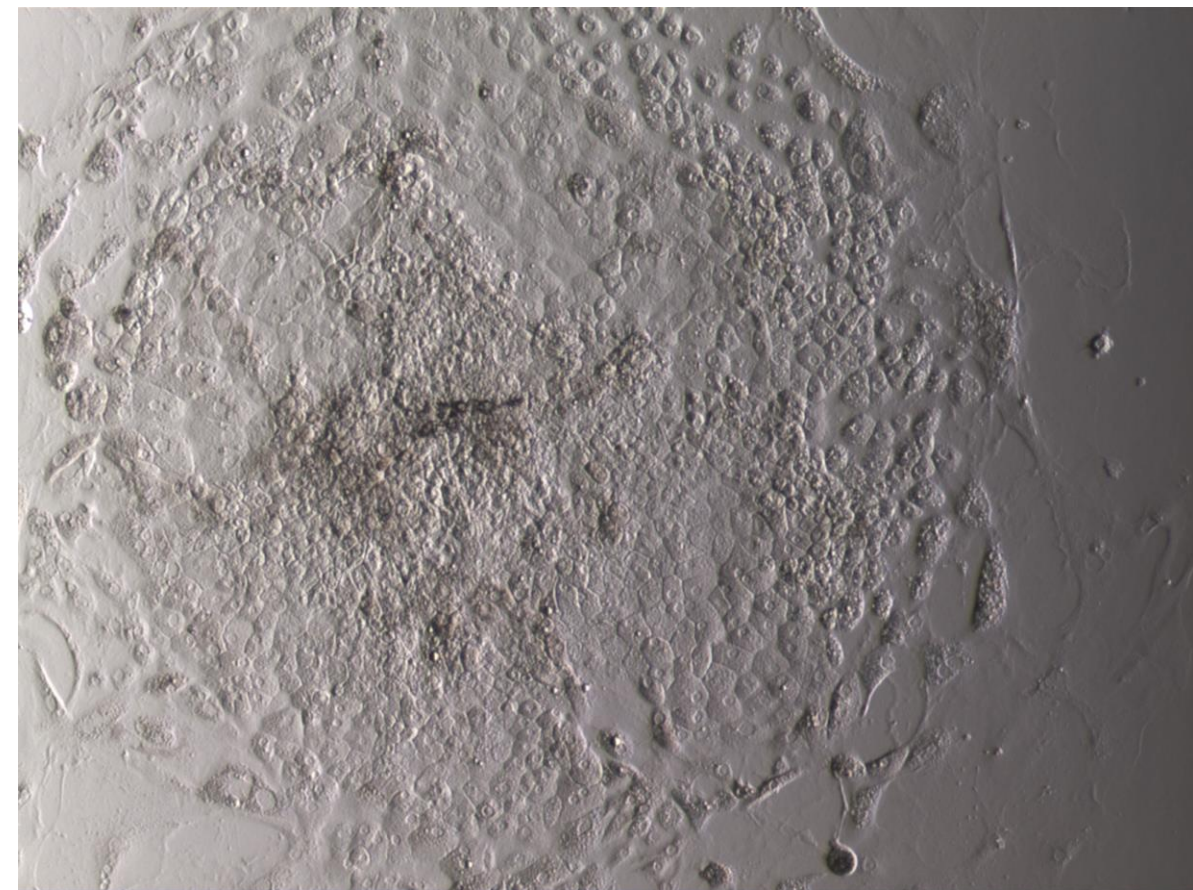




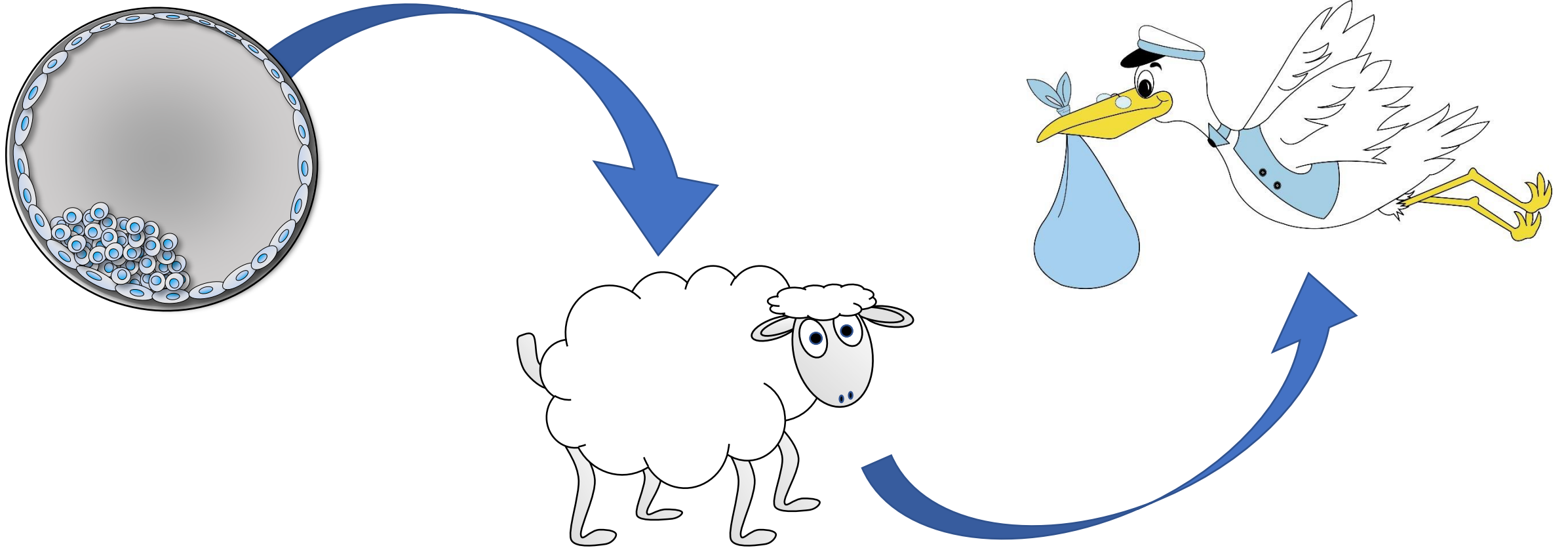
Experimental design



Cell line from whole blastocyst



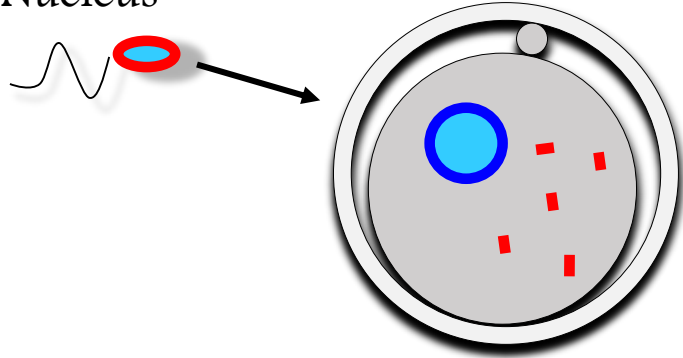
From dry to life



Efficient cloning in terms of births: inadequate nuclear remodeling

Fertilization

Spz. Nucleus

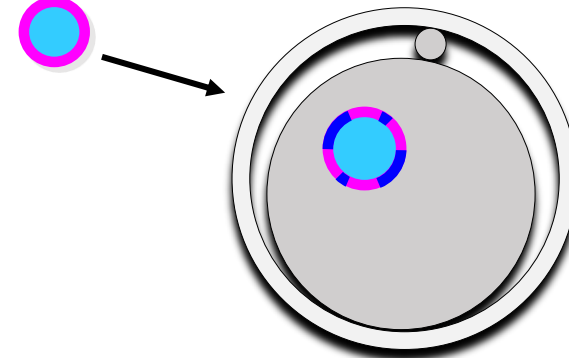


Complete replacement Protamin with maternal histones



Cloning

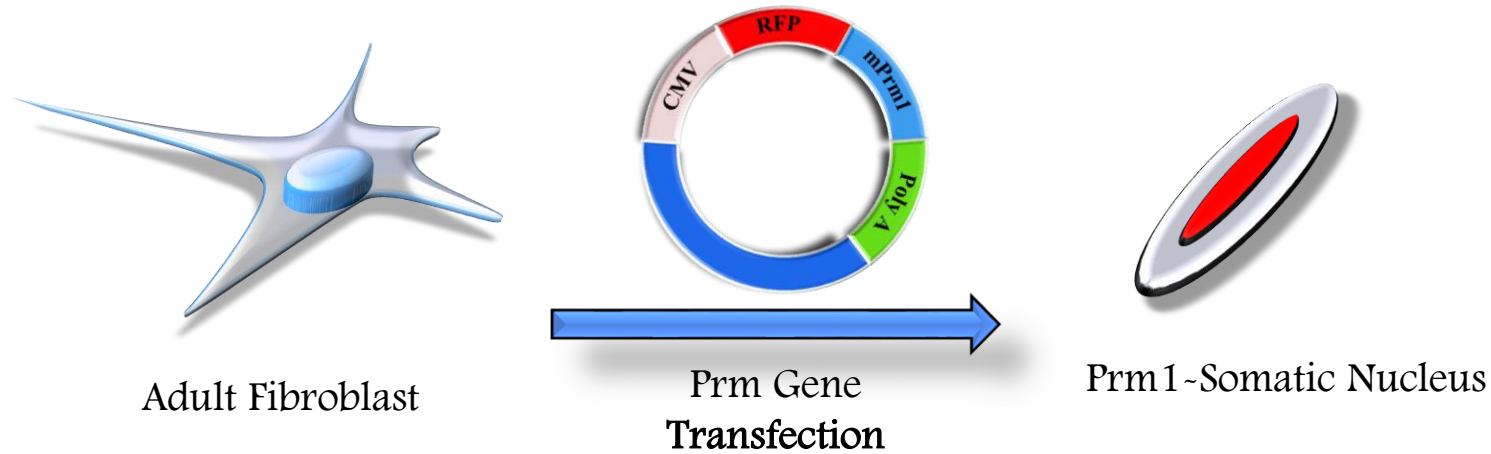
Somatic Nucleus



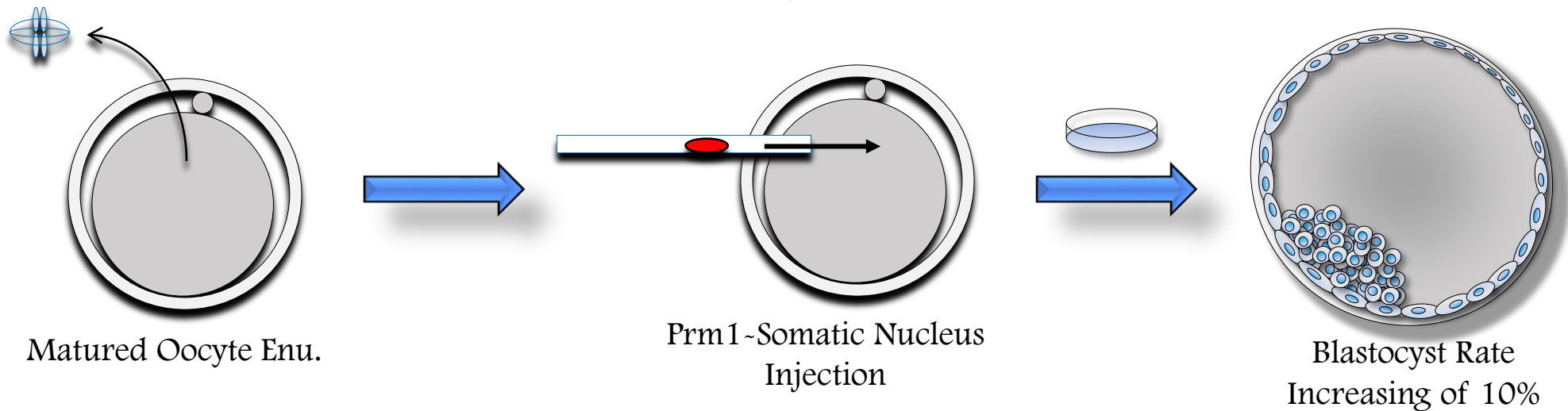
Somatic Histone Memory



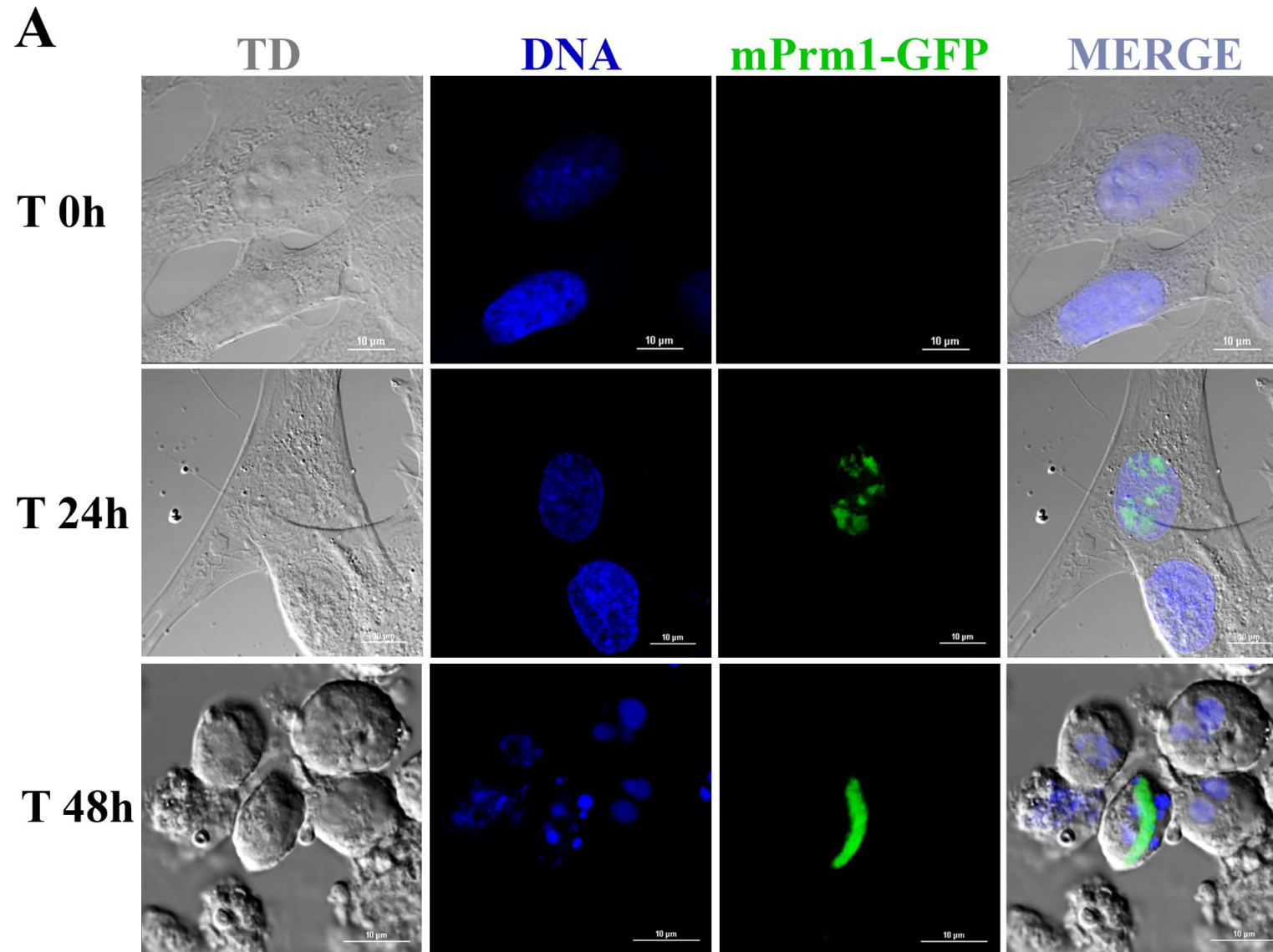
Somatic Cell Protaminization



Somatic Cell Nuclear Transfer (SCNT) with Prm1-Somatic Nucleus



In Vitro Protaminization







ARTICLE



<https://doi.org/10.1038/s41467-022-31216-4>

OPEN

Healthy cloned offspring derived from freeze-dried somatic cells

Sayaka Wakayama ^{1,2}✉, Daiyu Ito ¹, Erika Hayashi ¹, Takashi Ishiuchi¹ & Teruhiko Wakayama ^{1,2}✉

*How to protect DNA during
Freeze-dry?*



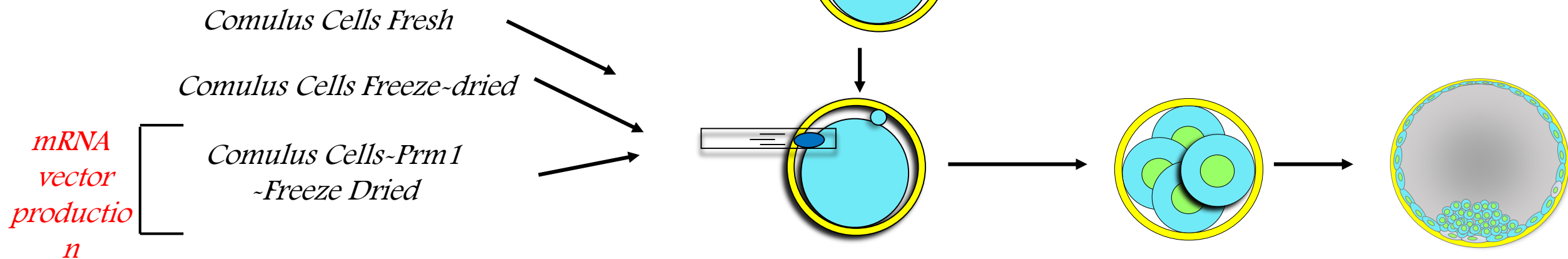
山梨大学 生命環境学部生命工学科

UNIVERSITY OF YAMANASHI

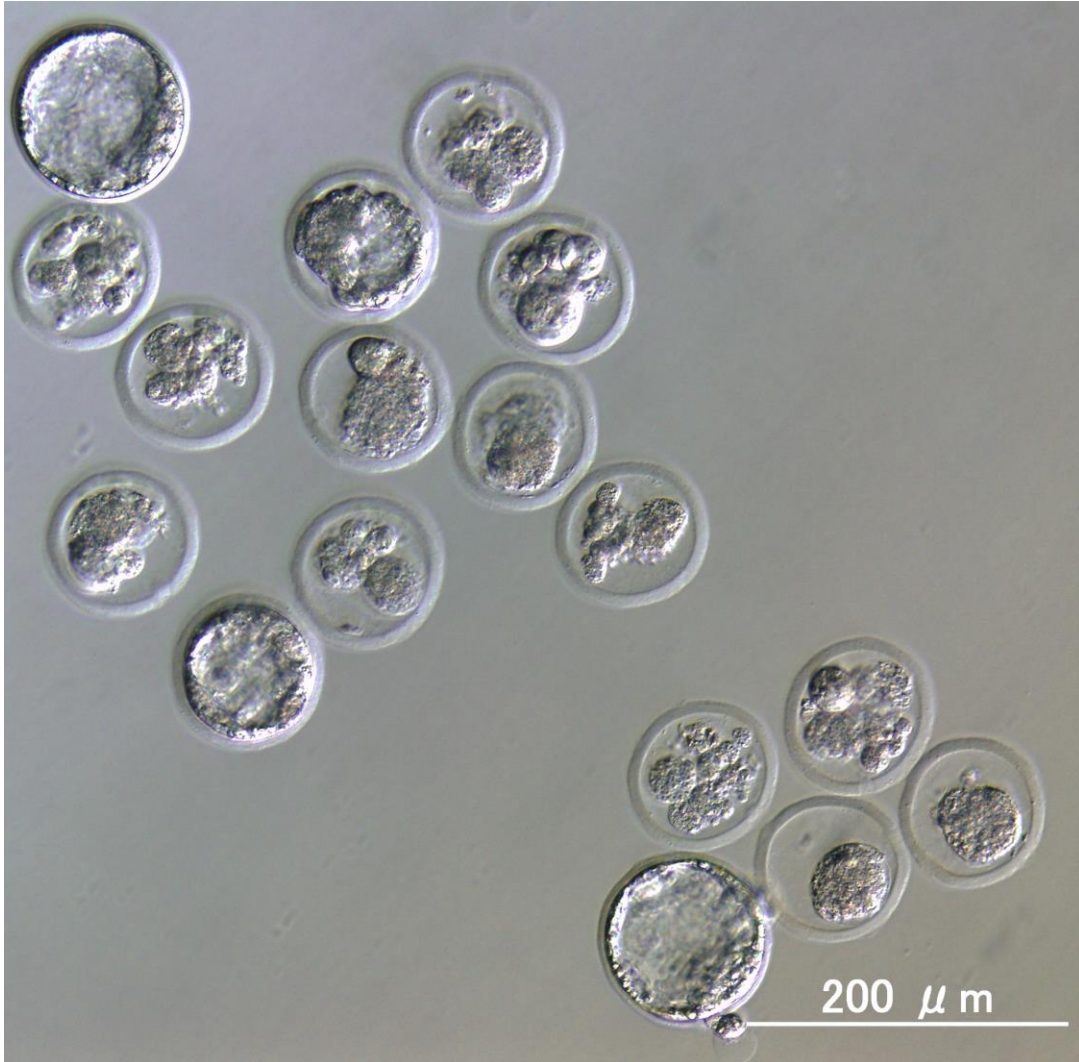
Department of Biotechnology, Faculty of Life and Environmental Sciences



Experimental Design

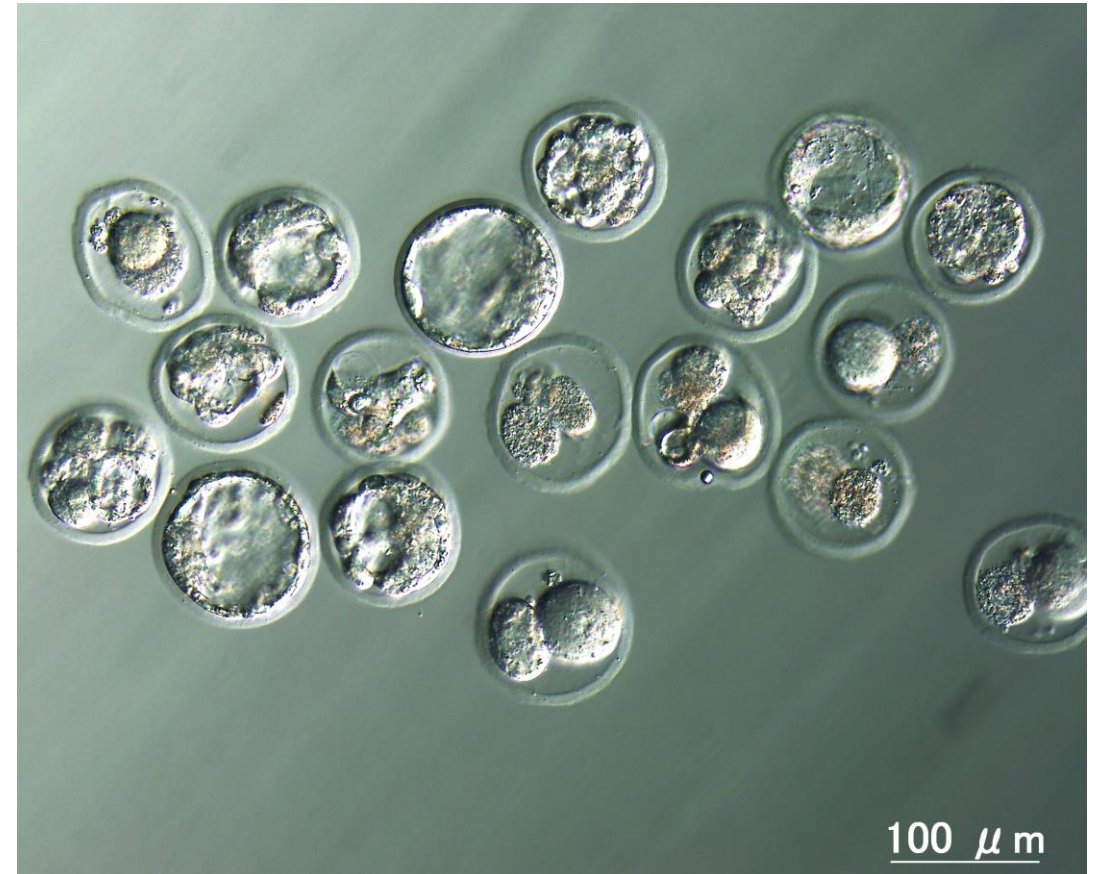
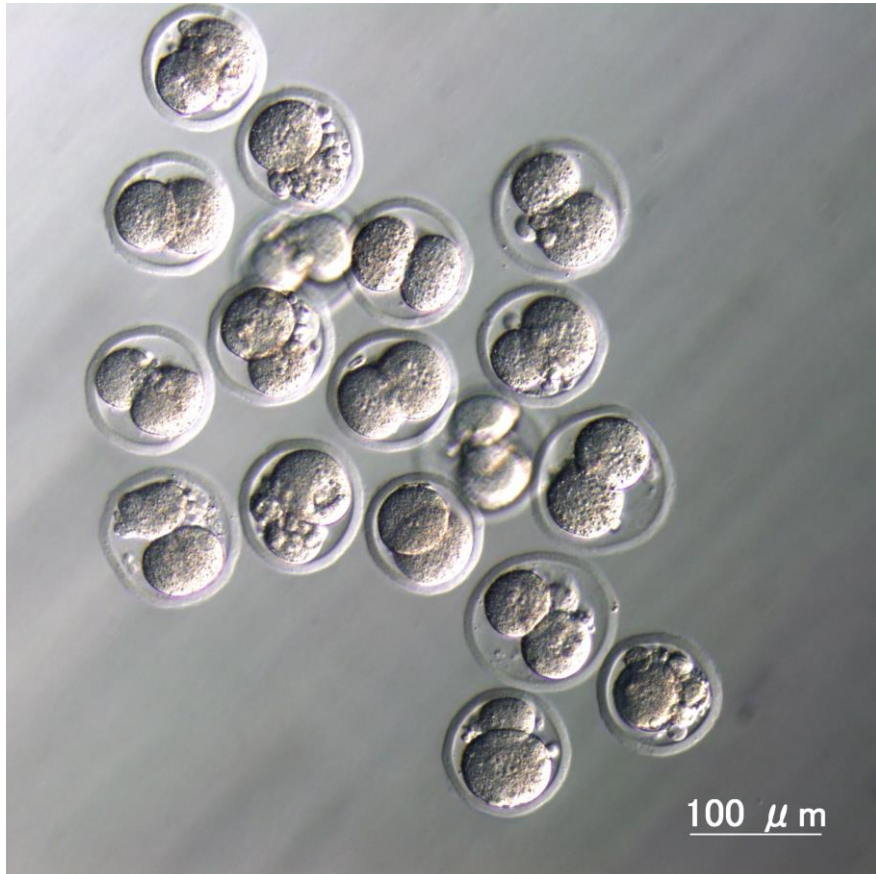


It's not so easy!



*3 months of training to produce
clone blastocyst in mouse*

GROUPS	1 PN (%)	2 CELL (%)	BLASTOCYST (%)
24h POST-TRANSFECTION	47,69	74,19	38,71
48h POST-TRANSFECTION	50,00	39,47	18,42
CTR	80,00	50,00	25,00

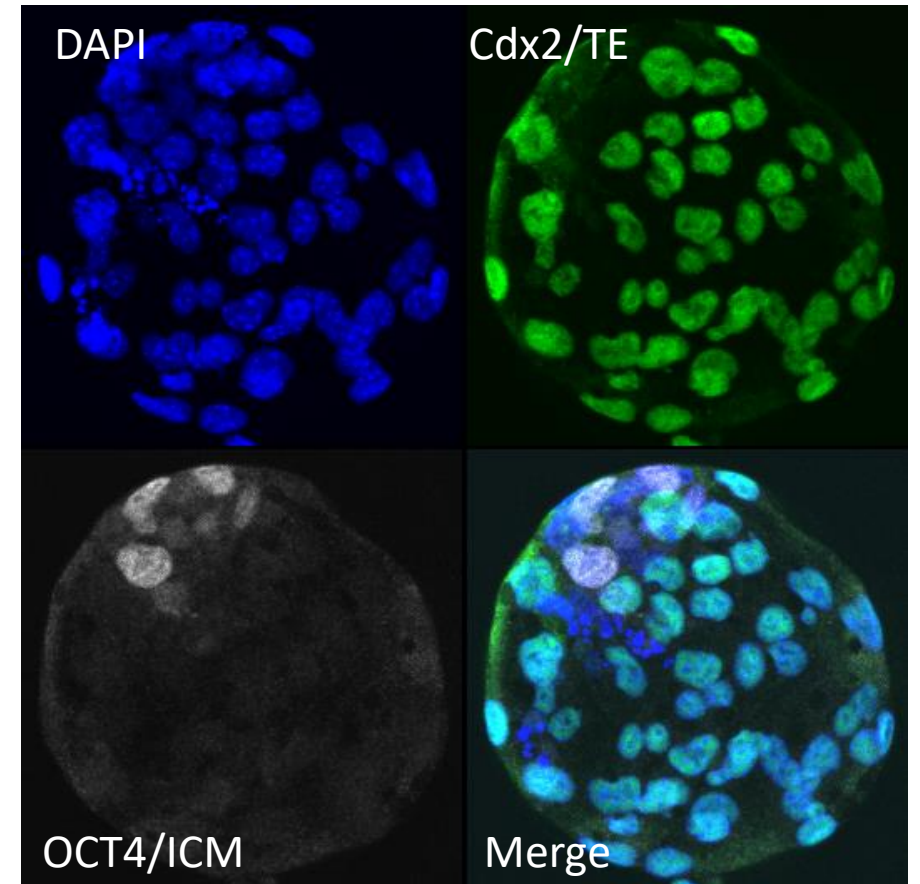
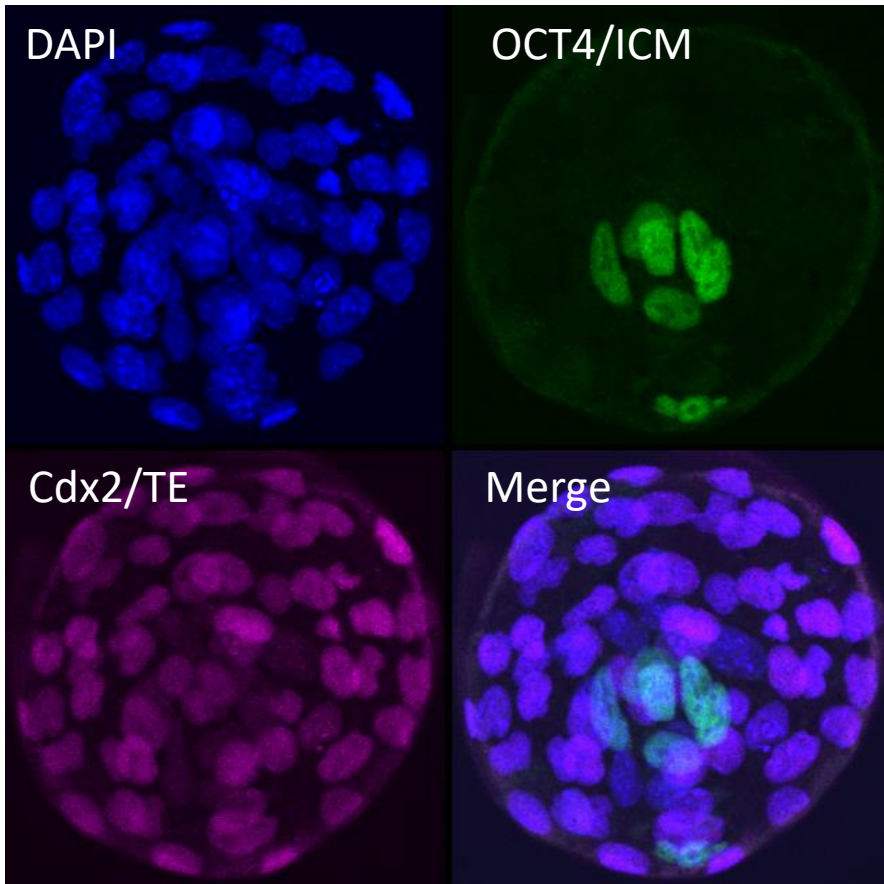


**BLASTO FROM CELL 24h POST-
TRANSFCETION**

BLASTO #	ICM	TE	TOT.
1	7	46	53
2	8	105	113
3	7	73	80
4	8	78	86
5	8	35	43
AVERAGE	7,6	67,4	75

**BLASTO FROM CELL 48h POST-
TRANSFCETION**

BLASTO #	ICM	TE	TOT.
1	4	89	93
2	10	73	83
3	9	66	75
AVERAGE	7,67	76,00	83,67



Thanks for the attention



Acknowledgments

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