Czech University of Life Sciences Prague

Biotechnology in small ruminant reproduction: an international experience - 2025

Advances in ram sperm storage

UNIVERSITÁ DEGLI STUDI DI TERAMO

FACOLTÀ MEDICINA VETERINARIA TERAMO

Dr. Luca Palazzese Ph.D.

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	3	**156**
Italy	**100**	**67**	**15**	**0**	**6**	**0**	**2**	**25**	**0**	**215**
France	91	55	132	10	24	0	5	72	6	**395**
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**

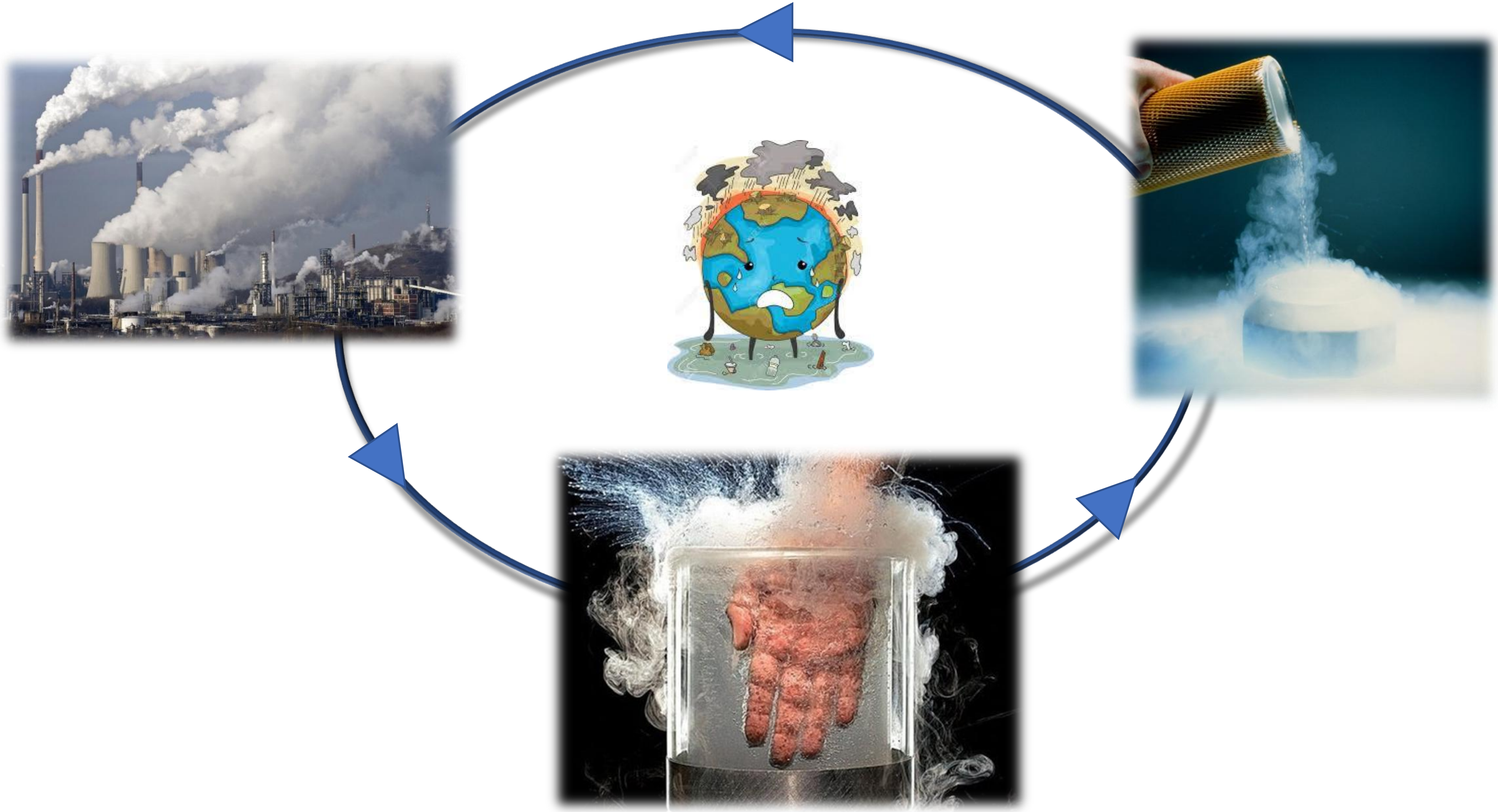
Conservation Methods

Conservation methods and their potential to contribute to various objectives

Objective	Type of conservation (if implemented as a stand-alone measure)		
	In situ	*Ex situ in vivo*	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?

1998 Mouse

RESEARCH

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

Teruhiko Wakayama[1,2] and Ryuzo Yanagimachi[1]*

[1]Department of Anatomy and Reproductive Biology, University of Hawaii School of Medicine, Honolulu, HI. [2]Department 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

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 Rabbits[1]

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]

Department of Animal Science/Center for Regenerative Biology,[5] University of Connecticut, Storrs, Connecticut 06269
Institute for Biogenesis Research,[6] University of Hawaii Medical School, Honolulu, Hawaii 96822
Faculty of Agriculture and Life Sciences,[7] Hirosaki University, Hirosaki 036-8561, Japan

2008 Rat

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

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

SHINICHI HOCHI,[1]* KAORI WATANABE,[1] MEGUMI KATO,[2] AND MASUMI HIRABAYASHI[2,3]**
[1]Faculty of Textile Science and Technology, Shinshu University, Ueda, Nagano, Japan
[2]National Institute for Physiological Sciences, Okazaki, Aichi, Japan
[3]The Graduate University of Advanced Studies, Okazaki, Aichi, Japan

«2011 Horse»

REPRODUCTION
RESEARCH

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

Y H Choi[1], D D Varner[2], C C Love[2], D L Hartman[3] and K Hinrichs[1,2]

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

2017 Mice from the space

PNAS

Check for updates

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

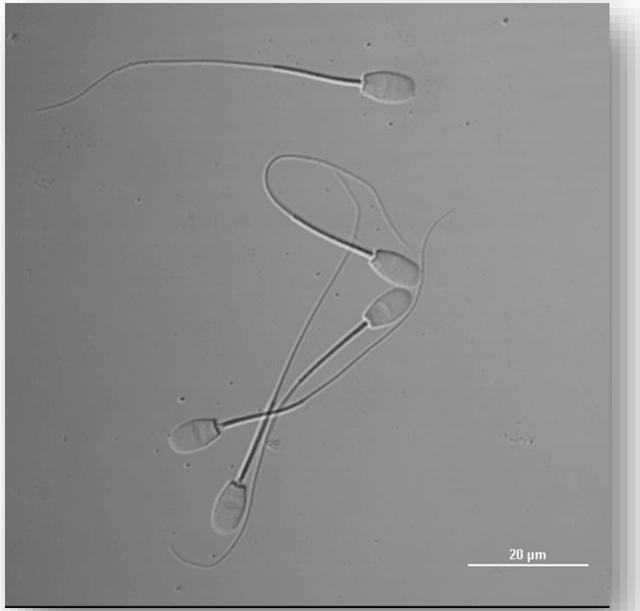
Sayaka Wakayama[a,1], Yuko Kamada[b], Kaori Yamanaka[c], Takashi Kohda[d], Hiromi Suzuki[e], Toru Shimazu[e], Motoki N. Tada[f], Ikuko Osada[f], Aiko Nagamatsu[g], Satoshi Kamimura[b], Hiroaki Nagatomo[a,h], Eiji Mizutani[b], Fumitoshi Ishino[d], Sachiko Yano[g], and Teruhiko Wakayama[a,b,1]

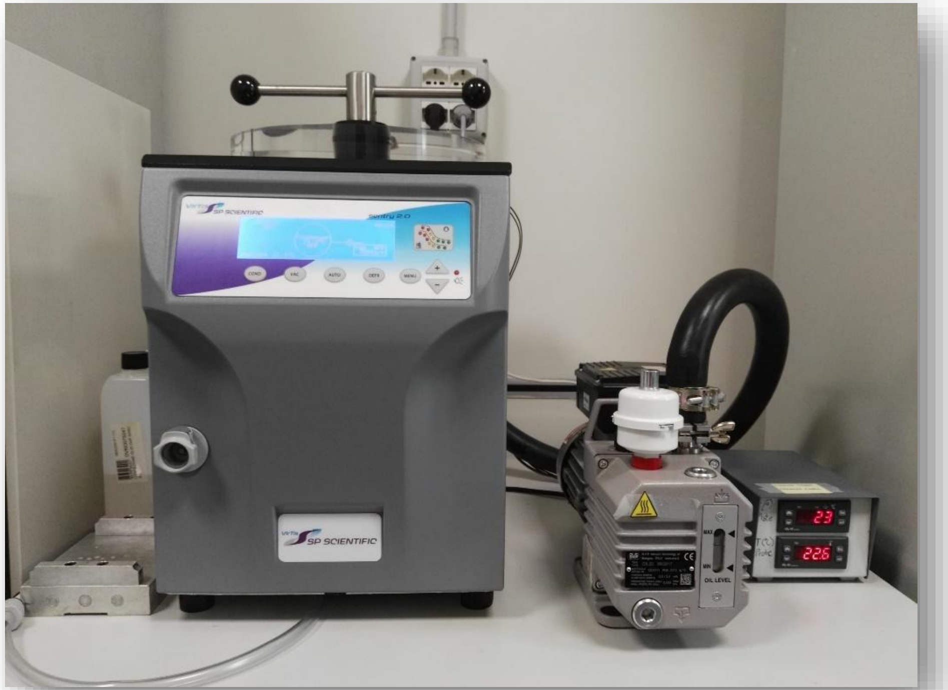
Pagliarola breed

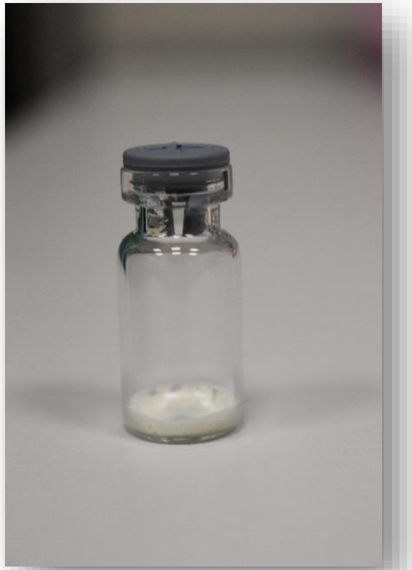
"…il contadino le somministrava quanto vi ha più di scadente fra le materie diverse che si usano e servono come foraggio: le fronde di potatura dell'olivo, le foglie verdi o secche, gli sterpi, le radici, l'erbe raccolte nei fossi o lungo le prode dei campi, la gramigna, lo strame, la paglia…"

Giovanni Savazzini (1910) *Le Pagliarole*

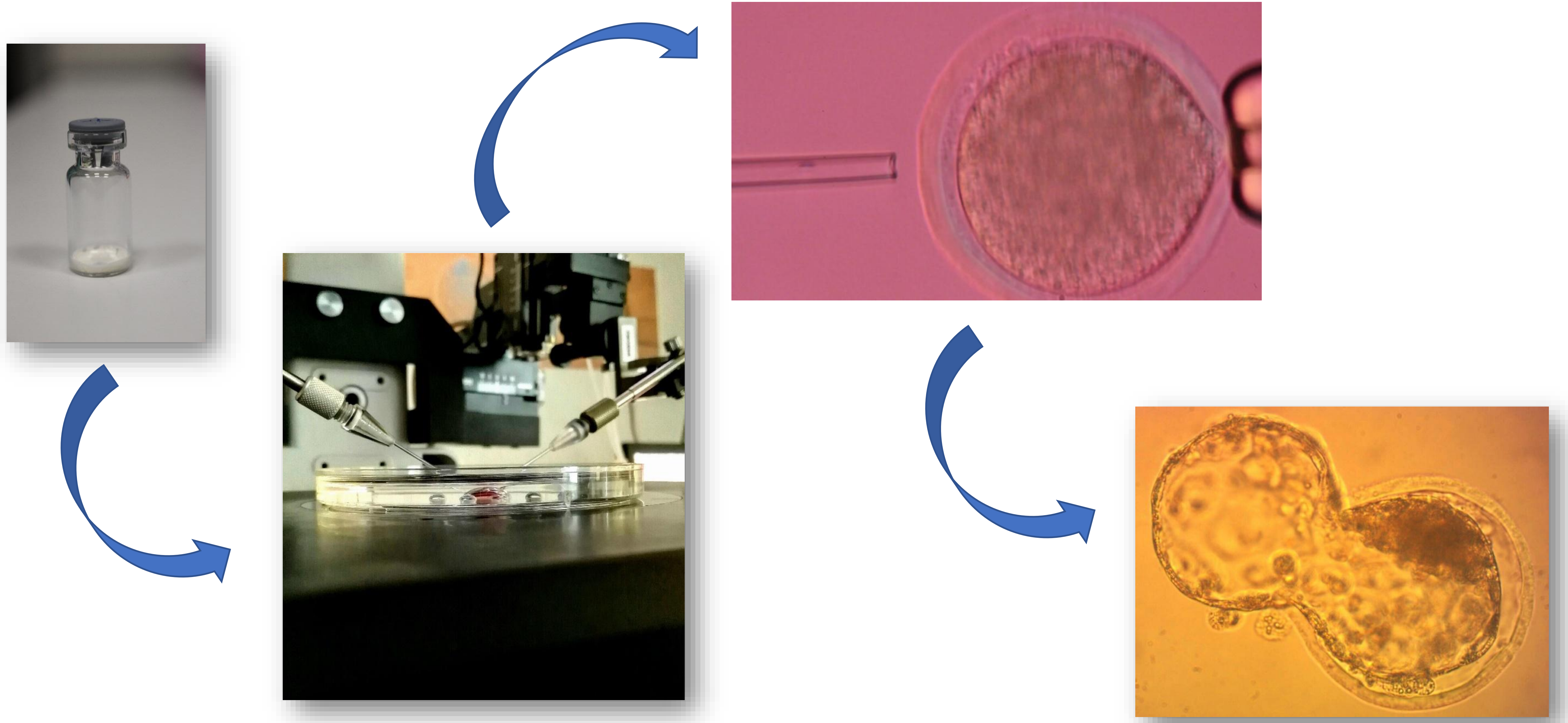
Freeze-dry Ram Spermatozoa



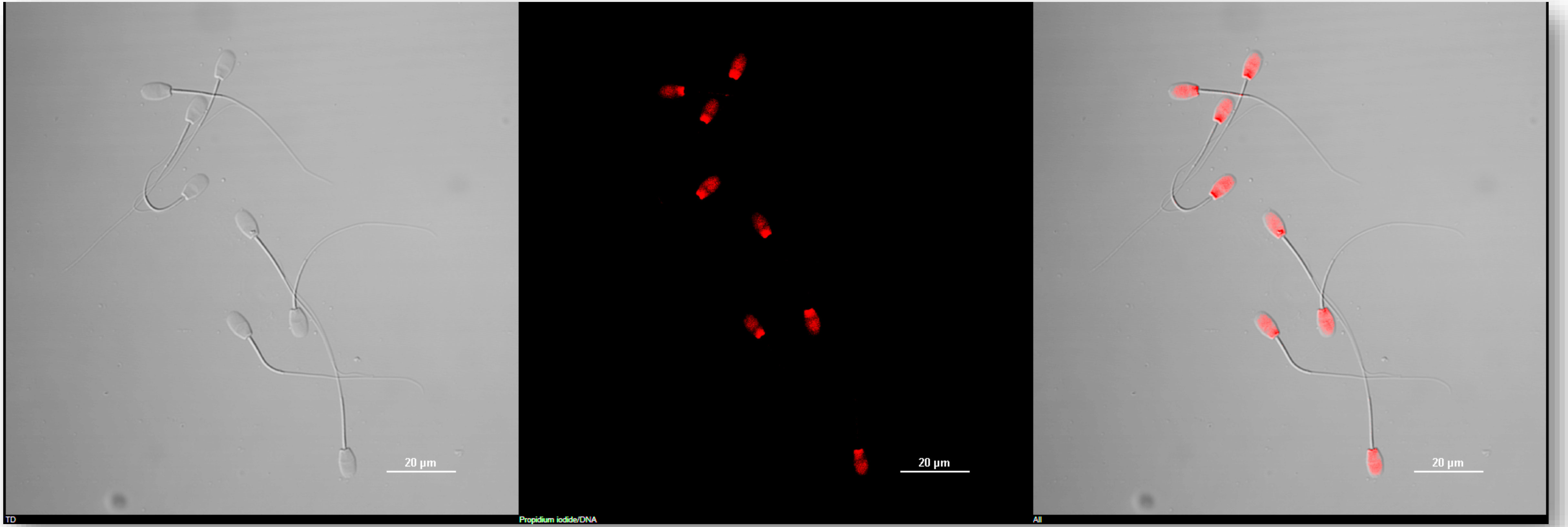




ICSI with freeze-dried semen



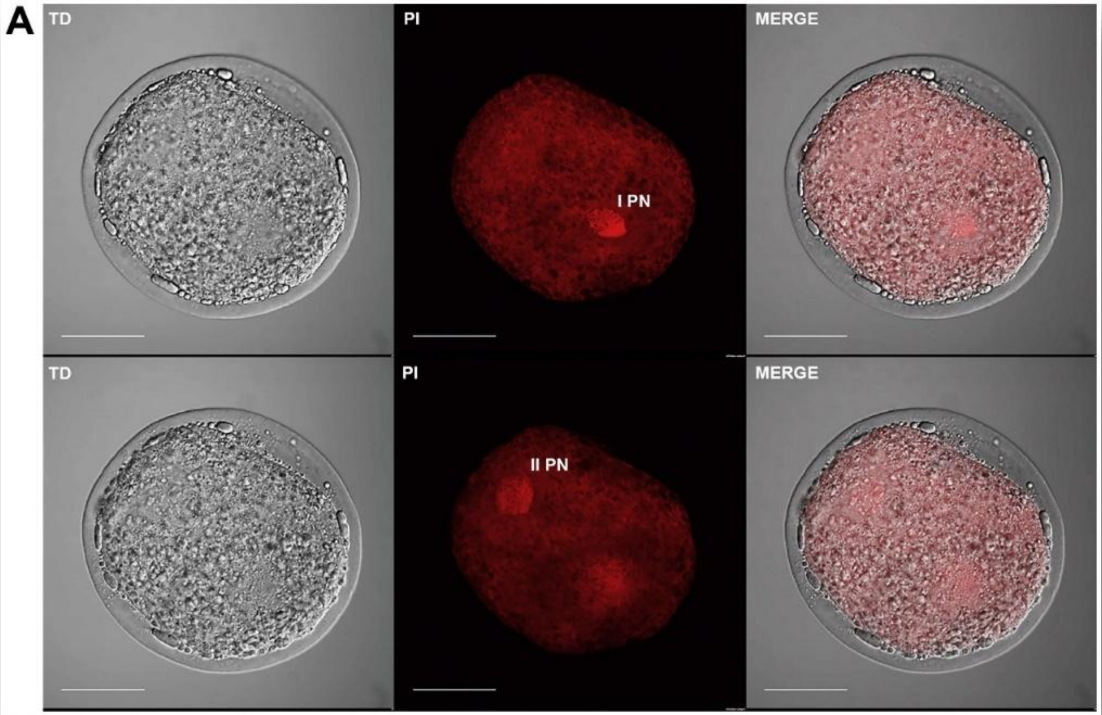
The lyophilized spz preserves the morphology post-rehydration

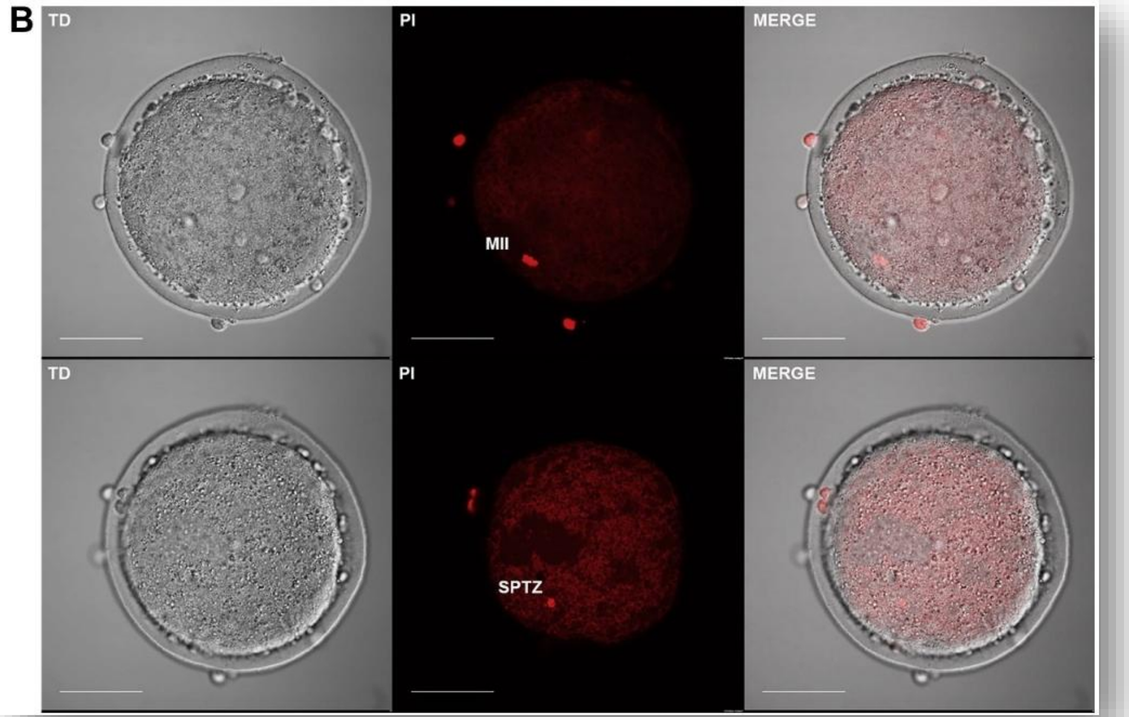


Anzalone et al. 2018

ICSI with FD spz requires chemical activation

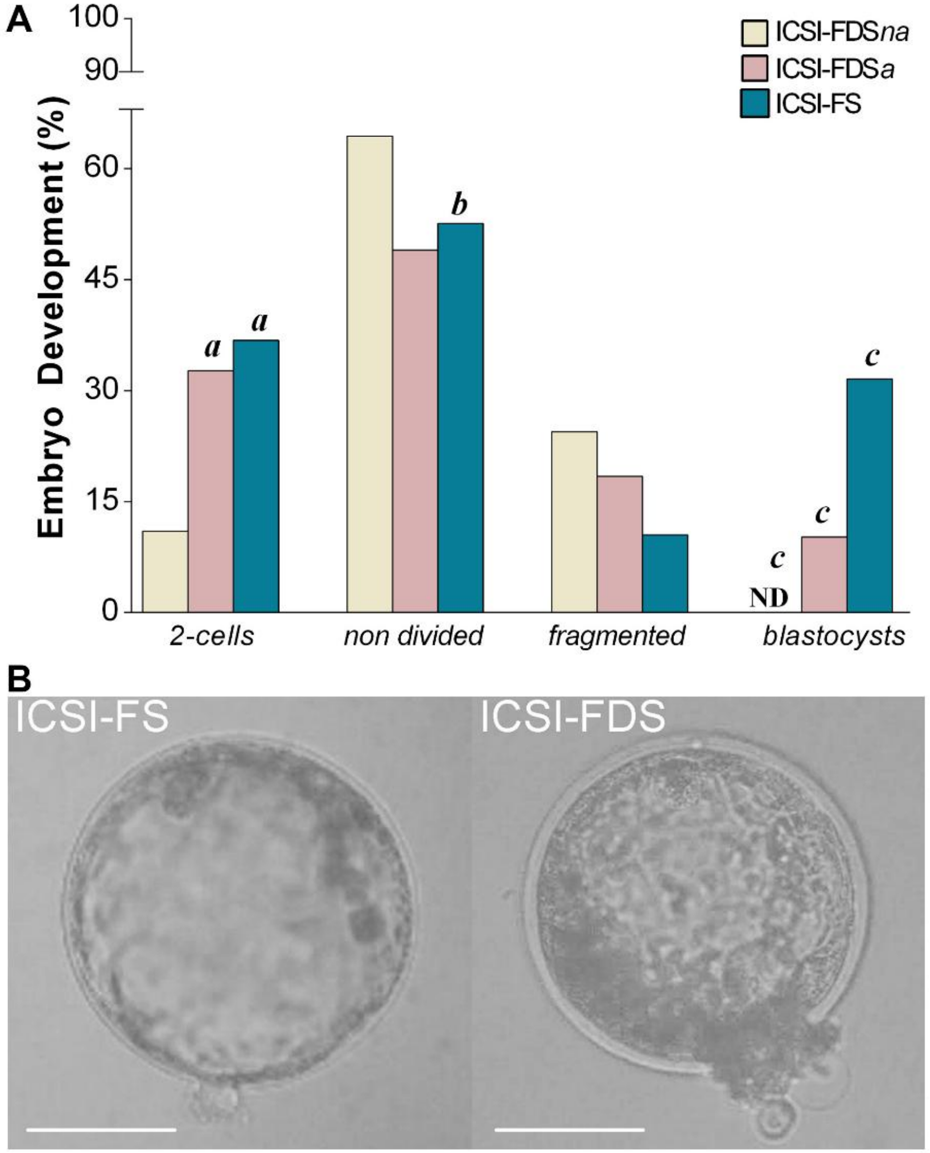
Groups	Nr. Oocytes	Lysed (%)	2-Cells (%)	Non divided (%)	Fragmented (%)	Blastocyst (%)
ICSI Frozen	22	3/22 (13,6)	7/19 (36,8)	10/19 (52,6)	2/19 (10,5)[c]	6/19 (31,57)
ICSI-FDSna	52	7/52 (13,5)	5/45 (11)[a]	29/45 (64,4)	11/45 (24,4)	0/45 (0)
ICSI-FDSa	42	4/42 (9,5)	12/38 (31,6)	15/38 (39,5)[b]	11/38 (29)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





Anzalone et al. 2018

Freeze-dried spermatozoa: an alternative biobank for endangered species



Anzalone et al. 2018

Lyophilization of the epididymal sperm

RESEARCH

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

Teruhiko Wakayama[1,2] and Ryuzo Yanagimachi[1]*

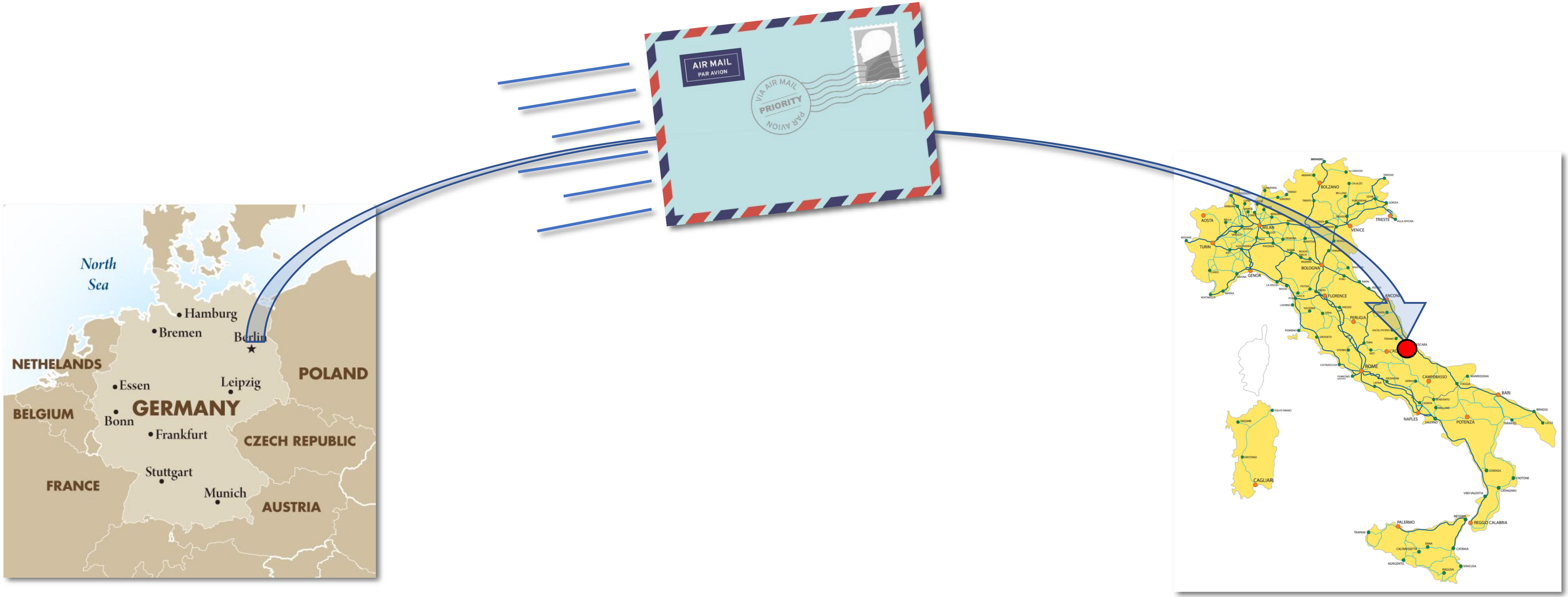
[1]Department of Anatomy and Reproductive Biology, University of Hawaii School of Medicine, Honolulu, HI. [2]Department of Veterinary Anatomy, Faculty of Agriculture, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan. *Corresponding author (e-mail: yana@hawaii.edu).

Received 16 April 1998; accepted 3 June 1998

Cases of male infertility (eg Azoospermia)

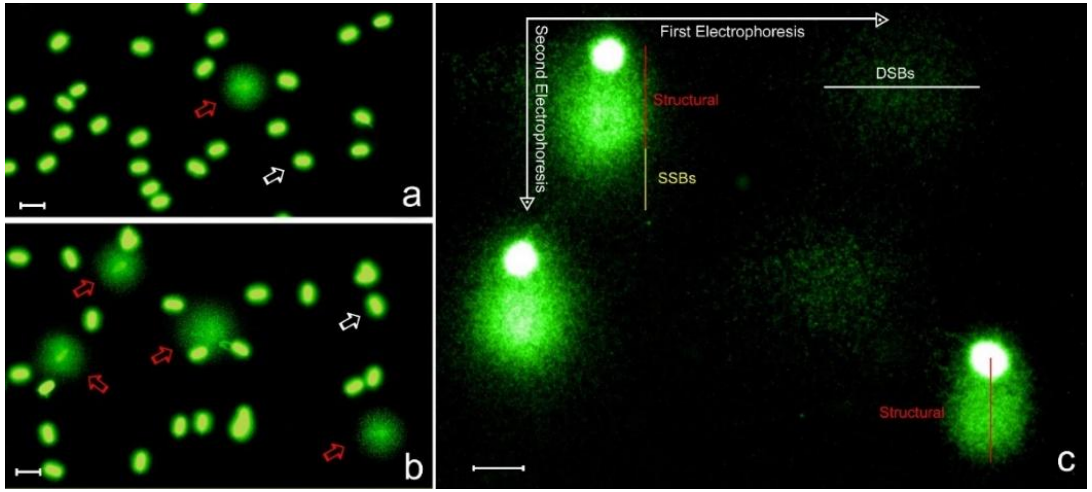
Epididymal spermatozoa

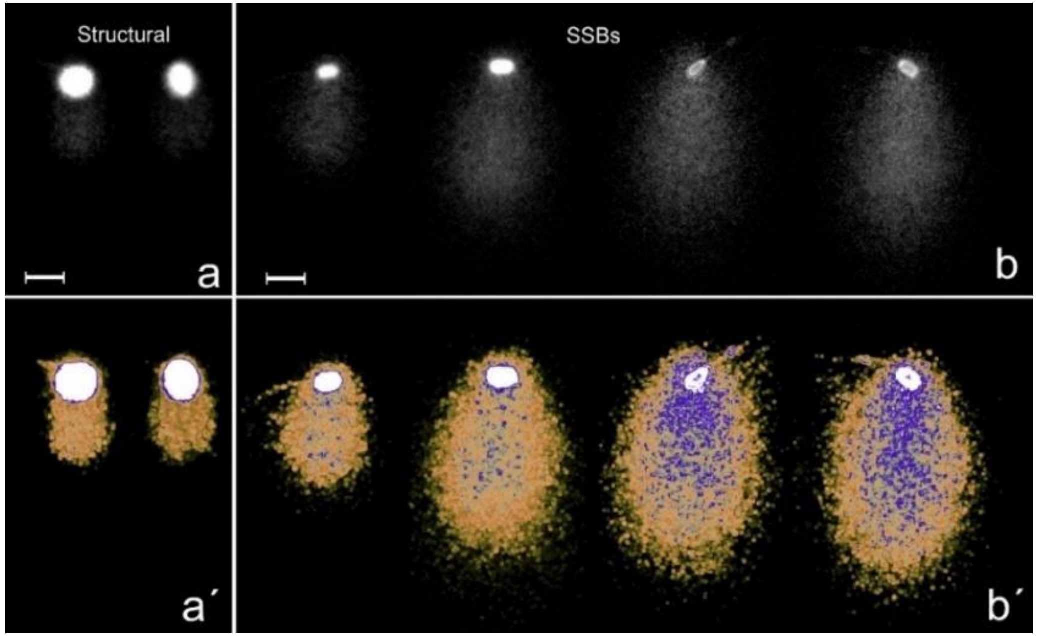
Lyophilization of the epididymal sperm



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

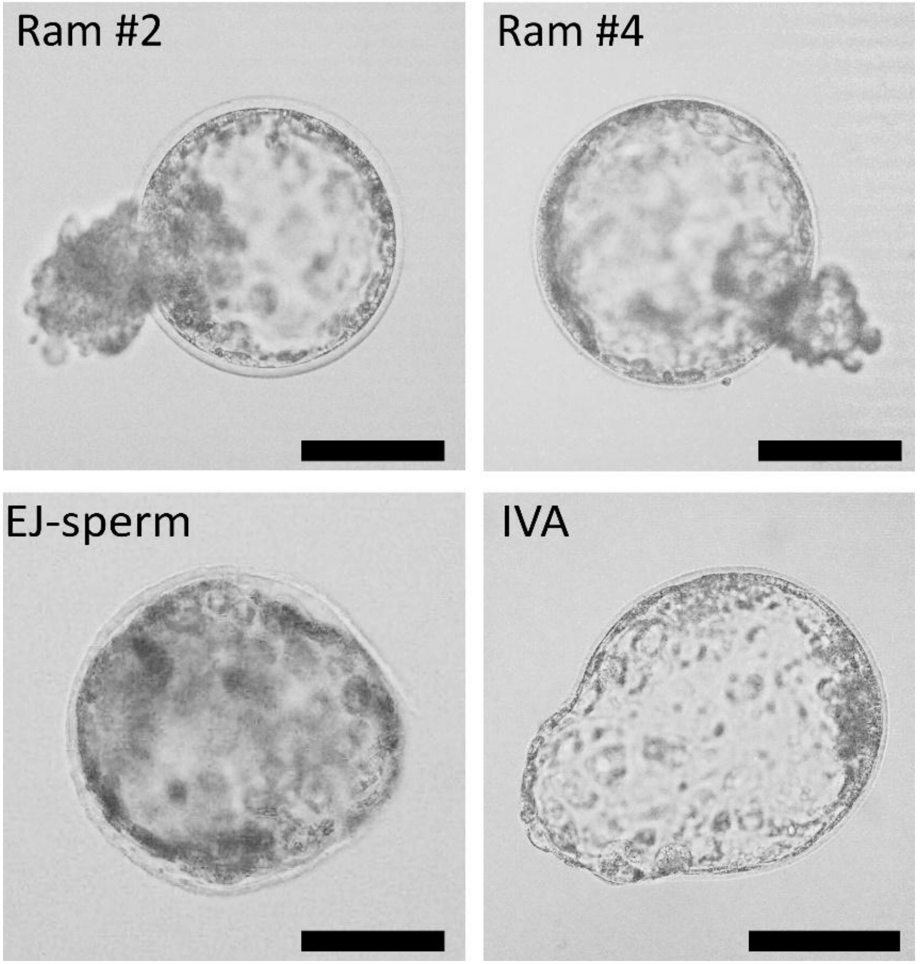




Palazzese et al. 2018

The DNA fragmentation of the epididymal ram spz affects embryonic development

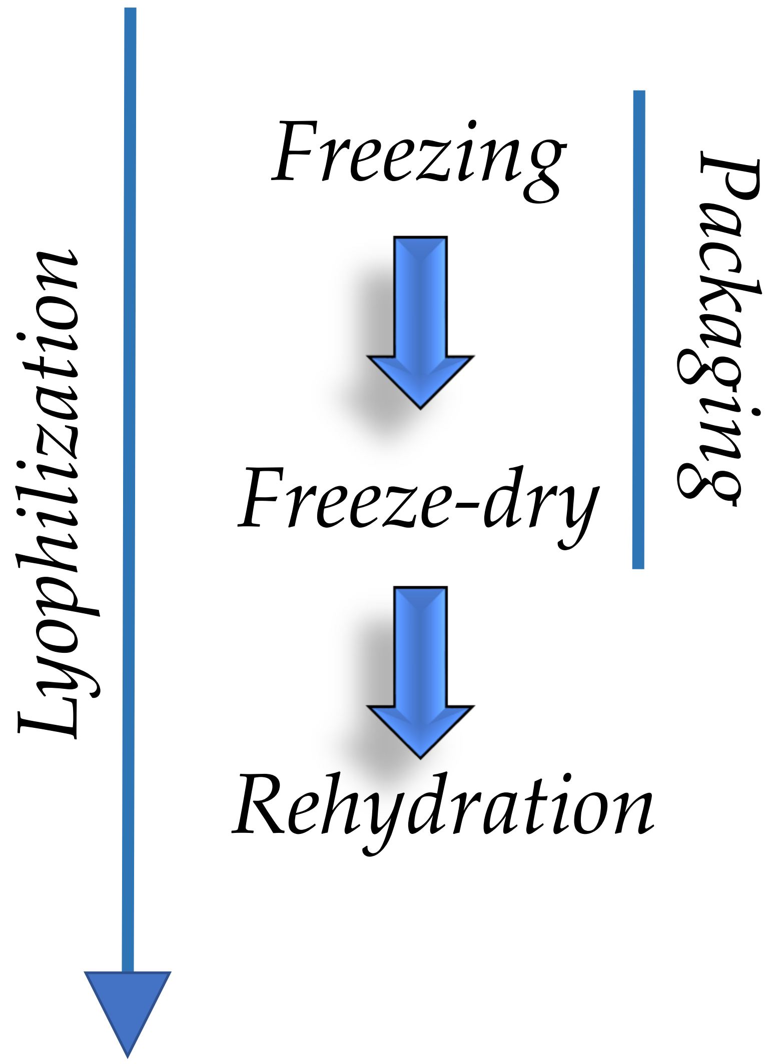
	N. oocytes	Fragmented (%)	Not-Divided (%)	2-Cells (%)	Expanded Blastocyst (%)
Ram #1	72	6 (8.3)[a]	57 (79.2)[c]	9 (12.5)	0 (0)
Ram #2	83	22 (26.5)	38 (45.8)	23 (27.7)	5 (6.0)[g]
Ram #3	58	12 (20.7)	29 (50.0)	17 (29.3)	0 (0)
Ram #4	64	16 (25.0)	36 (56.3)	12 (18.8)	4 (6.3)[h]
EJ-sperm	242	57 (23.6)[b]	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]



Palazzese et al. 2018

How to improve the freeze drying protocol?

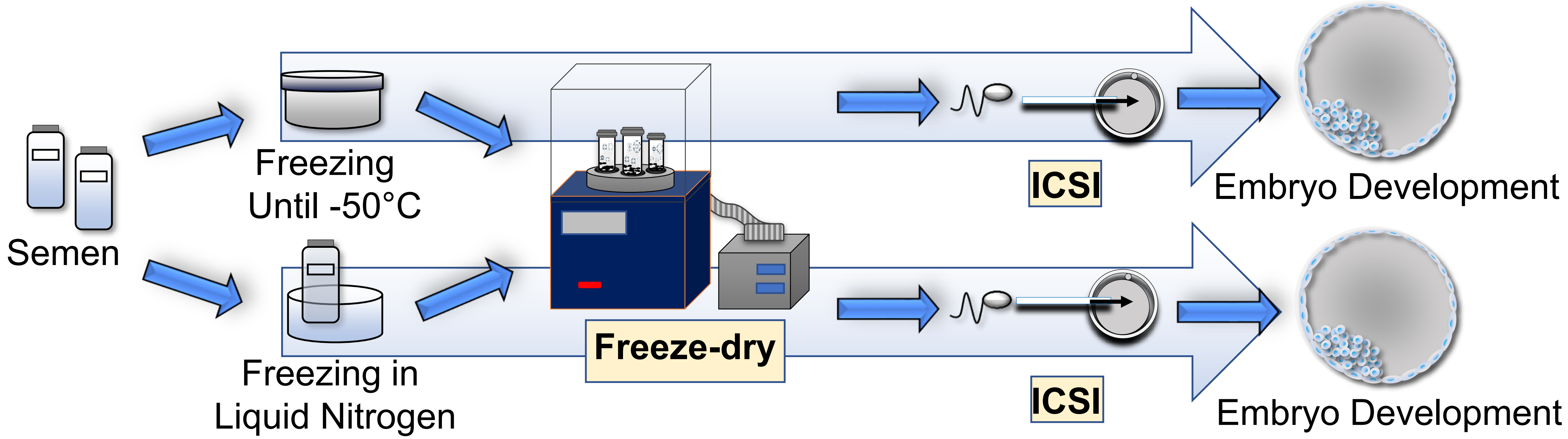
Understanding the lyophilization Step by step



Elimination of liquid nitrogen

Economical, safety and environmental issues?
Can we use milder sub-zero temperature before sublimation?

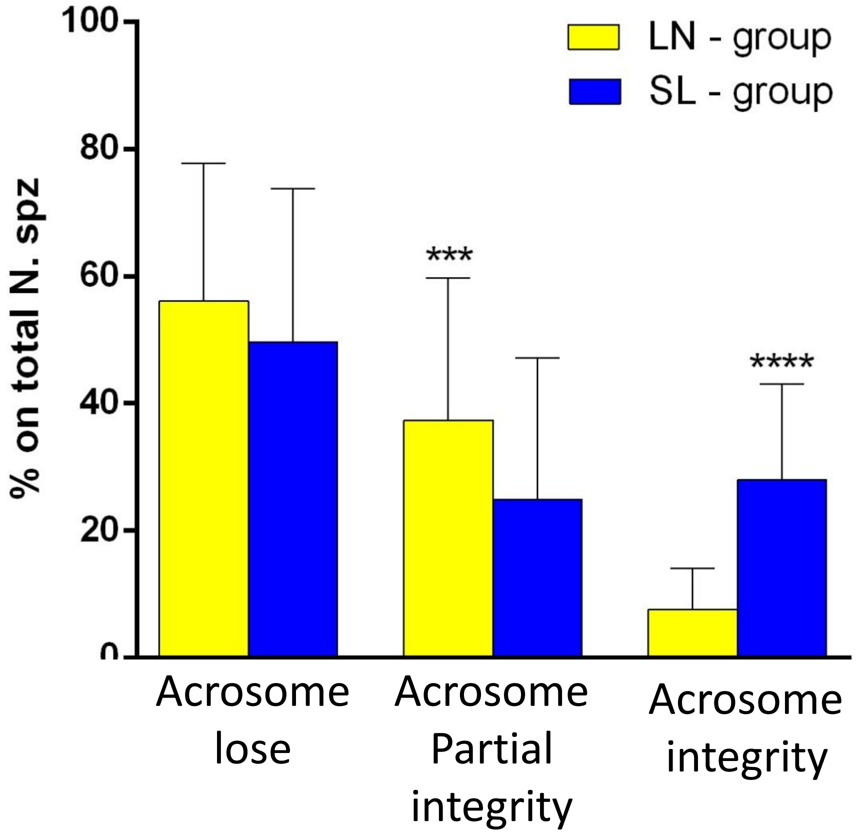
Experimental Design



Slow freezing better preserve acrosome integrity

LN-group

SF-group



Palazzese et al. 2020

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

Palazzese et al. 2020

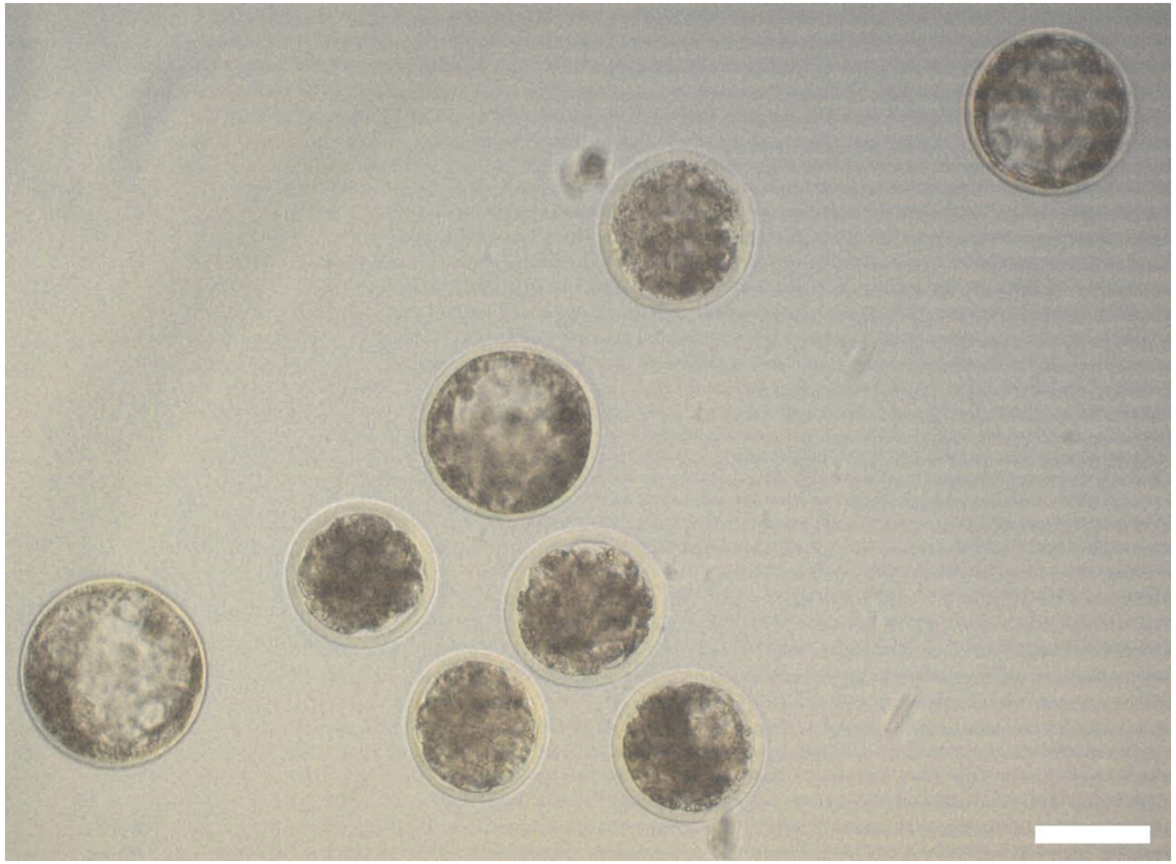
ICSI outcome

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)



Palazzese et al. 2020

scientific reports

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 Turri[2], Marco Faieta[3], Anna Donnadio[4], Flavia Pizzi[2], Paola Pittia[3], Kazutsugu Matsukawa[5] & Pasqualino Loi[1]✉

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 KAWASE[1)] and Hiroshi SUZUKI[2)]

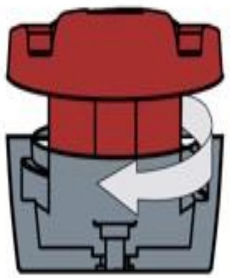
[1)]Chugai Research Institute for Medical Science, Inc., Gotemba 412-8513 and [2)]Research Unit for Functional Genomics, National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro 080-8555, Japan

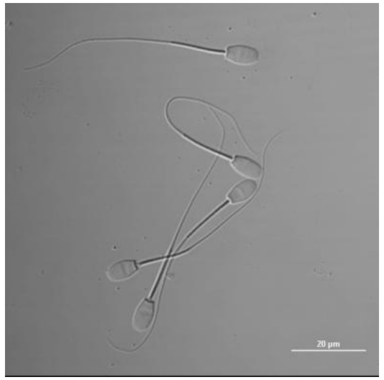
VACUUM DRY ENCAPSULATION (VDE)

DIFFERENT **TECHNOLOGICAL PROCESS** AND **PACKAGING**

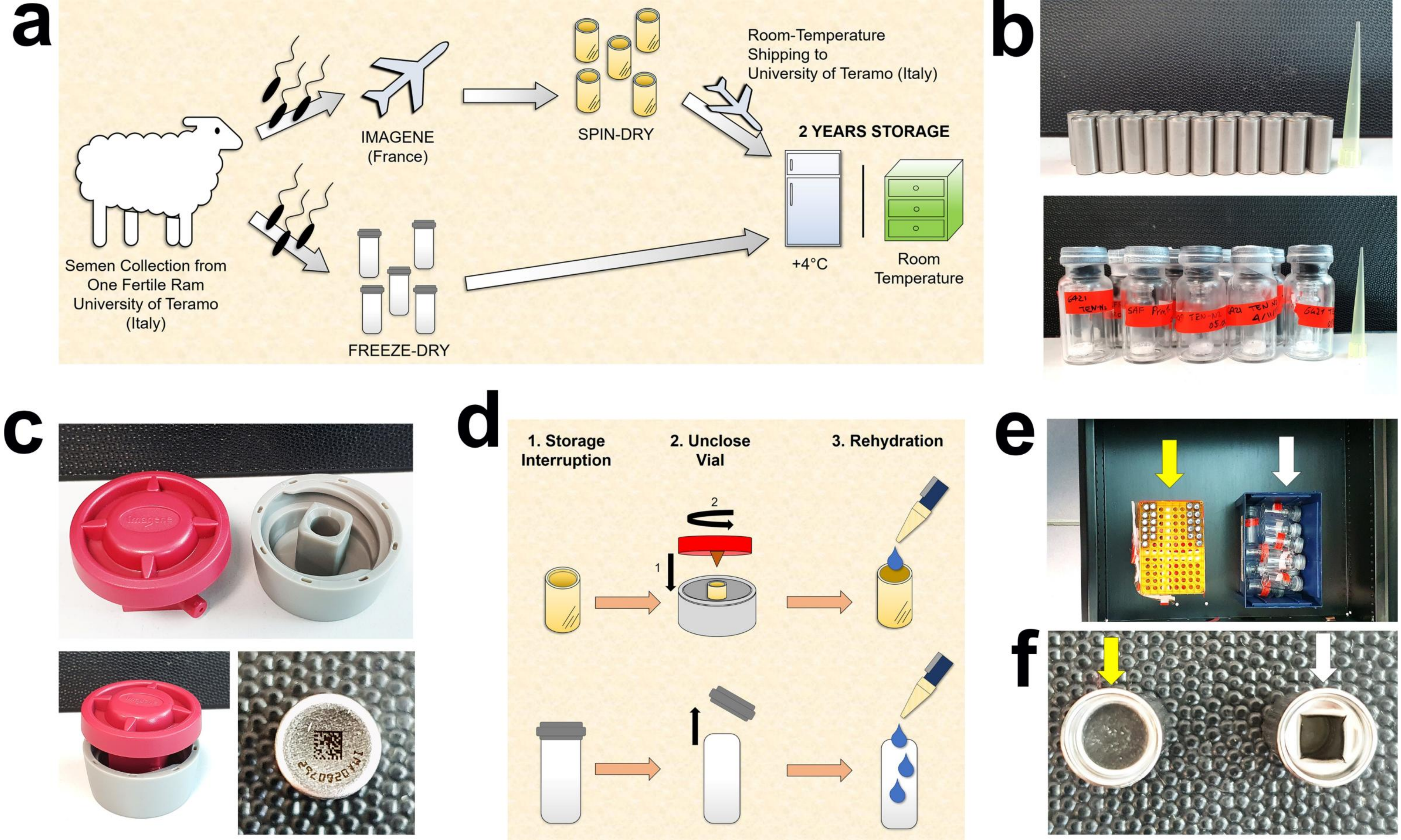
Secret ingredient by the company

SHELLOPENER



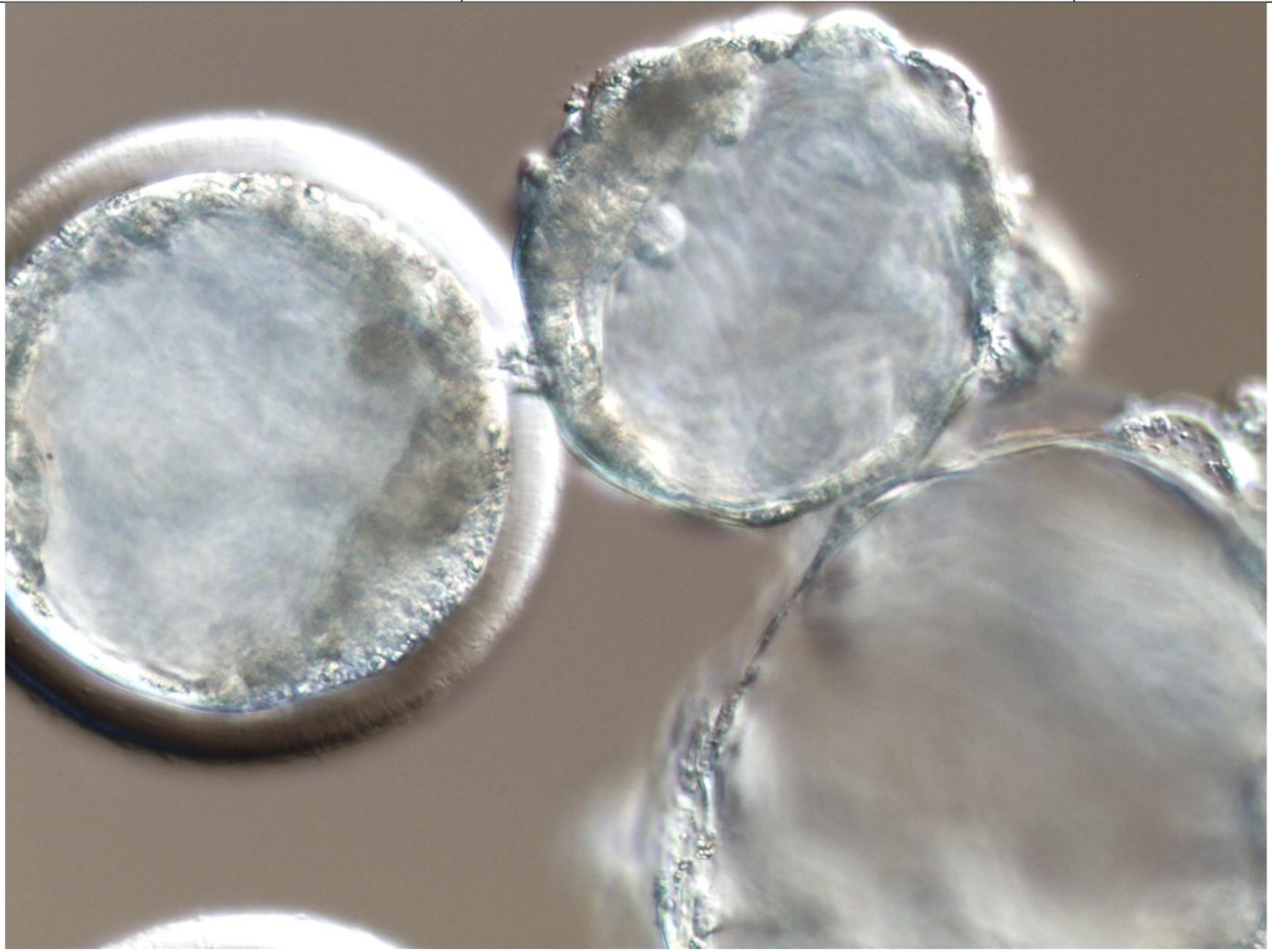


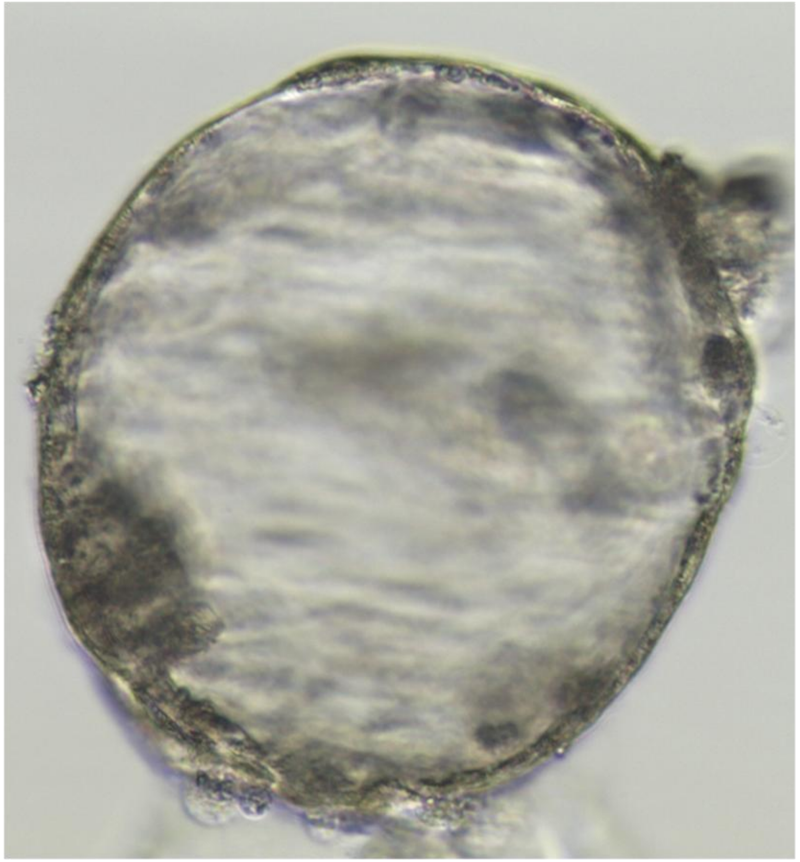
POSSIBILITY TO STORE AT **ROOM TEMPERATURE (RT)**?



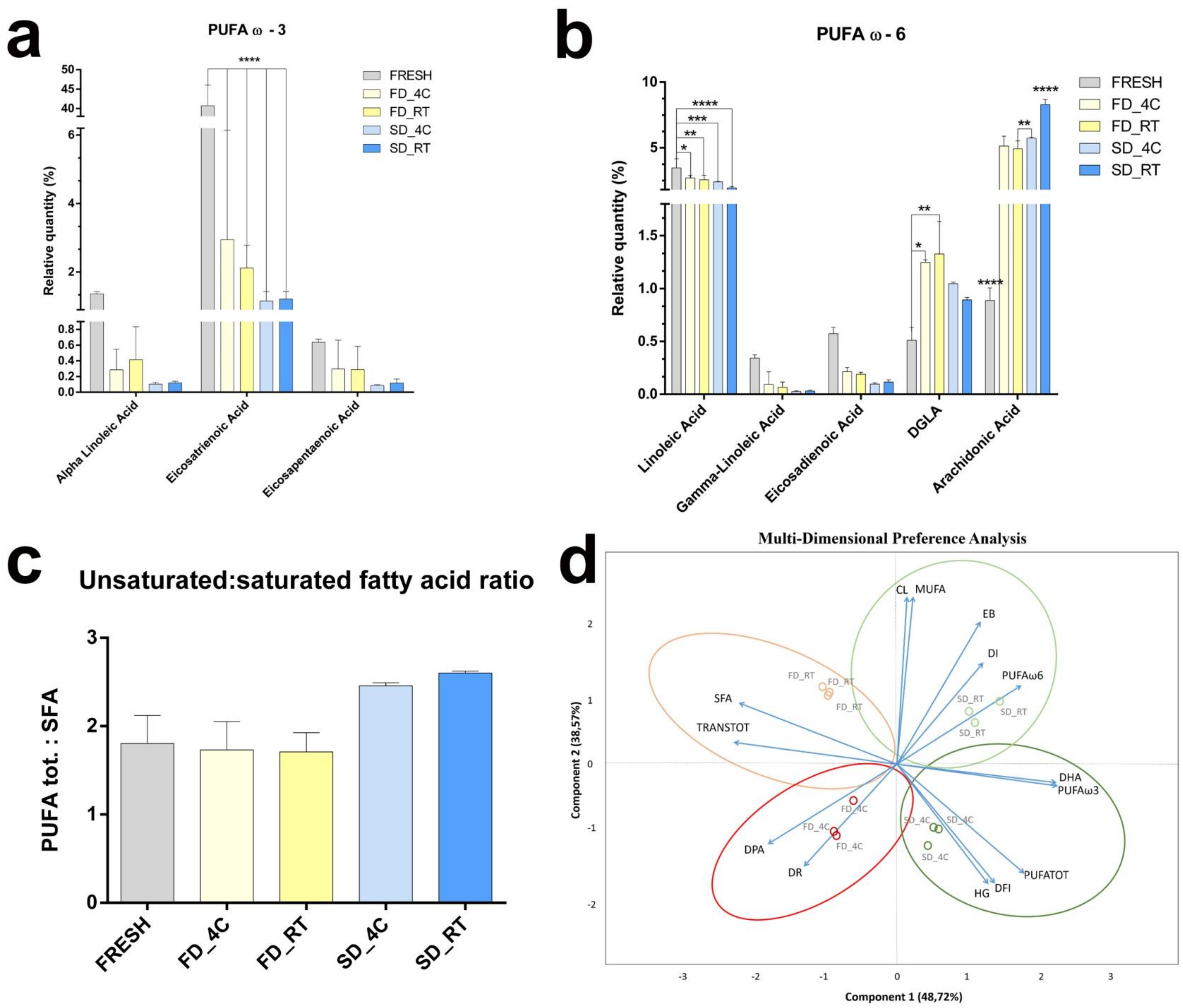
Palalzzese et al., 2023

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

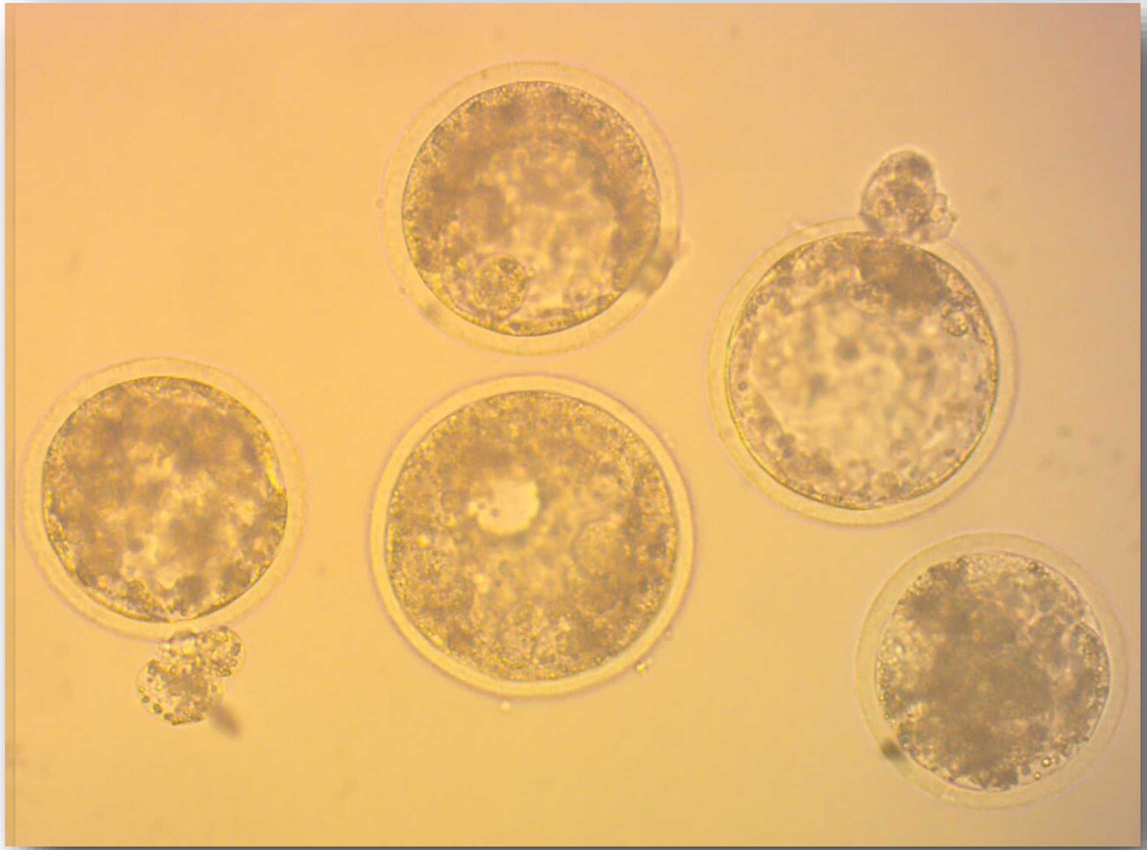




Palalzzese et al., 2023

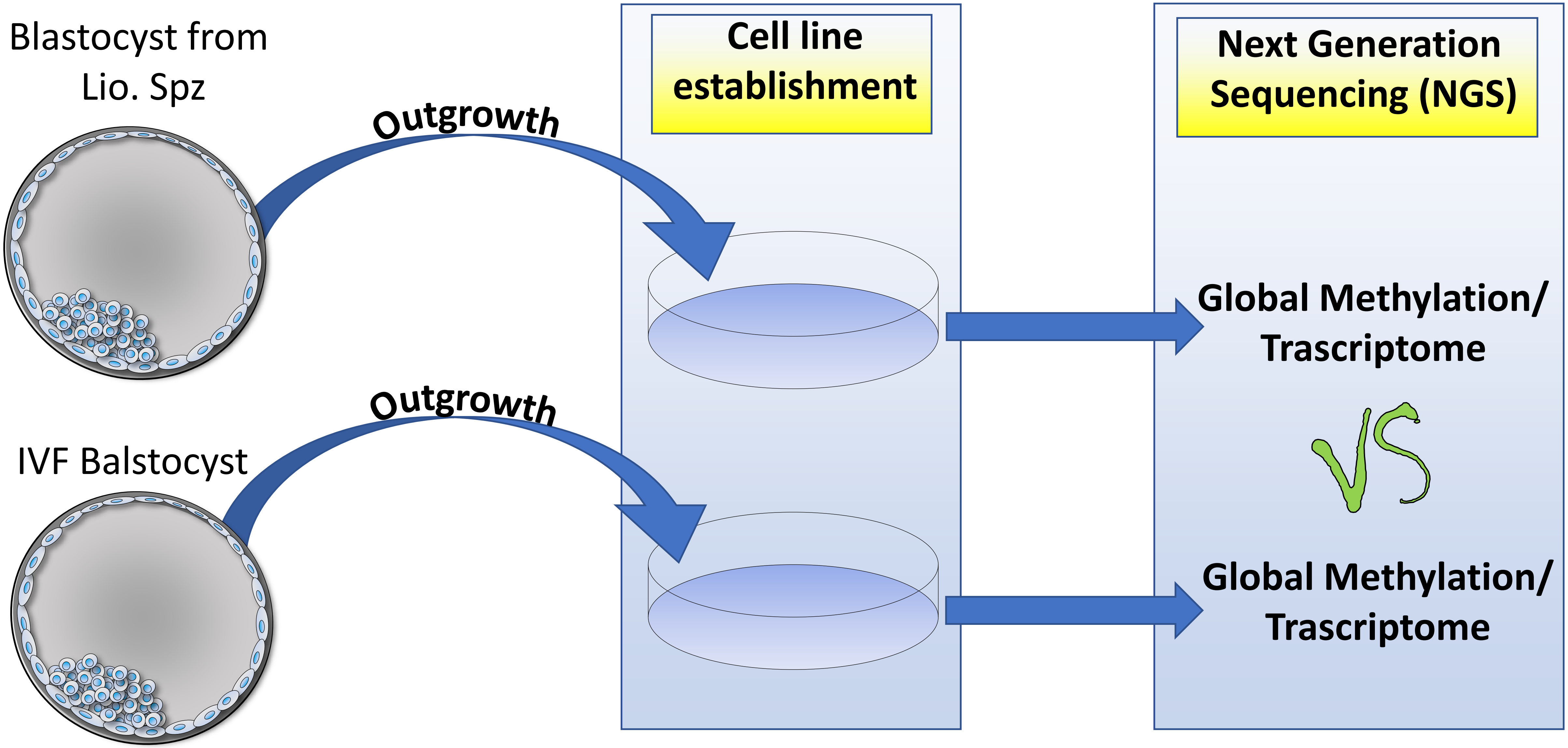


Palalzzese et al., 2023

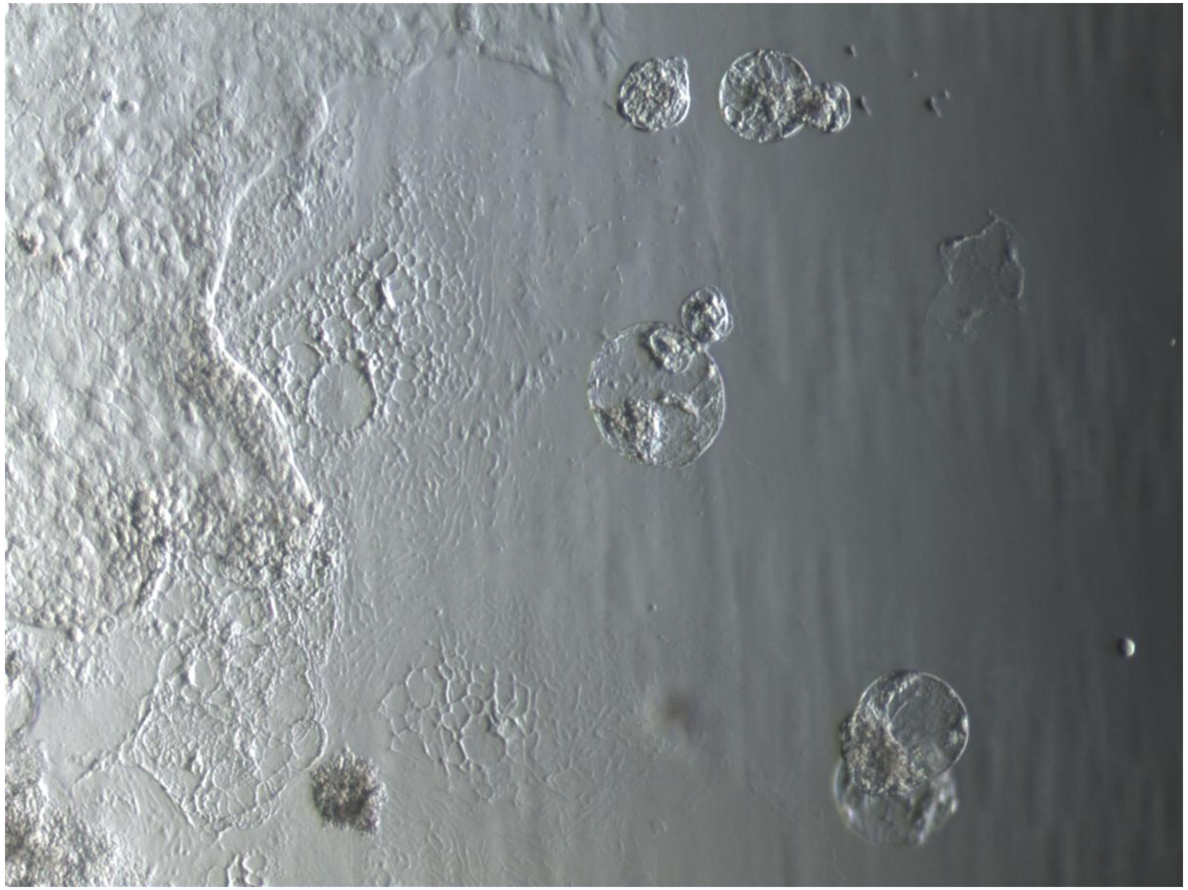


?

Experimental design

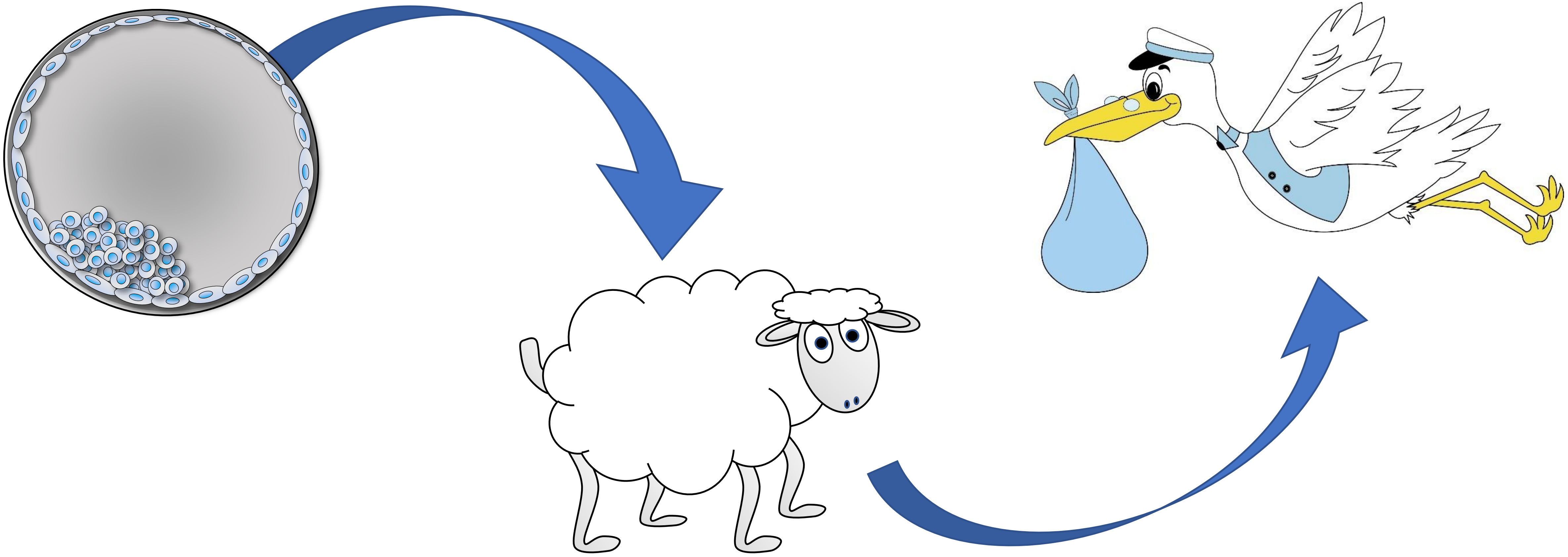


Cell line from whole blastocyst



Unpublished

From dry to life



nature COMMUNICATIONS

ARTICLE

Check for updates

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 Ishiuchi [1] & 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

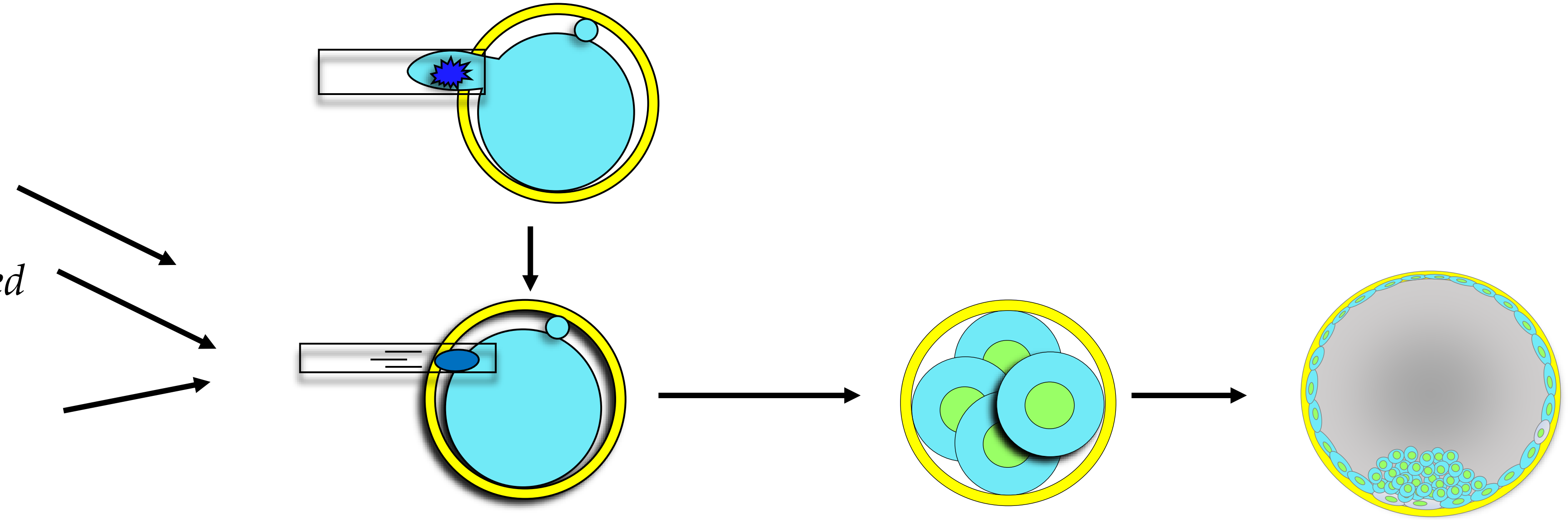
Experimental Design

Comulus Cells Fresh

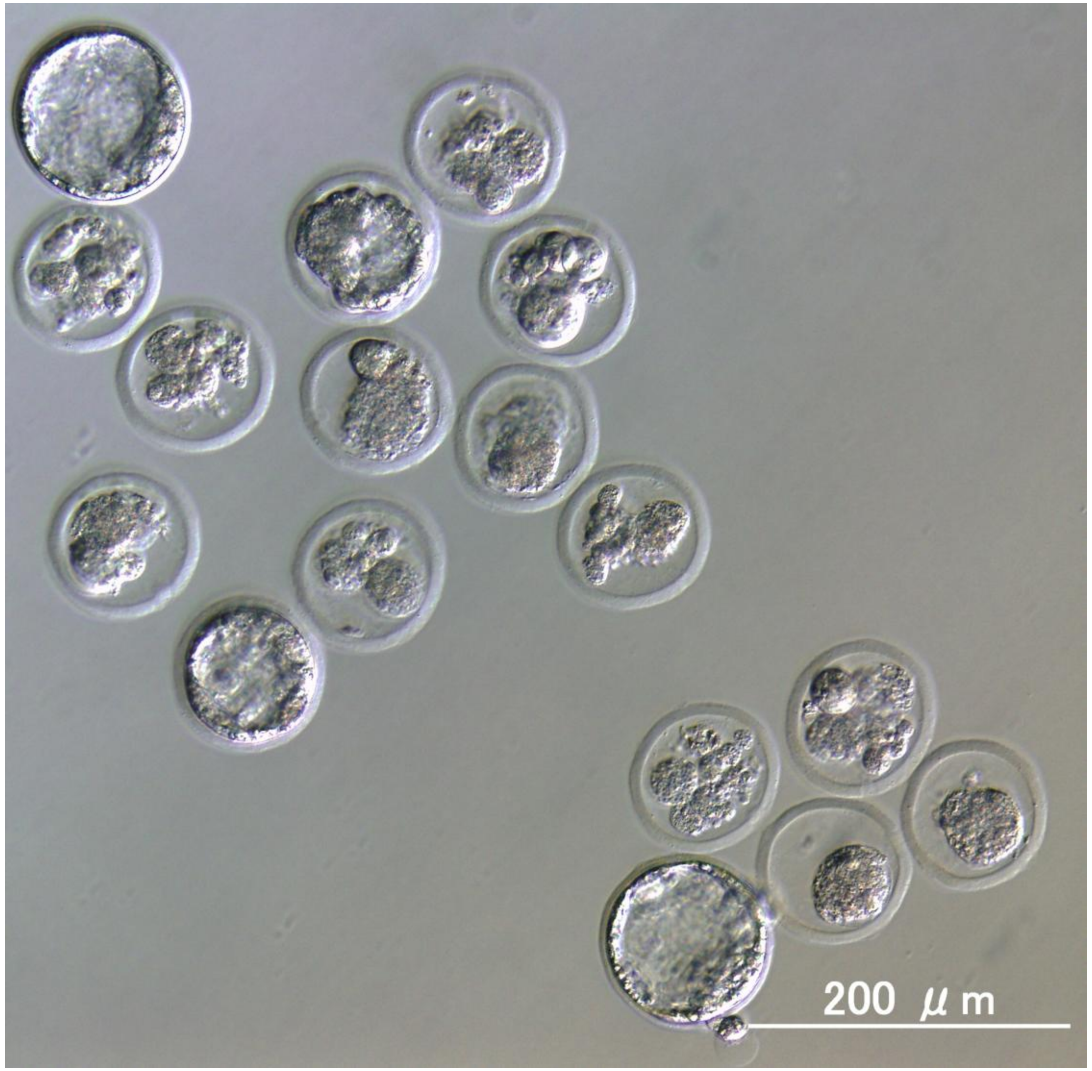
Comulus Cells Freeze-dried

mRNA vector production

Comulus Cells-Prm1 -Freeze Dried

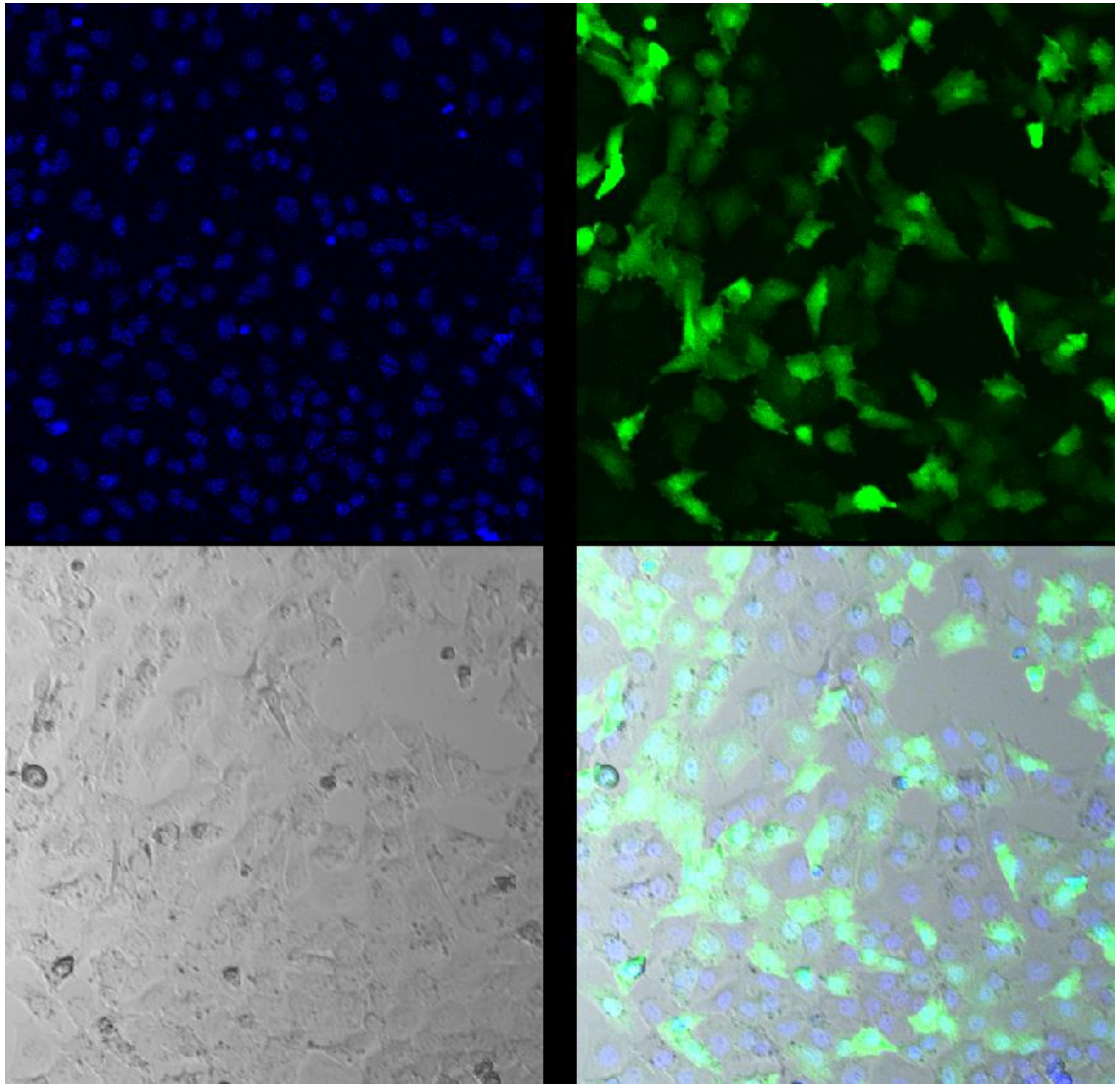


It's not so easy!



3 months of training to produce clone blastocyst in mouse

Prm1-EGFP cumulus cells



	n. Nuclei	n. EGFP-Cells	Efficiency (%)
	174	140	80,46
	133	95	71,43
	68	42	61,76
Tot.	375	277	**73,87**

CUMULUS CELLS 24h POST-TRANSFECTION WITH mRNA Prm1-2A-EGFP

	N. Oocytes	N. Reconstructed Embryos	1 PN	2 CELL	BLASTOCYST	BLASTOCYST %
	63	35	8	6	5	62,5
	53	30	23	17	7	30,43
TOT.	**116**	**65**	**31**	**23**	**12**	

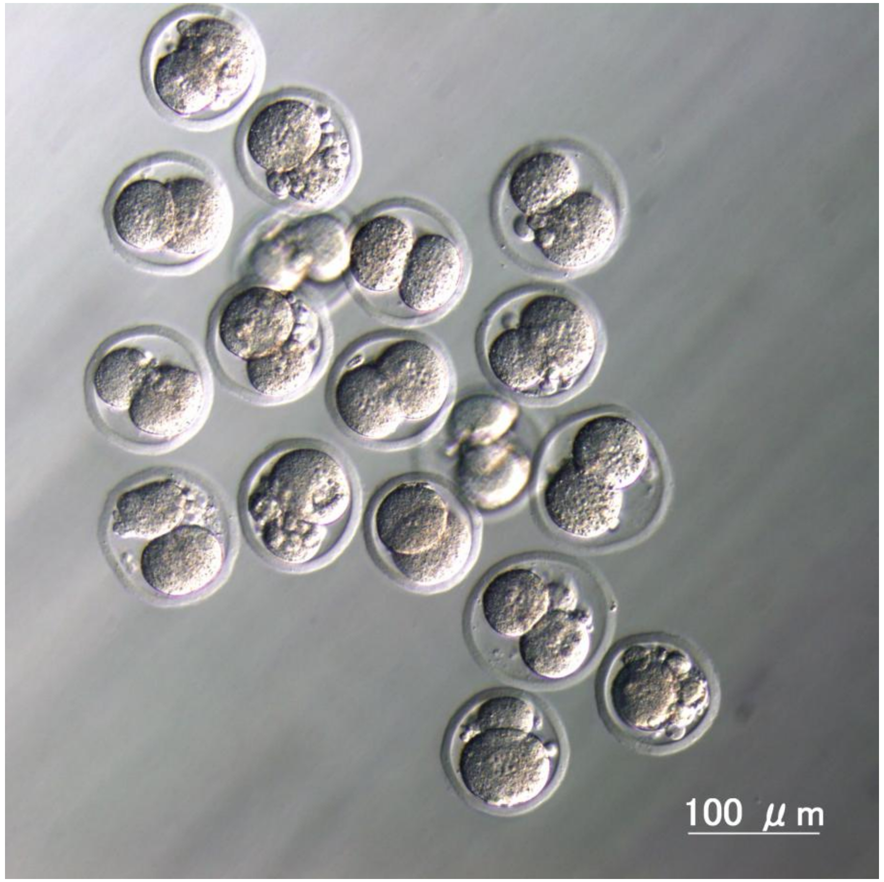
CUMULUS CELLS 48h POST-TRANSFECTION WITH mRNA Prm1-2A-EGFP

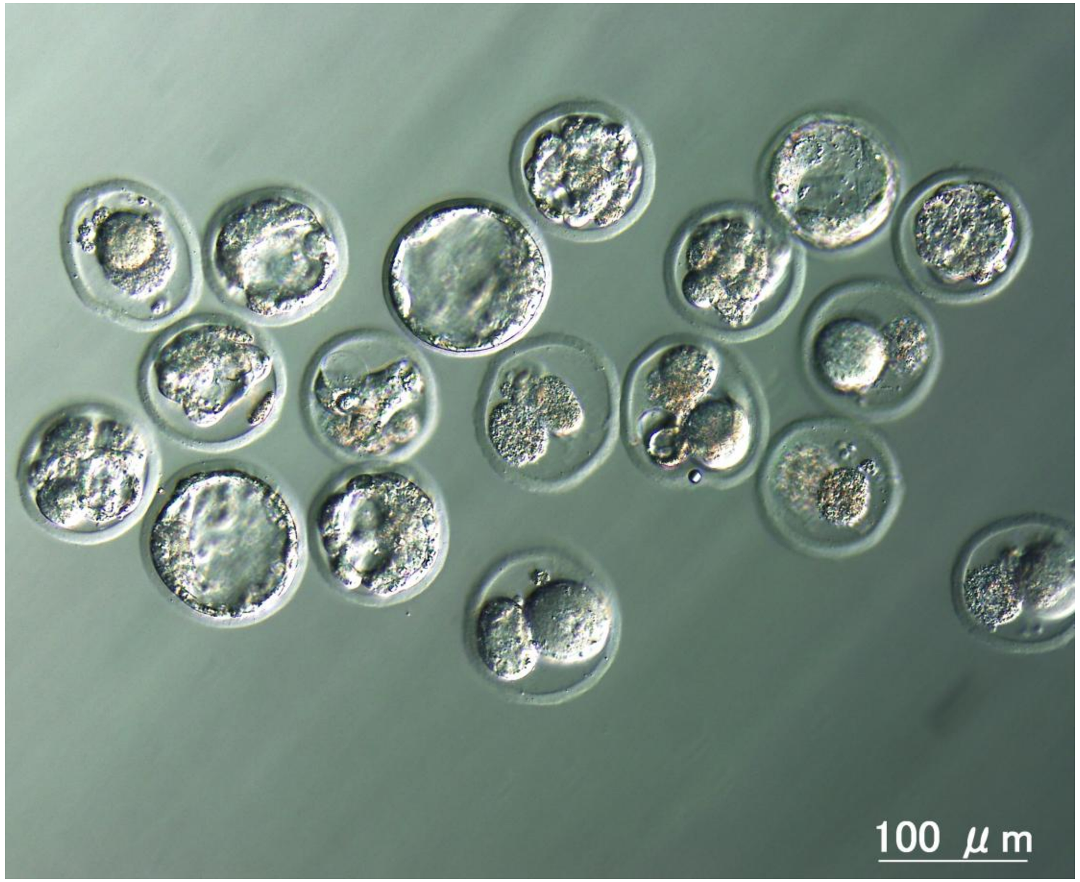
	N. Oocytes	N. Reconstructed Embryos	1 PN	2 CELL	BLASTOCYST	BLSTOCYST %
	135	69	34	12	4	11,76
	25	7	4	3	3	75,00
TOT.	**160**	**76**	**38**	**15**	**7**	

NON TRANSFECTED CUMULUS CELL (OVERNIGHT CULTERED)

	N. Oocytes	N. Reconstructed Embryos	1 PN	2 CELL	BLASTOCYST	BLASSTOCYST %
	21	5	4	2	1	25
TOT.	**21**	**5**	**4**	**2**	**1**	

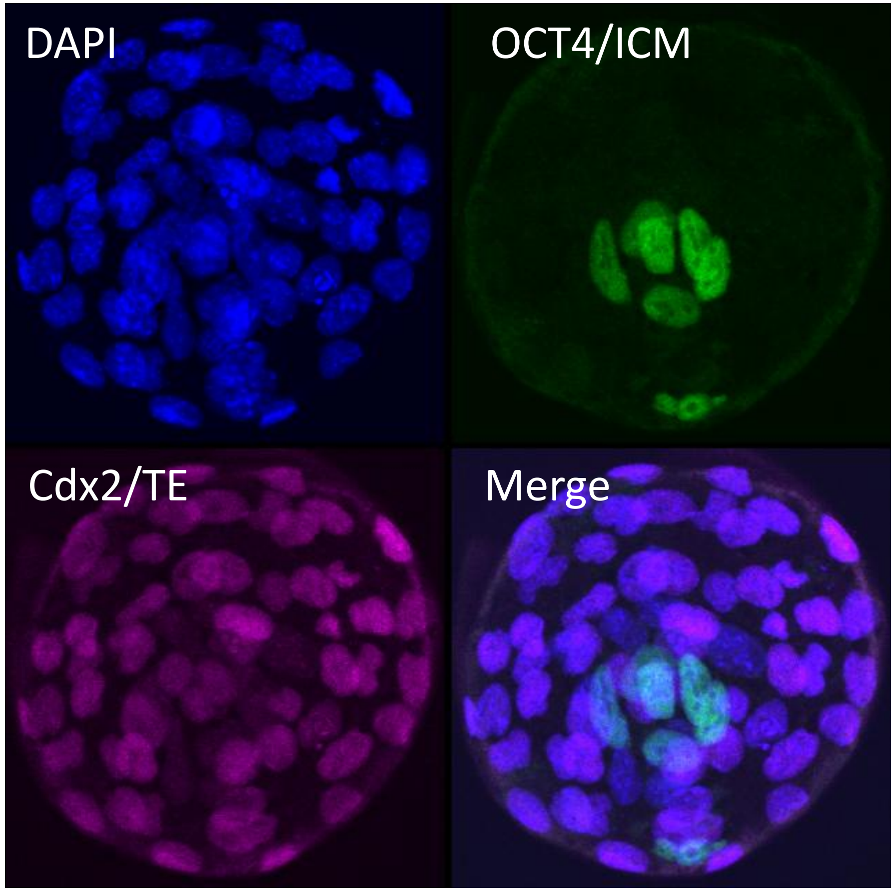
GROUPS	1 PN (%)	2 CELL (%)	BLASTOCYST (%)
24h POST-TRANSFECTION	47,69	74,19	38,71
48h POST-TRANSFECTION	50,00	39,47	18,42
CTR	80,00	50,00	25,00





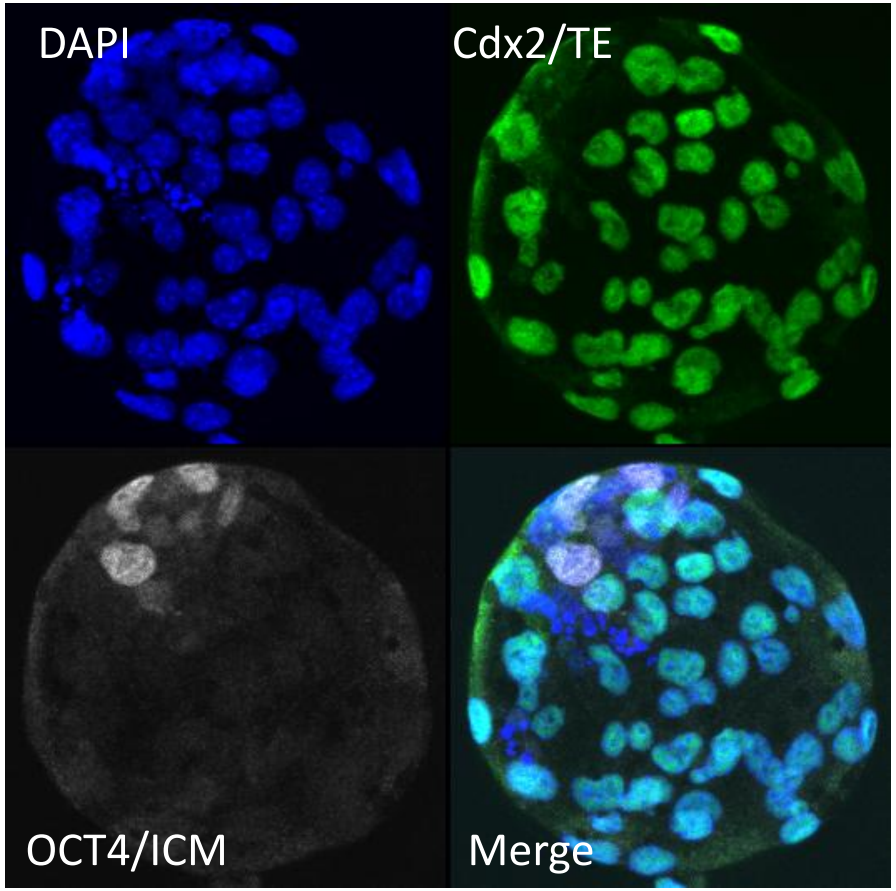
BLASTO FROM CELL 24h POST-TRANSFCETION

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

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