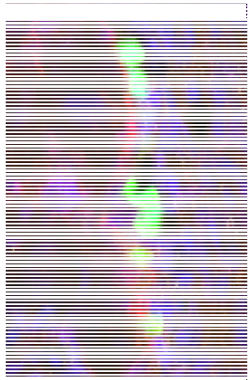
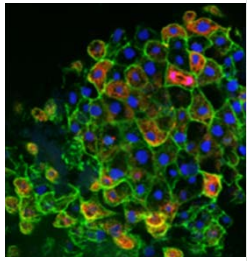
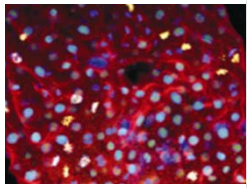
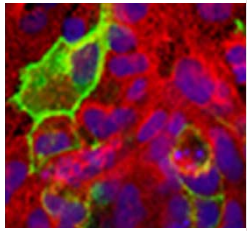
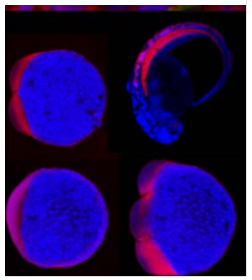










Fish PGC cryopreservation: is it a realistic tool for fish conservation biology?

V. Robles, D.G. Valcarce and M.F. Riesco

INSTITUTO ESPAÑOL DE OCEANOGRAFÍA 1914

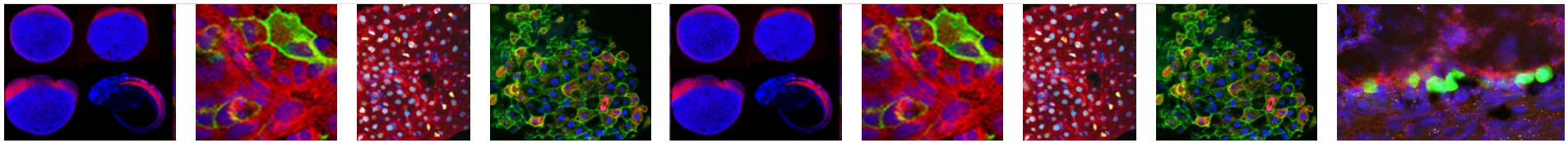
cost
EUROPEAN COOPERATION
IN SCIENCE AND TECHNOLOGY

AQUAGAMETE

5th Training School, Valencia

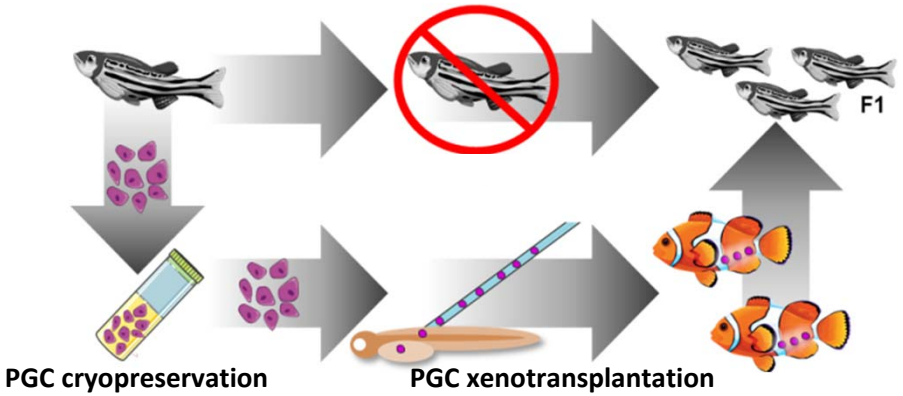
Contents:

- For what purpose should we cryopreserve PGCs?
- PGC cryopreservation
  - -PGC visualization
  - -Cryopreservation protocols
  - -How to evaluate the success of a PGC cryopreservation method?
- Limitations and future perspectives



Why and what for?

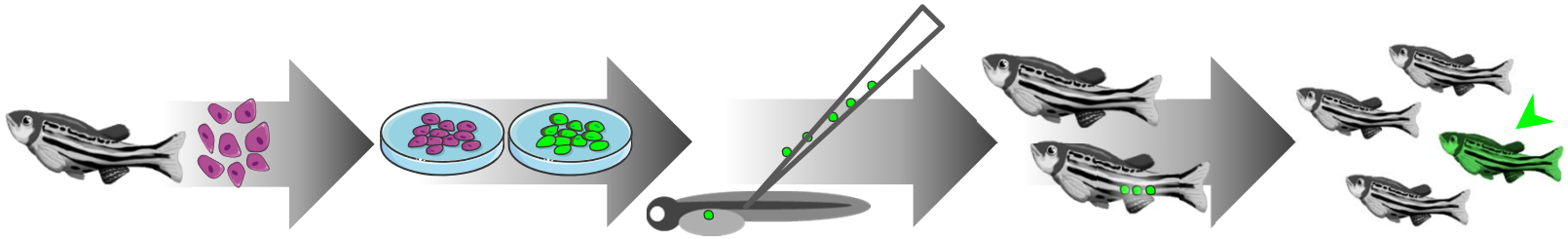
Conservation of endangered species



Host sterilization improves efficiency

Surrogate production

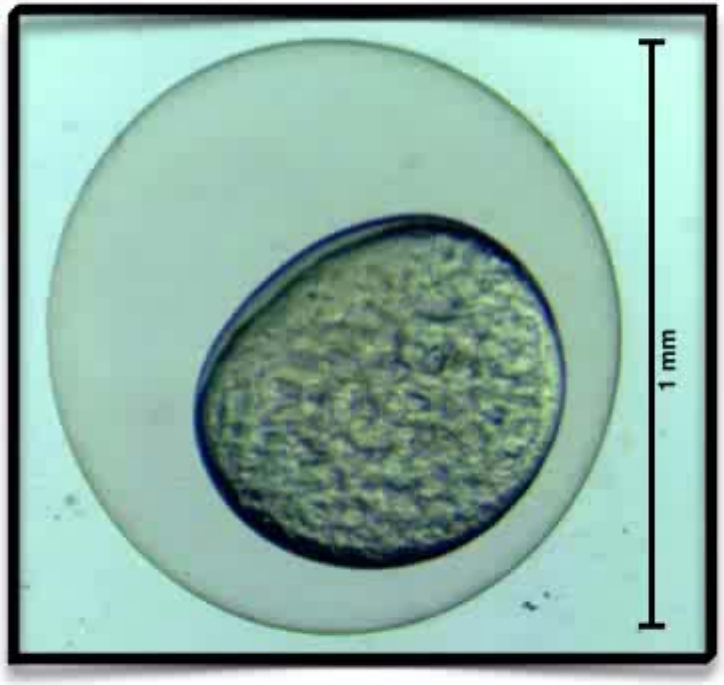
Biotechnological applications



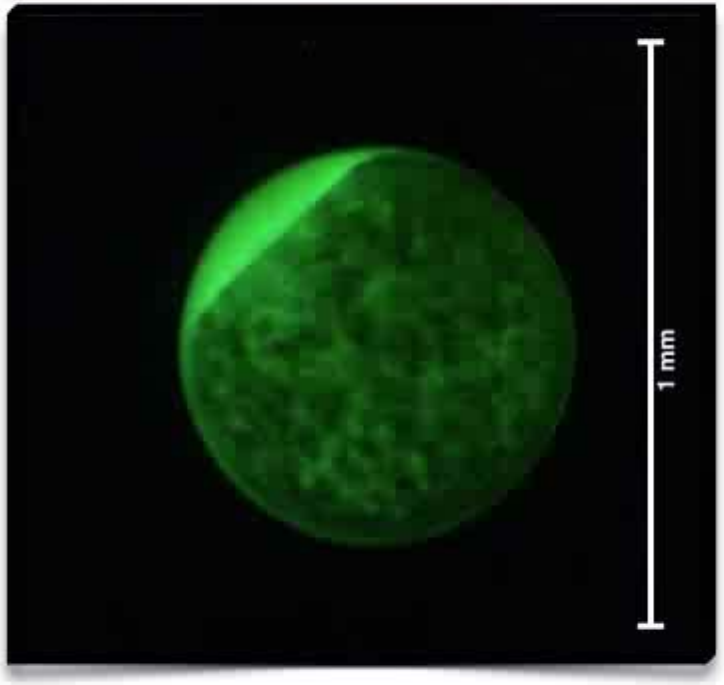
PGC cryopreservation: tools for PGC visualization

TIME LAPSE

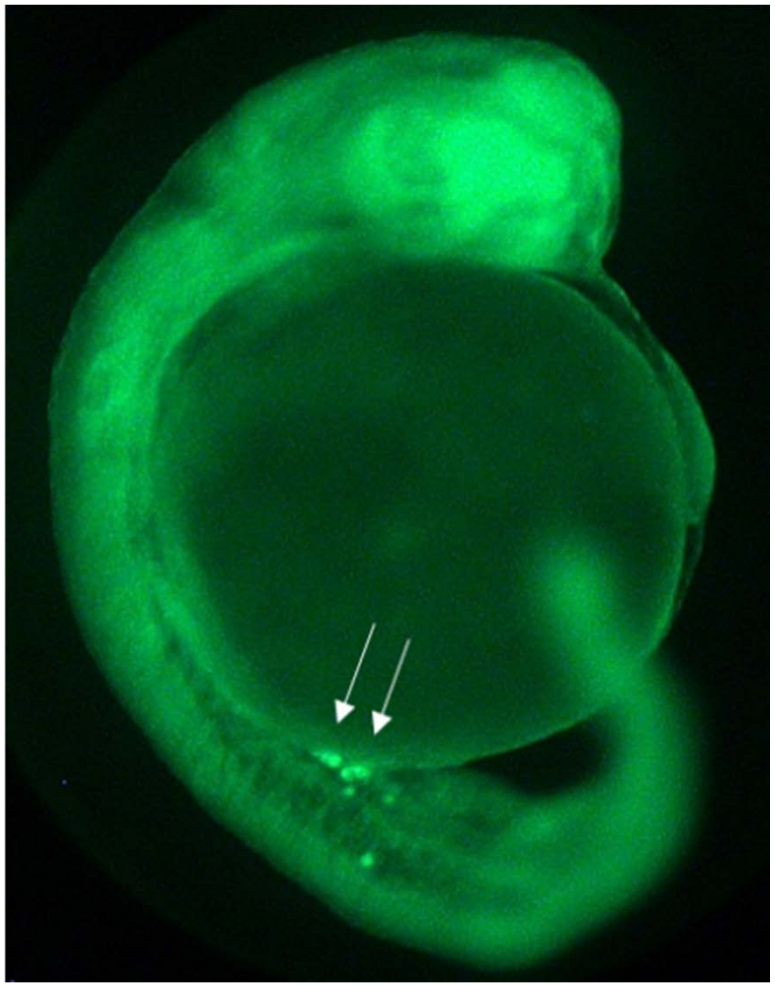
Bright field



Fluorescence field



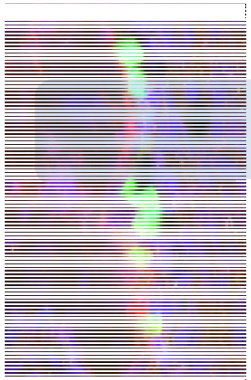
PGC cryopreservation: tools for PGC visualization

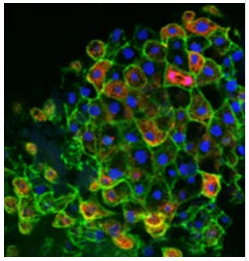


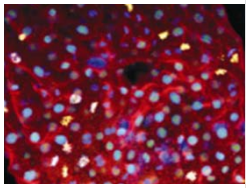
Riesco and Robles 2015

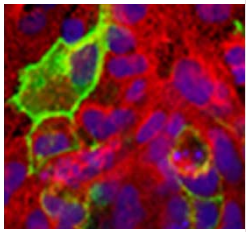
There are alternative non-transgenic methods

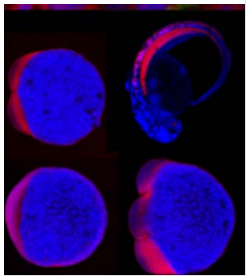
- GFP-nos 3'UTR mRNA
- FITC (for sturgeon) *Saito et al. 2014*







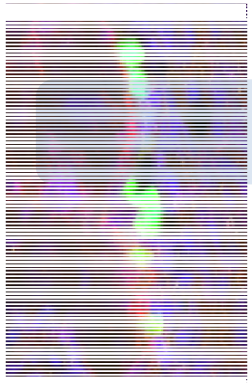


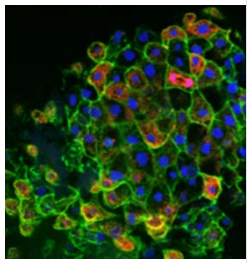


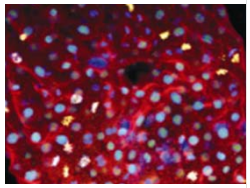
PGC cryopreservation: protocols

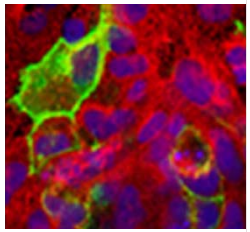
Table of PGCs cryopreservation protocols in different teleost species

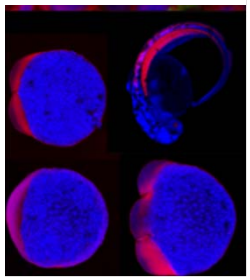
n.	SPECIES	BIOLOGICAL SAMPLE	PROTOCOL AND SURVIVAL EFFICIENCY	EVALUATION METHOD
	Anguilla japonica		Cryoprotectants / Loading containers and survival efficiency	
1	Kawakami et al., 2012a	YIE: Yolk-intact embryos YDE: Yolk-depleted embryos	PS: 1.5 M EG — VS: 3 M EG Time ratio VS/PS (min) 1) 1/10 — 4) 1/20 — 7) 1/30 2) 5/10 — 5) 5/20 — 8) 5/30 3) 10/10 — 6) 10/20 — 9) 10/30 Vitrification on a nylon mesh and stored into a cryogenic vial Survival PGCs rate YIE: - - - - - - YDE: 0% 0% 0% 0% 0% ≈ 76% YIE: 0% 0% 0% YDE: 0% 0% 0%	EGFP positive and negative cells for trypan blue
	Cyprinus carpio		Cryoprotectants / Loading containers and survival efficiency	
2	Kawakami et al., 2012b	YDE: Yolk-depleted embryos	EG: PS: 1.5 M, VS: 3 M — DMSO: PS: 1.5 M, VS: 3 M Time ratio VS/PS (min) 1) 5/30 — 3) 20/30 — 5) 10/50 — 7) 30/50 2) 10/30 — 4) 30/30 — 6) 20/50 Vitrification on a nylon mesh and stored into a cryogenic vial Survival PGCs rate EG: 1) ≈ 71% 2) ≈ 77% 3) ≈ 74% 4) ≈ 48% 5) ≈ 48% 6) ≈ 38% 7) 0% DMSO: 1) ≈ 9% 2) ≈ 44% 3) ≈ 54%	EGFP positive and negative cells for trypan blue
	Danio rerio		Cryoprotectants (VS) / Loading containers and survival efficiency	
3	Higaki et al., 2010	EM: Embryos	PS: A) 2 M, B) 3 M — VS: 5 M Time ratio VS/PS (min) 1) 10/20 — 3) 10/30 2) 20/20 — 4) 20/30 Vitrification on a nylon mesh and stored into a cryogenic vial No. Of membrane-intact PGCs per embryo EG: 1 2 3 4 A) 0 ≈ 1.3 ≈ 0.2 ≈ 3.3 B) ≈ 0.3 ≈ 3.5 ≈ 3.7 ≈ 4.2 DMSO: 1 2 3 4 A) 0 ≈ 0.2 0 ≈ 1 B) 0 ≈ 1.5 ≈ 0.2 ≈ 2	EGFP positive and negative cells for trypan blue
4	Riesco et al., 2012	GR: Genital ridges EM: Embryos DC: Dissociated cells	1) (EM) 5 M DMSO + 1 M EG + 4% PVP 2) (GR) 5 M DMSO + 1 M EG + 4% PVP 3) (GR) 5 M DMSO + 1 M EG + 4% PVP + 10 mg/mL AFP 4) (GR) 5 M DMSO + 1 M EG + 4% PVP + 20 mg/mL AFP 5) (DC) 5 M DMSO + 1 M EG + 4% PVP Survival PGCs rate 0.5 mL straw: 1) ≈ 90% 2) ≈ 90% 3) ≈ 90% 4) ≈ 50% 5) - Cryovial: 1) ≈ 75% 2) ≈ 75% 3) - 4) - 5) - Microdrop: 1) - 2) ≈ 95% 3) - 4) - 5) - Microcapsule: 1) - 2) - 3) - 4) - 5) ≈ 25%	EGFP positive and negative cells for trypan blue and pseudopoda emission
5	Higaki et al., 2013	YDE: Yolk-depleted embryos	PS: A) 2 M EG + 1 M DMSO; B) 2 M EG + 1 M PG; C) 2 M DMSO + 1 M EG; D) 2 M DMSO + 1 M PG; E) 2 M PG + 1 M DMSO; F) 2 M PG + 1 M EG VS: A) 3 M EG + 2 M DMSO; B) 3 M EG + 2 M PG; C) 3 M DMSO + 2 M EG; D) 3 M DMSO + 2 M PG; E) 3 M PG + 2 M DMSO; F) 3 M PG + 2 M EG Time ratio VS/PS (min) 1) 5/20 — 4) 5/30 2) 10/20 — 5) 10/30 3) 20/20 — 6) 20/30 Vitrification on a nylon mesh and stored into a cryogenic vial YDE (No. of membrane-intact PGCs per embryo) 1) - - - - - - 2) ≈ 9 ≈ 6.5 ≈ 8 ≈ 6 ≈ 5 - 3) ≈ 9 ≈ 6.5 ≈ 7.5 ≈ 7.5 ≈ 7.5 ≈ 6.5 4) ≈ 7.5 ≈ 3.5 ≈ 3 ≈ 3 - - 5) ≈ 9 ≈ 6.5 ≈ 7.5 ≈ 6.5 ≈ 7.5 ≈ 4 6) ≈ 9 ≈ 7 ≈ 7 ≈ 5 ≈ 7.5 ≈ 8	EGFP positive and negative cells for trypan blue and pseudopoda emission
	Oncorhynchus mykiss		Cryoprotectants / Loading containers and survival efficiency	
6	Kobayashi et al., 2007	GR: Genital ridges	PBS-based medium 0.5% BSA + 5.5 mM D-glucose and: 1) 1.5 M DMSO — 5) 1.2 M EG 2) 1.5 M Gly — 6) 1.8 M EG 3) 1.5 M PG — 7) 2.1 M EG Cryotubes (survival PGCs rate) 1) 0% — 5) ≈ 40% 2) 0% — 6) ≈ 40% 3) 0% — 7) ≈ 40%	EGFP positive and negative cells for trypan blue











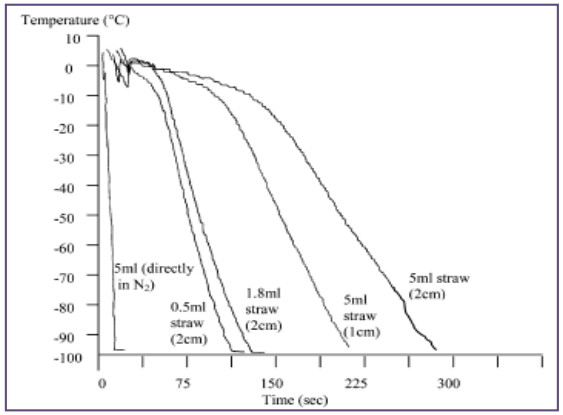
PGC cryopreservation: protocols

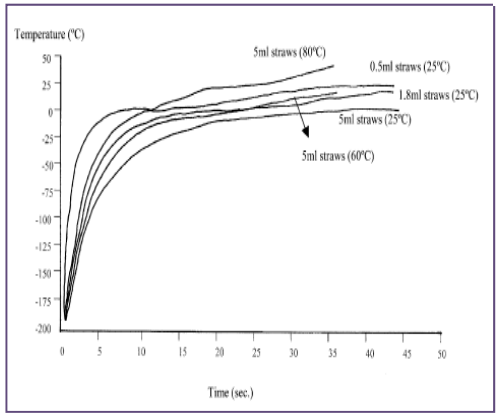
GR: Genital ridges
EM: Embryos
DC: Dissociated cells

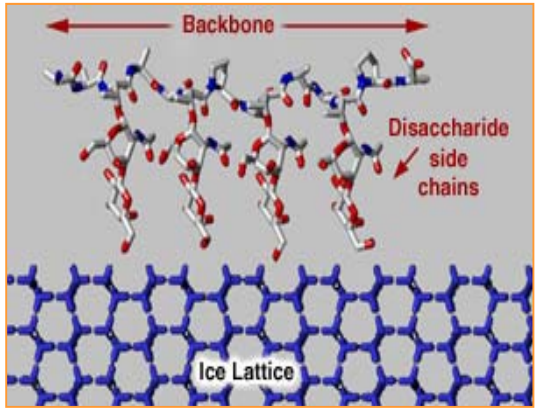
1) (EM) 5 M DMSO + 1 M EG + 4% PVP
2) (GR) 5 M DMSO + 1 M EG + 4% PVP
3) (GR) 5 M DMSO + 1 M EG + 4% PVP + 10 mg/mL AFP
4) (GR) 5 M DMSO + 1 M EG + 4% PVP + 20 mg/mL AFP
5) (DC) 5 M DMSO + 1 M EG + 4% PVP

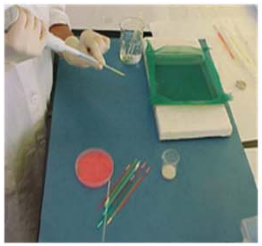
	Survival PGCs rate					EGFP positive
0.5 mL straw	1) ≈ 90%	2) ≈ 90%	3) ≈ 90%	4) ≈ 50%	5) -	and negative cells
Cryovial	1) ≈ 75%	2) ≈ 75%	3) -	4) -	5) -	for trypan blue
Microdrop	1) -	2) ≈ 95%	3) -	4) -	5) -	and pseudopoda
Microcapsule	1) -	2) -	3) -	4) -	5) ≈ 25%	emission

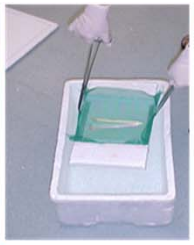
DMSO 2 M, EG 0,5 M (10 min)
DMSO 5 M, EG 1M (2min)
DMSO 5 M, EG 1 M, PVP 4 % (2min)
(AFP 10mg/mL o AFP 20mg/mL)



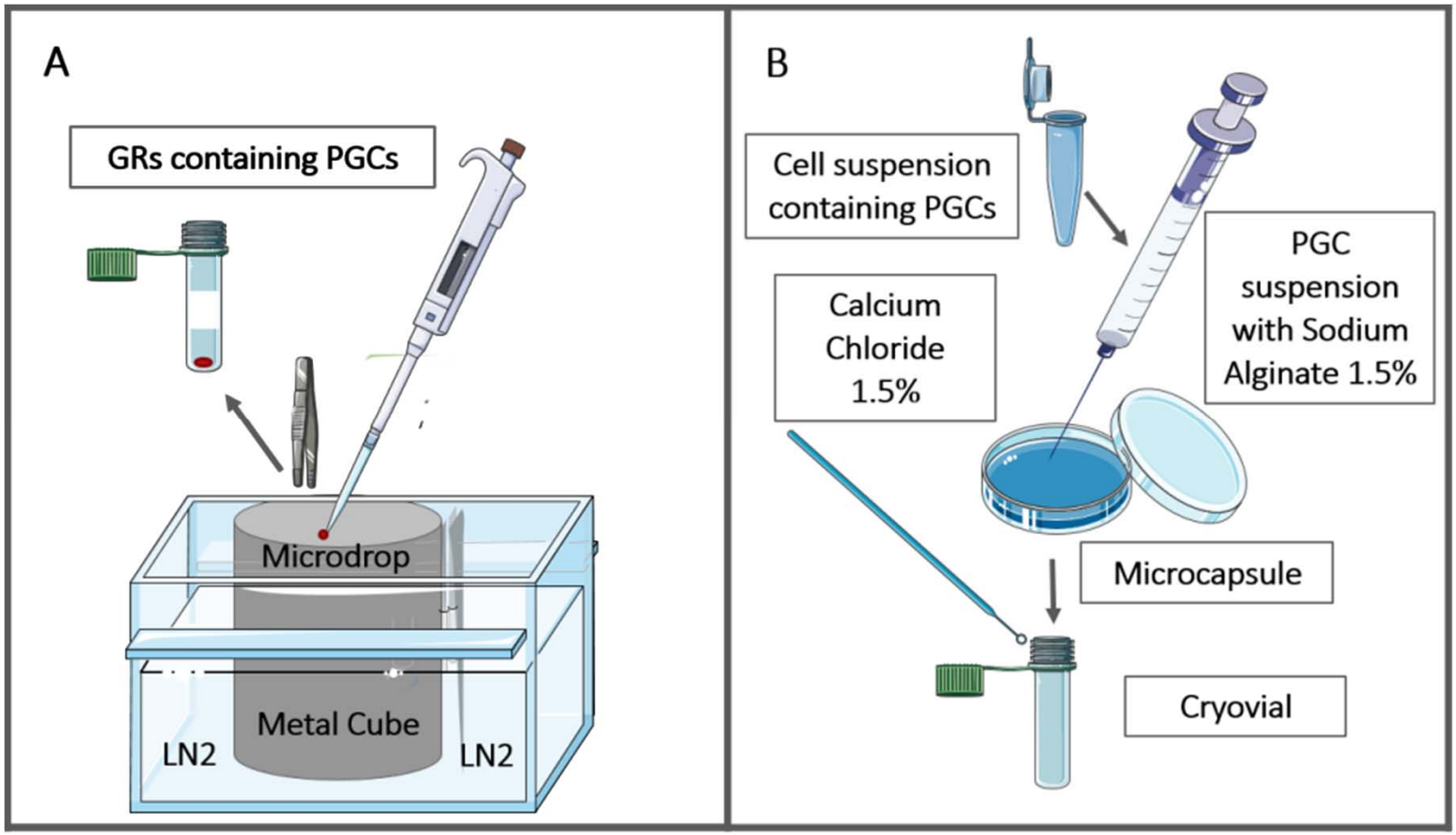








PGC cryopreservation: protocols

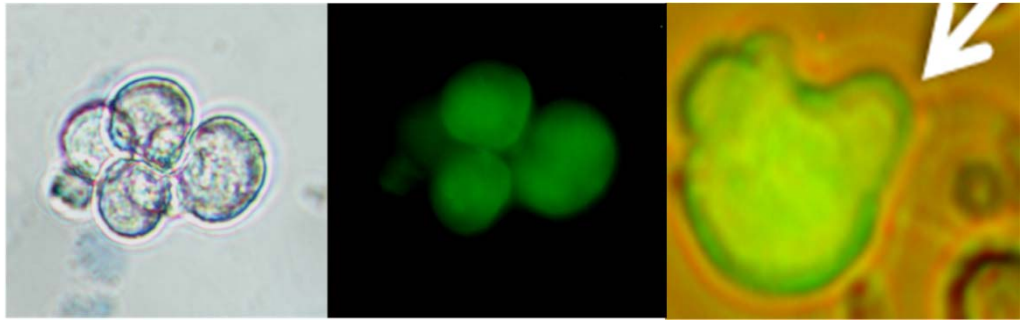


Riesco, Martínez-Pastor, Chereguini and Robles 2012. Theriogenology 77:122-130

PGC cryopreservation: evaluation after thawing

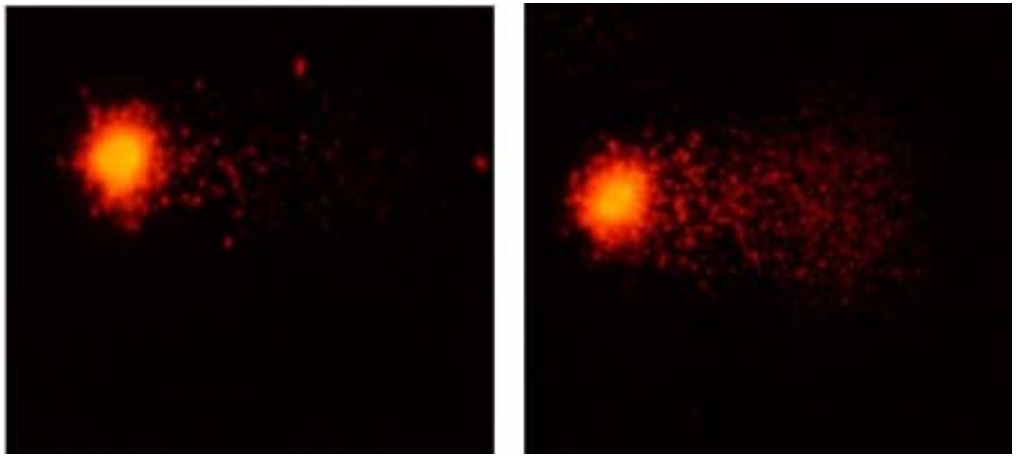
Viability
Pseudopodial emission

90% survival



DNA integrity
(Comet Assay)

‹10% (≈ control)



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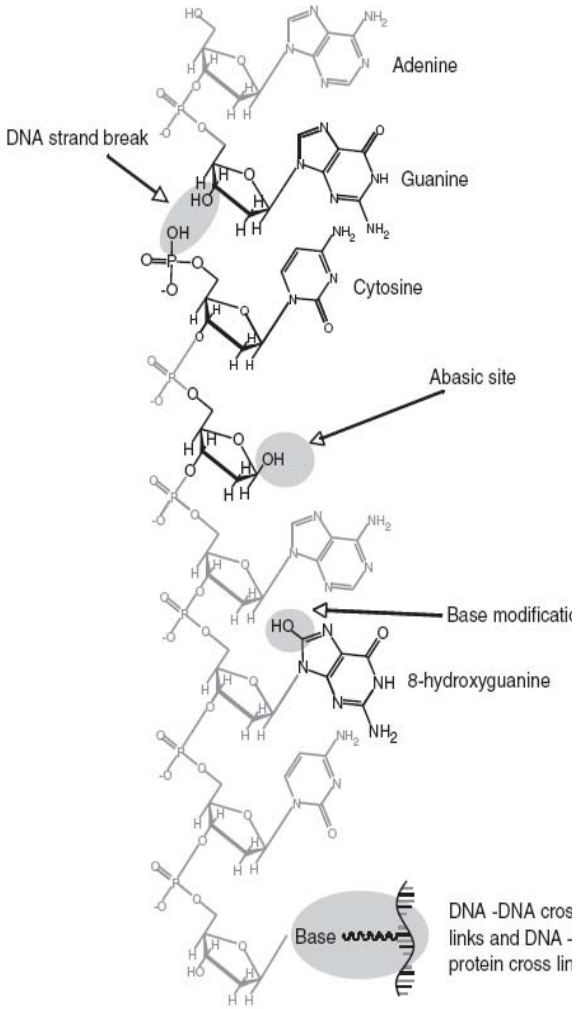
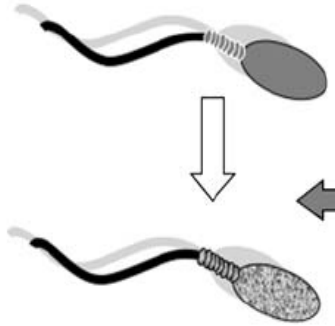
PGC cryopreservation: evaluation after thawing



Human spermatozoa are vulnerable to oxidative stress because:

- They contain high concentrations of unsaturated fatty acids
- Possess nuclear DNA that is incompletely protaminated and poorly compacted.
- Cannot effect DNA repair
- Cannot undergo apoptosis
- Possess low levels of cytosolic antioxidant enzymes
- Can generate reactive oxygen species via their mitochondria, specialized free radical generating systems (NOX5) and the redox cycling of xenobiotics
- Must spend several days as isolated cells in both the male and female reproductive tracts.

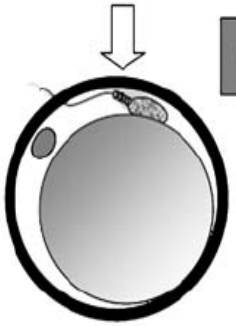
Aitken et al. 2008



DNA damage

- Excessive ROS generation by virtue of:
  - Redox cycling xenobiotics
  - Aberrant mitochondrial function
  - Excess cytoplasmic retention
  - Inadequate control of ROS generation
  - Testicular heating
  - Cryopreservation
  - Centrifugation
  - Leukocyte contamination
- Inadequate antioxidant protection
- Impaired DNA repair

Lipid peroxidation and DNA damage



Poor fertilization

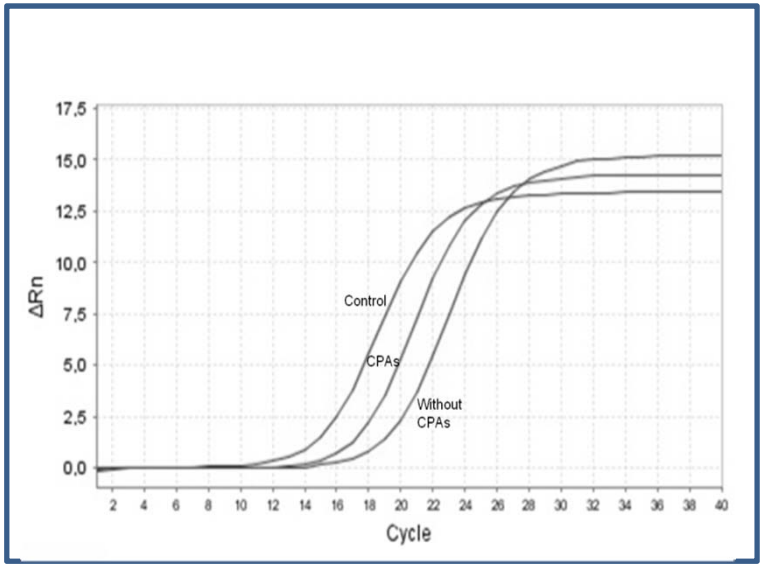
Aberrant DNA repair in the fertilized oocyte/early embryo

Impaired embryonic development | Increased abortion | Childhood disease

Lewis and Aitken 2005

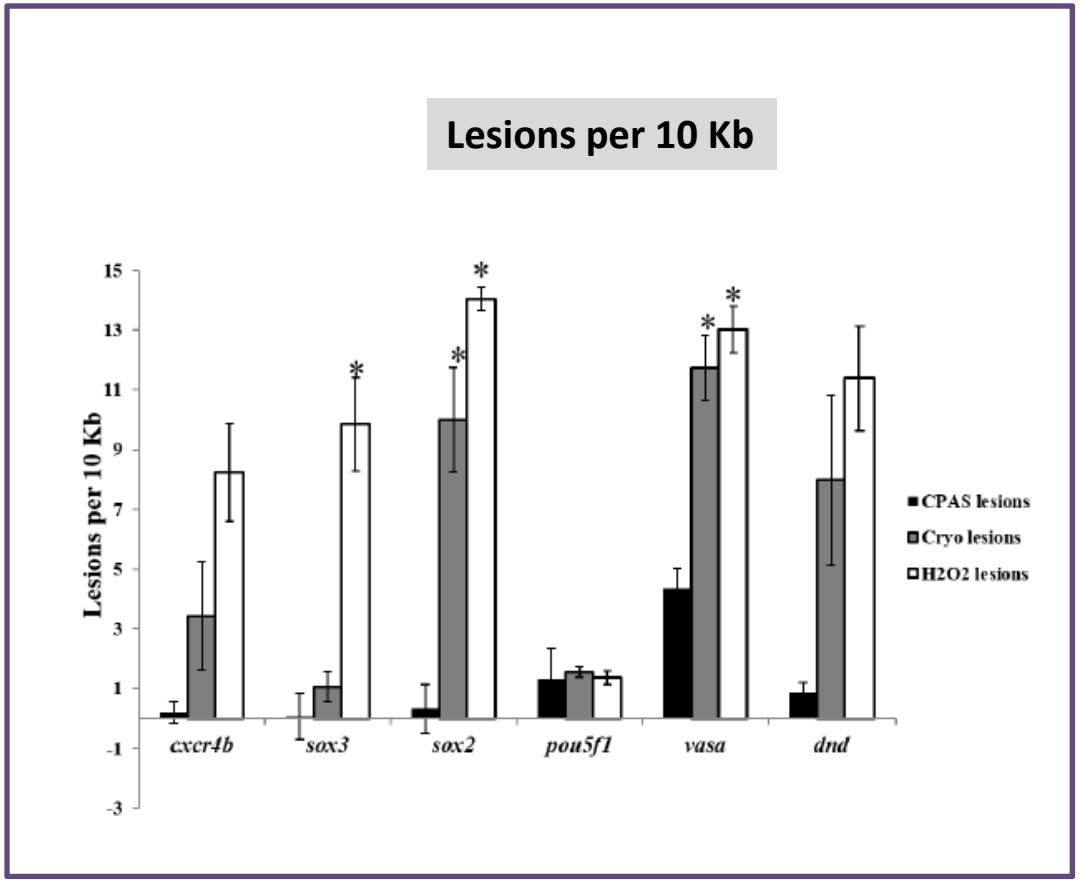
PGC cryopreservation: evaluation after thawing

Quantification of lessions in specific genes and genome regions



$$\left(1 - 2^{-(\Delta_{\text{long}} - \Delta_{\text{short}})}\right) \times \frac{10000\,[\text{bp}]}{\text{size of long fragment [bp]}}$$

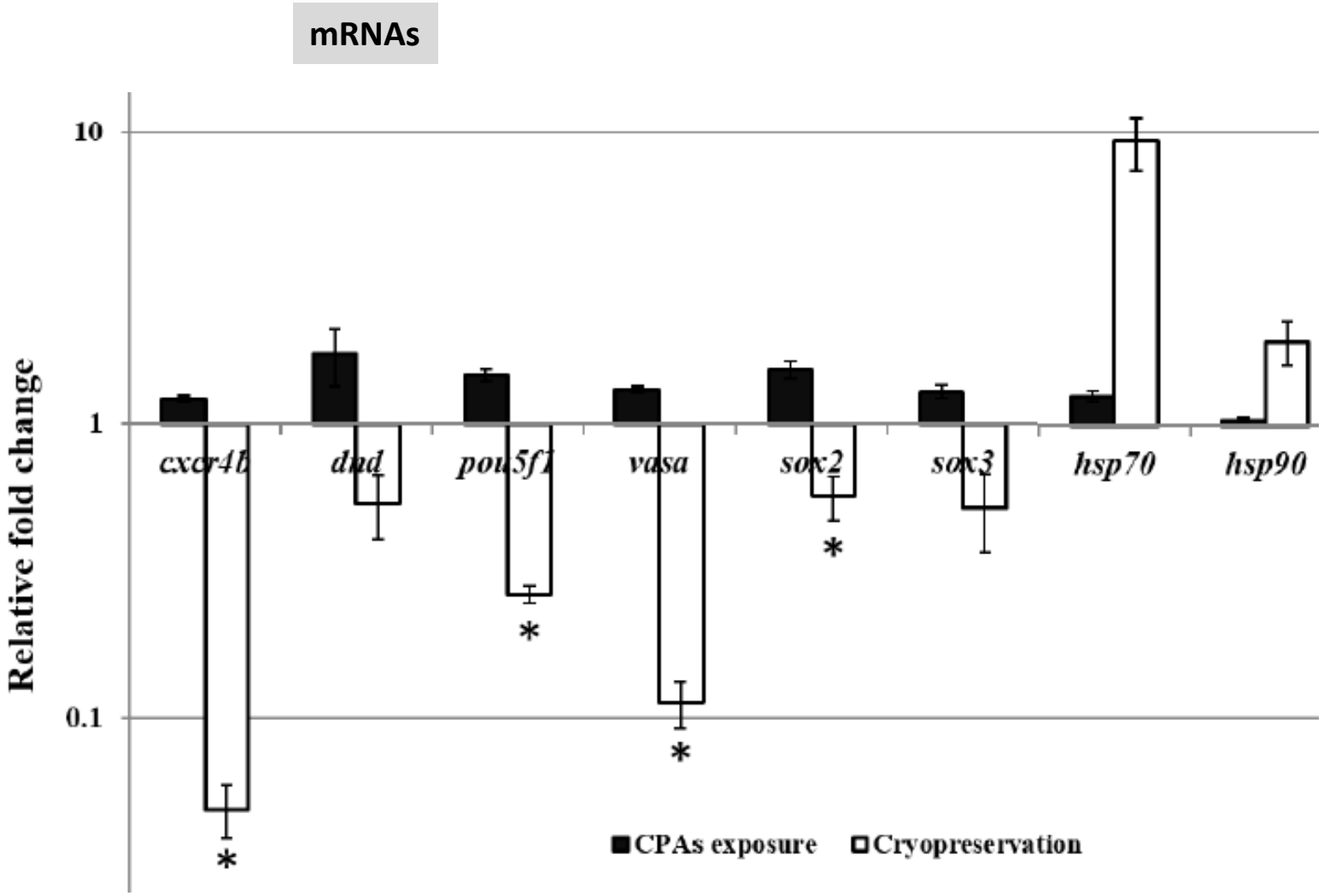
Rothfuss et al. Nucleic Acids Research, 2010



Riesco and Robles 2012 J. Appl. Ichthyol. 28 (2012), 925–929

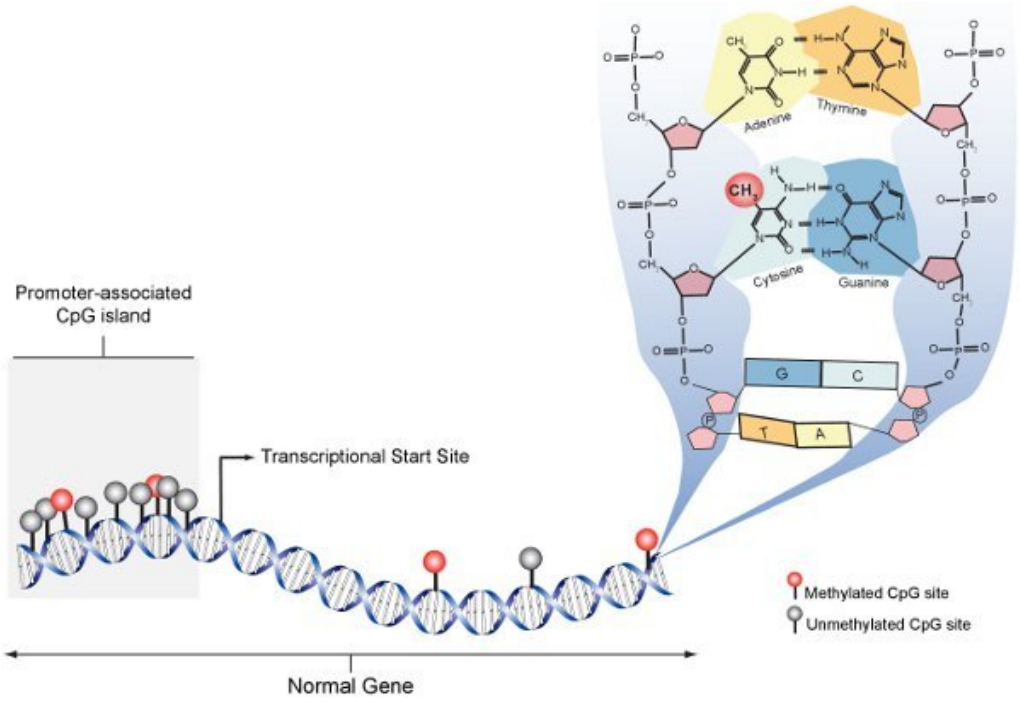
Riesco and Robles 2012 PLOS ONE (2013)

PGC cryopreservation: evaluation after thawing

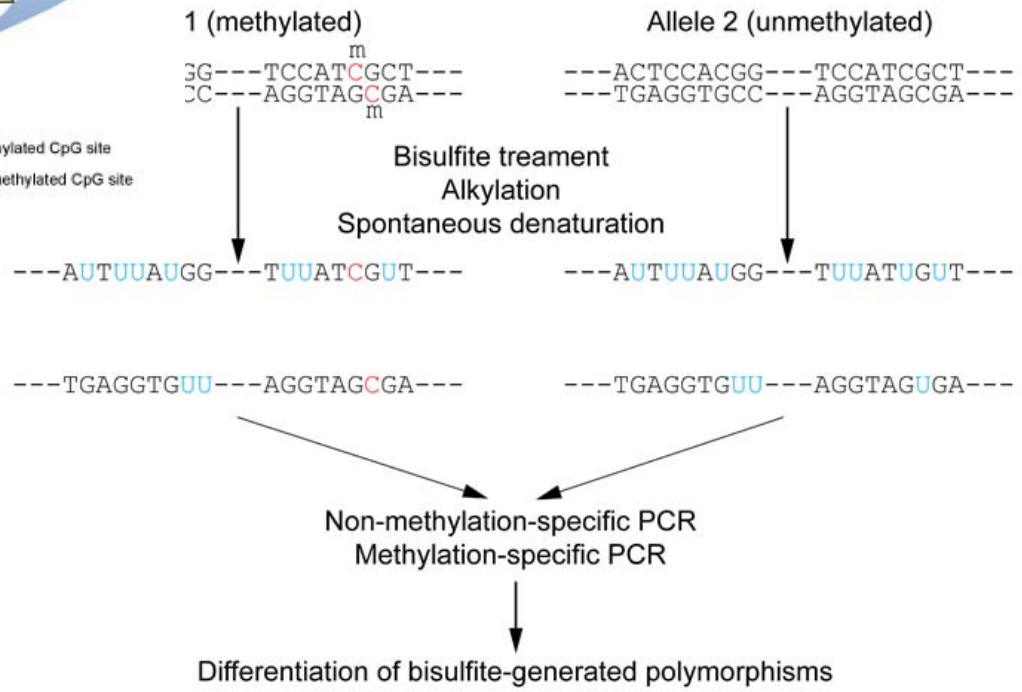


Riesco and Robles PLOS ONE (2013)

PGC cryopreservation: evaluation after thawing



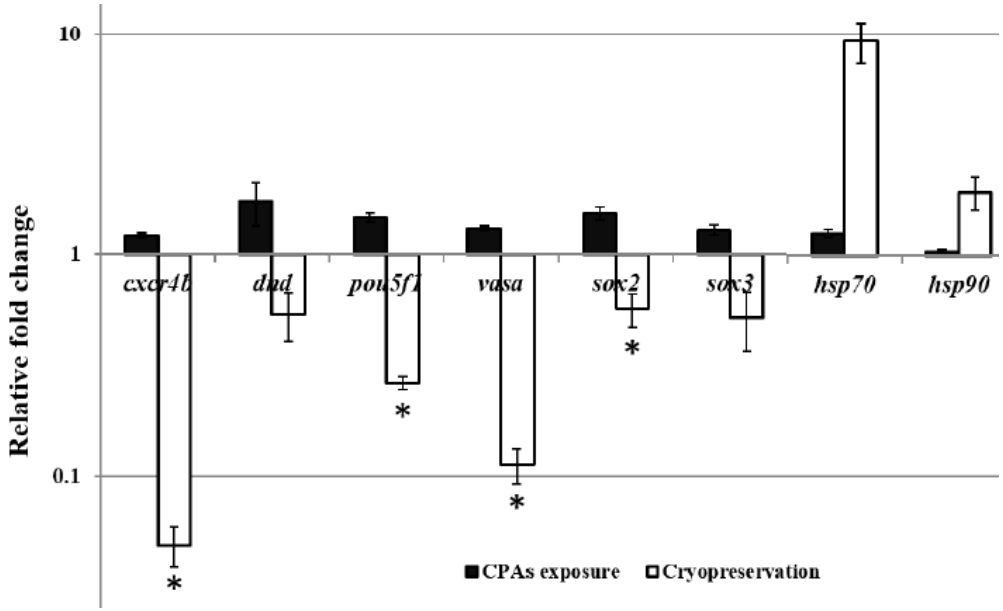
Promoter methylation

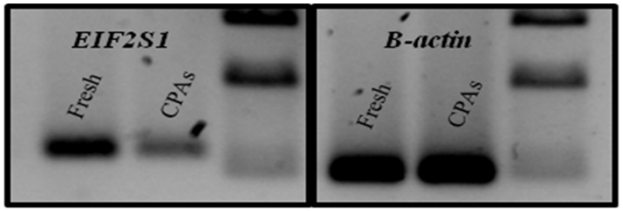


Patterson et al. J Vis Exp. (2011)

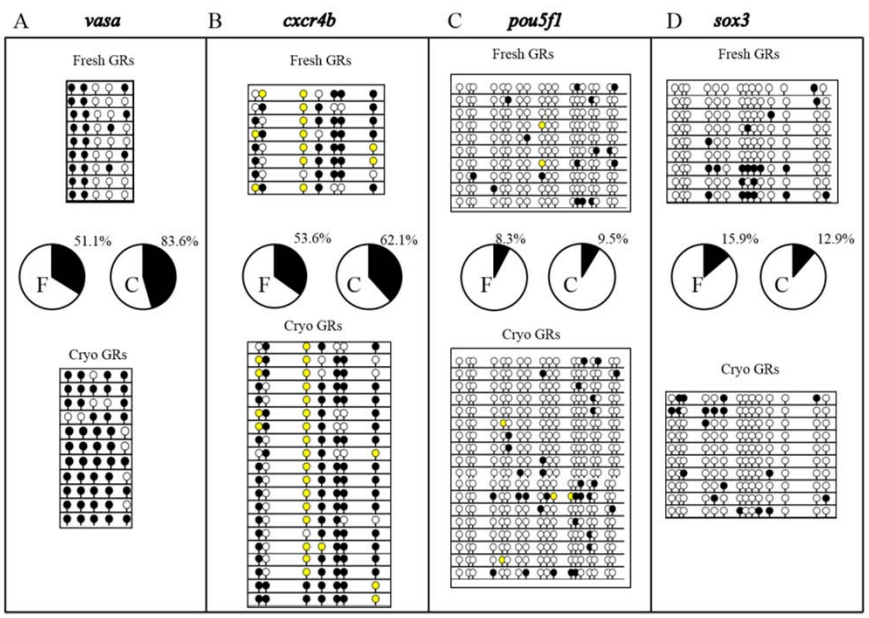
PGC cryopreservation: evaluation after thawing

mRNAs



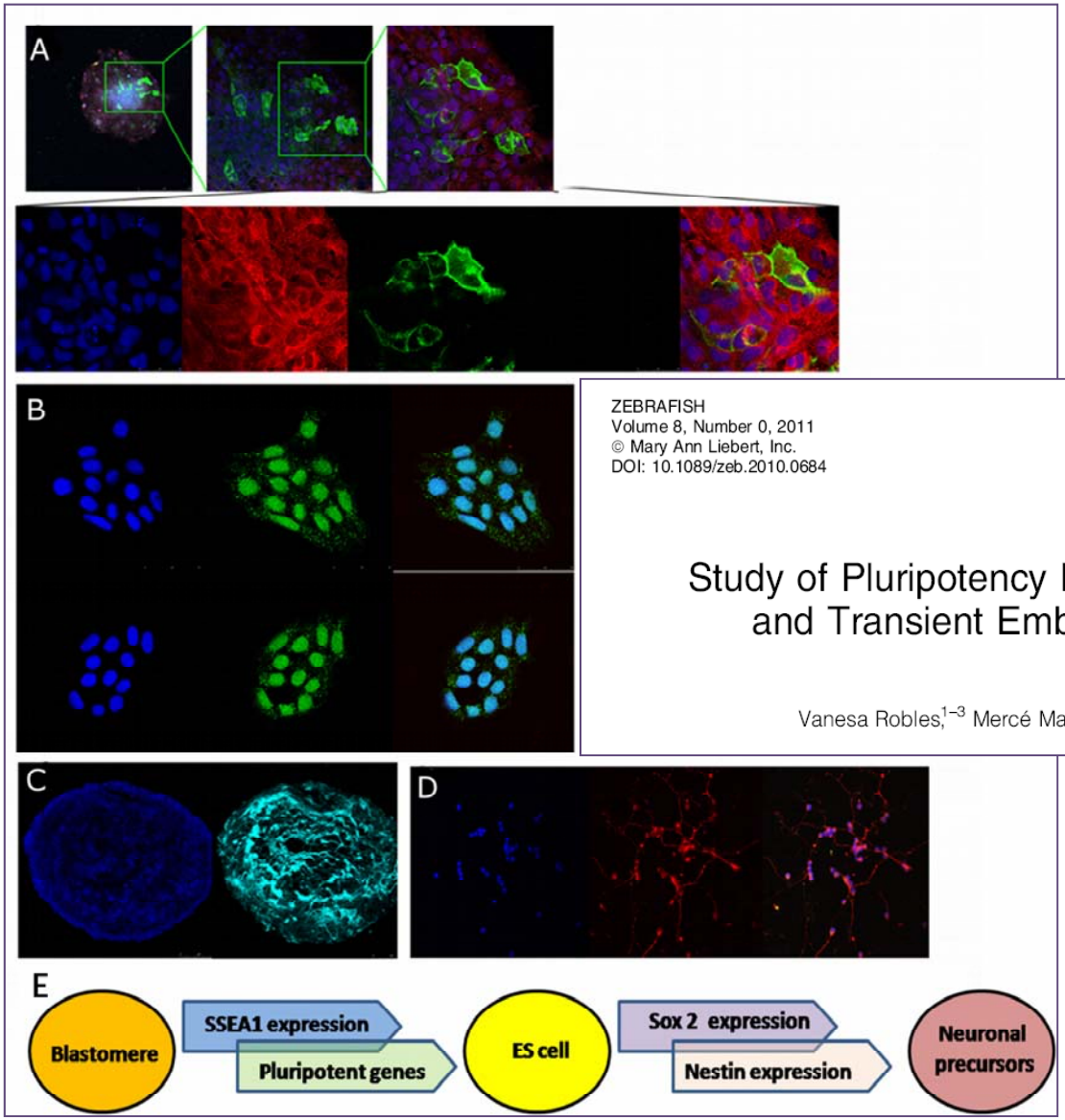


Promoter methylation



Riesco and Robles 2012 PLOS ONE (2013)

Limitations and future perspectives: PGC *in vitro* generation



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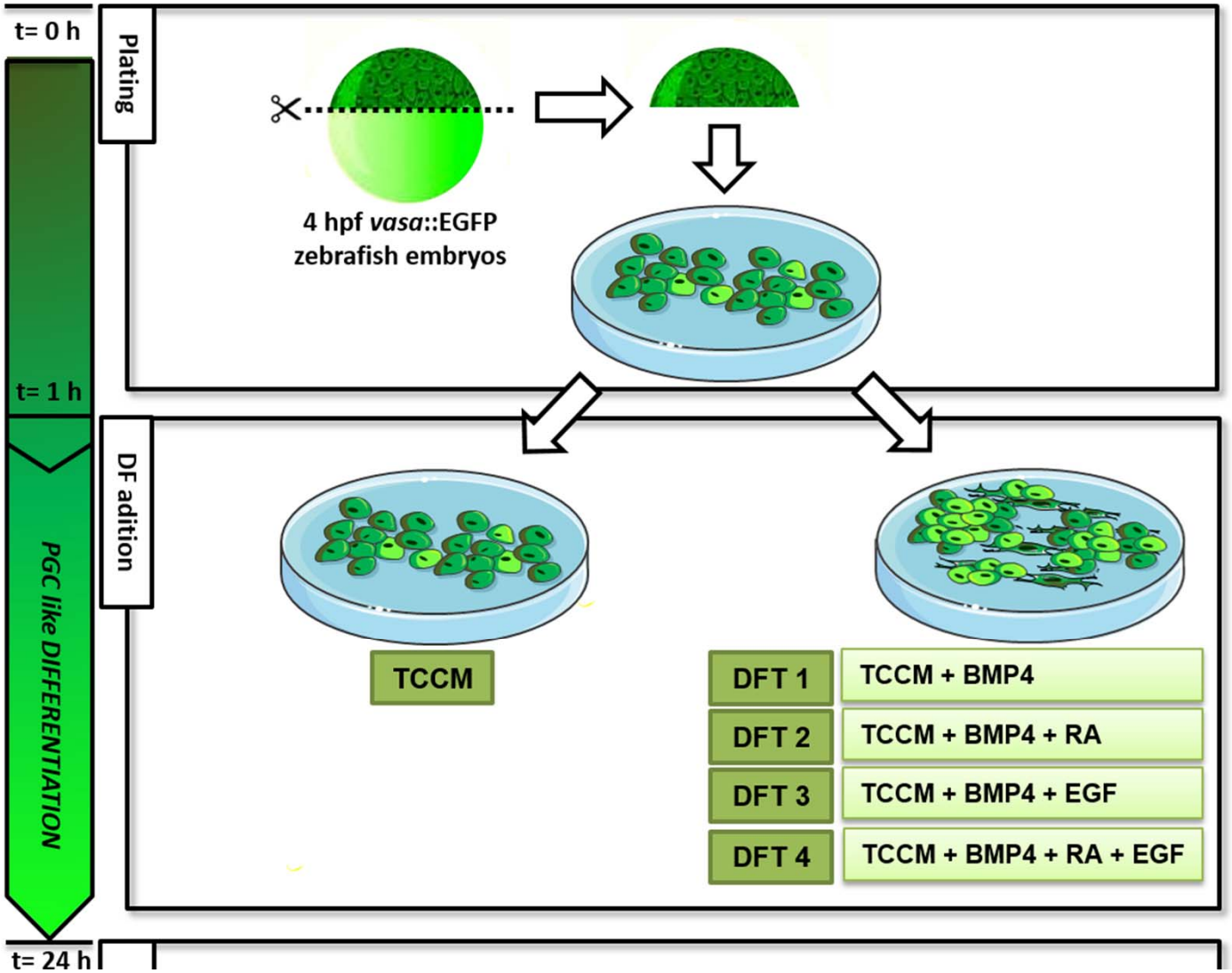
Original Article

Study of Pluripotency Markers in Zebrafish Embryos and Transient Embryonic Stem Cell Cultures

Vanesa Robles,[1–3] Mercé Martí,[1] and Juan Carlos Izpisua Belmonte[1,4,5]

Limitations and future perspectives: PGC *in vitro* generation



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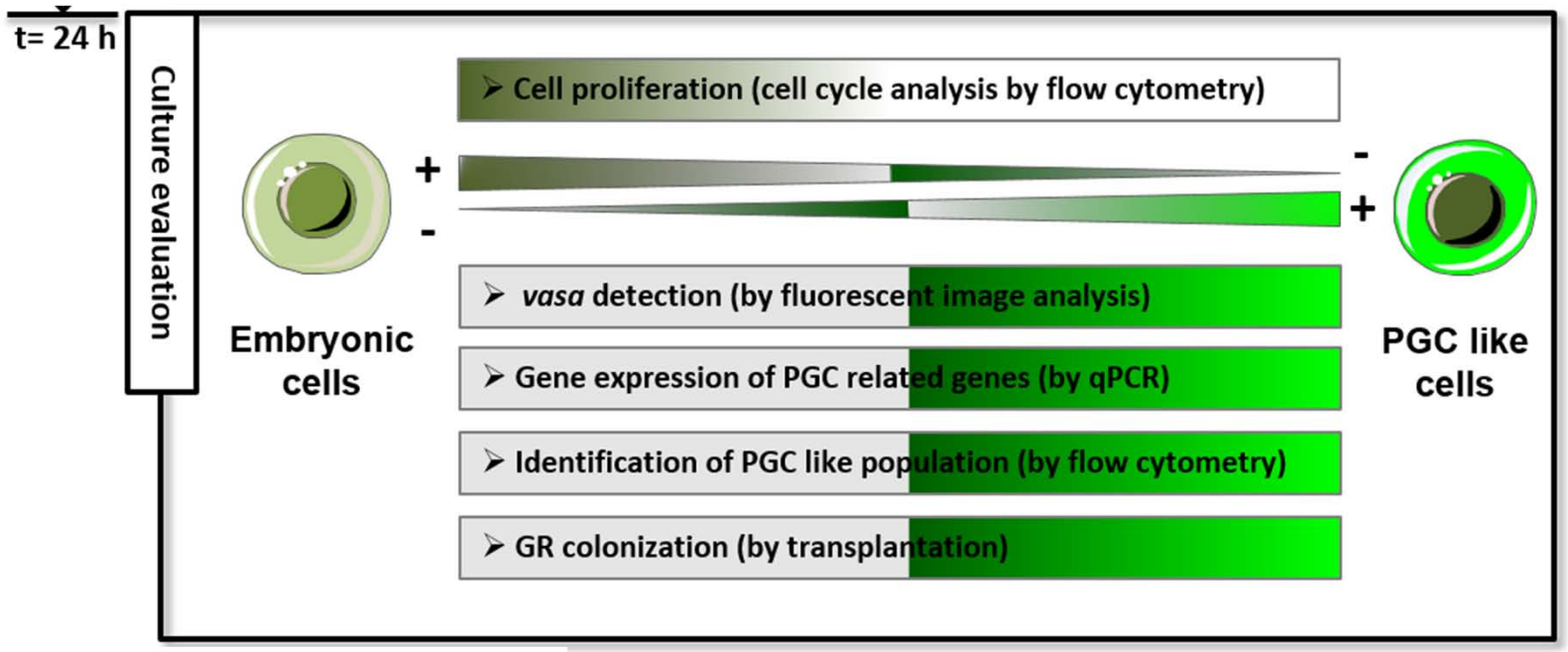

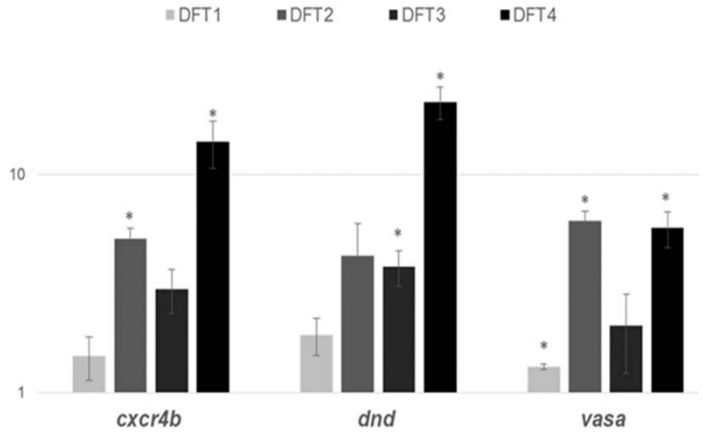

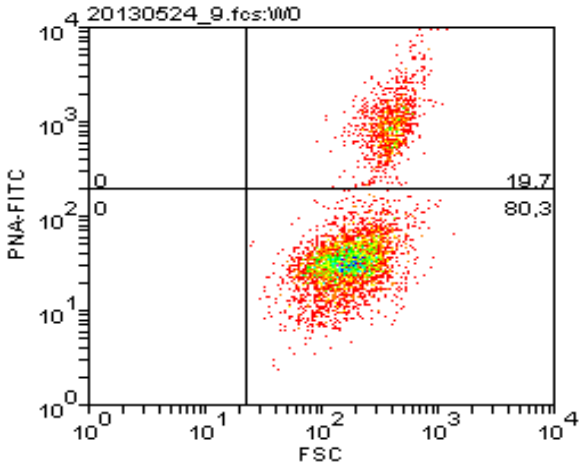

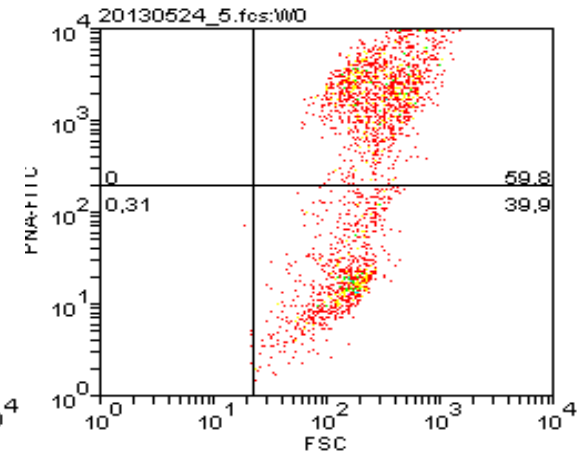

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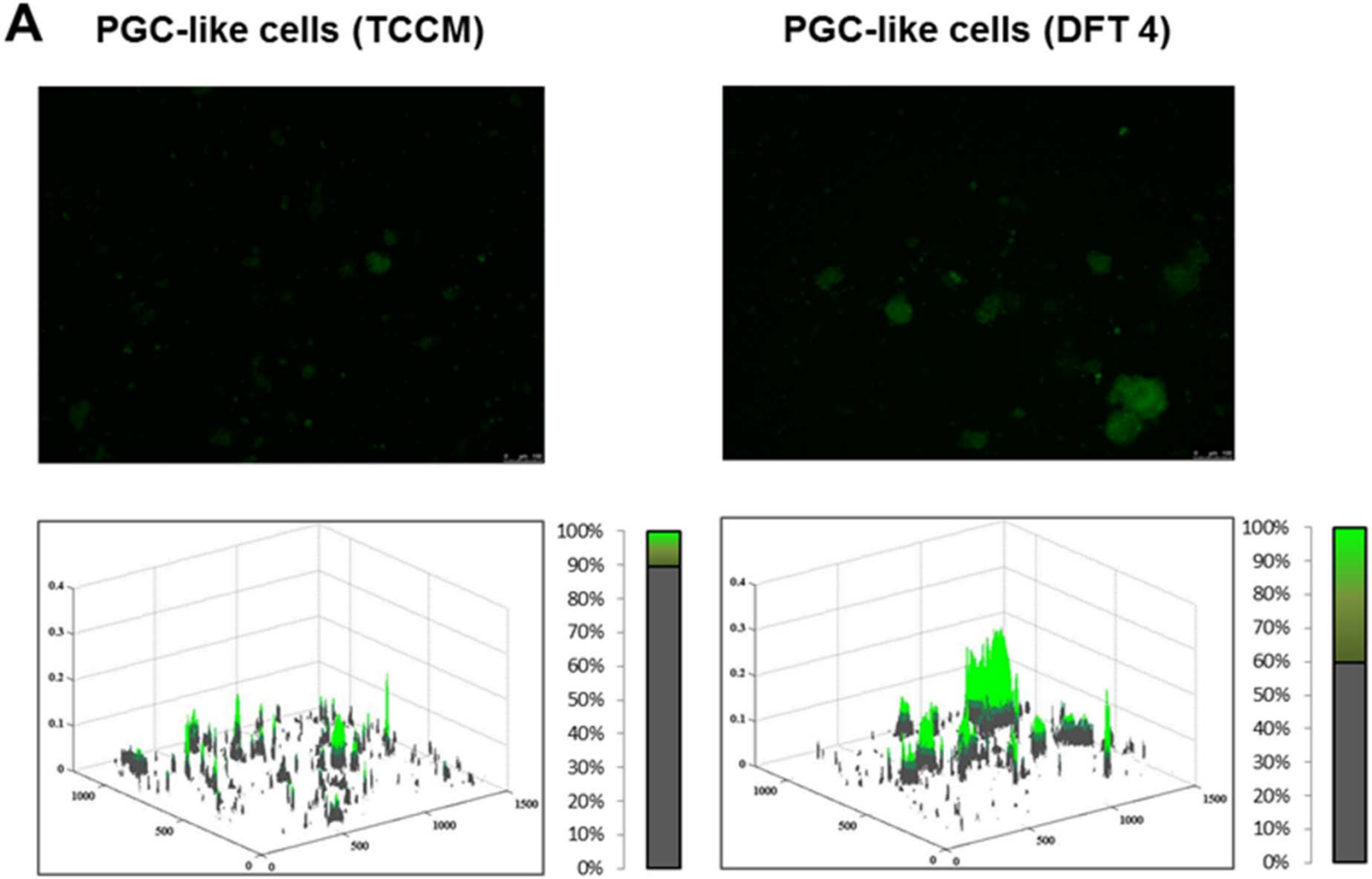

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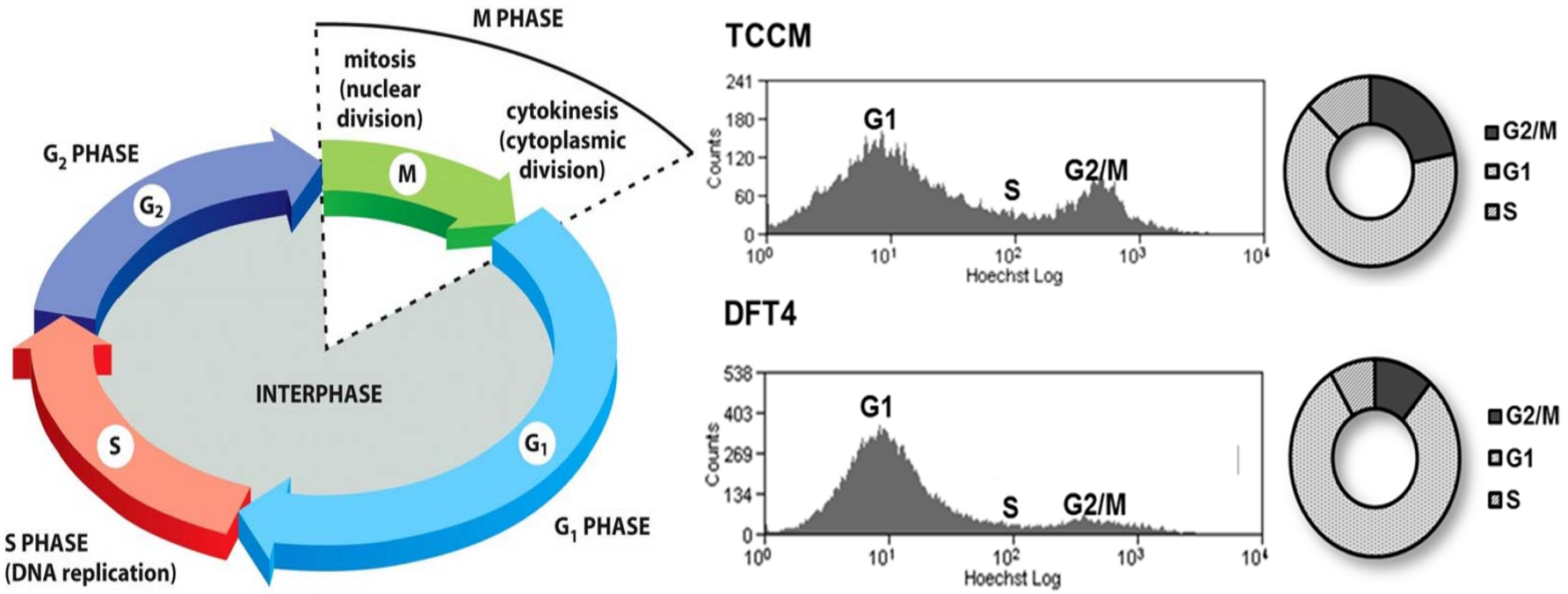

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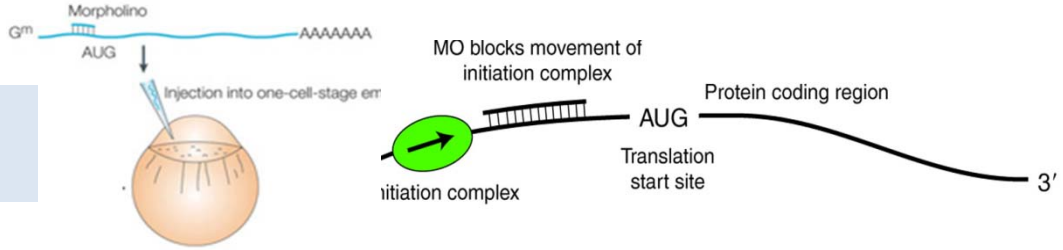
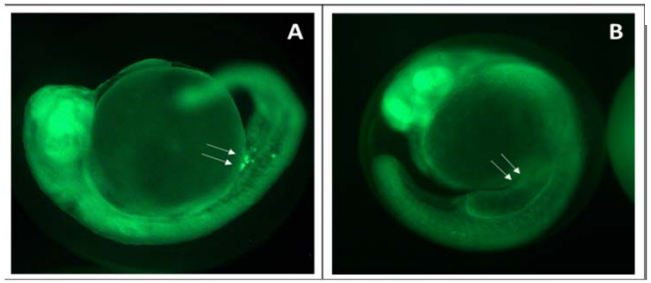
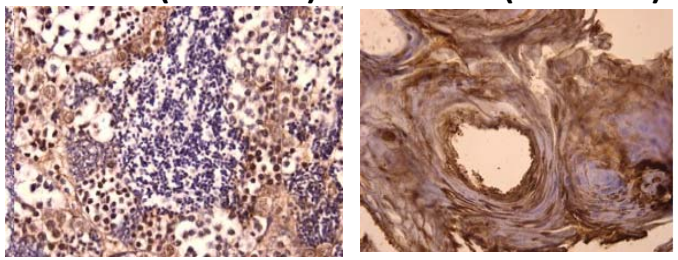
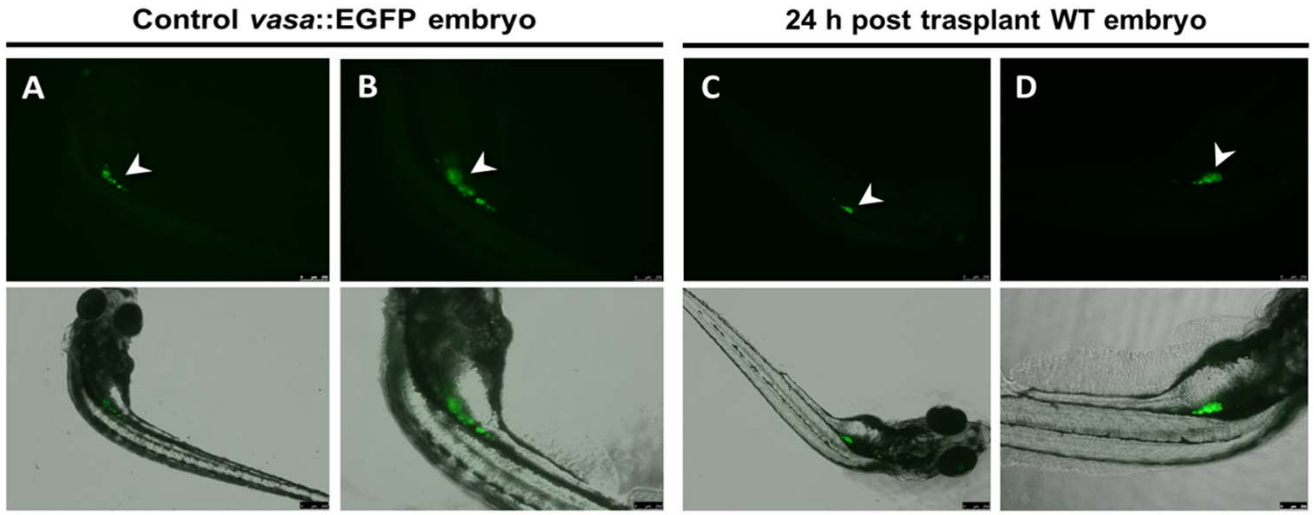
Germline contribution









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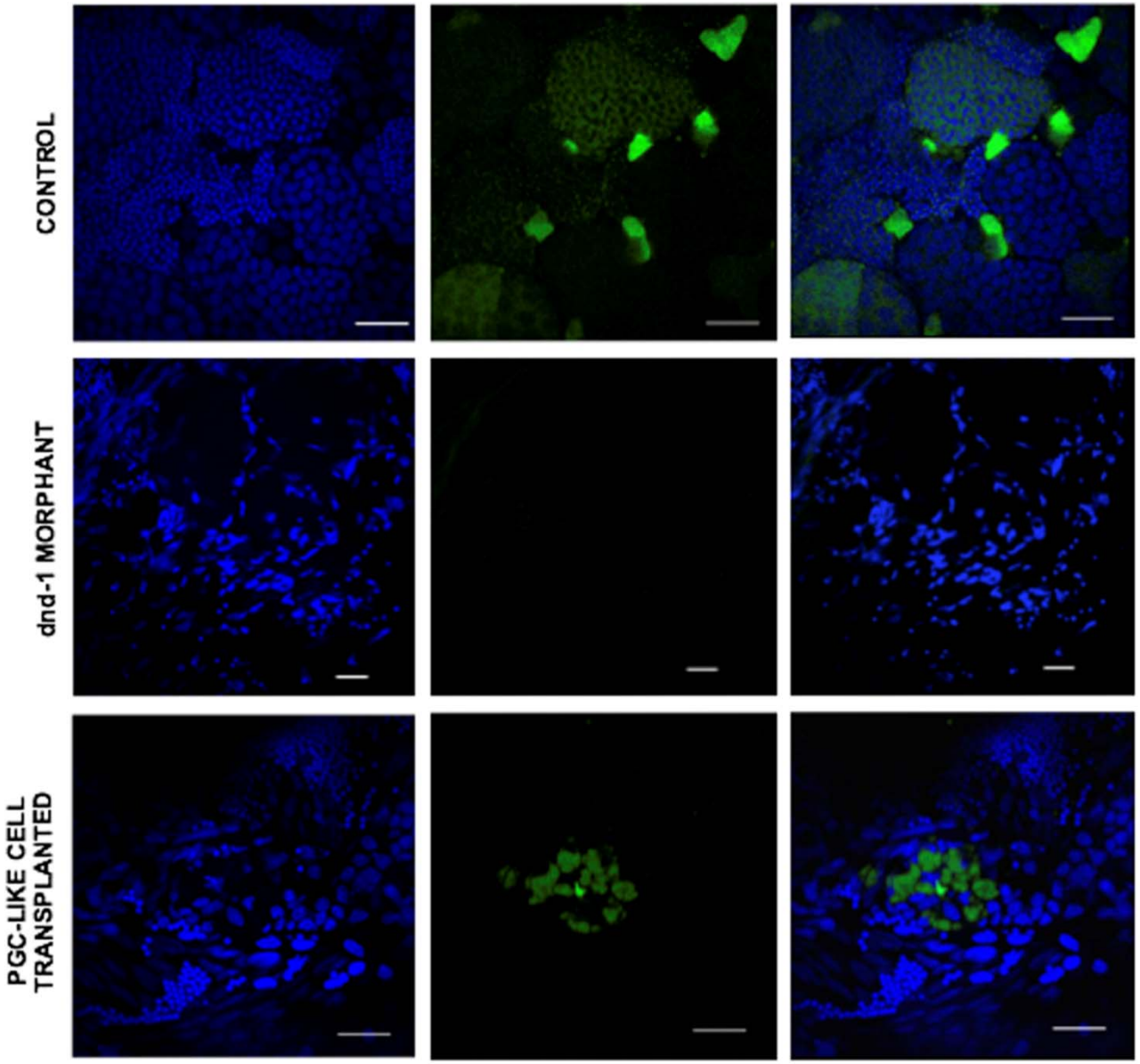

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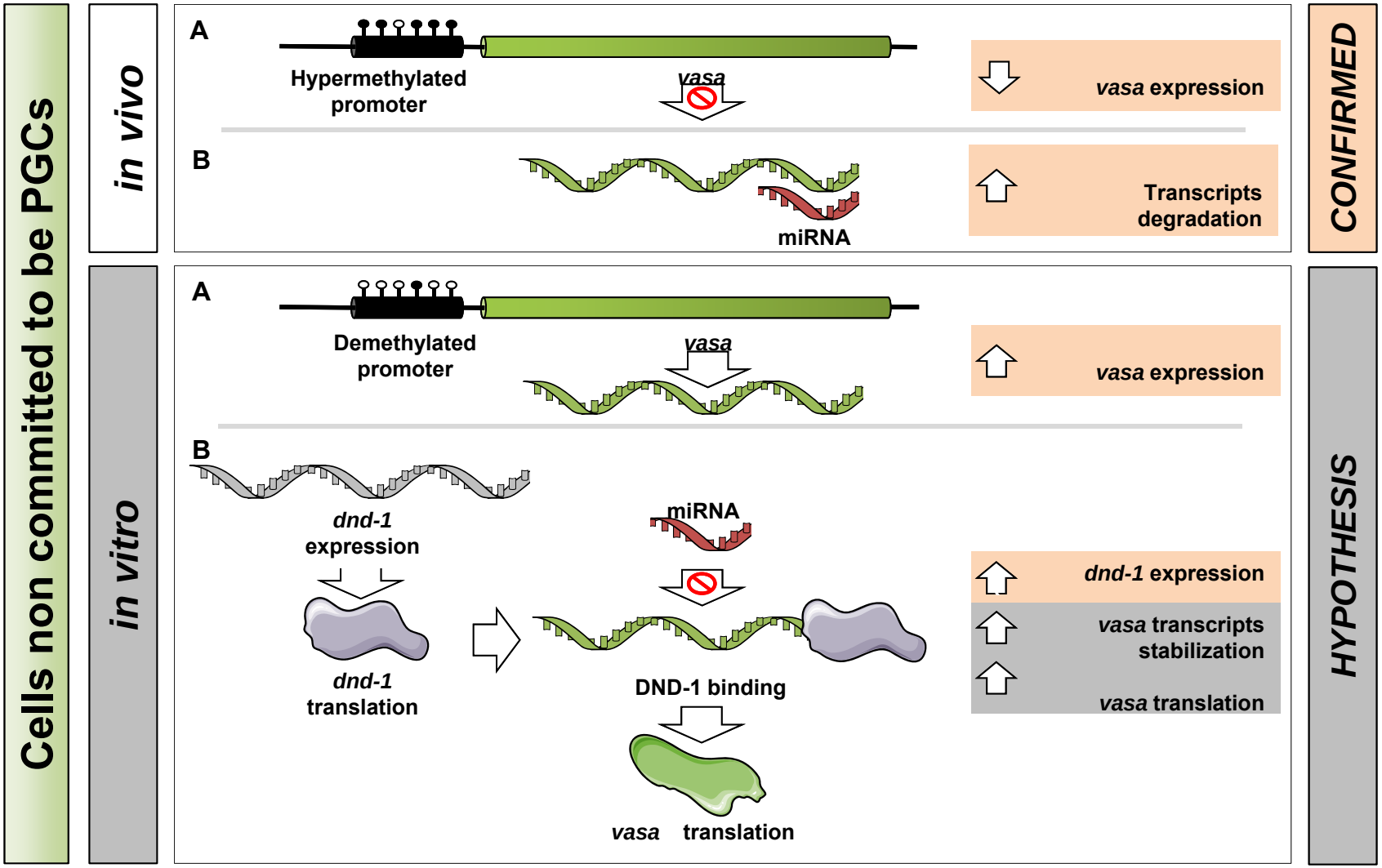

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Conclusions

PGCs can be successfully cryopreserved and successfully transplanted into host sterile embryos

Molecular analysis after cryopreservation is crucial to guarantee the success of a cryopreservation protocol and avoid undesirable effects in fertilization and early embryo development.

PGCs can be generated *in vitro* from embryonic cells

Thank you!

cost
EUROPEAN COOPERATION
IN SCIENCE AND TECHNOLOGY

AQUAGAMETE

FUNDACION RAMON ARECES

GOBIERNO DE ESPAÑA
MINISTERIO DE CIENCIA E INNOVACIÓN

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