HKUST TRANSGENIC SERVICES

JOANNE TAM

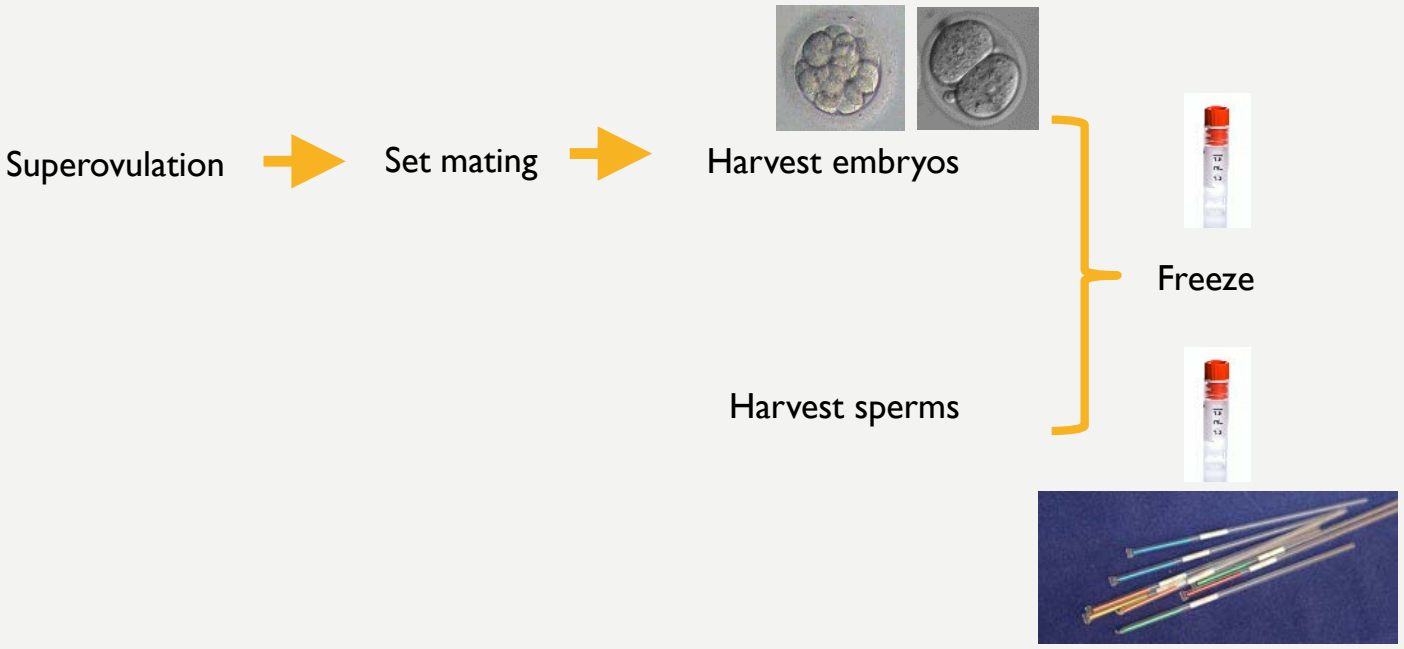
CONTENT

- Introducing service types
  - Mousse embryo cryopreservation
  - Mouse sperm cryopreservation
  - Mouse line rederivation
  - In vitro fertilization
  - Mouse zygote microinjection
- Logistics between different zones (Cryopreservation and Line Rederivation)
- Fabrication of tools
- Quality control steps

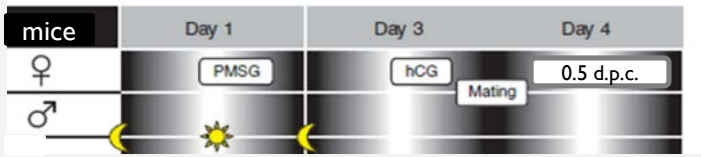
THE SERVICES

TWO APPROACHES OF CRYOPRESERVATION

- Free housing space for mouse lines not in use
- Keep backup for precious lines



CRYOPRESERVATION – EMBRYOS COLLECTION



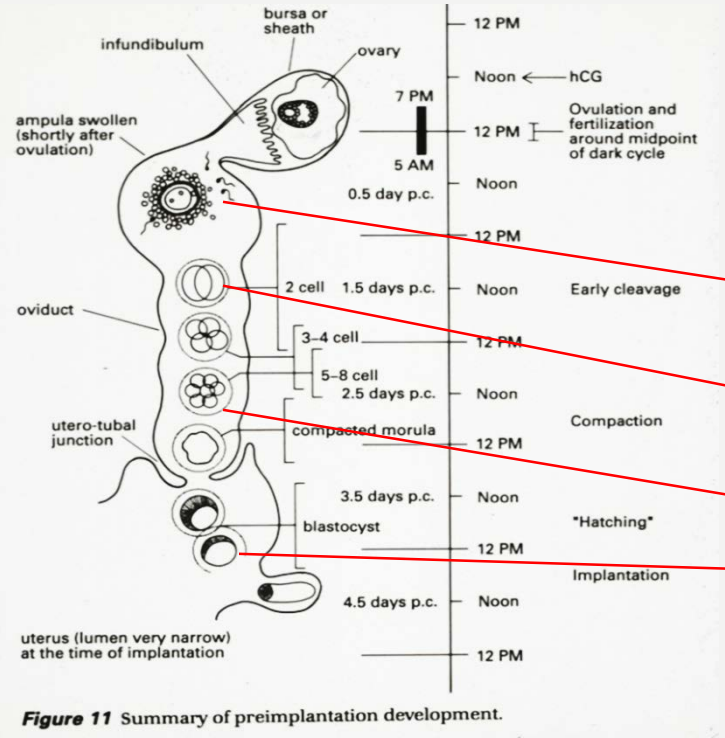


Figure 11 Summary of preimplantation development.

Days after fertilization and stage	Collection from:
0.5 dpc zygote/oocytes	Breaking Ampullae
1.5 dpc 2-cell	Flushing oviduct
2.5 dpc morula	Flushing oviduct & utero-tubal junction
3.5 dpc blastocyst	Flushing uterus

SLOW FREEZING AND VITRIFICATION

Conventional	Vitrification
Embryos dehydrated gradually by lowering the extracellular osmolarity by cryoprotectants. When water leaves the cells→ concentration intracellular solute increases → lower freezing point **Equillibration→ extracellar ice forms→ further decreases the extracellular osmolarity** **High salt concentration in the cell →toxic but safe to cells due to low termperature.** **Need careful control of cooling rate.**	High concentration of cyoprotectants increases viscosity of intracellular water. At high cooling rate, cell content solidifies before water molecules rearrange to form destructive ice crystal structure. The mechanical damages caused by ice crystals are minimized → high survival rate. Key to successful vitrification is to achieve a high cooling rate and a high but tolerable subtoxic concentration of cryoprotactants. (Tsang and Chow, 2010)

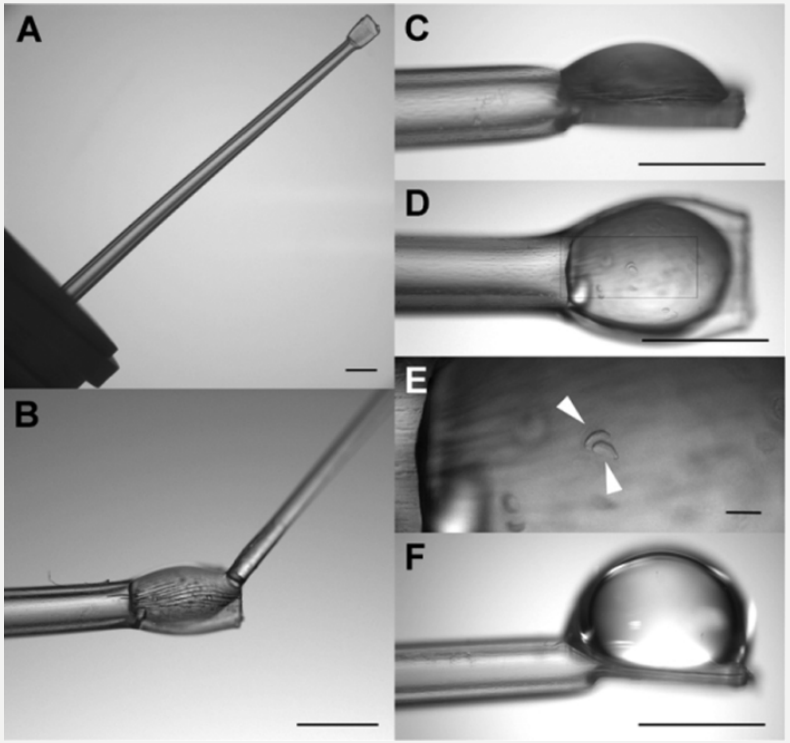
Slow freezing:	Vitrification:
• Complicate procedures • Relatively low efficacy • Requirement of a programmable controlled system	• Simple procedures • High viability of recovered embryos. • Cost effective in setup

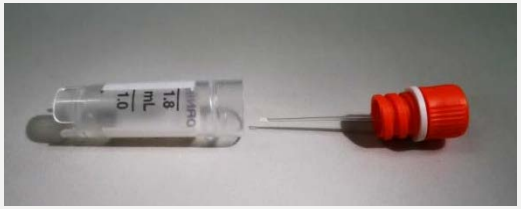
CRYOPROTENTANT (CRYOPROTECTIVE AGENT, CPA)

- Increase the total concentration of all solutes in the system
- Reduce the amount of ice formed at any given temperature
- Biologically acceptable, able to penetrate into the cells and low toxicity.
- Penetrable and non-penetrable cryoprotectant

Type of CPAs	Examples
Permeating	Glycerol
	Ethylene Glycol
	Propylene Glycol
	Formamide
	Propenediol
	DMSO
	Adonitol
Non-Permeating	Lactose
	Raffinose
	Sucrose
	Trehalose
	D-mannitol

CRYOPRESERVATION – VITRIFICATION spectula



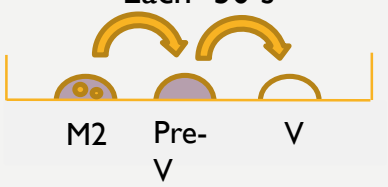


- Tsang and Chow 2009

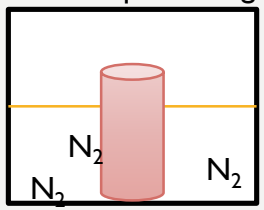
VITRIFICATION USING SPATULA-EQUIPPED CRYOTUBE

Embryos from M16 medium

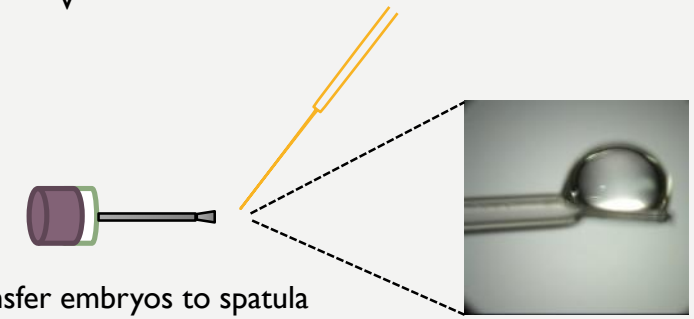
Each 30 s



Pre-chill cryotube in liquid nitrogen without cap

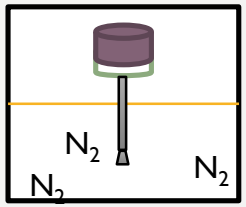


Pre-vitrification buffer
Vitrification buffer

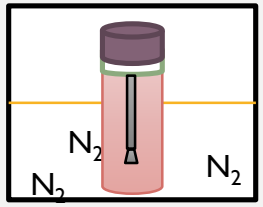


Transfer embryos to spatula
Under stereo microscope

Embryos in droplet

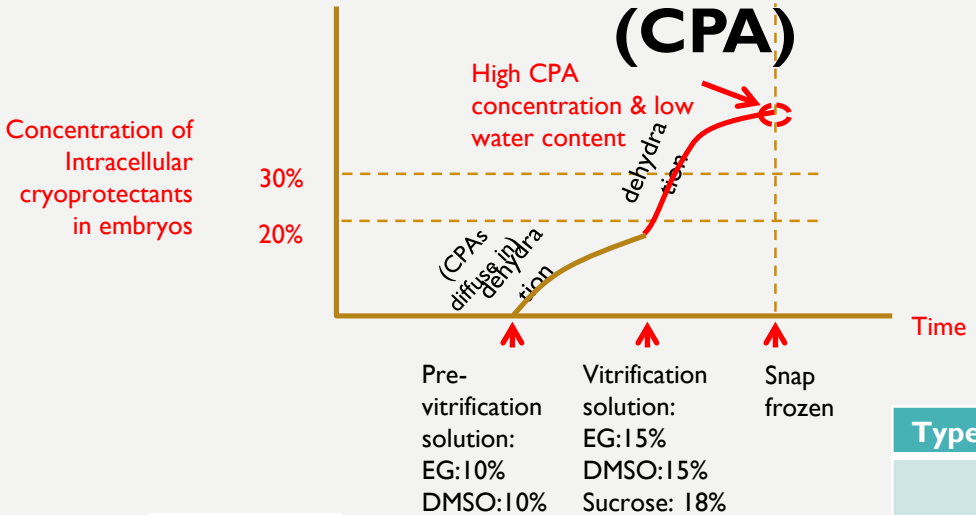


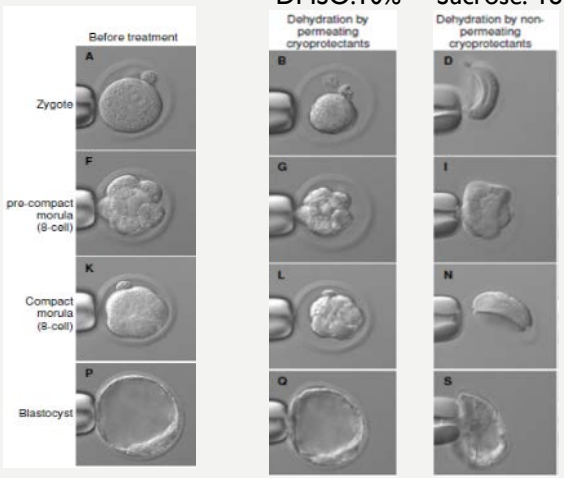
Quickly chill spatula with embryos in liquid nitrogen



Recap cryotube and keep in liquid nitrogen storage

Dynamic Changes of intracellular cryoprotecting agents (CPA)



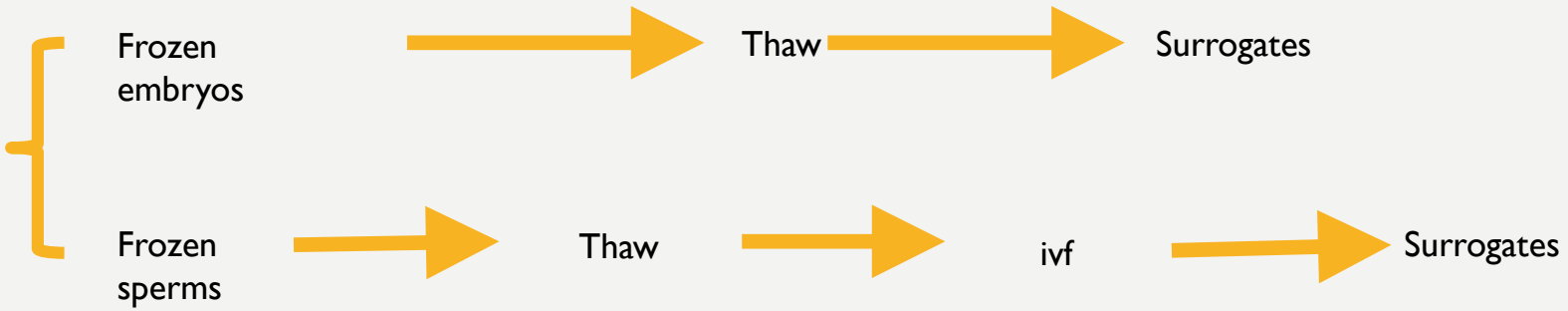


Type of CPAs	Examples
Permeating	Glycerol
	Ethylene Glycol
	Propylene Glycol
	Formamide
	Propenediol
	DMSO
	Adonitol
Non-Permeating	Lactose
	Raffinose
	Sucrose
	Trehalose
	D-mannitol

KEY TO SUCCESSFUL VITRIFICATION

1. Quick in every step
2. Pipette smaller volume
3. Change transfer pipette for each buffer
4. Refill new transfer pipette with buffer for the next incubation step
5. All these steps make sure to reduce water content inside the embryo as much as possible

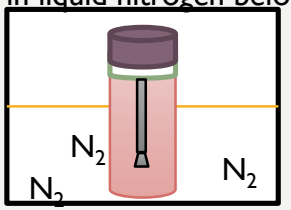
REVIVAL

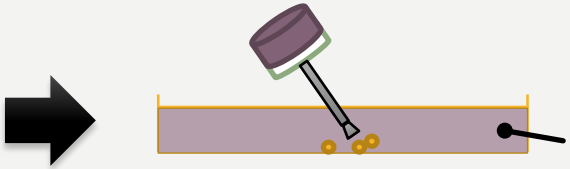

Frozen embryos
Thaw
Surrogates
Frozen sperms
Thaw
ivf
Surrogates

THAWING VITRIFIED EMBYROS FROM CRYOTUBE WITH SPATULA

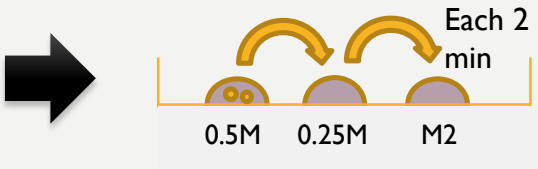
Keep in liquid nitrogen before thawing

Cryotube from
liquid nitrogen storage

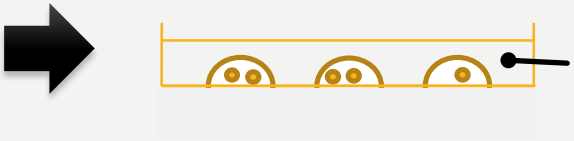




0.5M sucrose/M2 medium, 2 min



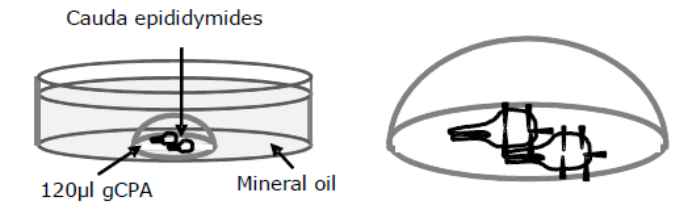
0.5M sucrose/M2 medium
0.25M sucrose/M2 medium

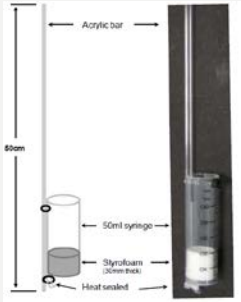


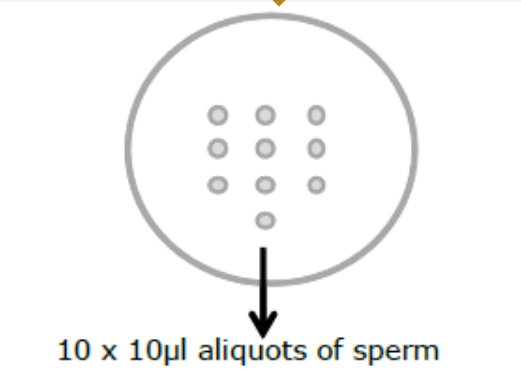
KSOM drop culture covered with light mineral oil.
Keep in 37°C CO_2 incubator.

CRYOPRESERVATION - SPERMS

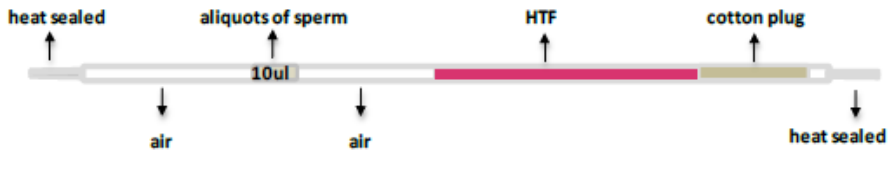
Harvest sperms



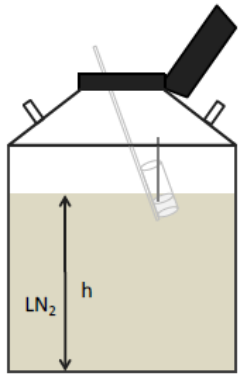




Position of sperm in straw



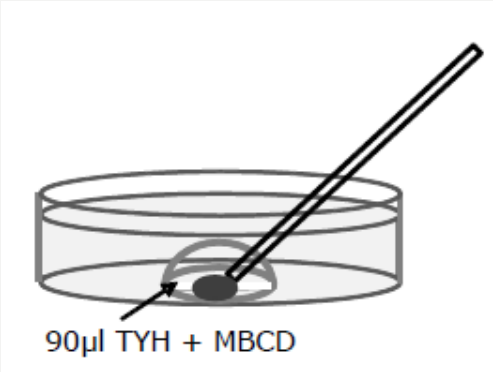
Freezing



Storage

REVIVAL - SPERMS

sperm revival



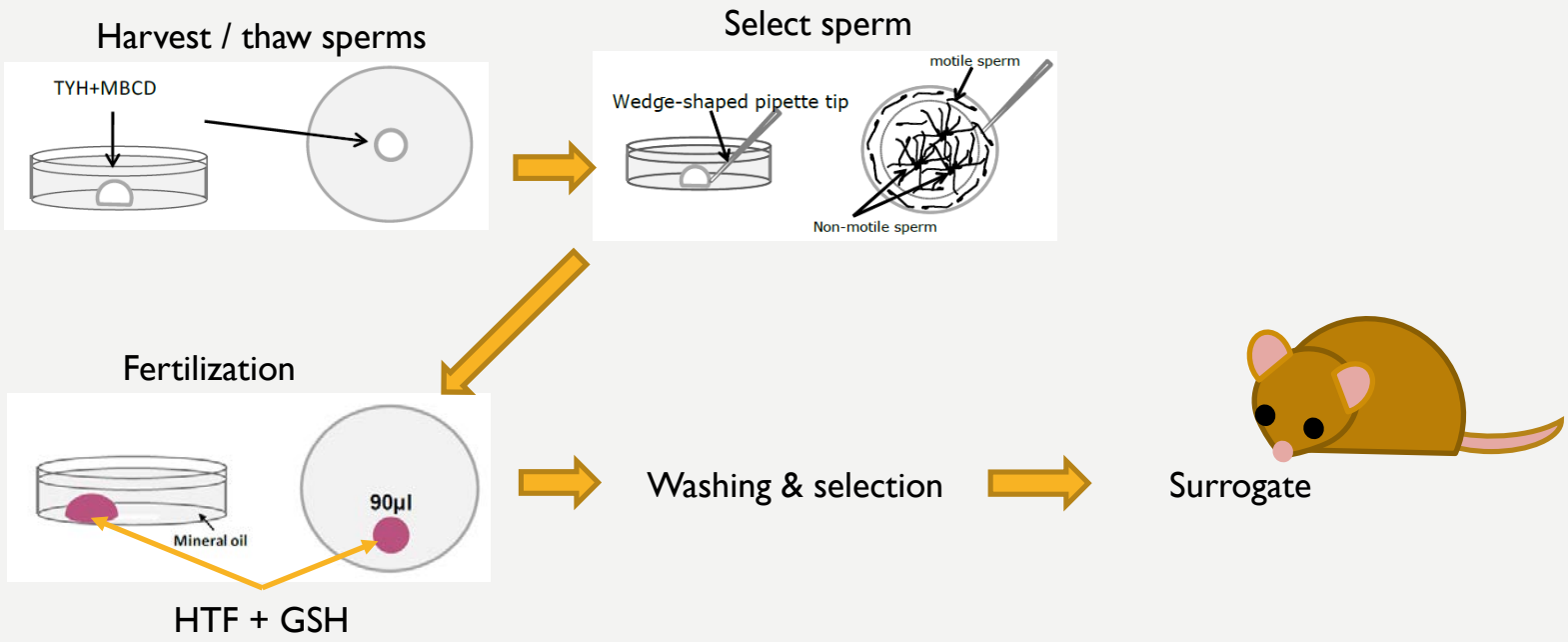
in vitro fertilization

IN VITRO FERTILIZATION

- To revive frozen stock
- To import mouse strains from consortiums
- To sustain some mutant lines (e.g. aging or disabilities in mating…etc)

IN VITRO FERTILIZATION

- Day 1: Injection of PMSG
- Day 3: Injection of Chorullon
- Day 4: Sacrifice male/thaw sperms and perform ivf
- Day 5: Select fertilized embryos for transferring into surrogates

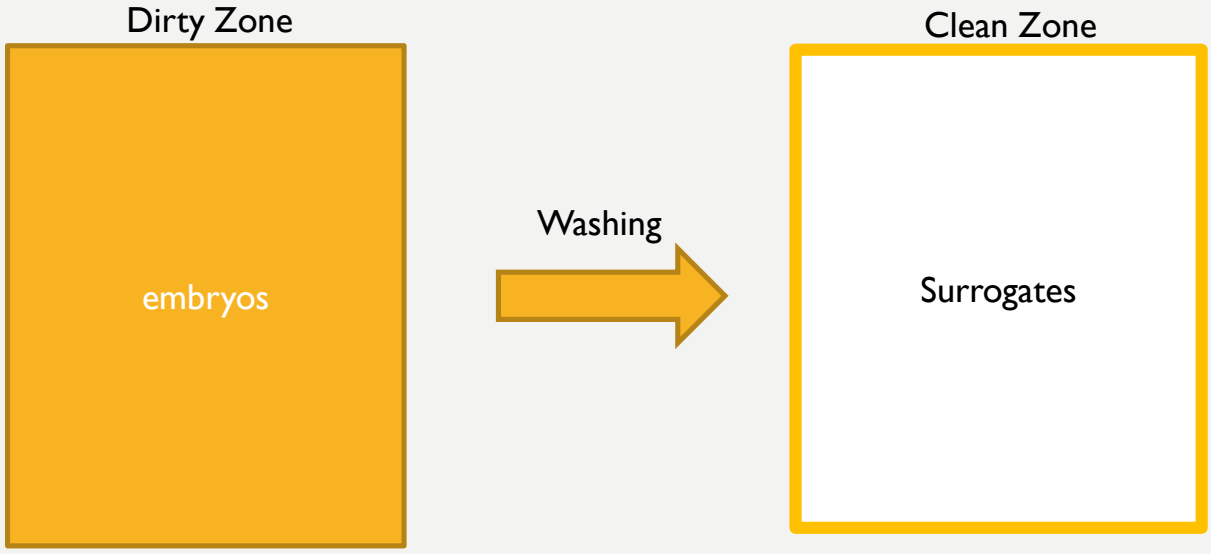


MBCD = methyl-β-cyclodextrin

GSH = reduced glutathione

LINE REDERIVATION

- To transfer mice from dirty zone to clean zone without passing the infectants



CONTRIBUTIONS OF TRANSGENIC SERVICE

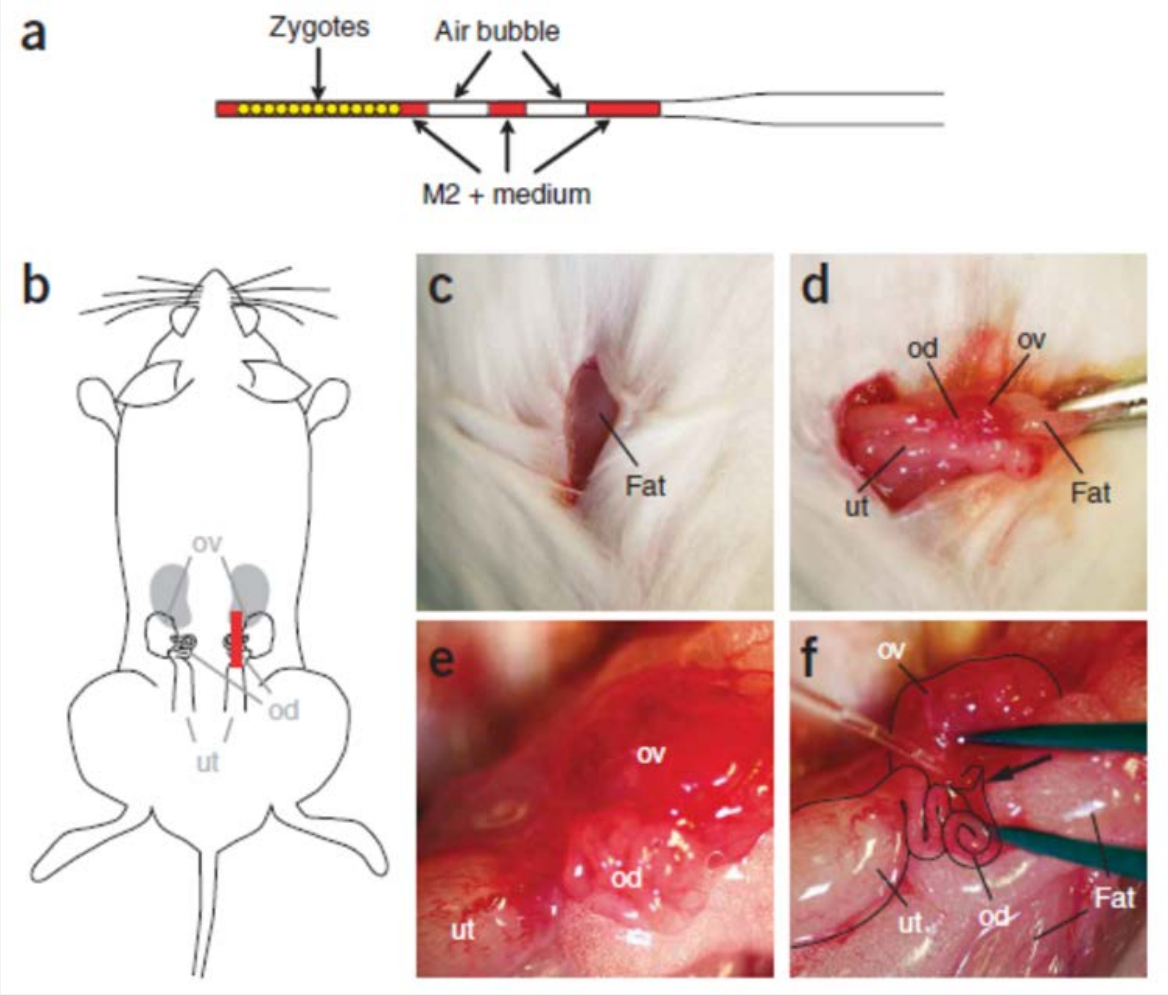
- 2014-2018

No. of strains:	75 strains	
Potential no. of cage vacated:	750 cages	(10 cage/strain)
Equivalent floor area vacated:	29 sqm	(9.4 IVC racks; 25.8 cage/sqm)
Equivalent construction cost:	2,177,000 HKD	(50K HKD/sqm)
Labor cost on colony management:	270,000 HKD/year	(15kHKD/month/FTE, 50 strains/FTE)
Labor cost on care & husbandry:	600,000 HKD/year (20kHKD/month/FTE, 300 cage/FTE, technical /husbandry/supporting staff; administration; FMteams and other university supports not included)	

MICROINJECTION

- DNA random integration
- CRISPR Cas9 KO/KI
  - Cas9 (100-200ng/ul)
  - sgRNA (50-100ng/ul)
  - ssDNA (100-200ng/ul)
- From 2014-2018: about 60 cases

EMBRYO TRANSFER TO SURROGATE

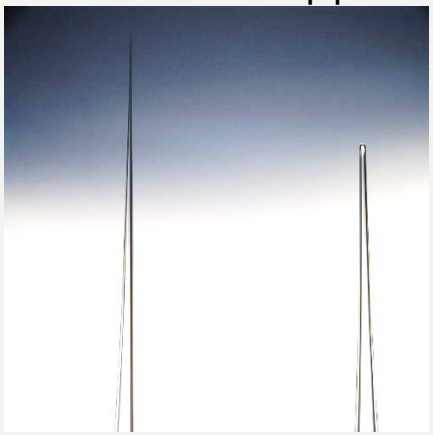


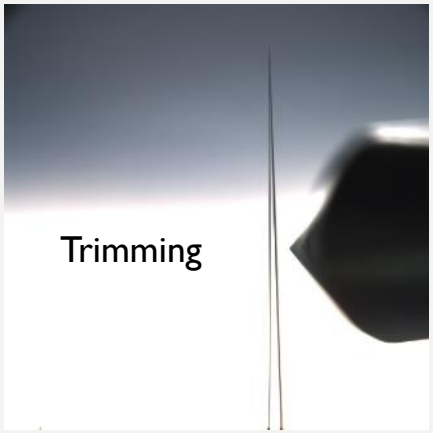
FRABRICATION OF TOOLS

HOLDING PIPETTE

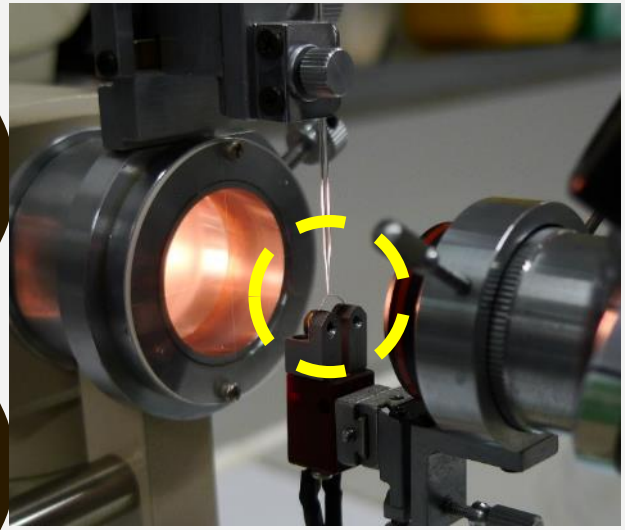
Pulled "needle"

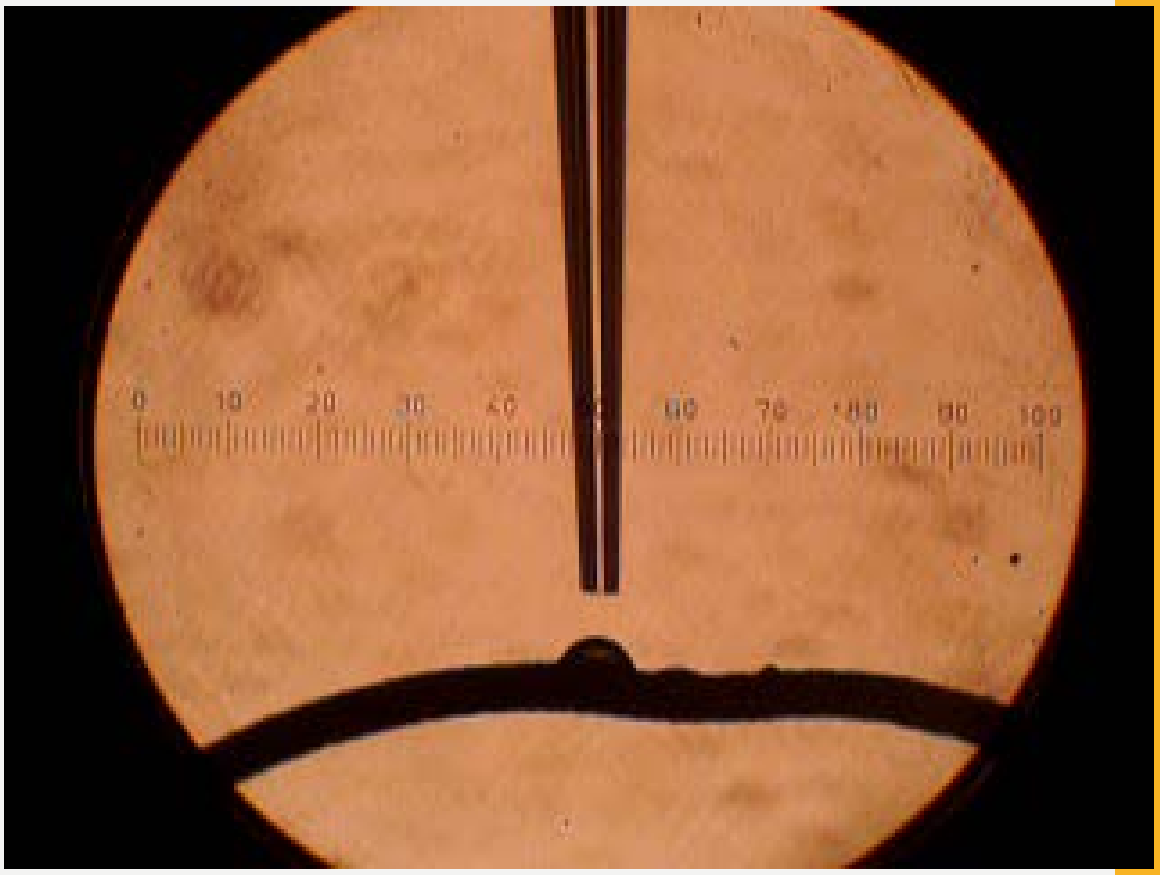
Holding pipette



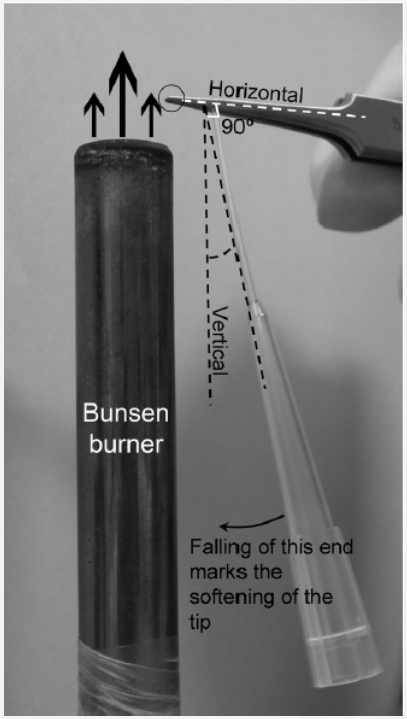


FRABRICATION OF HOLDING PIPETTE

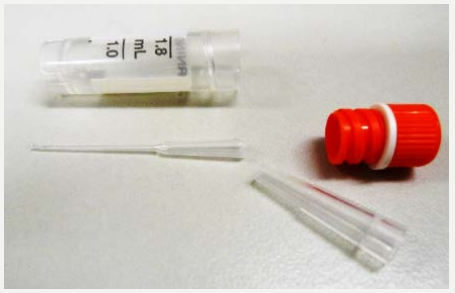


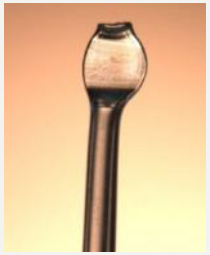


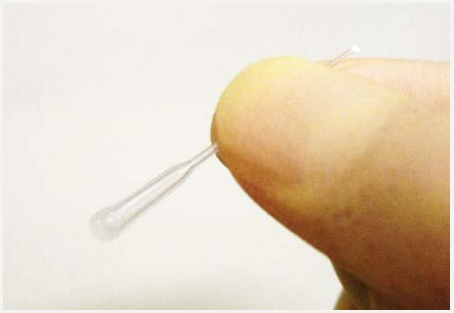
VITRIFICATION SPATULA

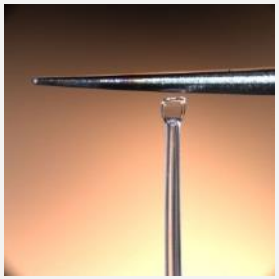


Tsang and Chow, 2009









SURVIVAL RATES
OF USING VITRIFICATION SPATULA

Table 1. In Vitro Recovery of Vitrified Embryos

	1-cell		Morula		Blastocyst	
	Vitrified	Unvitrified	Vitrified	Unvitrified	Vitrified	Unvitrified
1-cell	119 (100%)	123 (100%)	NA	NA	NA	NA
2-cell	117 (98.3%)	121 (98.4%)	NA	NA	NA	NA
Morula	117 (98.3)	119 (96.7%)	106 (100%)	112 (100%)	NA	NA
Expanded blastocyst	117 (98.3%)	119 (96.7%)	104 (98.1%)	112 (100%)	80 (66.7%[a])	126 (100%)
Hatched blastocyst	49 (41.2%)	81 (65.9%)	36 (34.0%)	68 (60.7%)	57 (71.3%[b])	103 (81.7%)

[a]Percentage of re-expanded blastocysts after warming of vitrified blastocysts (n = 120); [b]percentage base on the re-expanded blastocyst after warming (n = 80). NA, not applicable.

Table 2. In Vivo Recovery of Vitrified Embryos

	1-cell		Morula		Blastocyst	
	Vitrified	Unvitrified	Vitrified	Unvitrified	Vitrified	Unvitrified
Transferred embryos	128	119	120	134	121	106
Born pups	61 (47.7%)	54 (45.4%)	78 (65%)	90 (67.2%)	82 (67.8%)	75 (70.8%)
Average litter sizes*	7.3	ND	7.0	6.7	7.5	ND

*Average litter sizes of 15 pairs of animals recovered from each vitrified embryo group. ND, not determined.

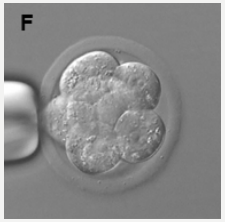
Tsang, *et. al.* 2009

QUALITY REASSURANCE

THAWING TEST

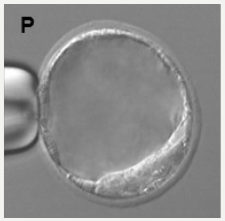
Thawing test for cryopreserved embryos

Thaw and revive



incubation

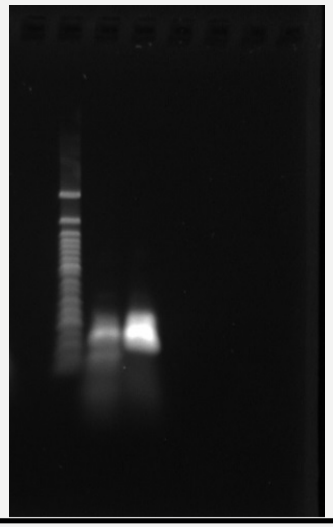
Check survival

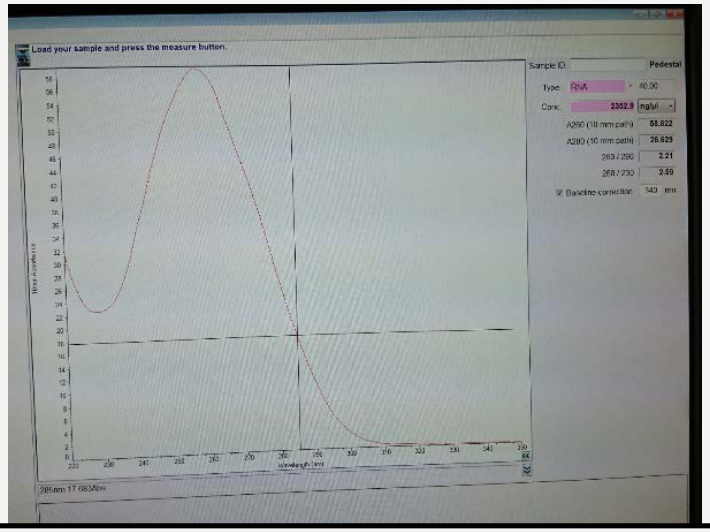


Blastocyst

QUALITY REASSURANCE

sgRNA synthesis QC





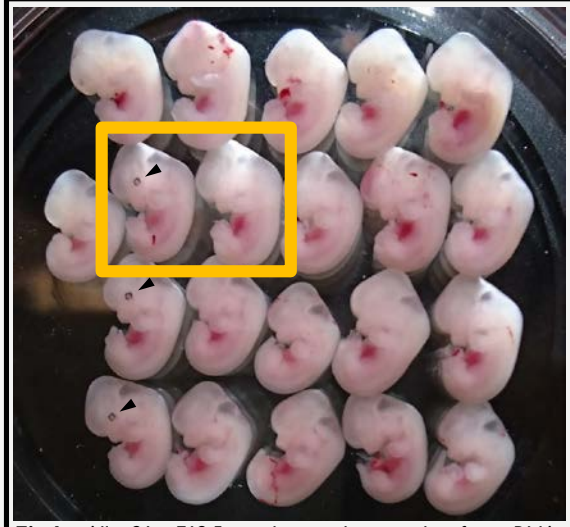


Fig.1 All 21 E12.5 embryos harvested after RNA microinjection. Normal retinal pigmentation (indicated by arrowheads) is observed in 3 embryos only. The rest of the 18 embryos lost retinal pigmentation.

Fig.2 A pair of embryos magnified from the inset in Fig.1. The right embryo shows the loss of pigmentation in its eye (open arrowhead), presumably as the result of *Tyr* mutation. Whereas, the left embryo shows normal retinal pigmentation (close arrowhead).

Performance Assessment on RNA Microinjection for CRISPR/cas9 Genome Editing (Transgenic Service)

Joanne Tam & Siva Tsang, March2017

Introduction and strategy

This work serves as an assessment on the quality of services provided to research groups to generate CRISPR/cas9 genome edited mice. To minimize the influence of variables generated by genotyping and for easy estimation of knockout efficiency, mutagenesis was generated in *Tyrosinase* (*Tyr*) on chromosome 7 which is responsible to black coat color and eye pigmentation. A well characterized and efficient CRSPR/cas9 knock out strategy was adopted from Chen *et al.* (2016) to ensure the assessment outcome is closely associated with the service performance to be assessed. Lastly, to reduce the involvement of more sensile animals (the 3Rs principles) and to speed up the assessment process, E12.5 embryos, instead of postnatal pups, developed from manipulated zygotes were harvested to obtain the assessment outcome. The assessment outcome was quantified by the proportion of embryos losing retinal pigmentation, presumably as the results of detrimental mutations in *Tyr*. (ref: PMID: 27151215)

Date of Microinjection: 9thMarch2017

Materials

Host strain:	C57BL/6J
Cas9 mRNA:	100ng/ul working (GeneArt™ CRISPR nuclease mRNA, Invitrogen)
gRNA: gRNA	100ng/ul working (Synthesized and purified with GeneArt™ Precision Synthesis Kit, Invitrogen)

Results:

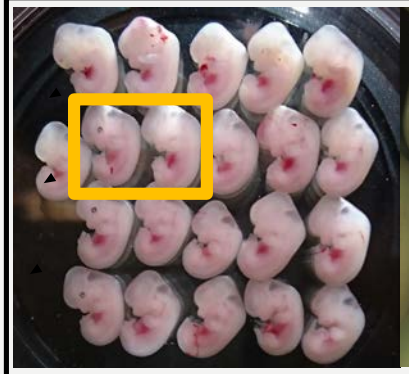


Fig.1 All 21 E12.5 embryos harvested after RNA microinjection. Normal retinal pigmentation (indicated by arrowheads) is observed in 3 embryos only. The rest of the 18 embryos lost retinal pigmentation.

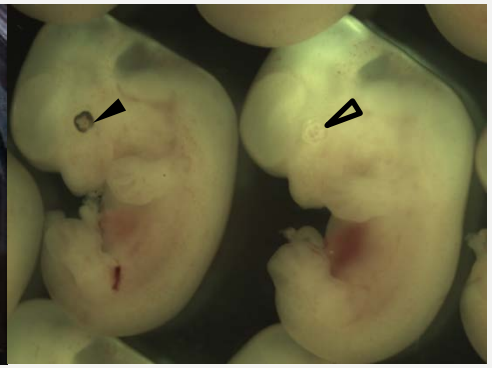


Fig.2 A pair of embryos magnified from the inset in Fig.1. The right embryo shows the loss of pigmentation in its eye (open arrowhead), presumably as the result of *Tyr* mutation. Whereas, the left embryo shows normal retinal pigmentation (close arrowhead).

Conclusion:

The functional knockout efficiency from this microinjection experiment is 86% (18/21)

THANK YOU!