WP 1.5

Aseptic and automatable vitrification of human embryonic stem cells using defined media

ULg - Glycomar

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Introduction: objectives

- Definition of hESCs cryopreservation conditions:

1. allowing recovery of **live and biologically intact** human embryonic cells (hESCs)
2. working in **aseptic conditions** (EC directive 2004/23/EC)
3. using **chemically defined media** without human & animal serum (mTeSR1®)
4. **compatible with automation**

Definition of the optimal cryopreservation procedure

- Comparison of two methods of cryopreservation

- « Conventional » Slow Freezing (SLF) in 1 ml cryotubes

- Aseptic vitrification (Vit) in french straws

Prevalidation steps

- **All conditions have been validated on:**
  - **mouse embryos:**
    - Submitted paper : (work performed on zygotes)

Vitrification succeeds with lower intracellular concentration of cryoprotectants (ICCP) as compared to slow freezing, despite exposure to higher concentrations of cryoprotectant solutions

Vanderzwalmen P, Connan D, Grobet L, Zech NH, Wirleitner B, Vanderzwalmen S, Nagy P, Ectors F.

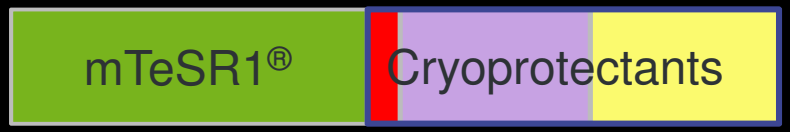
  - **mESCs**
    - cf: BEST presentation @ Lisbon (6/12/2011)

Definition of the optimal cryopreservation procedure

❑ Comparison of two methods of cryopreservation on **hESCs**

- « Conventional » Slow Freezing (SLF) in 1 ml cryotubes

- Aseptic vitrification (Vit) in 0.16 ml french straws



SLF as usually used for hESC

- ~ 10^6 cells/ml
- 10% DMSO – 40% KO-SR in mTeSR1®
- In cryotubes of **1 to 2 ml**
- Cooling rate: -1 to -2˚C/min until -80˚C, plunge in LN2

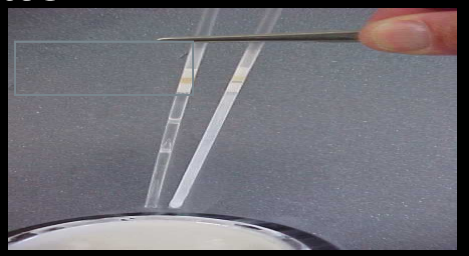
From the bench and from the literature:

•Advantages:
- Easy !!!
- Universal

•Drawbacks:
- Leaky to liquid N2 (>< to EU Tissue and Cells Directive 2004/23/EC)
- Poor control of supercooling → impair cell viability

Vitrification of hESCs

- Vitrification = extreme increase of viscosity upon very high speed cooling & warming (~1200˚C/min)
- Ultimately results in a solid amorphous state



From the bench and from the literature:

- Advantages
  - No ice crystal formation
  - No need of specific device for cooling & warming
- Drawbacks
  - Use of high extracellular concentrations of cryoprotectants (but low intracellular [CPs])

Our vitrification procedure:

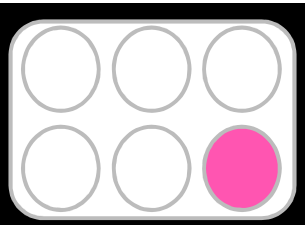
In defined & serum-free medium

- hESCs cultured in defined serum-free medium: mTeSR1®

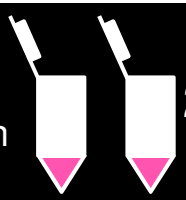
Aseptic

- Sealed straw: no contact with LN2
  (in compliance with EC recommendations)

Aseptic vitrification method: **cooling**



500 g, 5 min



2 to 4 tubes
140 µL

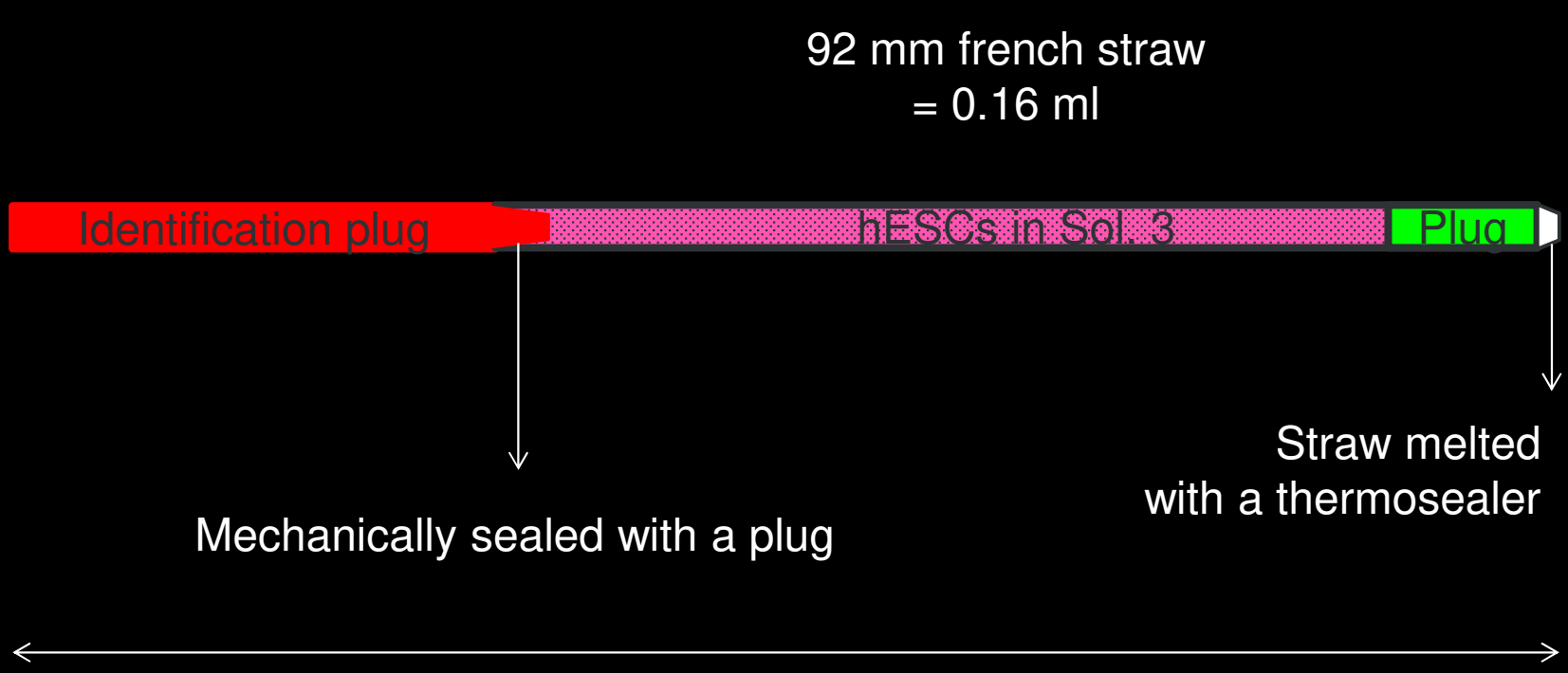
1.: + 140 µL of Sol. 1 = 280 µL; **incomplete** equilibration

2.: + 280 µL of Sol. 2 ; **incomplete** equilibration
Centrifugation @ 5000 g
Supernatant (560µL) removed

3.: Pellet re-suspended in 160 µL of Sol. 3; **no** equilibration
Fill in straw immediately; seal at both ends
Direct plunge in LN2

Aseptically vitrified straw

hESCs straw design:



Aseptic vitrification method: **warming**

Straw thawed in 37ºC water bath

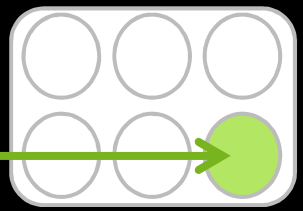
Straw emptied in 15ml tube prefilled w/ 1 ml of Suc1M in mTeSR1®

1.:  15 sec after thawing: + 1 mL of mTeSR1® = 2 mL of Suc 0.5M

2.:  30 sec after thawing: + 2 mL of mTeSR1® = 4 mL of Suc 0.25M

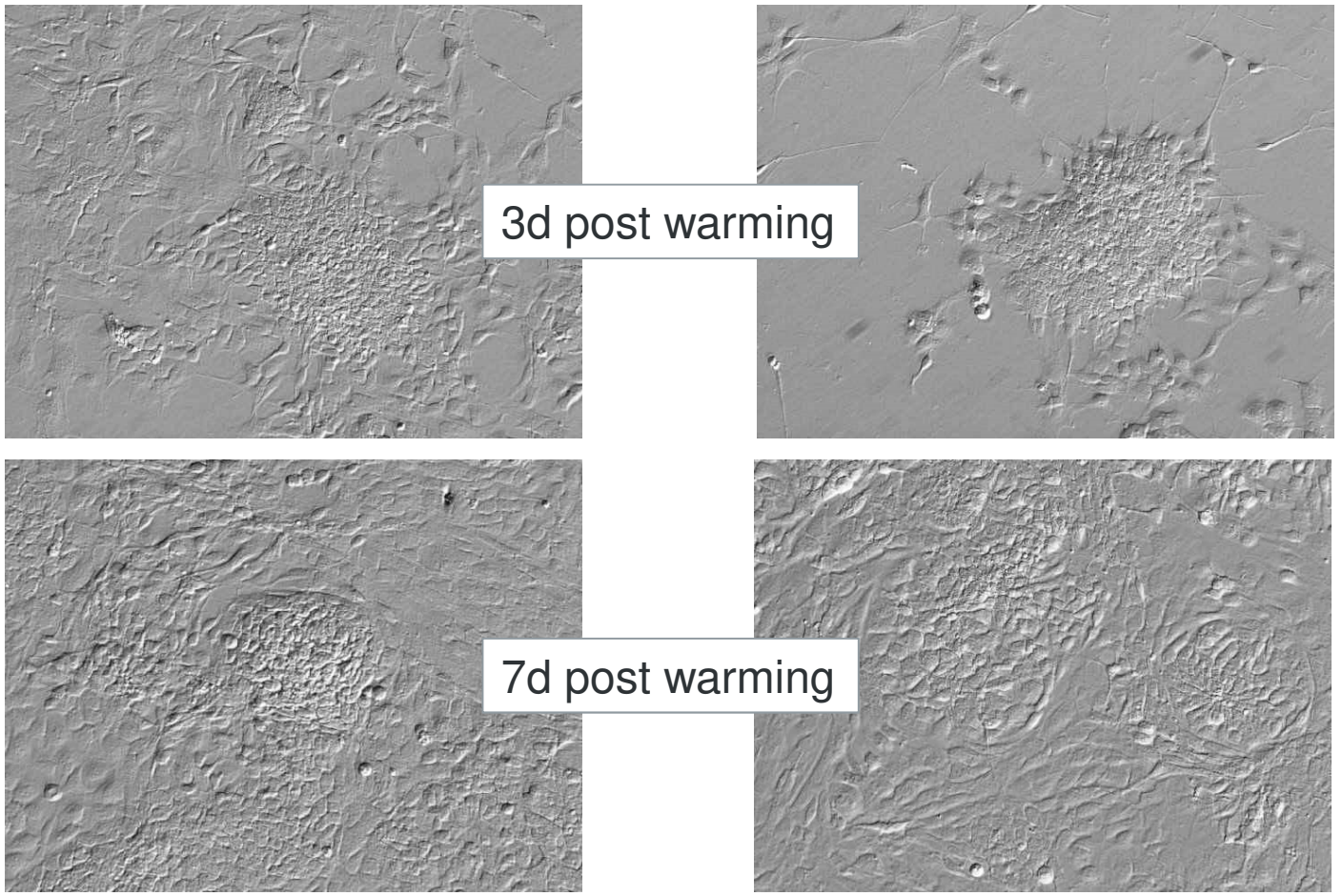
3.: 

60 sec after thawing: + 4 mL of mTeSR1® = 8 mL of Suc 0.125M
500 g during 5 min
Supernatant removed, add 2 mL of mTeSR1®



Results: Post-vitrification hESCs characteristics

- Morphology of colonies
- Morphometric analysis
- Karyotype
- Immuno-histochemistry
- Teratoma formation

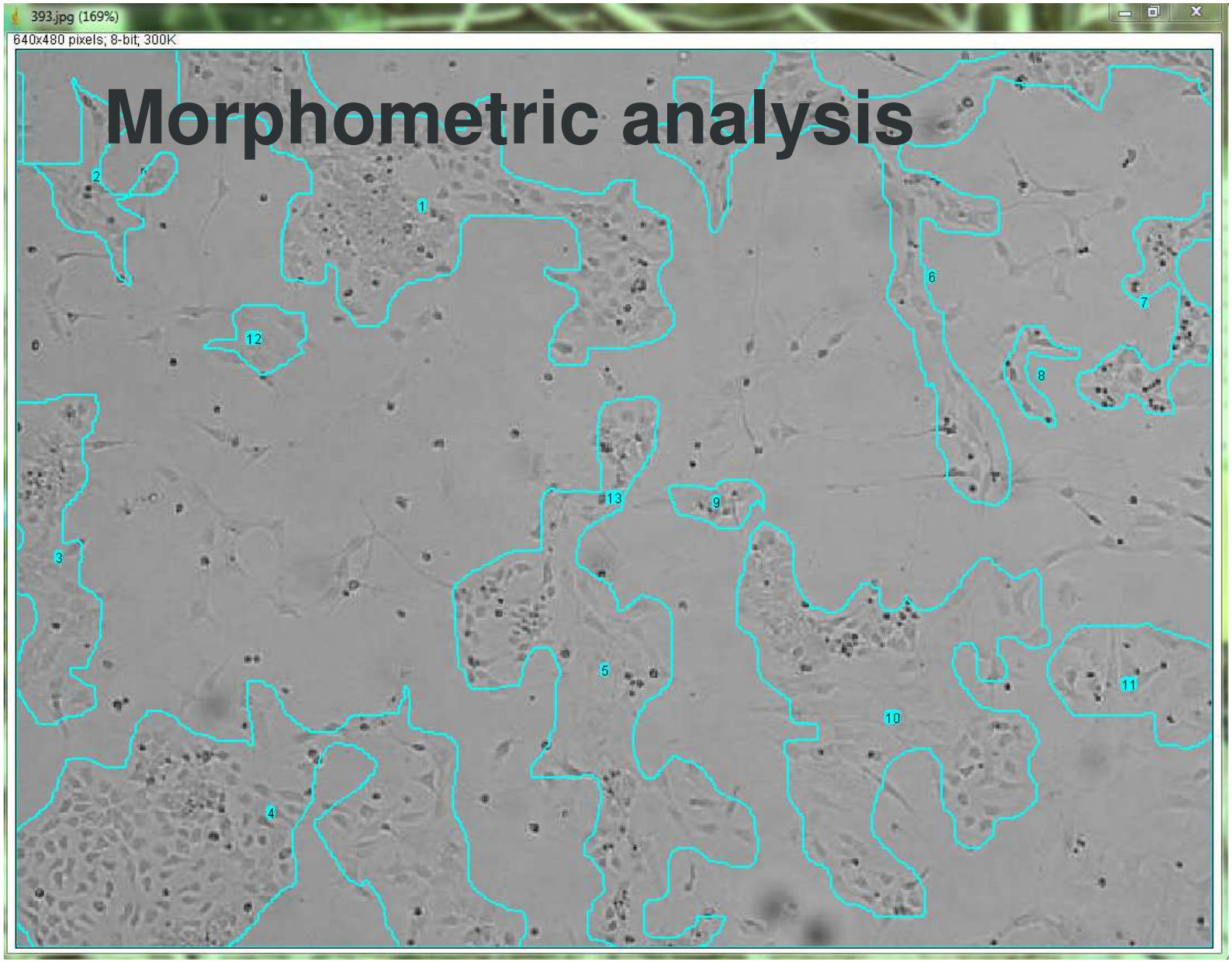


Morphology of colonies

3 & 7 d post-warming

Hoffman modulation contrast; x100

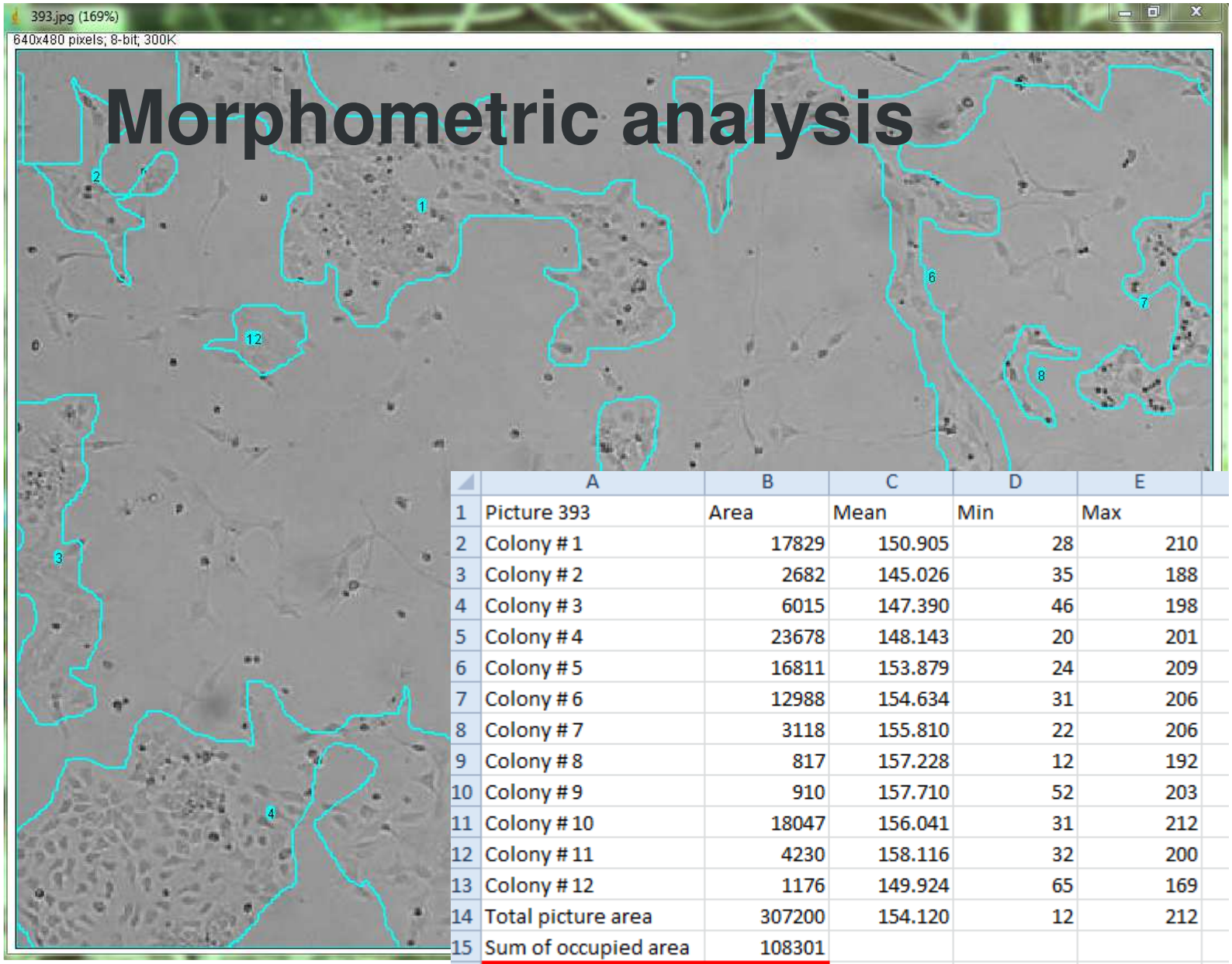
Morphometric analysis



Vitrified hESCs, d5, x40, picture #393

Pictures taken from the center of the well

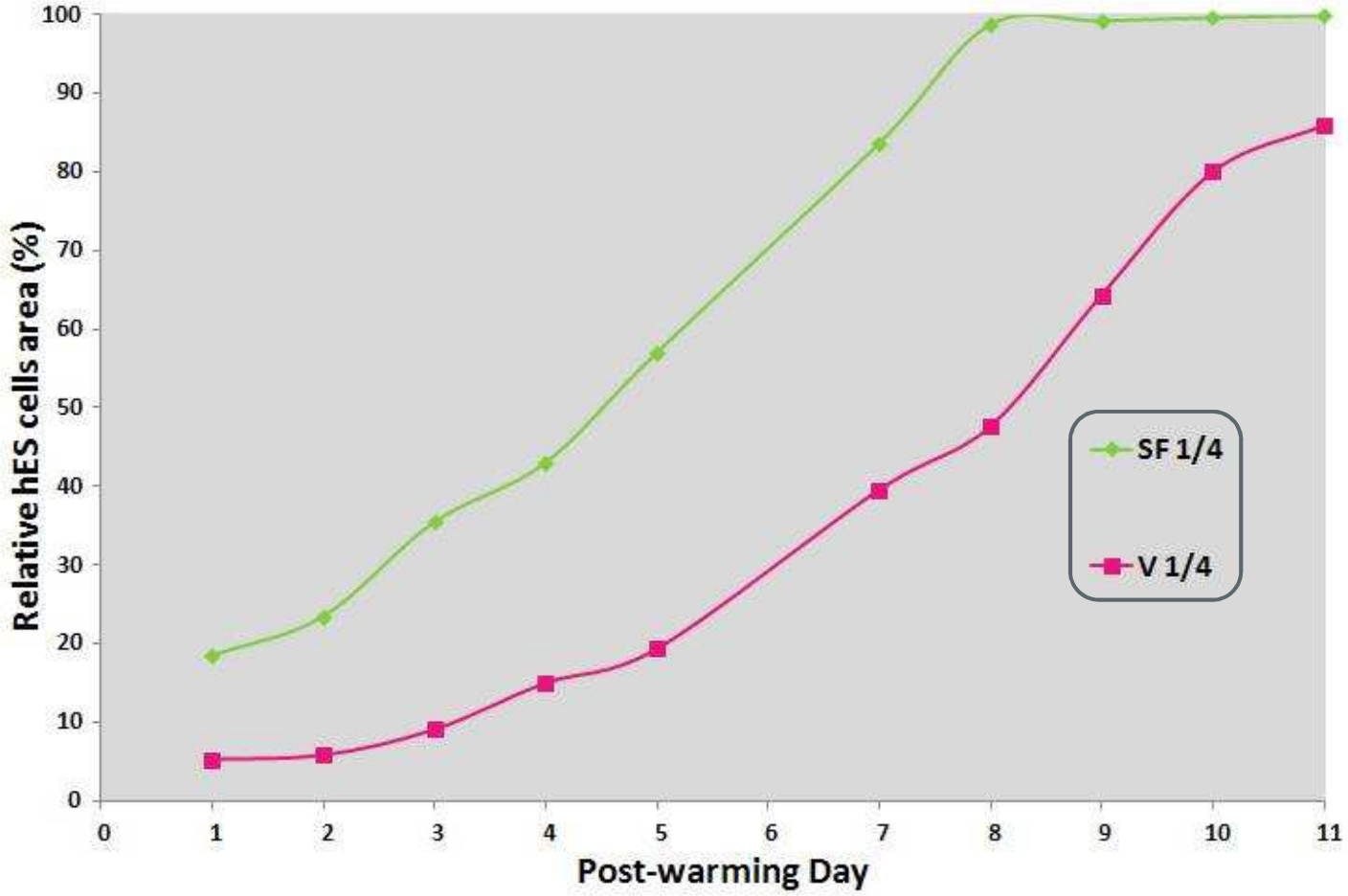
Morphometric analysis



	A	B	C	D	E
1	Picture 393	Area	Mean	Min	Max
2	Colony # 1	17829	150.905	28	210
3	Colony # 2	2682	145.026	35	188
4	Colony # 3	6015	147.390	46	198
5	Colony # 4	23678	148.143	20	201
6	Colony # 5	16811	153.879	24	209
7	Colony # 6	12988	154.634	31	206
8	Colony # 7	3118	155.810	22	206
9	Colony # 8	817	157.228	12	192
10	Colony # 9	910	157.710	52	203
11	Colony # 10	18047	156.041	31	212
12	Colony # 11	4230	158.116	32	200
13	Colony # 12	1176	149.924	65	169
14	Total picture area	307200	154.120	12	212
15	Sum of occupied area	108301			
16	Relative cells area	0,35254232			
17	1/2 well; Vit, d5				

Vitrified hESCs, d5, x40

Picture taken from the center of the well

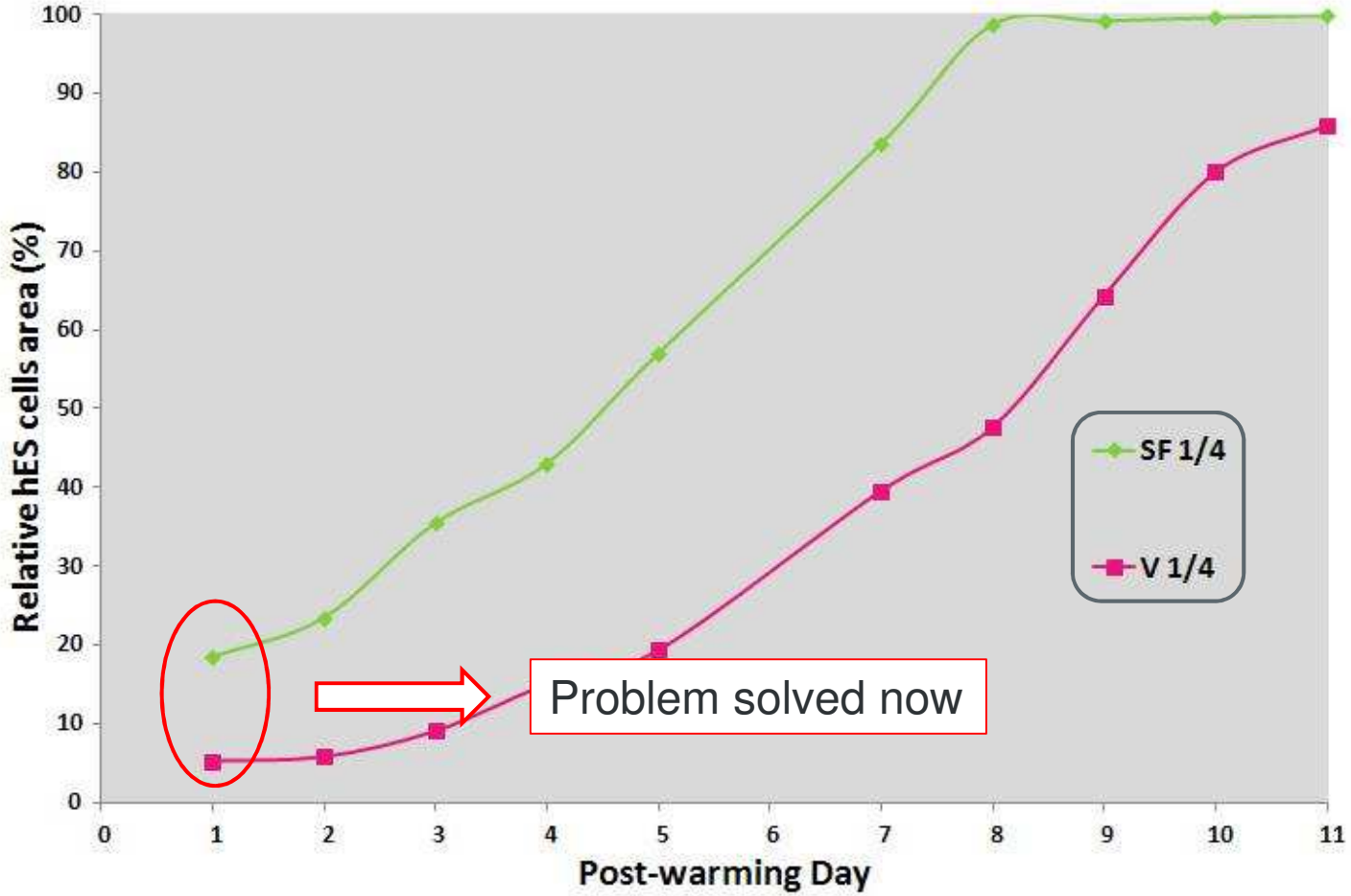


hESCs proliferation curves after SF or V

Morphometric analysis:

Proliferation curves after warming of slow frozen vs vitrified cells

¼ well of a 6-well plate



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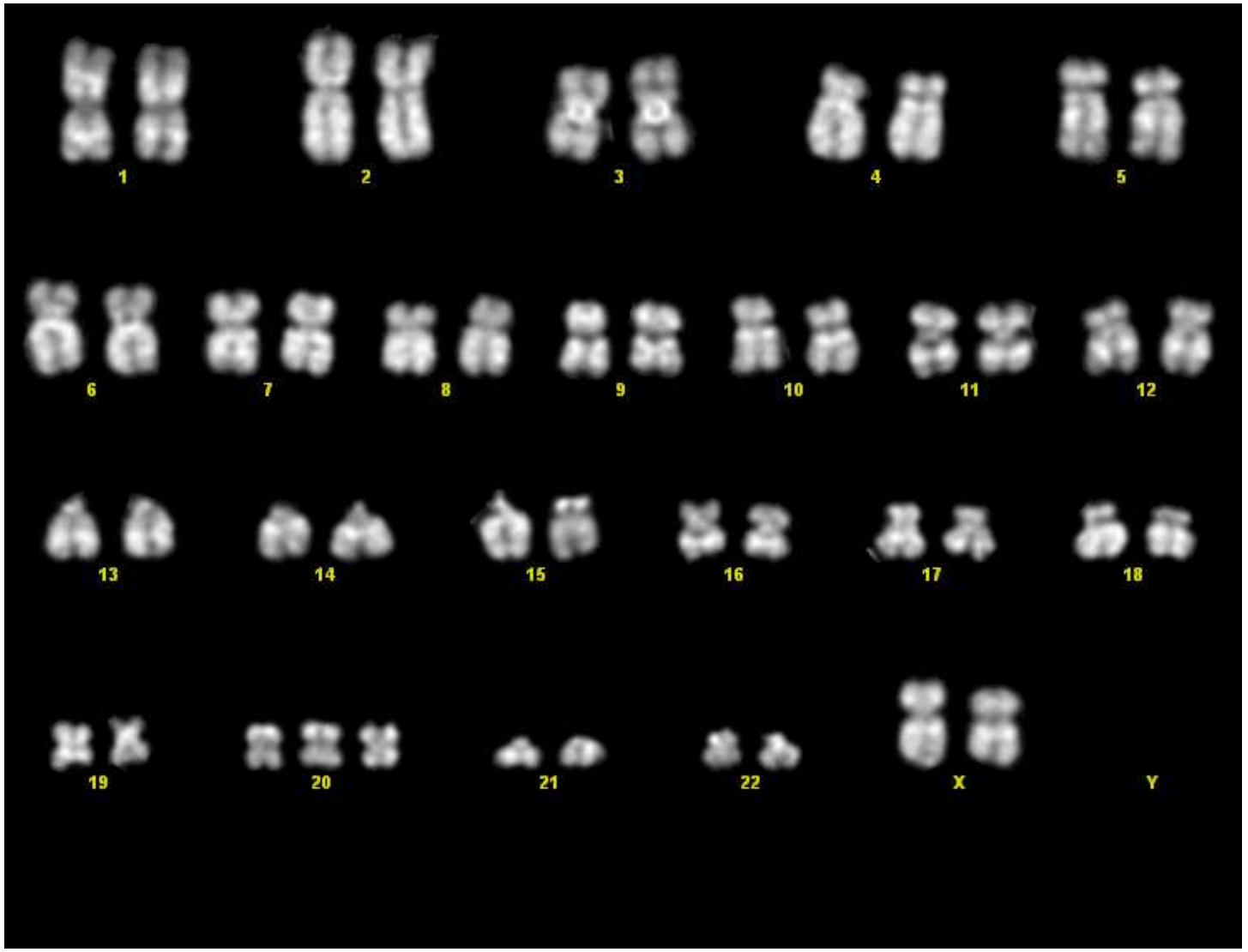
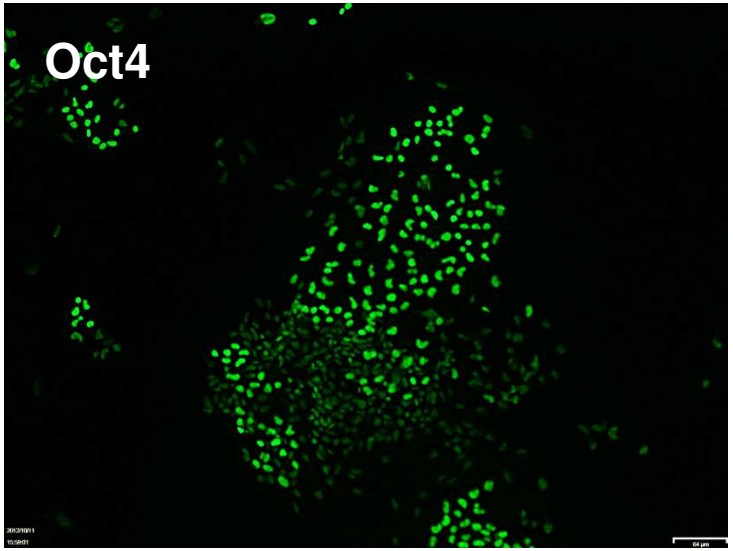
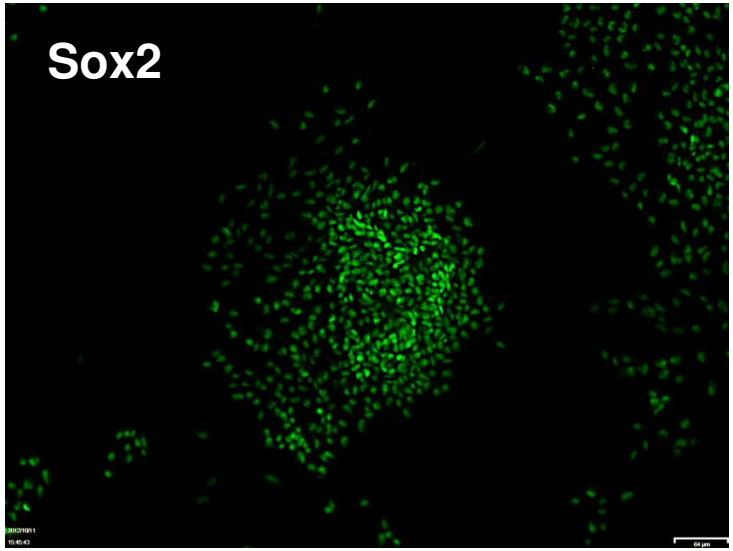
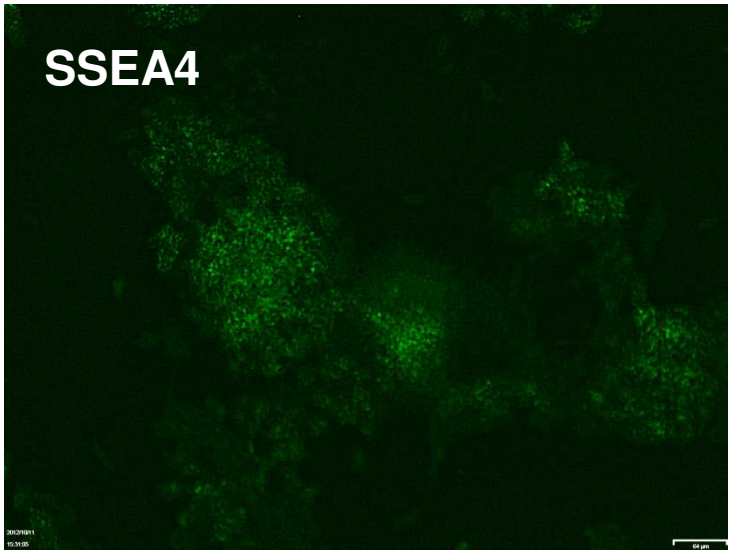
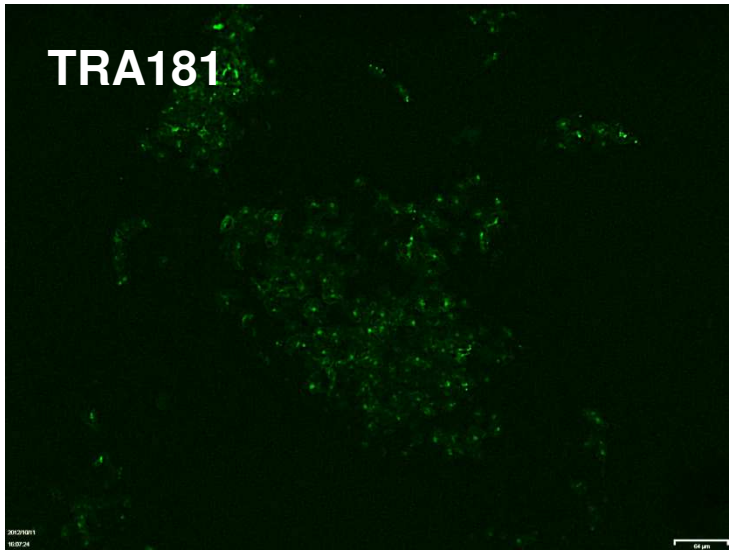


Karyotype analysis post-vitrification: 46XX



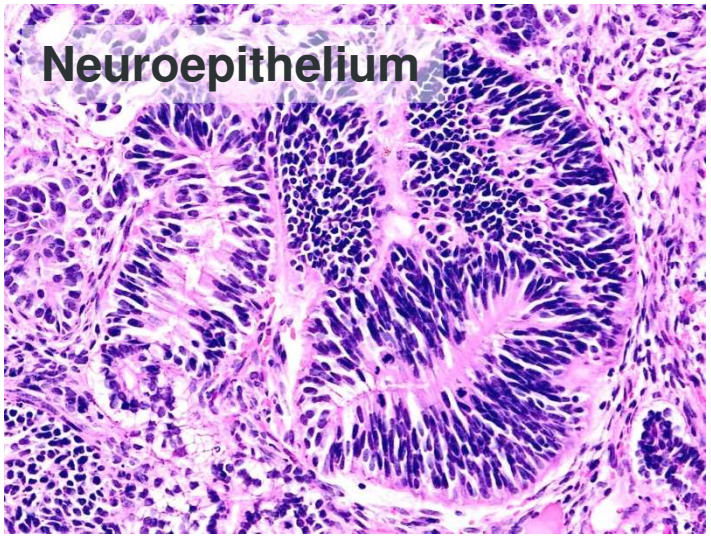


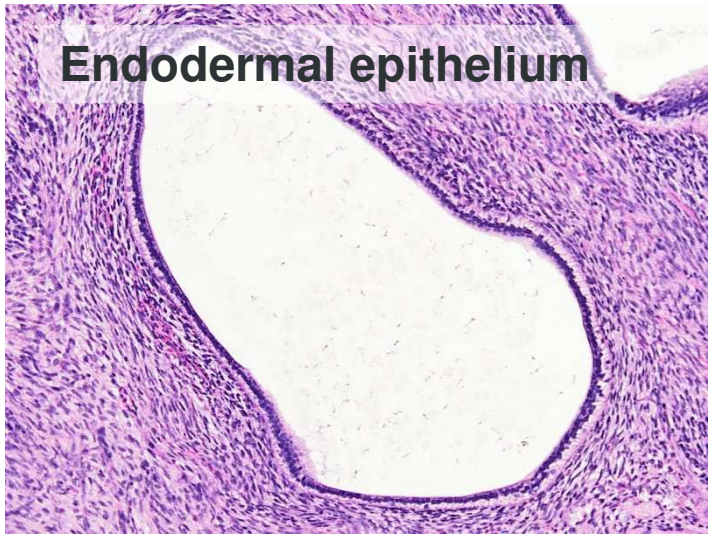


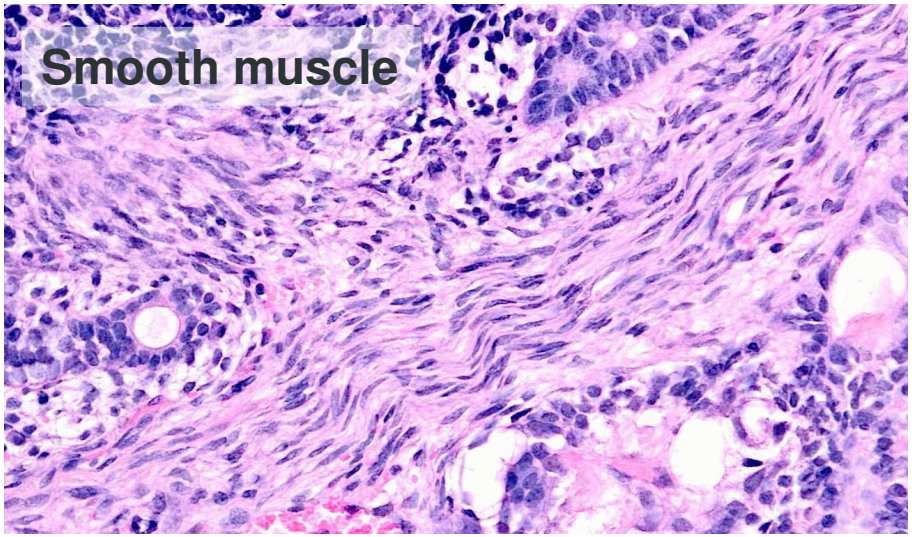


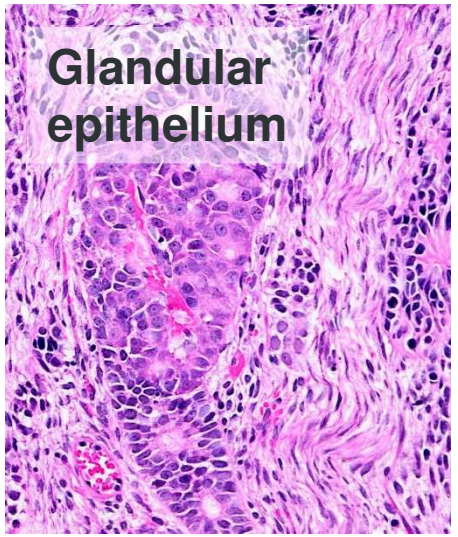
Immuno-histochemistry

hESCs RCM1: **p11 after vitrification** (x10)









Hematoxilin - Eosine

Teratoma formation post-vitrification (x20)

Conclusions

- Aseptic vitrification of hESCs in defined media w/o animal / human serum
- Stepwise addition and dilution of cryoprotectants before cooling and after warming ➔ Automation
- Vitrified RCM1 cells maintain their stem state

Perspectives

- Multiple steps of vitrification
- Method should be tested on other hES cell lines

Thanks to:

- Pierre Vanderzwalmen
- Joëlle Piret
- Nadine Antoine

- Thank you for your attention