**Immunostaining with cryosample**

Remove cryosections from -80C and let air dry slightly for 5 minutes at RT

Draw line surrounding sample with pap pen

Fix with 4% paraformaldehyde (4C or RT, 10min), wash 1-2x with PBS.

Fix with EtOH or Aceton or MeOH for 30 minutes at -20

Wash 2X with PBS (each wash step should be 2-3 min)

Permeabilize with 0.1% Triton-X100 in PBS 30min RT

Wash 2X in PBS

Block with 5% goat serum 30 min RT

Incubate primary Ab O/N at cold room and shaking PBS+5% goat serum + 0.1% tween-20

|  |  |  |  |
| --- | --- | --- | --- |
| Endoderm | Mesoderm | Ectoderm |  |
| a-fetoprotein (AFP) (A0008, DAKO) | a-sarcomeric actin | Tuj1  | Belmonte group\_Nature\_2009 |
| Foxa2 | Gata4 | GFAP |
| rabbit anti-Pdx1(1:200) (a kind gift from Dr. C. Wright | mouse anti-cardiac troponinT (CT3) (1:200) | mouse anti-bIII-Tubulin (Tuj1) (1:500)(Covance Research Products Inc., Denver, PA | ShengDing group\_Cell Stem Cell\_2008 |
| rabbit anti-albumin (DAKO |   |   |
| anti-afetoproteinpolyclonal antibody (N1501, Dako) | antia-smooth muscle actin monoclonal antibody (N1584, Dako | anti-bIII tubulinmonoclonal antibody (CBL412, Abcam) | Yamanaka group\_Cell\_2006 |

Wash 3X in PBST (0.1% tween-20)

Incubate secondary Ab (Cy3\_goat anti mouse) for RT 30min (1:300) in darkness

Wash 3X in PBS (0.1% tween-20)

Incubate DAPI in PBST for 5 min (1:500)

Wash 3X in PBST (0.1% tween-20)

Mount with slowfade gold and seal with nail polish

Store the slides in -20C freezer.

Cryosection IF

* Remove cryosections from -80C and let air dry slightly for 5 minutes at RT
	+ (fresh sections are best)
* Using pre-cooled MeOH, fix tissue for 30 minutes @-20C
* Rehydrate/wash 3-4X in PBS (each wash step should be 5-10 minutes)
* Wash 2X in 0.01% PBST (most standard protocols use Triton-X over Tween-20, but tween might be better in this case)
* Block in 5% horse serum + 0.01% PBST for 30 min @RT
* Incubate primary antibody O/N @4C, shaking (in 5% horse serum + 0.01% PBST)
	+ Ectoderm: βIII Tub/Tuj1 – Abcam suggestion of 1:200 for IHC-Fr on whole embryo; YC tested 1:500
	+ Mesoderm: T/Brachyury
	+ Endoderm:
* Wash 3-4X in PBST
* Incubate secondary for 30 min @RT (1:300)
* Wash 3-4X in PBST
* Incubate DAPI for 5-10 min @RT (1:500)
* Wash 3-4X in PBST
* Mount in Slowfade Gold