FACS Protocol

Capel Group, GUDMAP Consortium

1. Embryos were removed at 11.5, 12.5, and 13.5 dpc from wild type CD-1 females that were time-mated to the following reporter lines, with the following exceptions:

a. *Sry-EGFP*: labels supporting cells in the male at 11.5 dpc, and in the female at all stages (Albrecht and Eicher, 2001).
b. *Sox9-ECFP*: labels male supporting cells at 12.5 and 13.5 dpc (Kim et al., 2007).
c. *αSma-EYFP*: labels interstitial cells. CD-1 females were not used, and instead homozygous *αSma-EYFP* FVB females were used to increase the intensity of the fluorescent label (Cool et al., 2008).
d. *Mafb-EGFP*: labels interstitial cells. This is a knock in of GFP into the *Mafb* locus. A heterozygous male was mated to a wild type CD-1, and only embryos with GFP-positive gonads were used for sorting. Thus, all the embryos used were heterozygous for *Mafb* (Moriguchi et al., 2006).
e. *Flk1-mcherry*: labels endothelial cells. CD-1 females homozygous for the *Flk1-mcherry* transgene were used to increase the intensity of the fluorescent label (Larina et al., 2009).
f. *Oct4-EGFP*: labels germ cells (Szabo et al., 2002).

2. For 12.5-13.5 dpc embryos, the sex of the gonad is obvious, thus no genotyping was required, and we proceeded with the dissection. For 11.5 dpc embryos, where sex is not apparent, the tails were removed for genotyping to determine sex while the embryos were stored at 4ºC in a 24 well plate in PBS.

a. Each tail was placed in 200 µl of 50 mM NaOH and heated to 95°C for 10 minutes, then vortexed briefly.
b. 50 µl of 1M Tris HCl pH 7.6 was added and the samples were vortexed briefly.
c. PCR was performed with an annealing temperature of 55ºC to detect Kdm5c (X chromosome) and Kdm5d (Y chromosome) with the primers 5′-TGAAGCTTTTGGCTTTGAG-3′ and 5′-CCGCTGCCAAATTCTTTGG-3′. Females have a single band of 320 bp, and males have two bands of 320 bp and 280 bp. These bands are resolved on a 2.5% agarose gel.

3. The urogential ridge and dorsal aorta were removed from the embryo, and then the gonad/mesonephric complex was removed. The gonad was then removed from the mesonephros, with the following exceptions:

> **a.** For *Oct4-EGFP* sorts, the mesonephros was left attached. *Oct4* expression is highly specific to the germ cells, and so removing the mesonephros provided no benefit.
> **b.** For 11.5 dpc *Flk1-mcherry* sorts, only the anterior and posterior portions of the mesonephros were removed by cutting at a 45° angle from the end of the gonad. The vasculature in the gonad arises from a plexus in the mesonephros. Because we believe this is one population of endothelial cells at 11.5 dpc, the plexus was included.

4. All the male gonads were pooled into one 1.7mL tube, and the female gonads were pooled into a separate 1.7 mL tube.

5. The gonads were incubated in 250 µl 0.25% Trypsin EDTA (Gibco #25200) at 37°C for 5-10 minutes.

6. As much Trypsin as possible was removed without disturbing the tissue, which naturally settles to the bottom of the tube.

7. We added 400 µl PBS (without Ca/Mg) with 4 µl RNase-free DNase (Promega #M6101) to each tube. However, DNase was not used for the supporting cells (*Sry-EGFP* and -*Sox9-ECFP*-) because this reduced the yield.

8. The cells were dissociated by pipetting up and down with a P200 set at 100 µl, and then pulling the cells through a 27 gauge needle until no clumps were visible (around 5x).

9. The cells were passed through a cell strainer (BD Falcon #352235) by tapping, and then centrifuging for 1 second at 1000 rpm to collect the remaining liquid.

10. The cells were taken to the Duke Comprehensive Cancer Center Flow Cytometry Shared Resource for FACS sorting.

11. The positive fraction from the cell sort was pelleted at 6000 rpm for 5 minutes, and the liquid was removed.

12. The cells were immediately frozen at -80°C.

Citations:

- Albrecht, K.H., and Eicher, E.M. (2001). Evidence that Sry is expressed in pre-Sertoli cells and Sertoli and granulosa cells have a common precursor. Dev Biol *240*, 92-107.
- Cool, J., Carmona, F.D., Szucsik, J.C., and Capel, B. (2008). Peritubular myoid cells are not the migrating population required for testis cord formation in the XY gonad. Sex Dev *2*, 128-133.
- Kim, Y., Bingham, N., Sekido, R., Parker, K.L., Lovell-Badge, R., and Capel, B. (2007). Fibroblast growth factor receptor 2 regulates proliferation and Sertoli differentiation during male sex determination. Proc Natl Acad Sci U S A *104*, 16558-16563.
- Larina, I.V., Shen, W., Kelly, O.G., Hadjantonakis, A.K., Baron, M.H., and Dickinson, M.E. (2009). A membrane associated mCherry fluorescent reporter line for studying vascular remodeling and cardiac function during murine embryonic development. Anat Rec (Hoboken) *292*, 333-341.
- Moriguchi, T., Hamada, M., Morito, N., Terunuma, T., Hasegawa, K., Zhang, C., Yokomizo, T., Esaki, R., Kuroda, E., Yoh, K.*, et al.*(2006). MafB is essential for renal development and F4/80 expression in macrophages. Mol Cell Biol *26*, 5715-5727.
- Szabo, P.E., Hubner, K., Scholer, H., and Mann, J.R. (2002). Allele-specific expression of imprinted genes in mouse migratory primordial germ cells. Mech Dev *115*, 157-160.
-