**Analyzing Atherosclerotic Lesions in BCA**

Notes about taking pictures of sections:

* Use bright field
* Take z-stacks (approx. 8-10) based on Acta2 and eYFP staining, not DAPI
* Keep z-stack number consistent across sections to ensure data is not lost or gained (no less than 8)

Notes about analysis:

* Perform quality control on slides based on Acta2/eYFP staining within media. Media should be eYFP+ (~100% cells), Acta2+ (external medial layer). Sometimes you need to go through z-stack to see eYFP staining in all cells
* Be CONSISTENT with consideration to positive cell populations and BLIND for genotype (WT vs KO)

Setup:

1. Open image, un-click all fields except brightfield and/or DAPI
2. Click “Overlay” button and check “show all”
3. Click the rectangle drawing tool (default color is red), draw five total boxes per lesion measuring 400x400 (may be split up between two files)
4. Five boxes may not overlap. Two boxes encompass the **shoulder** and touch the media; two boxes encompass part of the **lesion** and touch the media; one box encompasses part of the **fibrous cap**, may touch media or lesion
5. Number first box using the “font” tool, unclick “show all”

Counting Lesions:

1. Widen screen enough to move “Overlay” tab next to “Dimensions”
2. In “Dimensions”, move z-stack to 1, make sure only DAPI is selected
3. In “Overlay”, click the **rectangle** and make sure it is **red**
4. Click once (do not make a rectangle) on a DAPI+ cell to mark it, you will have to click the rectangle drawing tool each time you mark a new cell

**NOTE:** do not count cells in the media, only cells in the lesion, do not count RBC (they are usually crescent shaped and autofluoresce with each laser)

1. Continue through all z-stacks in the section with DAPI
2. In “Dimensions” with DAPI still selected, select eYFP
3. In “Overlay”, click the **arrow** and change the color to **white**
4. Click once (do not make an arrow) on an DAPI+ cell that has eYFP expression (ensure eYFP+ mark co-stains with DAPI and is not on an entirely different plane)
5. In “Dimensions” with DAPI still selected, select EXP (fourth channel marker)
6. In “Overlay”, click the **circle** and change color to **green** (11)or **white** (12)
7. Draw green circles around eYFP+ DAPI+ cells (red and white dots)
8. Draw white circles around eYFP- DAPI+ cells (red dots only)
9. In “Dimensions” with DAPI still selected, select Acta2
10. In “Overlay”, click the **ellipse** and change color to **green** (15)or **white** (16)
11. Draw green ellipses around eYFP+ DAPI+ cells (red and white dots)
12. Draw white ellipses around eYFP- DAPI+ cells (red dots only)

**Note:** there will be cells that look like planets (both white and green)

Counting Fibrous Cap:

1. Widen screen enough to move “Overlay” tab next to “Dimensions”
2. In “Overlay”, check “show all”, check “show measurement” and select the **line** tool
3. Draw a line 30 +/- 0.2um perpendicular to the fibrous cap edge at that point
4. Draw enough lines for the shape of the cap
5. Zoom out enough to visualize all of fibrous cap, select the **closed poly** tool and connect the lines drawn with enough independent points to be able to manipulate the shape once zoomed in (make a new point every few um)
6. Note Area and Line values
7. Unclick “show measurements” and hide individual lines, unclick “show all”
8. In “Dimensions”, move z-stack to 1, make sure only DAPI is selected
9. In “Overlay”, click the **circle** and change the color to **green** (10)or **white** (11)
10. Draw green circles around eYFP+ DAPI+ cells (red and white dots)
11. Draw white circles around eYFP- DAPI+ cells (red dots only)
12. In “Dimensions” with DAPI still selected, select Acta2
13. In “Overlay”, click the **ellipse** and change the color to **green** (14)or **white** (15)
14. Draw green ellipses around eYFP+ DAPI+ cells (red and white dots)
15. Draw white ellipses around eYFP- DAPI+ cells (red dots only)

**Note:** there will be cells that look like planets (both white and green)

Analysis:

* Analysis will be based on individual stains
* Key Lab Metrics (average values in control non-KO mice:

1. eYFP+/DAPI (about 25-35%)
2. eYFP+Acta2-/eYFP+ (about 80-90%)
3. eYFP+Acta2+/eYFP+ (about 10-20%, inverse of 2)

* Other common values:

1. eYFP+Acta2- EXP+/eYFP+
2. eYFP+Acta2- EXP+/EXP+ (fraction of cells with respective marker/protein of interest that are eYFP+ (SMC-derived)
3. eYFP+Acta2- EXP+/DAPI (fraction of total lesion cells that have your phenotype)
4. eYFP- EXP+/eYFP- (fraction of cells with respective marker/protein of interest that are eYFP- (non-SMC-derived))