# Sample Procedure:
# Immunofluoresent Staining of Mouse Embryonic Fibroblasts for F-actin, cortactin and histone deacetylase 6 (HDAC6)

## Background and purposes of the experiment

Cortactin is important to initiate actin polymerization for membrane ruffling and cell migration. When cortactin is acetylated, its activity is dampened. The acetylation of cortaction is reversible and is catalyzed by HDAC6. The main purposes of this experiment is to examine how the absence of HDAC6 will affect (1) dorsal ruffle formation in number and morphology; (2) cortactin and F-actin localization to the ruffles or F-actin spikes (regarded as precursors of a complete ruffle).

(1). Put three 12 mm round glass coverslips (#1.5 from FisherScientific or VWR) in the wells of a 24-well plate.

Coverslip #1 for wild-type MEF
Coverslip #2 for HDAC6-null MEF
Coverslip #3 for wild-type MEF, but will be used as a '(-) anti-cortactin, (-) anti-mHDAC6' control

(2). Put the plate in the tissue culture hood and UV expose the coverslips for 30 minutes.

The UV light should be less than one foot in front of the coverslips and there should be no blockage between the coverslips and the UV light.

(3). Split the cells and add cells to the wells (0.5 ml total).

It is very often that air bubbles are trapped underneath the coverslips. Push down the coverslips to expel the air bubbles.

Rock the plate back and forth, left and right (± 45$^{o}$ angles) a couple of times to get even distribution of cells. Do not swirl the plate or move back and forth the plate without tilting it. That will cause the cells to concentrate at the center of the coverslips.

(4). Culture the cells for at least 12 hours to let the cells fully spread. The cells should be about 40-70% confluent.

(5). Wash the cells once with 37$^{o}$C pre-warmed serum-free medium and add 0.5 ml serum-free medium to the cells. 37$^{o}$C serum-starve the cells for 12-20 hours.

(6). Treatment: change the medium to 37$^{o}$C pre-warmed serum-free medium (0.25 ml is enough, this will save the expensive PDGF-BB) + 20 ng/ml PDGF-BB, and stimulate the cells for 10 minutes.

(7). Aspirate the medium and rinse the wells quickly with 4$^{o}$C PBS 2-3 times.

(8). Fixing: immediately add 1 ml 4% paraformaldehyde to the wells to fix the cells. Sit the plate on bench for 15 minutes.

> 4% paraformaldehyde can be freshly made or from -20$^{o}$C aliquoted stock. The later needs to be thawed in 37$^{o}$C waterbath and cooled to room temperature before use. Please do not reuse the left-over solution in future experiments.
>
> PBS washed coverslips tend to dry out quickly. Please act quickly.

(9). Aspirate the fixative and rinse the wells 2 times with 1 ml PBS.

(10). Add 1 ml 10 mM $NH_4Cl$-PBS to the wells and stay 10-30 minutes to quench the free aldehyde groups of the fixative.

(11). Wash the cells with PBS, 2 min X 3

(12). Permeabilization: add 0.5 ml 0.1% Triton X-100 (in PBS) to each well. Stay on bench for 10 minutes to permeabilize the cells.

(13). Wash the cells with PBS for 5 min X 2 and PBS-0.05% Tween-20 (PBS-T) 5 min X 1

(14). Without removing the solution in the wells, using sharp and bent tweezer to transfer the coverslips to the humidified chamber and put them on top of the parafilm with cells side up.

> The humidified chamber shall have several layers of distilled water-soaked filters at the bottom and a sheet of parafilm on top.

(15). Aspirate the solution on the coverslips as much as you can by tilting the humidified chamber a little bit, but do not let the coverslips dry out.

(16). Blocking: block for 5-10 min in 50-100 μl 5% normal goat serum made in PBS-T (v/v).

> The blocking solution should be 0.45 μm filtered and 0.05% $NaN_3$ can be added to the solution. Blocking solution can be stored at 4$^{o}$C for several weeks.

(17). Optional blocking for 5-10 min in 0.5% fish skin gelatin (available from Sigma-Adrich)-PBS-T (also filtered).

(18). Dilute primary antibodies in 5% goat blocking solution.

Antibody #1: 200 μl anti-cortactin mAb (1mg/ml) from Millipore, 1:100 dilution
Antibody #2: 200 μl anti-mouse HDAC6 antibody (home-made, affinity purified, about 20 μg/ml), 1:10 dilution.

Mix 20 μl of each antibody solution. The final concentration of anti-cortactin is 5 μg/ml and anti-mouse HDAC6 is 1 μg/ml.

The antibody solutions are made more than needed for one experiment. The reasons are: (1) it is more accurately to dilute antibodies in larger amount for consistent results among different experiments. (2) the antibody solutions can be stored at 4$^{o}$C for a couple of months without drastic activity drop, thus easing your workload.

(19). Spin the antibody solution at 14,000 rpm on a desk-top centrifuge for 10 min at 4$^{o}$C.

(20). Primary antibody: add 20 μl of the antibody mixture to coverslip #1 and #2 (wild-type MEF and HDAC6-null MEF); Add 20 μl of goat serum blocking solution to coverslip #3 (wild-type MEF) as control. Put the chamber in the drawer under the bench. Incubate for 1 hour at room temperature or for overnight at 4$^{o}$C. Intermittent mixing by swirling is good, but is not absolutely necessary.

Antibody solution should be added to one side of the coverslip. With the help of detergent in the solution, it spreads over the coverslip. Swirl the humidified chamber a little bit to ensure complete coverage of the coverslip.

(21). Washing: aspirate antibody solution from the edge of the coverslip. Tilting the chamber a little bit will help leave much less antibody solution on the coverslip. Add 100-200 μl PBS-T (filtered) at one side of the coverslip. Sit the humidified chamber on bench for 5 min (rotation is not necessary), and aspirate the solution from the opposite side of the coverslip. Repeat PBS-T washing 3 more times.

(22). Blocking: as in step (16) and (17).

(23). Prepare secondary antibodies in the goat blocking solution.

200 μl 1:200 Goat-anti-mouse IgG (H+L)-Alexa Fluor 488 *highly cross-adsorbed* (Invitrogen, cat no A-11029)

200 μl 1:200 Goat-anti-rabbit IgG (H+L)-Cy5, AffiniPure (Jackson ImmunoResearch, cat no 111-175-144), 1:200
200 μl 1:100 Phalloidin-Rhodamine (Cytoskeleton, Inc)

Mix 20 μl of each and spin at 14,000 rpm in a desk-top centrifuge for 10 min at 4°C. The final dilution of the antibodies is 1:600 and phalloidin-Rhodamine is 1:300.

(24). Secondary antibodies: apply 20 μl of secondary antibody solution to each coverslip. Swirl the humidified chamber to let the antibody solution cover the coverslips. Sit the whole chamber in the drawer (with drawer closed) for 45min-60min, or at 4°C overnight.

(25). Washing: as in step (21) for 5 times total.

(26). Prepare mounting: a standard glass slide can hold two 12mm round coverslips. Add a small drop (about 5-10 μl) of mounting medium (containing anti-fade reagent) on the slide for each coverslip.

(27). Mounting the coverslips on the slide. Try to sit the coverslips at the center of the slide. Many microscopic objectives can't be positioned to the ends of the slide.

Before mounting, dip the coverslip in $H_2O$ a couple of times to wash out the salt from the washing solution. Touch the edge of the coverslip on a piece of tissue and adsorb the liquid as much as possible. Turn the coverslip with cell-side down. Contact the mounting medium first with the distal side of the coverslip, gently release the tweeter. The coverslip will fall on the slide. Don't try to move the coverslip once it is in place. Most of the time, only few air bubbles might be trapped between the coverslip and the slide. Some times, more air bubbles are caught inbetween. Just let it go as it is and never try to squeeze the air out. That will damage the structure of the cells and cause high fluorescent background.

(28). Draw of excess mountant with filter paper and let the mounting medium solidifies for 1 to 2 hours before they are examined on microscope.

**The results**

(1). Minus anti-cortactin and anti-mouse HDAC6 control shows very faint background signal, suggesting the high specificity of the anti-mouse and anti-rabbit secondary antibodies.
(2). In HDAC6 knock-out cells, there is significantly less ruffle formation (by quantification of dorsal (ring) ruffle formation in 1000 cells from each group), suggesting that HDAC6 is required for efficient dorsal ruffle formation in MEFs.
(3). Dorsal ruffles formed in HDAC6-null cells are similar to those in wild-type cells in morphology.
(4). Localization of cortactin to the F-actin-enriched ruffles and smaller F-actin spikes are similar in both cell types, suggesting that acetylation of cortactin does not interfere with its association with these F-actin-containing structures.
(5). In HDAC6-null MEFs, anti-mouse HDAC6 antibody still shows weak (though much weaker comparing to wild-type cells) amorphous staining in the cytoplasm and in the nucleus, suggesting the existence of residual non-specific reaction from this affinity-purified antibody, which may affect critical analysis of some results using this antibody.

*Any questions about this sample procedure? Please contact: Dr. Yasheng Gao (ygao@duke.edu)*