Fiber Combing Protocol (Protocol from Paul Chastain)
Modified by Jason Stewart 5/21/12 (Changes/Additions in Blue)

Do the experiment first thing in the morning – there's a 4 to 6 h drying step towards the end of the first day of processing.

Experiment
Plate cells so that they are in log growth the day of the experiment. Plate HeLa1.2.11 cells ~5 x 10 cells/6 cm plate for each condition.

Remove medium from plates, take ½ of the medium and reserve it for the CldU pulse (CldU reserve) and add IdU to the other ½ at a final concentration of 50 µM, mix, add the IdU containing medium back to plates. Place CldU reserve media at 37°C to keep pre-warmed. Add CldU to this media just prior to "CldU pulse". Place cells in incubator for duration of "IdU pulse."

IdU and CldU can be obtained from Sigma (**I7125-5G and C6891-100MG**, respectively)

Remove medium, wash 1x with PBS, remove PBS, and expose cells to UV (only expose the cells to UV if you are studying how UV influences replication dynamics. Otherwise, just wash the cells with PBS and skip the UV exposure).

Cover cells with the CldU reserve containing 100 µM CldU. Place cells in incubator for duration of "CldU pulse."

For replication restart experiments, the cells are treated with 2 mM HU for 2 hrs, after the "IdU pulse". The media is then removed, the cells washed three times with serum-free media and media containing 100 µM CldU is added to the plates for various times (30 and 60 min).

Processing for Combing

Day 1
Wash cells with PBS, trypsinize, add 5 ml of media to plate and collect the cells. Spin at 1200 rpm for 4 min. Remove media and resuspend in 1 ml of ice-cold PBS. Determine the cell numbers and then dilute cells to ~ 200 cells to 400/µl.

If possible limit the time cells sit on ice. This limited time on ice may help in obtaining a bead of cell solution along the top of the slide. If they are kept on ice please keep them on less than ½ hour (longer may work, but we haven't tried). I have left the cells up to 1hr on ice and the fibers still looked good.

Slides should be made in an area with limited overhead light exposure. Take 2 µl of cells and draw line across slide (**Silane-Prep slides, S4651-72EA from Sigma**), just below label (hold pipettor at ~ 45° angle). Let cells dry to tackiness (shorter for dry days and longer for humid days). Tackiness, in my view, is when the line of cells is almost opaque just before they begin to

really dry out. This takes about 6-10 min (usually around 8 min) in our lab. After this the line will start to dry completely and will turn white from the SDS drying. In my experience, it is better to error on the side of having the line of cells on the "wet" side rather than the "dry" side. Either should still give you fibers but the spreading of the fibers will be affected. This step is important for good spreading and requires careful attention to the drying of the solution on the slides and the proper timing of when you draw your lines of cells on the slide. Also, you can make multiple lines of the cells on each slide, which will give you a more fibers/slide and have to do less staining. (I currently do 3 lines on the top half of the slide with the first being just below the "Starforst" written on the slide.) I usually make 5 slides/condition.

When just dry, overlay cells with 15 µl of spreading (lysis) buffer per each line of cells, but DO NOT let pipette tip touch the slide (if you do, then every place you touch the slide, you will see what looks like the grand canyon after the immunofluorescence staining step). Wait for 10 min. Tilt slide at 25° angle against rack for microcentrifuge tubes (bottom part of label lines up with edge of tube rack). Let the buffer run to the bottom of the slide. If needed, add some more solution to get the drop to the bottom. Whether the drop goes fast or slow down the slide does not seem to affect the fibers. The slides can then be bumped off the rack so that they lie flat. Although, Paul says that some people have left them tilted to dry and still get good fibers.

Lysis Solution:

2 mL	1M Tris pH 7.4
1 mL	0.5 M EDTA
0.5 mL	10% SDS

ddH_20 to 10 mL

Allow spreads to air dry. Minimum of 4 h from the time the last slides was made. 4 to 12 hrs is good for the drying time. However, do not let them dry overnight. If you allow them to dry overnight, you will have a lot of "relaxed" DNA, but very few straight and aligned DNA fibers.

Fix in 3:1 methanol: acetic acid for 2 min - preferably under a hood (If you do this step on a bench top, then you will smell like a salt and vinegar potato chip for the remainder of the day). Let the slides dry overnight in an area with limited light exposure or cover slides with tin foil tent.

Day 2

Place the slides in a glass slide carrier. Place slides in -20 °C for a minimum of 24 h. This step improves the resolution or "crispness" of the image.

Day 3

Before the treated with HCl, prepare "humidified chambers" by putting water in tip boxes, and placing the boxes in the 37 °C incubator.

Treat slides with freshly prepared 2.5 M HCl for 30 min (coplin jar).

HCl Solution:

20.6 mL HCl

dd$H_2$0 to 100 mL

Wash 1x with PBS plus Tween (PBS + 0.1% Tween (final concentration)), then 2x with PBS for 3 min each @ RT

Block in 5% BSA in PBS for 30 min at "RT" (place the slides in the pre-warmed tip boxes and apply 2 ml of 3% BSA in PBS directly to the slide).

After blocking, remove the blocking solution and blot the slide on a paper towel (tap the slide on the towel!) to remove excess blocking solution.

Incubate with 140 µl of 1° antibodies (diluted in 5% BSA in PBS + 0.1% Tween) for 1 h at RT
Drip 1° antibody solution across length of slide, placing more towards the top of the slide where the fibers are located. "Coverslip" the slides using pre-cut pieces of the plastic used to bag Western blots, then place in tip box. Make sure you cover the slides up with the lid.

1° antibodies = mouse α-BrdU (1:500), rat α-BrdU (1:500). Antibodies can be obtained from **Becton Dickinson *(347580) and Accurate Chemical and Scientific Corporation (OBT0030)**, respectively.

Place slides in stringency buffer for **10-15** min (stringency buffer can be made 2 to 3 days ahead of time). Stringency buffer will reduce non-specific binding. I currently incubate for 13 min but this step should be determined empirically as it is important to reduce cross-reactivity but not diminish the continuity of signal of the DNA fiber tracks. You have to find a fine balance between the two to get good staining.

Stringency Buffer:

1 mL 1M Tris pH 7.4
8 mL 5M NaCl
0.2 mL 10% Tween
0.2 mL 10% NP40

dd$H_2$0 to **100** mL

Wash slides 2x in PBS (3 min each @ RT)

Block again in 5% BSA in PBS for 30 min at "RT" (place the slides in boxes and apply 2 ml of 5% BSA in PBS directly to the slide). Note: you do not need to keep the tip box at 37 degrees during the wash steps, leave it at RT.

Incubate with 140 µl of 2° antibodies (diluted in 5% BSA in PBS + 0.1% Tween) for 30 min at RT.

2° antibodies = Alexafluor 594-conjugated rabbit α-mouse (1:1000), Alexafluor 488-conjugated chicken α-rat (1:750).

These antibodies can be obtained from Invitrogen (rabbit anti mouse (A-11062), chicken anti-rat (A-21470).

Wash 1x with PBS plus Tween (PBS + .1% Tween (final concentration)), then 2x with PBS. for 3 min each @ RT

Block again in 5% **BSA** in PBS for 30 min at "RT".

Incubate with 140 µl of 3° antibodies (diluted in 5% BSA in PBS + 0.1% Tween) for 30 min at RT.

3° antibodies = Alexafluor 594-conjugated goat α-rabbit (1:1000), Alexafluor 488-conjugated goat α-chicken (1:750).

These antibodies can be obtained from Invitrogen (goat anti rabbit (A-11037), goat anti chicken (A-11039)).

Wash 1x with PBS plus Tween (PBS + .1% Tween (final concentration)), then 2x with PBS for 3 min each @ RT

Dehydrate the slides with EtOH series (70%, 90% and 100%). 2 min each. (This step is optional.)

Coverslip with 24 x 60 mm coverslips using 50 µl of antifade from Invitrogen (**P36930**)

Additional Methods to try if this method does not work for you.
DNA Fiber Analysis: Mind the Gap! Quinet A., Carvajal-Maldonado D., Lemacon D., Vindigni A. (2017) *Methods in Enzymology*, 591 , pp. 55-82.