CO-FISH protocol

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based on protocol from de Lange lab

BrdU/C incorporation and Metaphase spreads

- Seed cells in 6 or 10 cm dish
- 16-20 hours before experiment, remove medium and add medium containing 5'-BrdU:5'- BrdC (1:1000 dilution of stock containing 7.5 mM BrdU + 2.5 mM BrdC).
- Keep cells covered and reduce their exposure to light as much as possible.
- 2.5 h before harvesting add Colcemid (KaryoMax, Life Technologies) to a final concentration of 0.1 µg/ml (1:100 dilution of 10 µg/ml stock) to accumulate mitotic cells
- Harvesting: shake the culture flask vigorously by tapping on the bench, take off medium and save in falcon. Wash cells once in PBS, then trypsinize shortly and collect the rest of the cells (not all cells need to be collected!)
- Count cells
- Pellet cells for 5 min at 1000 rpm
- Resuspend cells in 2 ml medium, then slowly add 6 ml prewarmed 75 mM KCl solution while tapping the falcon to ensure constant mixture
- incubate the cells in the KCl solution at 37 °C for 40 min (invert the tube every 10 minute to keep the cells suspended)
- pellet cells for 5 min at 1000 rpm
- decant supernatant and Resuspend cells in the small volume KCl that's left by tapping the tube
- slowly and dropwise add ice-cold MeOH-acetic acid (3:1); few µl first, then more and more until ~5 ml, mix continuously by tapping or vortexing
- cells can be kept at this stage at 4 °C for several weeks to months
- before dropping cells: pellet cells and resuspend in fresh MeOH-acetic acid
- manual dropping
  Concentration: 1*10^6 cells/ml
  drop ~40-60 µl on a 12 well coverslip or on a glass slide
  put coverslip on wet paper towel on heat block at 42°C for 1 min
  let dry O/N in the dark

Degradation of newly synthesized strand

- Rehydrate slides in PBS for 5 min at RT
- Treat slides with 0.5 mg/ml RNase A in PBS for 10 min at 37°C (50 µl drops on parafilm in wet chamber)
- Stain slides with 0.5 µg/ml Hoechst 33258 (Sigma) in 2x SSC for 15 min at RT (Hoechst Stock 1 mg/ml from Katharina in cell culture fridge → 1:2000 in 2X SSC)

- Place slides in a 12 well plate and add enough 2x SSC to cover the slides
  - Expose slides to 365 nm UV light at RT for 30 min (equivalent to $5.4x10^3$ J/m^2) → UV linker @AG Grummt: setting a) 5400 x 100 µJ/m^2 (→ takes only ~5 min) → setting b) set time to 30 min (do time series: 15, 25 and 30 min)
  - Digest the BrdU/BrdC-substituted DNA strands:
    ExoIII (Thermo, 200U/µl) final conc 10 U/µl in Exo III buffer, 50 µl per slide on parafilm in wet chamber for 10 min @ RT
  - Wash in PBS for 5 min
  - Dehydrate in ethanol series 70%, 95%, 100% at RT and air dry slides (slides can be stored at RT)

FISH

- Hybridize slides at RT 1.5 h with PNA probe **TelG-Cy3 1:1000 in hybridization buffer** (75% formamid pH 7.4, 0.1% BSA, 20 mmM NaCl, 20 mM Tris); 20 µl per slide on parafilm in wet chamber
- Wash quickly in 70% formamide, 10 mM Tris pH 7.4
- Hybridize slides at RT 1.5 h with PNA probe **TelC-FAM 1:1000 in hybridization buffer** (75% formamid pH 7.4, 0.1% BSA, 20 mmM NaCl, 20 mM Tris); 30 µl per slide on parafilm in wet chamber
- Wash 2x 15 min in 70% formamide, 10 mM Tris pH 7.4
- Wash 1 min in 2X SSC
- Wash 1x 5 min in 0.1XSSC @ 55°C
- Wash 2x 5 min in 2X SSC, 0.05% Tween20
- Dehydrate slides in 100% EtOH
- Mount slides with Prolong + DAPI