Immunofluorescence on tobacco BY-2 suspension cells (FA fix)

Cells should be happy and healthy. *total time needed*

PEI coat wells on microscope slide:

add 10 µl of 0.1% polyethyleneimine per well, incubate in moist chamber for ≥ 1 h. ('moist chamber' is a petridish with wet filter paper – keep bottom of slide dry by propping it up on toothpicks, straws, etc) let water evaporate before loading cells (the draft at the chemical hood helps to speed this up)

take approx. 300 µl cell culture (30 to 100 µl packed cells), add MSBG to 1200 µl, add 300 µl 20% PFA. (final conc. 4%)

incubate for 1 hr on "labquake" rotator. *1 h*

wash 3x with MSBG *1.5 h*

how to wash: spin 2 min @ 250 rpm w/ "soft stop" in Eppendorf microfuge (≈ 600 *x g*) – this may have to be repeated after the tubes have been turned 180° to get all cells down; carefully remove supernatant w/ pasteur pipette – remember to discard into PFA waste; fill tube with fresh buffer; on rotator for ~5 minutes

have cells at ~3x packed volume in MSBG, add 1:4 volume 5x enzymes, incubate for **5** minutes (keep this volume « 500 µl so the following dilution/wash step is more efficient)

dilute by filling tube w/ MSBG, wash 2x with MSBG (do 'double spin' to ensure individual cells aren't lost in the supernatant) *2 h*

let cells settle onto PEI-coated multi-well slide (use 12µl/well for 12-well slide) for 20 to 30 minutes *2.5 h*

wash cells 1x with PBS

how to wash on the slide: carefully remove ~10 µl (i.e. less than you loaded) of the supernatant from the cells – pipette slowly, making sure that most cells stay put and **cells are always covered with buffer!** Don't ever let them dry out! If you see little humps on the buffer surface they are already starting to plasmolyze! Add another 10 µl buffer (PBS) to the cells and incubate for 5 minutes.

add ~10 µl 1% Nonidet (in PBS) for 15 minutes

wash 3x with PBS *3 h*

add primary antibody (in PBS) for 0.5 to 1 h [for MTs use "4A1" anti-tubulin 1:40] *4 h*

wash 3x with PBS *4.5 h*

add secondary antibody (in PBS) for 1 h [for MTs use Alexa-a-mouse at 1:400] *5.5 h*

wash (at least) 3x with PBS *6 h*

remove most of the supernatant (but leave cells covered!), add 1-2 µl PBS/glycerol (1:1), view [fluorescence is stable for ≥ 1 week if coveslip is sealed and slide kept in dark (RT)]

Buffers etc:

20% paraformaldehyde (PFA):
— paraformaldehyde is a fixative and should be handled with gloves only —
weigh out enough PFA for final conc 20 mg/ml PFA (will need 300 μl per sample)
transfer from weighing boat into test tube (bring cap along)
add H_20 (can use this water to rinse PFA rest from weighing boat)
add 2 drops of 1M NaOH (per ml)
bring water bath to boil in Erlenmayer
in the hood place test tube into hot water bath and swirl around until PFA is in solution

MSBG (Microtubule Stabilizing Buffer w/ Glycerol):

100 mM PIPES, pH7.0	1.5 g (pH with ~1 ml 10M NaOH)*
2 mM MgCl2	2 ml 100 mM
5 mM EGTA	5 ml 100 mM (pH8.0)
2 % Glycerol	2 ml
	—> H2O to 100 ml

* PIPES doesn't go into solution below pH6..6.5. Add NaOH until solution starts to clear up, then add MgCl2 and EGTA **before** you adjust pH to 7.

PBS (Phosphate Buffered Saline):
(see Maniatis)
Keep it sterile so no bugs will grow.

5x Enzyme Mix
use cellulase (Worthington) and pectinase (Fluka) at a final concentration of 1%
to prepare 5x mix weigh out ~10 mg each of the enzymes and add ~200 μl MSBG
always prepare enzymes fresh (usually while washing PFA out)