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

cells should be happy and healthy. *total time needed*
take approx. 700 µl cell culture, add 10 µl glutaraldehyde (70%) **in the hood**
incubate for 15 min on "labquake" rotator. *0.25 h*

wash 3x with PBS **(in the hood)** *0.75 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 GA waste; fill tube with fresh buffer; on rotator for ~5 minutes

add washed cells to 0.1% $NaBH_4$ (sodium borohydride) in PBS an let sit over night **in the hood**

$NaBH_4$ has to be prepared fresh every time (best as the cells are being fixed). This step is supposed to remove the glutaraldehyde fluorescence

next morning:
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)

remove supernatant and discard (sink), transfer 400 µl cells w/ wide-bore tip to microfuge tube

have cells at ~3x packed volume in PBS, add 1:4 volume (i.e. 100 µl) 5x enzymes, incubate for **15 minutes** (keep this volume « 500 µl so the following dilution/wash step is more efficient)

dilute by filling tube w/ PBS, wash 2x with PBS (do 'double spin' to ensure individual cells aren't lost in the supernatant) *1h*

let cells settle onto PEI-coated multi-well slide (use 12µl/well for 12-well slide) for 20 to 30 minutes *1.5 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 *2 h*
add primary antibody (in PBS) for 0.5 to 1 h [for MTs use "4A1" anti-tubulin 1:40] *3h*
wash 3x with PBS *3.5 h*
add secondary antibody (in PBS) for 1 h [for MTs use Alexa-a-mouse at 1:400] *4.5 h*
wash (at least) 3x with PBS *5 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:

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)