There's several parts: 1) Fix-gest and perm, 2) hybridize, 3) wash and image.

Note on spinning cells: if you want to improve/optimize this protocol, an easy thing to consider would be centrifugation speed and times. It only takes a few hundred rcf for a few minutes to pellet yeast, depending on the viscosity, cell density, volume. Keep in mind that cells in different physical stages (digested or not) will pellet differently. This all could be improved, but the below seems to work.

The digest volume, enzyme, and time are not optimized as they are the first set of conditions that worked consistently. If you want more consistent spheroplasting, consider dialing down the enzyme and going longer as suggested here.

**Fixing, digest**

**Traditional formaldehyde, lyticase, ethanol method**

**Buffer B** - 1.2M sorbitol and 100mM KHP04, pH 7.4 works - Combine Xml monobasic and Xml dibasic potassium phosphate, add Xg sorbitol, and bring up to volume with nuclease free water. Make sure dissolved, and autoclave. Keep it on ice for the following.

1. Grow cells to exponential phase. In Nlim+Pro, I aim for 5e6 cells per ml.
2. Combine 45ml cells and 5ml 37% formaldehyde (aka formalin, methanol is OK). Make sure the formaldehyde isn't precipitating on the bottom. Fixation is not optimized.
3. Let incubate at RT 45min. Invert to mix at the very beginning and during the first 10 minutes. Inversion every 10 min works okay too, also a rocker obviously works.
4. Spin down cells in a swinging bucket centrifuge, 716g RT 5min.
5. Decant supernatant into waste. There shouldn't be more than ~200ul left at the bottom.
6. Use 1ml of cold Buffer B to transfer cells to a new tube. (You can pool multiple tubes at this stage, if need be)
7. Spin tube 3min at 3000g RT. Aspirate supernatant, resuspend gently with 1ml cold Buffer B.
8. Spin tube 3min at 3000g RT. Aspirate supernatant, resuspend gently with 1ml cold Buffer B.
9. Spin tube 3min at 3000g RT. Aspirate supernatant, resuspend gently with 1ml cold Buffer B. Put on ice or 4C.
10. Count cells, using hemacytometer or coulter. Hemacytometer is a good idea just to check that everything looks okay and budding (but fixed) as we would expect.
11. Make fresh spheroplasting buffer. 1ml is 898ul Buffer B, 100ul 200mM VRC, 2ul Beta-mercaptoethanol. Pre-warm to 37C.
12. Spin down 10 to 100 e6 fixed cells, 3min at 3000g RT.
13. Aspirate super, and resuspend in warm spheroplasting buffer (10ul per 1e6 cells, so 1ml is 100e6 cells).
14. Within a few minutes, add 1U lyticase (from 25U/ul stock in 1xPBS from Sigma L2524) per 1e6 cells, mix with tip.
15. After about 4 minutes, take about 1-2ul samples onto slides and view with coverslip under phase contrast objectives. You should see plenty of cells, mostly phase bright. Look around the whole sample, quickly. Over digested cells look like that, over digested, dark, and fragmenting. Then they'll start to look like ghosts when they're way over digested. You want to aim for about 2/3 phase dark cells. This will take optimization and fisher's luck, sorry.
16. When digestion is good enough, put the digest tubes into wet ice for 1min to slow the reaction.
17. Centrifuge 3min 800g RT.
18. Aspirate supernatant. Pellet is solid enough, but occupies lots of volume, so be careful. Resuspend gently in 1ml ice cold buffer B, using blue tips. Centrifuge 3min 800g RT.
19. Aspirate, resuspend more thoroughly but gently in ice cold buffer B, centrifuge 3min 800g RT.
20. Aspirate, resuspend in 1ml 70% ethanol, put into 4C at least overnight. Recommend -20C for long term storage before hybridizations, 4C seems to work for a couple of weeks.

**Hybridization**

Hybridization buffer - 2x SSC, 10% dextran sulfate w/v, 2mM VRC, 200ng/ml BSA, 10% formamide

Wash buffer - 10% formamide, 2x SSC, diluted with nuclease-free water

GLOX - 1ml is (880ul nuclease-free water, 10ul 1M Tris (pH ~7.4 works), 10ul 40% glucose, 100ul 20x SSC)

2x GLOXzymes - 100ul GLOX, add 2ul of glucose oxidase and 2ul of catalase. Make fresh? (Stale has not been not tested)

Regarding probe concentration, you should probably do a dilution series to estimate a good dilution of the probe stock for your conditions. In NLim+Pro with ::GFP Stellaris, I get good results with 1:100 and 1:300, so I use 1:100 dilution of the probe stock.

1. Spin down about 10-20e6 cells, as estimated from input of lyticase digestion, at 800g RT for 3-5min.
2. Resuspend in 100ul hybridization buffer, from a thawed (RT) aliquot of such. Make sure this has had time to warm up to RT.
Wash and image

1. Add 1ml wash buffer to the tube. Put back on 37°C roller drum 30 min.
2. Spin 10min 800g RT, aspirate supernatant.
3. Gently resuspend with 1ml wash buffer, add DAPI (5 ng/ml final). On roller 37°C for 30min.
4. Spin 5min 800g RT, aspirate supernatant.
5. Resuspend 1ml 2x SSC. Spin 5min 800g RT, aspirate supernatant.
6. Resuspend in GLOX buffer w/o enzymes, put on and let settle onto a poly-L treated coverslip for 20 minutes.
7. Before mounting, add equal volume 2x GLOXzymes.
8. Flip onto slide, press flat with a kimwipe to get excess, and nail polish it on there.