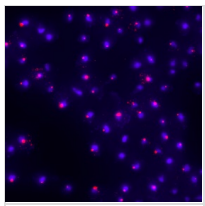

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May 17, 2022

Fixation of yeast cells for RNA-FISH V.2

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protocol .

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This protocol is for *S. cerevisiae* and is a modification of the one developed and used by Arjun Raj and his colleagues for:

Raj A, van den Bogaard P, Rifkin SA, van Oudenaarden A, Tyagi S. Imaging individual mRNA molecules using multiple singly labeled probes. Nature Methods. 2008 Oct;5(10):877–9.

The critical change is in the digestion of the cell wall with Zymolyase (or Lyticase if you prefer). Insufficient digestion will prevent full probe penetration and will result in a misleading outcome that transcription appears to be 'off' in some fraction of your cells, even if all are transcribing.

For the rest of the RNA-FISH protocol and for more details, please see Arjun's amazing website: https://sites.google.com/site/singlemoleculernafish.

Lenny Teytelman, Arjun Raj 2022. Fixation of yeast cells for RNA-FISH.
protocols.io
https://protocols.io/view/fixation-of-yeast-cells-for-rna-fish-b9ifr4bn
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Cell Wall Digestion Notes

It will take a bit of practice to get comfortable with the Zymolyase digestion and what to check for. If you are using a phase contrast microscope for the first time and not sure what you are looking for, do a 10x digestion control as Daniel Pollard advises. As a rule of thumb, I wait for 80%-90% of the cells to turn dark (Figure 1).

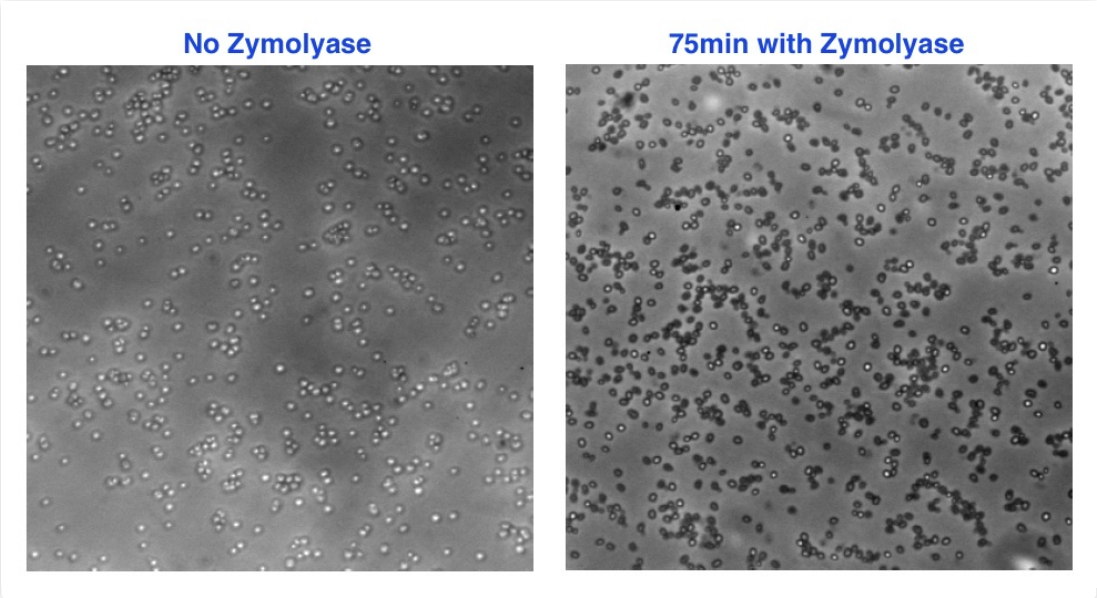

Figure 1. Phase contrast of fixed wild type cells, comparing no-Zymolyase to cells digested with Zymolyase for 75 minutes.

Under-digestion will block probe penetration into the cell. Over-digestion is also bad - cells deform, images come out very poor. There is a bit of trial-and-error required for this.

Controls for RNA-FISH

Just as with PCR, it is important to have controls for single-cell RNA-FISH.

Ideally, the control that will give the same single-cell mRNA distribution, regardless of the condition. Definitely not Actin or any other highly-expressed constitutive gene as that will result in fireballs and it will be impossible to quantify anything. Try using an inducible gene such as MET5 or some other gene with very low basal level. The truth is that virtually nothing is completely 'off'. All genes that I have ever looked at have a low basal level. That is perfect; check that the mRNA counts and spot intensities are consistent between conditions.

This is not simply about controlling for digestion and permeability. Many other biases can creep in. I was doing 30-second time course experiments to look at Pol II dynamics of transcription, fixing 10 induced samples at intervals, up to 15 minutes post-induction. While imaging the 10 samples, mRNA can degrade, anti-bleach buffer can lose effectiveness, the probe dye may quench. It is very reassuring to quickly image each sample with the control probe and simply check for consistency.

The story behind this protocol

It took me a year and a half of my postdoc to figure out that there was a problem with the way we were doing RNA-FISH and to understand how to change the Zymolyase step to get the probes in reliably.Below is the brief history of this effort. This experience is the motivation that inspired us to create protocols.io, starting in 2012.

I was trying to apply the RNA-FISH to Fred Winston's cryptic transcription mutants. Everything worked beautifully, but in the mutant, in contrast to the published northern blots, I saw zero spots (Figure 2).

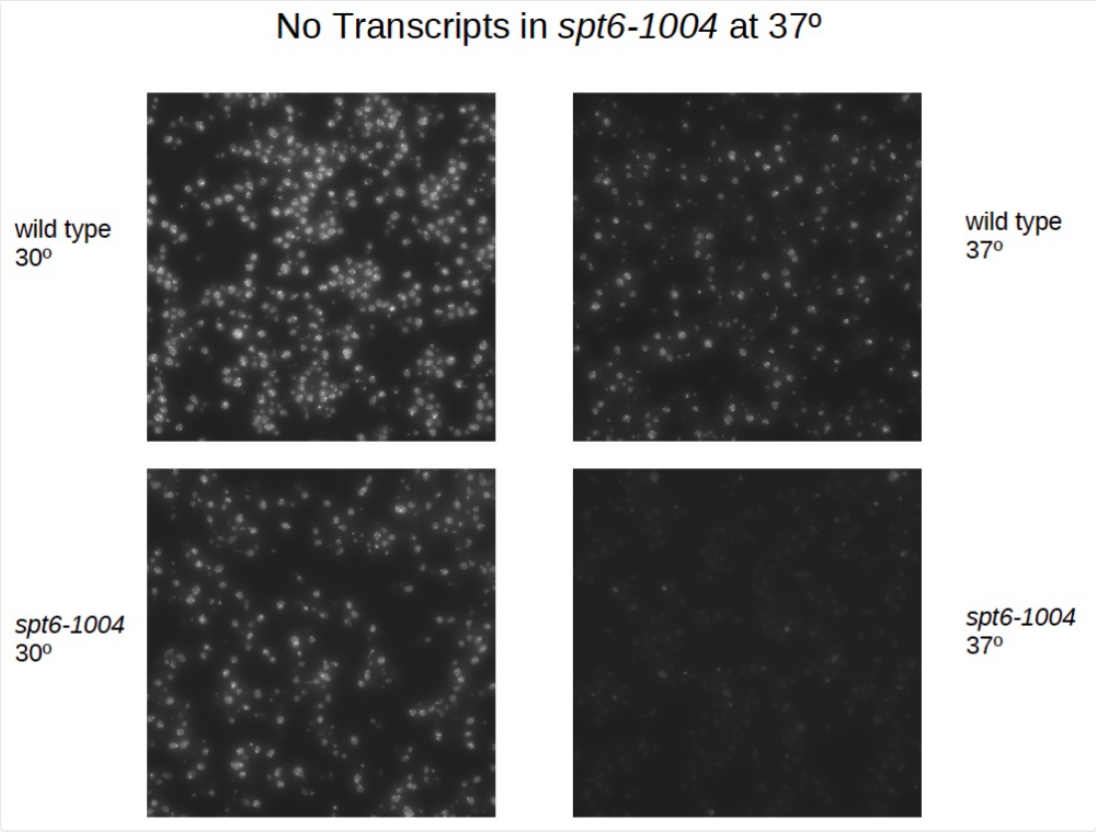

Figure 2. RNA-FISH for *FLO8* in wild type and mutant strains at permissive and restrictive temperatures.

After a year in the lab, I gave a group meeting where I mentioned that I saw no RNA signal in the mutant. A labmate then quipped, 'This wouldn't be the first time that a published result was wrong.' (I had spent months trying to reproduce Fred Winston's conditions to figure out what I am doing wrong and why I am not seeing their transcripts. I was encouraged to switch to another project.

I persevered because of a gut feeling that this needs to be solved. I split the cryptic mutant (spt6) sample in half and learned to do a Northern. That's when I established that the RNA is transcribed, but we are not seeing it under the scope (Figure 3; many thans to Dan Spatt for teaching me Northern Blotting).

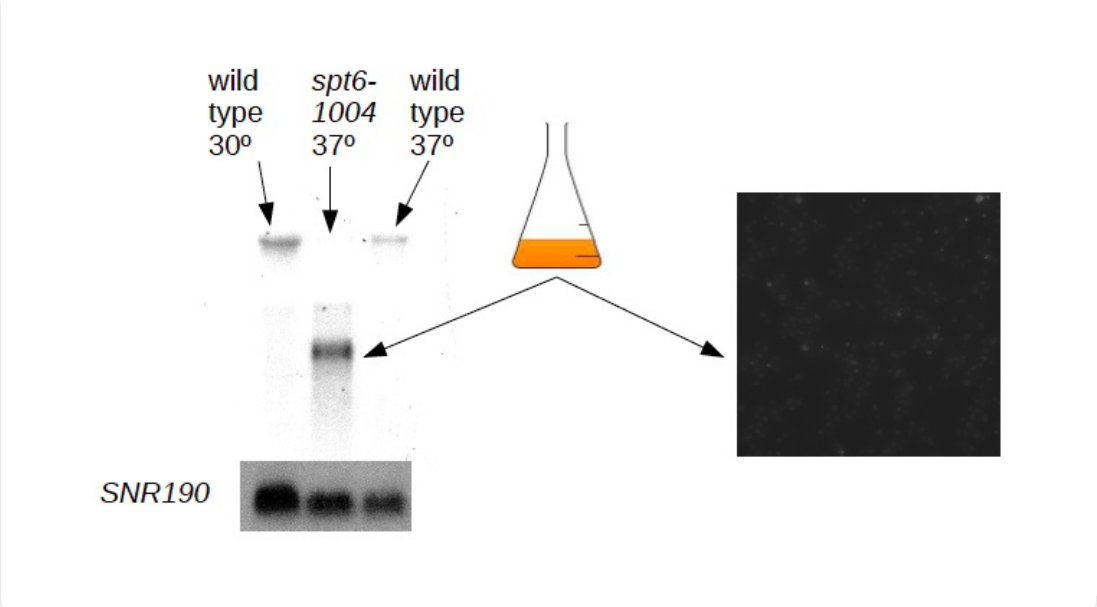

Figure 3. Cryptic *FLO8* is detectable by Northern Blot but not by RNA-FISH.

The above established that there was some problem with the RNA-FISH in the mutant strain at the restrictive temperature. To test if the issue was just with the *FLO8* or broader, I imaged for a highly-expressed constitutative PMA1 gene. It was also missing from the restrictive temperature in the *spt6* mutant (Figure 4).

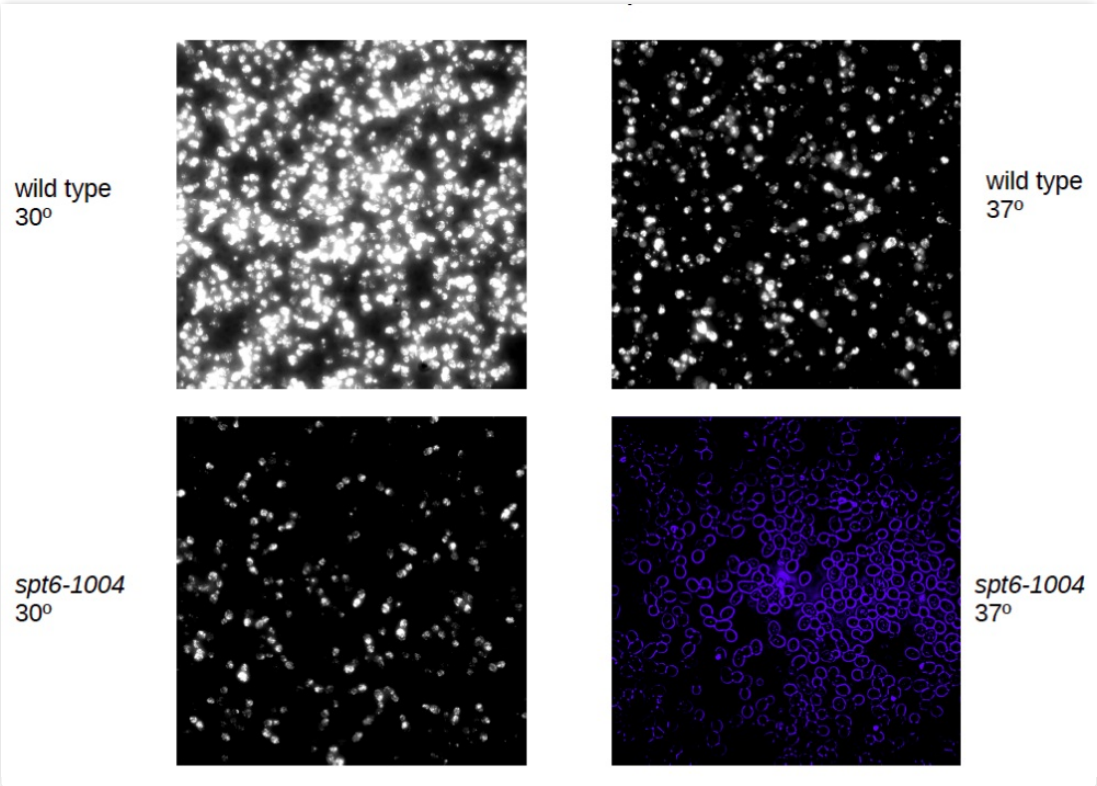

Figure 4. RNA-FISH for *PMA1* in wild type and mutant strains at permissive and restrictive temperatures.

That was progress, but the concern was that this RNA-FISH issue was some peculiar problem specific to the mutant strain. My hypothesis at that point was that the mutant is nearly-dead with thousands of genes miss-transcribed, and its structural integrity must be damaged with the RNA leaking out.

I started a new project, but the spt6 result continued to bother me. I kept analyzing my data and thinking about them, and I noticed that the DAPI signal was also lower in the mutants (Figure 5). This is when another labmate said that the problem was probe penetration rather than the RNA leaking out - chromosomes could not be escaping from the nucleus.

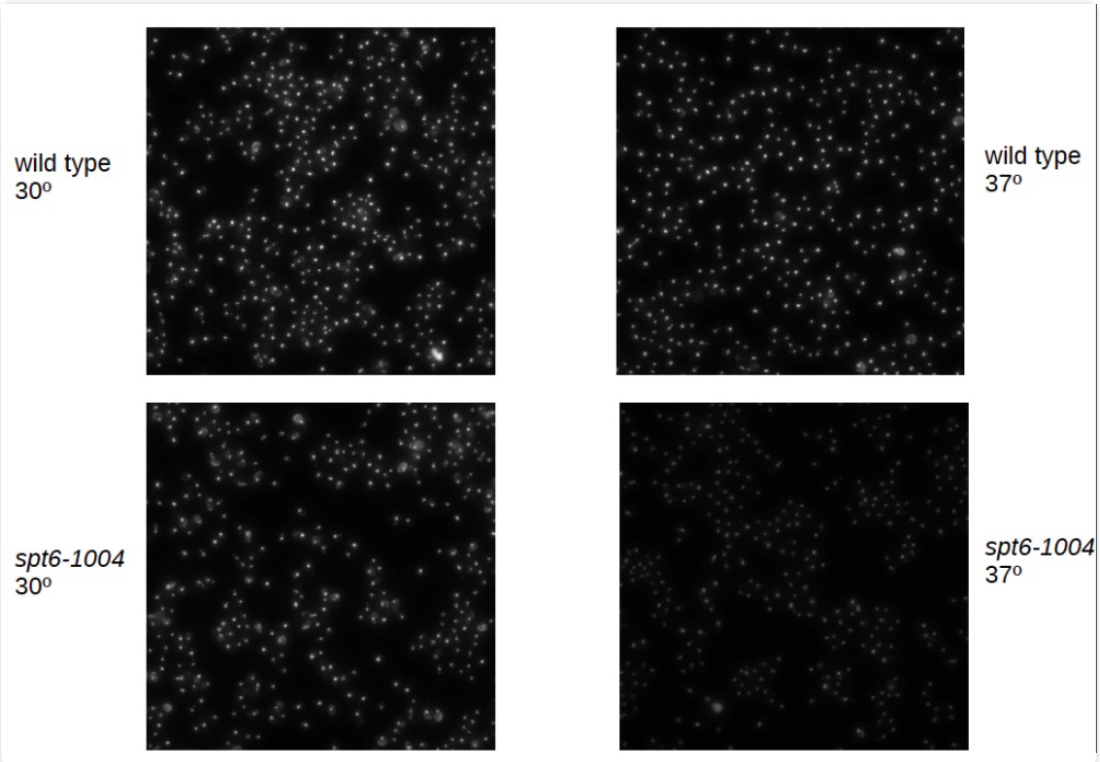

Figure 5. DAPI imaging in wild type and mutant strains at permissive and restrictive temperatures.

Once I knew that the problem is penetration, I talked to people in the Winston lab and found out that a student there was having issues with nucleosome preps in the *spt6* mutant in pombe, because of the resistance to zymolyase. I also learned from a student of David Botstein that zymolyase could play a big role in the RNA-FISH. I did SDS-OD experiments and showed that the mutant takes twice as long to digest with zymolyase (Figure 6).

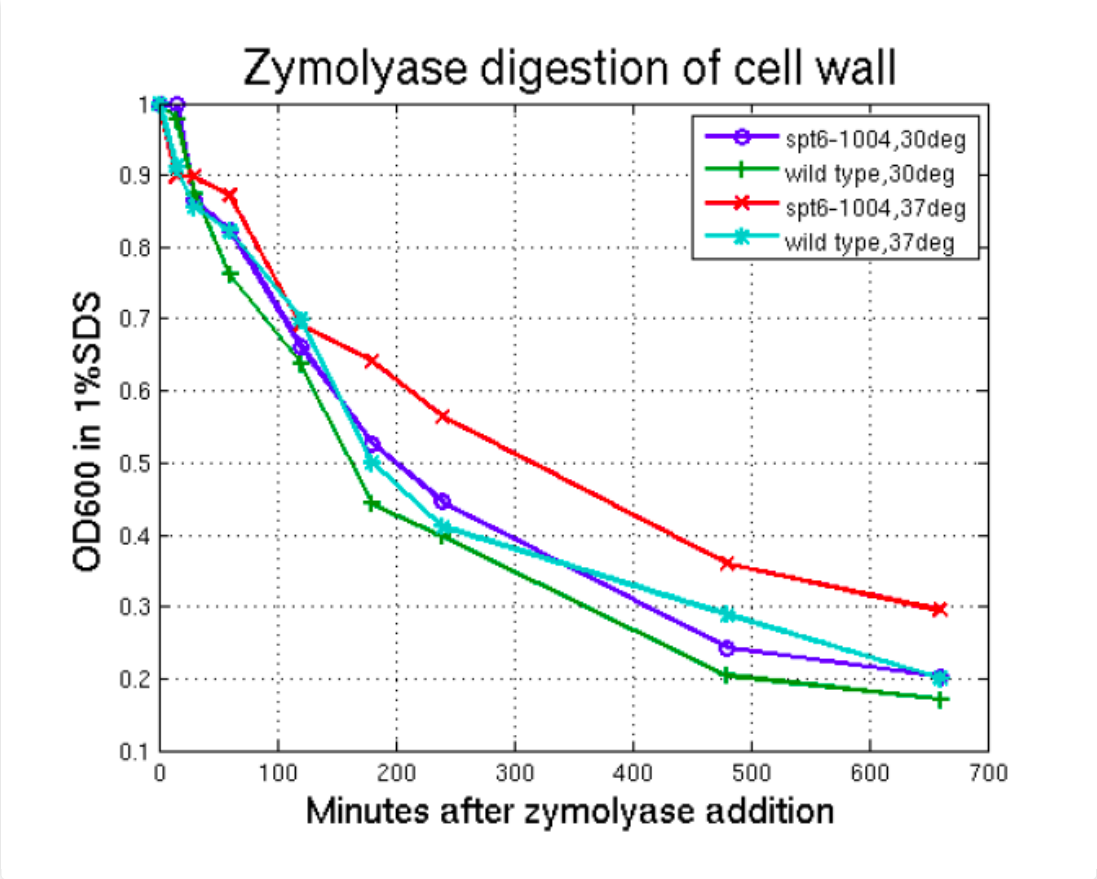

Figure 6. Resistance of *spt6* mutants at the restrictive temperature to Zymolyase cell wall digestion.

I figured out how to do the phase contrast check of the digestion. I did titrations on the mutant and finally saw signal for the first time.

All of the above was important, but the part that I am really proud of is in asking whether the zymolyase step is a problem in the wild type as well. It was possible that the *spt6* mutant is a strange case - an exception. I spent a few more weeks, doing titrations of zymolyase concentrations, digestion times, and testing as many genes as I could, to show that this is not a strain or gene-specific issue (Figure 7).

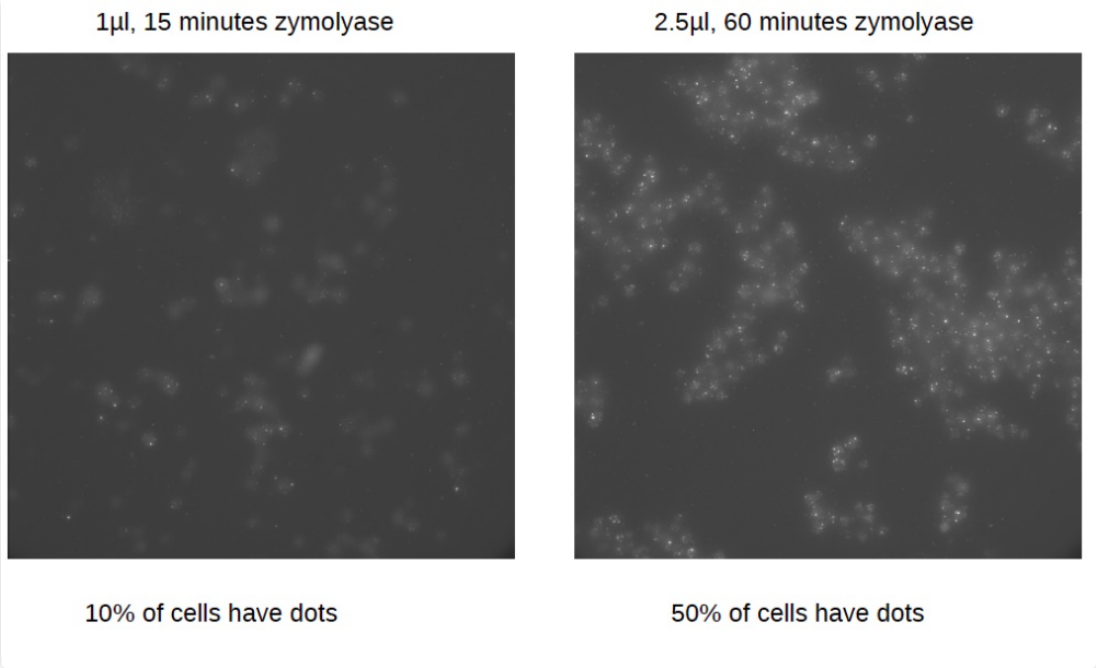

Figure 7. Standard Zymolyase treatment for wild type strains is insufficient for probe entry. RNA-FISH to the *FLO8* gene.

We were under-digesting by an order of magnitude. I gave a group meeting on this, warning people to do controls and not simply assume that RNA-FISH is working. Just as with qPCR we use Actin or another constitutive control, it is important to use a control probe set in RNA-FISH to make sure that strain and growth conditions are not affecting the microscopy signal.

The absurd part is that all of the above work was a change of a single step of a previously-published protocol. As such, it's not a new technique but just a correction. There was no simple way of sharing this, so I got no credit for the year and a half of work. More importantly, everyone else using the technique was either getting misleading results or had to spend another 1-2 years rediscovering what I already knew. Hence protocols.io...

For RNA-FISH, we want the cells continuously in exponential phase, never reaching stationary, so do not do overnight inoculation as in the transformation protocol.

Inoculation and Growth

1 Around 10am, start a cell culture in a 50ml tube, using 10ml of CSM.

2 Grow for 8-10 hours in a shaker at 30 °C.
08:00:00

3 Measure OD in the evening and dilute into 250ml glass flasks, starting 45ml of CSM for overnight growth. Aiming for OD 0.2-0.4 around 10am the next morning.
16:00:00

> Better to dilute more and wait till the next morning than to over-grow; don't want to fix at OD>0.5.

Fixation

4 Transfer to 50ml falcon tubes.

5 Add 5ml of Formaldehyde, invert a few times, set at benchtop for 45min.
00:45:00

> Optional: Transfer to gentle rocking overnight at 4C for 18-24 hours. (**This is NOT recommended per Anne Dodson, Marc Sherman, Lenny Teytelman.**)

6 Spin down at 3,000rpm for 4min.
00:04:00

Formaldehyde removal

7 Decant and wash by pipetting with 1ml of ice-cold BufferB. Transfer to 1.5ml tubes. (wash 1/2)

8 Spin down at 3,000rpm for 4min. (wash 1/2)
00:04:00

9 Wash once more with 1ml ice-cold BufferB. (wash 2/2)

10 Spin down at 3,000rpm for 4min. (wash 2/2)

00:04:00

11 Decant and resuspend in 1ml of BufferB.

Digestion

12 Add 2.5ul of Zymolyase and digest at 30 °C until most of the cells turn dark when checked by phase contrast microscope. (See more in the Guidelines)

In healthy W303 and S288C strains, Zymolyase digestion usually takes 45-90 minutes, depending on the cell concentration.

In some conditions and mutants, the required digestion time can double due to a much thicker cell wall.

NOTE (per Dan Pollard): if you're not sure what zymolyase treatment looks like, do a 10X positive control for 3 hours. Marc Sherman adds: if the positive control cells don't look *markedly* different (essentially black/opaque) from untreated control, try zymolyase treatment immediately after fixation.

13 Drop 1 µL of the cells onto a glass slide, **do not cover** (pressure makes it look like they digested). Use a 20x scope with a phase contrast ring.

14 Check digestion progress every 30 minutes. The cells turn black/grey from white/shiny. Aim for 80%-90% of the cells within the view to be digested. **Do not overdigest.**

00:30:00

15 Wash with 1ml ice-cold BufferB, spinning 5-6min at **2,000**rpm. (wash 1/2)

00:05:00

The cells are fragile now due to the digested cell wall. Be careful to spin at 2,000rpm as faster speed will deform and burst the cells.

16 Decant and wash again with 1ml ice-cold BufferB, spinning 5-6min at **2,000**rpm. (wash 2/2)

00:05:00

17 Resuspend in 1ml of 70% Ethanol overnight at 4 °C.

16:00:00