fixgestperm: paraformaldehyde fixation, lyticase digest, etOH permabilization

This protocol is suitable for fixing and permeabilizing yeast cells for doing hybridization-based cytometry. It has been optimized for flow cytometry with single molecule FISH probes. The protocol is based on McClean's and Botstein's mRNA FISH protocols, but departs from this in setting an exhaustive digest and optimizing the preliminary fixation with PFA.

**Sampling**

1. Filter approximately $5 \times 10^7 - 5 \times 10^8$ cells onto 25mm nylon filter. Put the filter in a 1.5ml eppendorf. Flash freeze in liquid nitrogen, as for RNA analysis. Samples can be stored at -80°C until ready for processing.

**Fixation**

1. Resuspend cells in 1mL 4% paraformaldehyde by vortexing: add 1ml, vortex about 10-15s in pulses with inversion, then immediately remove the filter using that pipette tip.

2. Incubate cells at RT in dark for 2 hours.
   
   *(note: Growth conditions affect fixation time. 1 hour is sufficient to allow N-starved (0.12 vol/hr) chemostat samples to survive Quantigene hybridization (with detergents), but 3 hours is necessary to get samples 30 min after an upshift to survive polyA hybridization. Also, YPD is more "fragile" than Nilm-Pro grown cells. You should do a time-series to optimize for the growth state you're interested in, then check that it isn't changing too much during your experiment. )*

3. Stop fixation by adding 200uL of 2.5M glycine and invert to mix. Immediately spin down at 3000g for 3 minutes RT.
   
   *(notes: This seems to be important, to stop the fix before spinning)*

4. Aspirate supernatant to non-hazardous waste, resuspend in 1x PBS. Spin 3000g 4 minutes RT.

5. Resuspend again with 1ml 1x PBS, spin 4 minutes at 3000g.

6. Aspirate supernatant to non-hazardous waste, resuspend in 1mL cold Buffer B. Invert to mix, let sit on ice.
   
   *(note: cells can be counted at this point.)*

**Digestion**

1. Spin down ~1e7 cells at 3000g 4 minutes RT.

2. Aspirate, resuspend in fresh spheroplasting buffer. Incubate at 37°C for one hour (we use a waterbath).
   
   *(note: To monitor digest, you can put cells under phase contrast microscopy. They should appear a bit darker, but shouldn't be fragmented, "ghost-like", or completely phase-black. To see what this looks like, try a time-series digest of under-fixed cells (i.e. 0.5 hours of fixation) to see the changes. Changes should start at 15 +/- 5 min.)*

3. Invert the digestion several times, then spin 1200g for 3min RT.

4. There should be a giant soft pellet filling ~200ul of the tube.
   
   *(note: if there is no pellet, it is most likely that you have underfixed with paraformaldehyde and thus overdigested, if the pellet is very small and tidy (looks like normal yeast pellet) then you may have overfixed and thus underdigested).*

   Aspirate as much of the supernatant as you can safely and reasonably take. Resuspend pellet with 1mL of cold Buffer B, mix with pipetting. Spin 5min 1200g.

5. Repeat aspiration, resuspension, and spins two more times.
   
   *( note: A 10ul sample can be taken following the last wash to count cells. )

6. Aspirate as much of the supernatant as you can safely remove. Resuspend pellet with 1mL of RT 80% etOH and incubate at 4C at least
overnight (16+ hours).

These cells should now be usable in various hybridizations. They should be tough enough to survive Quantigene’s detergents, but still have some morphology retained (at least nucleus vs cytoplasm). They should show a roughly log-normal distribution of polyA staining signal.

**Reagents**

4% paraformaldehyde

Mix 1 volume of 16% paraformaldehyde (EMS RT 15710, stored in ampules) with 3 volumes of 1x PBS.

*Solution should be made fresh for each series of fixations and then discarded.*

**Buffer B**

0.1M potassium phosphate, 1.2 M sorbitol, pH 7.5

*To make, combine:*

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1M KH$_2$PO$_4$</td>
<td>8ml</td>
</tr>
<tr>
<td>1M K$_2$HPO$_4$</td>
<td>41.5ml</td>
</tr>
<tr>
<td>2M sorbitol</td>
<td>30ml</td>
</tr>
</tbody>
</table>

*Solution seems to be stable indefinitely*

**Spheroblasting buffer**

*To make 1mL (scale up to make master mix) combine:*

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer B</td>
<td>898ul</td>
</tr>
<tr>
<td>200mM vanadyl ribonucleoside complex (NEB, denature freshly as in instructions)</td>
<td>100ul</td>
</tr>
<tr>
<td>14.3M Beta-mercaptoethanol (100%)</td>
<td>2ul</td>
</tr>
<tr>
<td>25U/ul lyticase in 1xPBS (Sigma, R/S bottle in 1x PBS and freeze aliquots -20C, thaw once)</td>
<td>4ul</td>
</tr>
</tbody>
</table>

*Make fresh just before use, mix well, keep at RT. Should probably be used within an hour.*