# Direct Synthesis of Gold Nanoparticles on Cysteine-rich Tags in Yeast Cells

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**Method Article**

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# Abstract

We developed a novel auto-nucleation suppressed mechanism (ANSM) for direct synthesis of EM-visible gold nanoparticles (AuNPs) on cysteine-rich tags (e.g., metallothionein) in cells for single-molecule detection with electron microscopy (it accompanies our Nature Method manuscript, Jiang et al. 2020 [1]). Both tagged-fusion proteins expressed in cells (*e.g.*bacteria, yeast and mammalian cells) and antigens stained with antibody-tag fusion proteins can be visualized by this protocol. Here we describe the typical protocols (both the chemical fixation and the high pressure freezing cases) developed for ANSM-based AuNP synthesis in yeast cells expressing metallothionein (MTn) tags (**Figure 1**). This approach should be useful for EM visualization of single-molecule in yeast cells, and easier adapted for bacterial cells.


# Introduction

# Reagents

EMMmedium[2], 2-mercaptoethanol (2-ME, Cat.0482-250ML, Amresco); $HAuCl_4$(Cat.4022-1G, Sigma); $NaBH_4$(Sigma); D-penicillamine (D-P) (Cat.P0147, TCI); 3,3'-dithiodipropionic acid (DTDPA)(Cat.D0947, TCI); PIPES(Sigma);25% glutaraldehyde solution (Cat.16220, Electron Microscopy Science); 10%glutaraldehyde in acetone (Cat.16530, Electron Microscopy Sciences);$OsO_4$ (Cat.19110, EMS) ; Uranyl acetate (Cat.22400, EMS); sorbitol (Sigma); glycine(Sigma); Triton X-100 (Sigma); Zymolyase-20T (Cat.320921, MP); Methanol; Acetone (>99.7%).

50mL polypropylene centrifuge tubes; Customized 3.05 mm x 0.66 mm specimen holders for HPF (Beijing Wulundes Biotech Ltd.); 3mmx0.16mm sapphire discs (Beijing Wulundes Biotech Ltd., or Engineering Office of M. Wohlwend GmbH);3mm 0.1mm/0.2mm,0.05mm/0.25mm, 0.025mm/0.275mm Aluminum carriers (Beijing Wulundes Biotech Ltd., Engineering Office of M. Wohlwend GmbH);2mL polypropylene screw cap microtubes (Cat. 81-0204, Biologix);1.5mL MaxyClear snaplock microtube (MCT150C, Axygen Scientific); Flat mold or flat-tip BEEM embedding capsule (Tedpella Inc.); 200 mesh hexagonal copper grid (G200HEX, Tedpella Inc.);SPI-Pon 812 resin (SPI Inc.);Formvar 15/95 resin (EMS);Foam cryobox; Forceps; Tweezers; Pasteur pipette; Liquid nitrogen ($LN_2$); 4L Dewar for $LN_2$;Alcohol lamp;2 pairs of 45-degree fine-tipped tweezers (Ted Pella, Inc.); Filter paper (Fisher Scientifi c, Whatman No. 1); Paper points (Ted Pella, Inc.);Fine syringe.

**Reagent Setup**:

**PBS-A buffer**: Dissolve $NaH_2PO_4$ (1.125 mM), $Na_2HPO_4$(3.867 mM), NaCl (100 mM) in 1L $ddH_2O$, adjust to pH 7.4.

**10X PBS buffer**: Dissolve $NaH_2PO_4$(100 mM), $K_2HPO_4$(18 mM), KCl (27 mM), NaCl (1.37 M) in 1 L $ddH_2O$.

**1x PBS buffer**: dilute 10X PBS to 1X PBS, and adjust pH to 7.4.

**0.2 M HEPES buffer**: Dissolve HEPES (0.2M) in 980mL $ddH_2O$, then add 10 mL of 100mM $MgCl_2$ and 10mL of 100mM $CaCl_2$ (final concentration 1mM) respectively, adjust pH to 7.4.

**50mM glycine in 0.2 M HEPES buffer**:Add 50mL of 1M glycine to 950 mL of 0.2M HEPES buffer (containing 1mM $CaCl_2$and 1mM $MgCl_2$).

**0.1M PIPES**: 0.1 M PIPES buffer with 1 mM $MgCl_2$, 1 mM $CaCl_2$and 0.1 M sorbitol (pH 6.8).

**10% UA in methanol**: 1 g uranyl acetate dissolved in 10 mL of methanol in a 15mL centrifuge tube wrap with aluminum foil, vortex to dissolve, filtered and store at -20°C.

**Fresh 10 mM $HAuCl_4$in $ddH_2O$**: make 10mM **$HAuCl_4$**in 1 mL of $ddH_2O$ in a 2mL polypropylene centrifuge tube, seal and wrap with aluminum foil, store at 4°C;

**1% $HAuCl_4$in acetone**: Dissolve 0.1g **$HAuCl_4$**in 1 mL of acetone in a 2mL microtube, seal and wrap with aluminum foil, store at -20°C.

**Fresh 10 mM $NaBH_4$ in acetone**: (1) Weight 0.04g $NaBH_4$ and sealed in a 2mL tube keep at -60°C; (2) Add 1mL of pre-cooled to -60 °C methanol to tube to make 1 M $NaBH_4$ in methanol; (3) Transfer 10 µL of 1 M $NaBH_4$ to 1 mL of cold acetone at

-60 °C to make 10 mM $NaBH_4$ in acetone. Freshly prepared for immediate use only.

**Fresh 100 mM $NaBH_4$ in $ddH_2O$**: (1) Weight 0.04 g $NaBH_4$ and sealed in a 2mL tube on ice; (2) Add 1 mL of $ddH_2O$ to tube to make 1 M $NaBH_4$ in $ddH_2O$; (3) Transfer 100 µL of 1 M $NaBH_4$ to 900 µL of $ddH_2O$ on ice to make 100 mM $NaBH_4$ in $ddH_2O$. Freshly prepared for immediate use only.

**1% $OsO_4$ + 0.1 % UA in acetone**: (1) Add 24.5 mL of acetone into a 50mL polypropylene centrifuge tube and keep the tube closed on ice; (2) Open an ampule with 0.25 g $OsO_4$ (wrap with several layers of paper towels); (3) Fetch 0.3 mL of acetone with a glass Pasteur pipette, add to the broken ampule and gently suck in and blow out to dissolve $OsO_4$, and then transfer the solution back to the 50mL tube. (3) Add 0.25 mL 10 % UA to the 50mL tube, and mix well; (4) Dispense the 1 % $OsO_4$+0.1 % UA in acetone into a 2mL polypropylene crew cap microtubes by a glass Pasteur pipette (about 1 mL per tube, the 1 mL position of tube can be pre-labelled); (5) Cap the tubes immediately and then immerse into $LN_2$ for freezing by holding the tube right-side up; (g) Store the frozen 1 % $OsO_4$+0.1 % UA fixative under $LN_2$ until ready to use.

# Equipment

Laminar flow cabinet; Biosafety level 2 cabinet;

High-pressure freezing machines (Wohlwend HPF Compact 01, Leica HPM 100);

Leica UC7/FC7 ultramicrotome (Leica Microsystem);

Leica AFS2 freeze-substitution machine;

FEI Spirit electron microscope (FEI Corp.);

4k x 4k 895 CCD camera (Gatan Corp.);

Plasma cleaner (Harrick Plasma);

Vacuum oven (20–200 °C);

UV-Vis spectrophotometer;

Dissecting microscope with fiber-optic light source;

# Procedure

1. ***S. pombe*cells and cell culture** (Timing 2 day)

The detailed information for preparing the stably fission yeast *Schizosaccharomyces pombe*(*S. pombe*)strains that expressing metallothionein (MTn) tags was described in our *Nature Method*manuscript (Jiang et al. 2020). The selected stable expression cells were cultured under selective growth conditions (*e.g.*liquid EMM medium[1]in the absence of thiamine at 30 °C).

1.1 Grow*S. pombe*cells in liquid EMM at 30 °C overnight;

1.2 Measure the OD600 reach to ~1.0 with a UV-Vis spectrophotometer;

1.3 Harvest 10 mL of the culture into a 15mL centrifuge tube for centrifugation at 2000 rpm for 2 min to obtain a cell pellet;

1.4 Disperse the cell pellet with 1mL of PBS buffer;

1.5 Transfer to a 1.5mL microtube for centrifugation at 2000 rpm for 2min to obtain a final cell pellet for further EM experiments.

2. **Oxidization and Fixation** (Timing 1-3 day)

The cell pellets obtained (from Step 1.5) could be further processed with Scheme 1 (“Cold MeOH”), or chemical fixation (Scheme 2), or cryo-fixation (*e.g.*, high pressure freezing (HPF)) (Scheme 3) before using for AuNP synthesis (Figure 1). The procedures for the three Schemes are described as following:

**Scheme 1(“Cold MeOH”): Cold methanol fixation** (Timing 1 h):

2.1 Fast inject 1 mL of -60 °C methanol into the microtube, disperse instantly by pipetting for 1 min (Note: or using -80 °C, -20 °C, 4 °C methanolinstead);

2.2 Centrifuged at 3000 rpm for 1 min to remove thesupernatant;

2.3 Disperse with 1 mL ofPBS-A; Ready for ANSM-based AuNP synthesis in the following step 3.1

**Scheme 2 (a, b, c): oxidization and chemical fixation** (Timing ~ 13 h):

2.1 Oxidize in 1 mL of 3 or 5 mM DTDPA in PBS (or 0.1M PIPES) in a 1.5mL microtube for 30 min at 4°C;

2.2 Chemical fixation

**Scheme 2a,2b**: Add 20 µL of 25% GA (0.5% final concentration) to the tube (from Step 2.1) for 30 min fixation at 4°C;

**Scheme 2c**: Centrifuge to remove the supernatant (at 2000 rpm for 2min); Add 1 mL of ice-cold 5 mM DTDPA and 4% PFA in PIPES buffer for 30 min oxidization and fixation at 4°C;

2.3 Centrifuge to remove the supernatant (at 2000 rpm for 2min);

2.4 Neutralize excess aldehyde with 1 mL of 50mM glycine in PBS (or in 0.1 M PIPES) at 4 °Covernight;

2.5 Centrifuge to obtain cellpellet (at 2000 rpm for 2min); Wash with PBS 3 X 5 min; Centrifuge to remove thesupernatant;

2.6 Permeabilization

**Scheme 2a**: Fast inject 1 mL of 4 °C methanol into the microtube, disperse the cell pellet instantly by pipetting for 1 min; Centrifuged at 3000 rpm for 1 min to remove thesupernatant;

**Scheme 2b,2c**: Incubate with 1 mL of 0.2 mg/mL zymolyase-20T in PBS (or in 0.1 M PIPES) for removing cellwall at RT for ~30 min (check under microscope to make sure cells properly digested as dull gray); Permeabilized with 1 mL of 0.1% triton X-100 in PBS for 2 min atRT; Wash with 1 mL of PBS-A for 5 min x3; Centrifuge at 2000rpm for 2 min for obtaining cell pellet;

2.7 Disperse cell pellet with 1 mLPBS-A; Ready for AuNP synthesis; Ready for ANSM-based AuNP synthesis in the following step 3.1.

**Scheme 3 (a,b) : high pressure freezing and freeze-substitution fixation (HPF/FSF)** (Timing 40-53 h):

2.1 high pressure freezing (Timing ~ 1 h)

(1) Removecellwallwith1mLof0.2mg/mLzymolyase-20TinPBSatRT forabout30min (check under microscope to make sure cells properly digested as dullgray);

(2) Centrifuge at 2000rpm x 2 min to obtain cell pellet;

(3) Fill the pre-cleaned 0.1 or 0.05 mm deep aluminum carrier with cell pellet;

(4) Cap a 3 mm x 0.16 mm sapphire disc (pre-soaked with 1-hexadence) quickly onto the aluminum carrier;

(5) Load the capped carrier into the customized HPF specimen holder for high pressure freezing (**Figure 2**);

(6) Unload the carrier under liquid nitrogen in the foam cryobox and stored in 2mL polypropylene microcentrifuge tubes in liquid nitrogen before use.

2.2 Freeze-substitution fixation (Timing 39-52 h)

Load 10-12 specimens (in aluminum carriers) into a 2mL polypropylene microtube with 1 mL of frozen freeze-substitution solvent (different for following **Scheme 3a,3b**) under liquid nitrogen($LN_2$) with a pair of pre-cooled forceps; Then transfer the microtube to the pre-cooled to -90°C chamber of a Leica AFS2 freeze-substitution machine for FSF processing.

The procedures for **Scheme 3a,3b**are described as following:

**Scheme 3a (HPF/FSF)** (Timing 35 h):

(1) Keep the specimens in 1 mL of acetone containing 3 or 5 mM DTDPA + 3% dd$H_2O$ at -90°C for 12h;

(2) Warm up gradually from 90°C to -30°C within 6h;

(3) Stay at -30°C for 2h;

(4) Add 50 µL of 10% GA (in acetone) to the microtube (still contains the 1mL DTDPA solution) at -30°Cfor 5 h fixation;

(5) Take out the aluminum carriers and sapphire discs (using forceps tip to separate cell pellet from carrier), leave the cells in the solution;

(6) Gradual rehydration (using a syringe or a pipette tip touch the tube just under the solution for gently adding or sucking off solution to avoid breaking the specimen):

a. Change to 1mL -30 °C 5% dd$H_2O$ in acetone, wait for 30 min;

b. Change to 1mL -30 °C 30% dd$H_2O$ in acetone, wait for 30 min;

c. Warm up gradually from -30 °C to -20 °C within 30 min;

d. Warm up gradually from -20 °C to 4 °C, add 3 x 100 μL of pre-frozen PBS drops (next drop addedupon previous drop dissolve completely) within 30 min;

e. Add 700 μL of ice-cold PBS for 10 min at 4 °C;

f. Centrifuge at 2000 rpm for 2 min to change pre-cooled 4 °C PBS for 10 min;

(7) Neutralization: change to 1 mL of 50mM glycine in PBS for neutralization at 4 °Covernight;

(8) Wash with 1 mL PBS-A for 5 min x3; Ready for ANSM-based AuNP synthesis in the following step 3.1.

**Scheme 3b (HPF/FSF**; **Gold enhancing)** (Timing 52 h):

(1) Keep the specimen in 1mL of acetone at -90 °C for 3h;

(2) Change the medium to 1 mL of acetone containing 50 mM DTDPA + 0.02% TA + 5% dd$H_2O$ at -90°C and stay for 20h;

(3) Warm up gradually from -90°C to -60°C within 5h;

(4) Stay at -60°C for 10h;

(5) Warm up gradually from -60°C to -35°C within 5h;

(6) Change to 1 mL of acetone containing 0.25% GA + 0.01% UA + 50mM DTDPA + 5% dd$H_2O$at -35°Cfor 5 h fixation;

(7) Detach cells from the carrier and sapphire disc, and take out the carrier and disc from the microtube with forceps.

(8) Gradual rehydration (using a syringe or a pipette tip touch the tube just under the solution for gently adding or sucking off solution to avoid breaking the specimen):

(a) Change to 1mL of acetone containing 30% 0.2M HEPES buffer at -35°C, wait for 30 min;

(b) Warm up from -35 °C to 0 °C within 10 min;

(c) Add 1 mL of RT 0.2 M HEPES buffer containing 50mM glycine;

(9) Neutralization: Keep in 1 mL of 50 mM glycine in PBS for neutralization at 4 °Covernight;

(10)Wash with 1 mL of PBS-A for 3 x 5 min (Note: centrifuge at 2000 rpm x 2 min for obtain cell pellet); Ready for ANSM-based AuNP synthesis in the following step 3.1.

**3. ANSM-based AuNP synthesis for yeast cells** (Timing 3 h)

3.1 Add 4.28 μL of 2-ME into the 2mL microtube containing yeast cells dispersed in 1 mL of PBS-A and incubate at RT for 1 h;

3.2 Add 50 μL of 10 mM $HAuCl_4$to the microtube, immediately vortex; Add 100 μL of 500 mM D-P, vortex; Then, incubate for 2h at 4 °C;

3.3 Add 20 μL of 100 mM $NaBH_4$, immediately shake to mix well, incubate for 5 min (Note: 100 mM $NaBH_4$must be freshly prepared at 4 °C for immediate use).

3.4 centrifuge at 2000 rpm for 2 min to obtain cell pellet;

3.5 Add 1 mL of PBS-A and keep at 4 °C.

**4. HPF/FSF** (Timing 56-58 h):

4.1 high pressure freezing (Timing 1 h)

(1) Centrifuge at 2000rpm x 2 min to obtain cell pellet;

**(2)** Fill the pre-cleaned 0.1 or 0.05 mm deep aluminum carrier with cell pellet;

**(3)** Cap a 3 mm x 0.16 mm sapphire disc (pre-soaked with 1-hexadence) quickly onto the aluminum carrier;

**(4)** Load the capped carrier into the HPF specimen holder for high pressure freezing (**Figure 2**);

**(5)** Unload the carrier under liquid nitrogen in the foam cryobox and stored in 2mL polypropylene microcentrifuge tubes in liquid nitrogen before use.

4.2 Freeze-substitution fixation and gold enhancing (Timing 56-58 h):

After the AuNP synthesis, the frozen specimens were either processed with standard 1% OsO4+0.1% UA in acetone freeze-substitution fixation directly or with optional FSF-based gold enhancement (**Scheme 3b**) prior to the standard 1% OsO4+0.1% UA in acetone freeze-substitution fixation.

**Standard FSF with 1% OsO4+0.1% UA in acetone** (Timing 56 h)**:**

(1) Load 1-4 specimens (on the aluminum carriers) into a 2mL polypropylene microtube with 1 mL of frozen acetone containing 1% $OsO_4$+0.1% UA under $LN_2$;

(2) Transfer the microtube to the pre-cooled to -90°C chamber of a Leica AFS2 freeze-substitution machine and stay for 24h at -90 °C;

(3) Warm up gradually from -90°C to -60°C within 5h;

(4) Hold at -60°C for 6h;

(5) Warm up gradually from -60°C to -20°C within 3h;

(6) Hold at -20°C for 18h;

(7) Warm up gradually from -20°C to 4°C within 2h;

(8) Wash with acetone 3x1h at 4°C;

(9) Warm up to RT for resin infiltration;

**Scheme 3b (FSF-based gold enhancing)** (Timing 58 h):

(1) Load 1-4 specimens (on the aluminum carriers) into a 2mL polypropylene microtube with 1 mL of frozen acetone containing 0.5% GA under $LN_2$;

(2) Transfer the microtube to the pre-cooled to -90°C chamber of a Leica AFS2 freeze-substitution machine and stay for 12h at -90°C;

(3) Warm up gradually from -90°C to -60°C within 6h;

(4) Wash with acetone for 3x1h at -60°C;

(5) Separate the specimen from aluminum carriers with a fine tip at -60 °C;

(6) FSF-based gold enhancing

(a) Add 10μL of 1% $HAuCl_4$(in acetone) to the microtube containing 1mL of acetone for 8h incubation at -60°C;

(b) Add 10 μL of fresh 10 mM $NaBH_4$(must be fresh prepared) to the solution for 20h gold enhancing at -60°C;

(c) Wash with acetone 3x1h at -60°C;

(7) Change to 1 mL of 1%OsO4+0.1% UA in acetone for 5h fixation at -60°C;

(8) Warm up gradually from -60°C to -30°C within 5h;

(9) Warm up gradually from -30°C to 4°C within 2h;

(10)Wash with acetone 3x1h at 4°C;

(11)Warm up to RT for resin infiltration;

**5. Resin infiltration*, embedding, polymerization, and thin sectioning** (Timing ~3 day):

5.1 Infiltrate the sample with 1:1 SPI-Pon 812 resin: acetone for 1.5h, 3:1 SPI-Pon 812 resin:acetone for 6-12h, 100% SPI-Pon 812 resin for 1 h, 100% SPI-Pon 812 resin for 3 h, and 100% SPI-Pon 812 resin for 1 h on a rotator.

5.2 Transfer the resin infiltrated sample attached on the sapphire disc to a flat-tip BEEM embedding capsule, incubate in fresh resin for 12 h polymerization at 45°C followed with 24-48h at 60°C.

5.3 Trim the polymerized specimen carefully to exposure the sapphire disc, dip into $LN_2$for 10 s to detach the cells from the disc and separate the disc from the block with forceps; then trim the block to trapezoidal shape for thin sectioning (e.g., 90nm) with a Lecia ultramicrtome.

5.4 Pick the sections on a 200-mesh hexagonal copper grid that coated with/without formvar.

*Note: For Scheme 2b and 2c, HM20 resin also applied (See the online methods in our Nature Method manuscript, Jiang et al. 2020 [1]for more details).

**6. EM imaging of cells** (Timing 6-24 h):

90 nm thin section on copper grids positive stained with 2% UA for 6 min or without positive staining were directly used for EM imaging on an FEI Spirit electron microscope (FEI Corp.) operating at 120kV. The images were recorded with a 4k x 4k 895 CCD camera (Gatan Corp.) under proper defocus values. Usually at magnification range from 11kX to 30kX are suitable for visualizing AuNPs in cells with proper defocus values to ensure 2-3 nm AuNPs visible.

# Troubleshooting

(1) $NaBH_4$should be freshly made before use.

(2) For ANSM-based AuNP synthesis, when add $HAuCl_4$requires immediately vortex to mix with 2-ME rapidly (because 2-ME reacts with $Au^{3+}$almost instantly, the local concentration does affect the ratio of thiolate/$Au^{3+}$, which might form polymeric forms of Au(I)SR compounds).

(3) The current ANSM-based AuNP synthesis protocol was optimized for 1 mL of solution, changing to other volume might need to re-optimize due to the reason mentioned in the above item (2).

(4) When no HPF/FSF equipment is available, the final HPF/FSF processes could be replaced by regular chemical fixation and dehydration or using freezer for FSF.

(5) This protocol also worked for bacteria except the centrifugation procedures using 3000 rpm for 5 min and no need of removing cellwall (see our Nature Method manuscript, Jiang et al. 2020 [1] for more details).

## Time Taken

The whole processes from cell culture to final EM imaging of the above protocol require 11-13 days.

## Anticipated Results

See our Nature Method manuscript, Jiang et al. 2020 [1] for more details.

## References

[1] Jiang,Z., Jin X., Li,Y., Liu, S.,Wang, Y.-Y., Zhao,P.,Cai,X., Liu Y., Tang,Y., Sun,X., Liu,Y., Hu,Y., Li,M., Cai,G., Qi,X., Chen,S., Du,L.-L. and He,W.,Direct synthesis of EM-visible gold nanoparticles on genetically encoded tags for single-molecule visualization in cells, *Nature Methods manuscript*(2020).

[2] Forsburg, S. L. & Rhind, N. Basic methods for fission yeast. Yeast 23, 173-183, doi:10.1002/yea.1347 (2006).

## Acknowledgements

## Figures

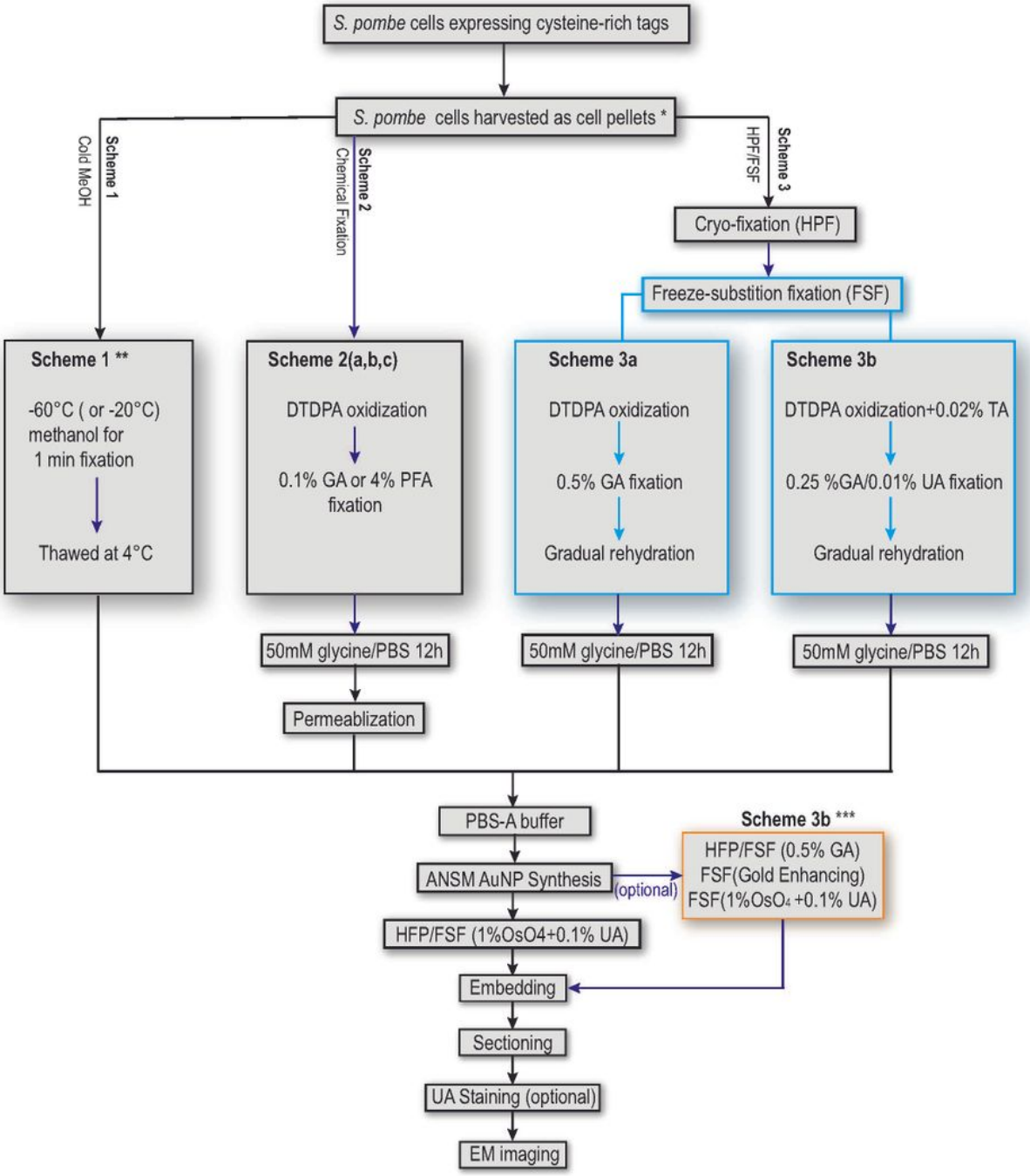


**Figure 1 Flow chart of the protocols for ANSM AuNP sysnthesis in yeast cells**

* For **Scheme 2 (a,b,c)** the cell wall of yeast cells were pre-digested by zymolyase-20T (*See* Methods online).
** **Scheme 1** was adapted from the **"Cold MeOH"** protocol for *E. coli*, so it is fully applicable for *E. coli*.
*** Only **Scheme 3b** had been adapted for FSF-based gold-enhancing test; Other scheme(s) could be adapted for the optional FSF-based gold-enhancing procedures as well.

## Figure 1

Figure 1 Flow chart of the protocols for ANSM AuNP sysnthesis in yeast cells

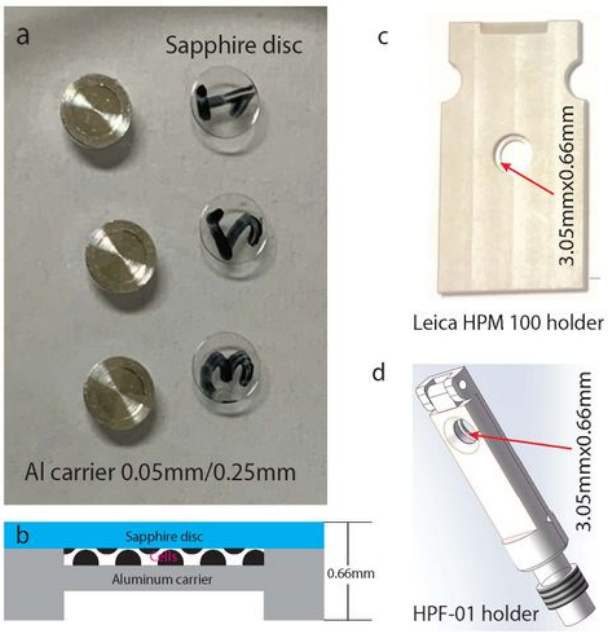


**Figure 2 Scheme for EM specimen preparation of yeast *S. pombe* cells**
(**a**) Aluminum carrier (0.05 mm/0.25 mm) for loading yeast cell pellet, then cap with a 3 mm x 0.16 mm sapphire discs for high pressure freezing; (**b**) Sketch of capping the sapphire disc on the 0.05 mm side of aluminum carrier ; (**d**) Customized 3.05 mm x 0.66 mm specimen holder with a step for loading the capped specimen on Leica HPM 100 or ICE high pressure freezing machine; (**e**) Customized 3.05 mm x 0.66 mm specimen holder with a step for loading the capped specimen on a Wohlwend HPF compact 01/02/03 high pressure freezing machine.

### Figure 2

Figure 2 Scheme for EM specimen preparation of yeast S. pombe cells

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplement1.pdf
- Figure2YeastProtocolv2.pdf
- supplement2.pdf
- Figure1YeastProtocolv2.pdf