Submission ID #: 61154
Scriptwriter Name: Susan Timberlake
Project Page Link: http://www.jove.com/files_upload.php?src=18652018

Title: Fabrication of Zero Mode Waveguides for High Concentration Single Molecule Microscopy

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Author Questionnaire

1. **Microscopy**: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **no**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **no**

3. **Filming location:** Will the filming need to take place in multiple locations? **no**

Protocol Length:
Steps: 22
Shots: 40

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Kevin Y Chen:** The nanosphere lithography protocol presented here for fabricating zero mode waveguides is an accessible, low-cost method that does not require any specialized fabrication tools or facilities.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested b-roll: 5.5.1 and 5.5.3*

1.2. **Kevin Y Chen:** This method will be useful for single molecule biophysics and allow more researchers to perform single molecule experiments at biologically relevant concentrations of fluorescent reagents.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Protocol

2. Glass Coverslip Cleaning

2.1. To create a clean surface for evaporative deposition of colloidal particles, place optical borosilicate glass coverslips in the grooved inserts of a coplin glass staining jar **[1].** Fill the staining jar with enough acetone to cover the coverslips and cover the jar **[2].** Sonicate the jar for 10 minutes at 40 degrees Celsius **[3].**

2.1.1. Talent places coverslips in grooved inserts of staining jar.
2.1.2. Talent adds acetone to jar and places cover on.
2.1.3. Talent places jar in sonicator.

2.2. Pour out the acetone and rinse the coverslips three times with distilled water **[1].**

2.2.1. Talent pours out acetone, adds water, and pours out water.

2.3. After repeating the sonication in acetone, fill the jar with enough potassium hydroxide to cover the coverslips **[1].** Sonicate the jar again, covered, for 20 minutes at 40 degrees Celsius **[2].**

2.3.1. Talent filling the jar with KOH, with the KOH container in the shot.

2.3.2. Talent placing the jar in the sonicator and starting sonication.

2.4. Rinse the coverslips with distilled water six times **[1],** then sonicate them in ethanol for 10 minutes at 40 degrees Celsius **[2].**

2.4.1. Talent begins rinsing the coverslips with distilled water.

2.4.2. Ethanol-filled staining jar in the sonicator.

2.5. After washing the coverslips 3 times with distilled water, gently pick up each coverslip at the edge using forceps **[1].** Dry each coverslip with nitrogen gas, and place each clean, dry coverslip in its own Petri dish **[2].**

2.5.1. Talent picks up coverslip with forceps.
2.5.2. Talent dries coverslip with nitrogen gas and places it in Petri dish.

3. Evaporative Deposition of Polystyrene Beads

3.1. Centrifuge 50 microliters of 1-micron, non-functionalized polystyrene beads **[1-TXT].** Discard the supernatant, leaving as little water remaining as possible **[2].** Resuspend the beads in 50 microliters of solvent, then mix thoroughly by pipetting **[3-TXT].**

3.1.1. Talent places tube of polystyrene beads in centrifuge. **TEXT: 15,000 x *g*; 25 °C; 5 min**

3.1.2. Talent removes and discards supernatant.

3.1.3. Talent adds solvent and pipettes up and down. **TEXT: Solvent: 1:400 Triton X-100:ethanol**

3.2. To set up a humidity chamber for deposition, arrange 6 Petri dishes in a row **[1].** Place one coverslip in each Petri dish, and leave the lids slightly ajar, with the coverslips positioned to be exposed to the environment **[2].**

3.2.1. Talent sets up six Petri dishes in a row.

3.2.2. Talent ~~places a coverslip in a Petri dish and~~ places the lid on the Petri dish so as to leave the coverslip exposed. NOTE: The coverslip was already placed in the petri dish in 2.5.2 so the shot 3.2.1 has the coverslips already in the petri dish. There are two Shots (3.2.2 Take 1). The first is a close up that shows where in the petri dish the coverslip and the second is taking the lids off the petri dish to leave them exposed.

3.3. Center a hygrometer and a small electric fan behind the Petri dishes **[1].** Then, record the relative humidity in the lab **[2].** Fill a 200-milliliter beaker with 150 to 200 milliliters of water at approximately 75 degrees Celsius and place the beaker behind the fan **[3].**

3.3.1. Talent places hygrometer and fan behind Petri dishes.

3.3.2. Talent looks at hygrometer and records relative humidity.

3.3.3. Talent places beaker of water behind the fan.

3.4. Turn on the fan, and then cover the Petri dishes, fan, beaker, and hygrometer with an overturned, transparent plastic storage container **[1].**

3.4.1. Talent turns on the fan and covers the entire set-up with a plastic storage container.

3.5. When the relative humidity reaches 70 to 75 percent, lift the plastic storage container slightly, and quickly close the lids of the Petri dishes to prevent over-wetting of the coverslips **[1-TXT].**

3.5.1. Talent lifts plastic storage container and closes the lids of the Petri dishes. **TEXT: Record the relative humidity in the chamber.**

3.6. When the relative humidity in the chamber reaches 85 percent, pipette 5 microliters of the bead suspension onto the center of each coverslip. Cover each Petri dish immediately after deposition and keep everything covered with the storage container between depositions **[1]**. *Videographer: This step is important!*

3.6.1. Talent slightly tips open storage container for access, opens one Petri dish, pipettes the bead suspension, immediately covers the Petri dish, and covers everything with the storage container.

3.7. **Kevin Y Chen**: Bead depositions should be completed as quickly as possible to minimize loss of humidity. If depositions are too dry or wet, chamber humidity is the most important variable to optimize.

3.7.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

3.8. When all 6 depositions are complete, record the relative humidity in the chamber, and let the bead droplets spread and dry for 5 minutes **[1]**.

3.8.1. Talent records relative humidity.

4. Bead Annealing for Reducing Pore Size

4.1. To provide a uniform temperature surface for annealing of the polystyrene beads, place a flat, milled aluminum plate on top of a standard ceramic hot plate, and set the temperature of the hot plate to 107 degrees Celsius, the glass transition temperature of polystyrene **[1]**.

4.1.1. Talent places aluminum plate on top of hot plate and sets the temperature of the hot plate.

4.2. Place a coverslip containing the bead template on the hot aluminum plate **[1]**. After annealing it for 20 seconds, remove the coverslip from the aluminum plate, and promptly place it on a room temperature aluminum surface to cool **[2]**. *Videographer: This step is important!*

4.2.1. Talent places coverslip on the aluminum plate and starts timer at the same time.

4.2.2. Talent moves the coverslip to an aluminum plate at room temperature. NOTE: File "5Y7A1954.MP3" was not on the protocol but figured a close up shot of the coverslip at this stage could be useful.

5. Nanofabrication of Aluminum Zero Mode Waveguides

5.1. Using thermal evaporative deposition, deposit 300 nanometers of copper at 2 Angstroms per second over coverslips containing the bead templates **[1]**. This will generate posts in the interstices between the beads **[2]**.

5.1.1. Talent places coverslip with beads in thermal deposition unit.

5.1.2. Added shot: Talent takes coverslips out of deposition unit and holds the samples for close-up shot. **TEXT: copper was deposited onto the bead templates beforehand for filming purposes.** NOTE: Files "5Y7A2004.MP4" and "5Y7A2005.MP4" is him taking the coverslips out of the deposition unit that is the completed process of 5.1.1 it was not in the protocol but he wanted to shoot it

5.2. To remove the excess copper from the beads, gently press a piece of tape onto the surface, and then slowly peel off the tape **[1]**. *Videographer: This step is important!*

5.2.1. Talent presses tape on the coverslip and then slowly removes it.

5.3. Dissolve the polystyrene beads by placing the coverslips in toluene and leaving them overnight **[1-TXT]**.

5.3.1. Talent places the coverslips in toluene and parafilm seals the container. **TEXT: Caution! Toluene fumes are toxic.**

5.4. Handling the coverslips carefully, rinse the them once with chloroform and twice with ethanol **[1-TXT]**. Dry the templates with nitrogen **[2]**. Place them in an oxygen plasma cleaner for 30 minutes to remove residual polymer and contaminants **[3]**.

5.4.1. Talent rinses coverslip with chloroform and begins rinse with ethanol. **TEXT: Caution! Chloroform fumes are toxic.**
5.4.2. Talent dries coverslip with nitrogen.
5.4.3. Talent places coverslips in an oxygen plasma cleaner.

5.5. Using thermal evaporative deposition, deposit 3 nanometers of a titanium adhesion layer at 1 Angstrom per second. Then, deposit 100 to 150 nanometers of aluminum at 4 Angstroms per second, around and on top of the copper posts **[1]**. Ensure that the samples are not rotating during deposition **[2]**, as is automatically done in some deposition units **[3].**

5.5.1. Talent places coverslip in thermal evaporative deposition unit, and begins deposition process.
5.5.2. Added shot: Talent highlights with cursor on deposition unit control screen the “rotation” parameter to emphasize the importance of having no rotation during deposition. **TEXT: Rotation should be disabled for all metal depositions, including copper deposition.**

NOTE: "5Y7A2008.MP4" was not in the protocol but talent thought the settings would be useful to show. 5Y7A2009.MP4 through 5Y7A2013.MP4 were not on the protocol and are him taking the coverslips out of the deposition unit and is the completion of step 5.5.1. this is done in both wide shot and close up shot of coverslips.

5.5.3. Added shot: Talent takes coverslips out of deposition unit and holds the samples for close-up shot.

5.6. To dissolve the copper posts, soak the coverslips in copper etchant for 2 hours **[1]**.

5.6.1. Talent places coverslips in copper etchant. **TEXT: Caution! Copper etchant can cause skin irritation.**

5.7. After rinsing the coverslips in distilled water, dry them with nitrogen **[1]**. Then, gently buff the surface with lens paper to expose any posts that are still covered in cladding

[2]. *Videographer: This step is important!*

5.7.1. Talent dries wet coverslips with nitrogen

5.7.2. Talent buffs coverslip with lens paper.

5.8. Soak the coverslips in copper etchant for another 2 hours **[1]**. Then, rinse them again with distilled water and dry them with nitrogen **[2]**.

5.8.1. Talent places coverslips in copper etchant. NOTE: FILES "5Y7A1991.MP4" and "5Y7A1992.MP4" are an insert shot for illustrative purposes that was not in protocol that Kevin thought would be useful

5.8.2. Talent rinses coverslips and dries them with nitrogen.

5.9. After surface passivation, zero mode waveguide coverslips can be paired with quartz slides using double-sided sticky tape to make microfluidic flow chambers for single molecule imaging **[1].**

5.9.1. Added shot: Talent holds an example of flow chambers for close-up shot. **TEXT: see reference 44 (Chandradoss et al., JoVE, 2014) in text for microfluidic chamber assembly protocol.**

Results

6. Results: ZMW Fabrication Quality Control

6.1. Success of the colloidal template self-assembly depends critically on the relative humidity **[1]**. In a well-formed template, the region of beads is circular, with borders defined by an opaque, multilayered ring **[2]**.

6.1.1. LAB MEDIA: Figure 3. *Video editor, show only Figure 3A, 3B, and 3C.*
6.1.2. LAB MEDIA: Figure 3. *Video editor, show only Figure 3A, 3B, and 3C, and emphasize 3A.*

6.2. In well-formed templates, most of the template should contain hexagonally-close packed beads. There will be some defects between grains due to jamming during the evaporative sedimentation **[1]**.

6.2.1. LAB MEDIA: Figure 3. *Video editor, show only Figure 3E and 3F.*

6.3. After copper deposition and dissolution of the polystyrene beads, copper posts should be below 150 nanometers in diameter **[1]**. For a 300-nanometer copper deposition depth, posts are around 250 nanometers tall **[2]**.

6.3.1. LAB MEDIA: Figure 4 *Video editor, show only Figure 4D, 4E, 4F, and 4G.*
6.3.2. LAB MEDIA: Figure 4 *Video editor, show only Figure 4D, 4E, 4F, and 4G, and emphasize 4F and 4G.*

6.4. After deposition of aluminum cladding and subsequent dissolution of the copper posts **[1]**, waveguides should be visible by atomic force microscopy and be spaced about 550 nanometers apart **[2]**.

6.4.1. LAB MEDIA: Figure 5. **TEXT: ZMW: Zero Mode Waveguide**
6.4.2. LAB MEDIA: Figure 5. *Video editor, zoom in on Figure 5C.*

6.5. Annealing the polystyrene templates for 20 seconds produced aluminum waveguides with a diameter of 118 nanometers, sufficiently small to cut off propagation of visible light **[1]**.

6.5.1. LAB MEDIA: Figure 5. *Video editor, show only Figure 5D and Figure 5E.*

6.6. A typical field of ZMWs for imaging contains 3000 waveguides in a 40- by 80-micron field of view **[1]**. Single-molecule FRET was performed to test for ZMW functionality **[2]**.

6.6.1. LAB MEDIA: Figure 6. *Video editor, show Figure 6B only.*
6.6.2. LAB MEDIA: Figure 6. *Video editor, show Figure 6A only.*

6.7. Single-molecule FRET traces were detectable at all concentrations of ambient Cy5 fluorophore tested. In comparison, single molecules would only be detectable in TIRF illumination with picomolar to low nanomolar concentrations **[1]**.

6.7.1. LAB MEDIA: Figure 6. *Video editor, show Figure 6C, 6D, 6E, and 6F.*

Conclusion

7. Conclusion Interview Statements

7.1. **Kevin Y Chen:** After ZMW fabrication, devices can be passivated with polymers to reduce non-specific interactions during single molecule imaging. The glass bottoms can also be etched slightly to enhance single molecule emission.

7.1.1. INTERVIEW: Named author says the statement above in an interview-style statement while looking slightly off-camera.

7.2. **Kevin Y Chen:** Overall, this new ZMW fabrication method will allow researchers to more easily investigate dynamic biochemical processes, such as transcription or translation, at concentrations and rates closer to those in the cell.

7.2.1. INTERVIEW: Named author says the statement above in an interview-style statement while looking slightly off-camera.