Submission ID #: 60910
Scriptwriter Name: Anastasia Gomez
Project Page Link: https://www.jove.com/account/file-uploader?src=18577598

Title: In Vivo Hydroxyl Radical Protein Footprinting for the Study of Protein Interactions in *Caenorhabditis elegans*

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Section - Protocol

2. Microfluidic Flow System Assembly

2.1. Begin the flow system assembly by cutting a 2-centimeter piece of fluorinated ethylene propylene, or FEP, tubing **[1-TXT]**. Use a clean dissecting needle to widen the inner diameter at one end of the tubing, creating a small crater about 50 millimeters in length **[2]**.

2.1.1. WIDE: Establishing shot of talent cutting the tubing. **TEXT: 1/16 inches o.d. X 0.020 inches i.d.**

2.1.2. Talent widening the end of the tubing.

2.2. To create the infusing lines of the flow system, cut two 15-centimeter pieces of 250-micrometer i.d. fused silica with a ceramic cutter **[1]** and tape the two capillaries together with self-adhesive tape, making sure that the ends are 100% flushed **[2]**.

2.2.1. Talent cutting the fused silica. *Videographer: Obtain multiple, reusable takes of this shot because it will be reused in 2.5.1.*

2.2.2. Talent taping the two capillaries together.

2.3. Insert the two capillaries into the crater of the FEP tubing, pushing them up to the very edge **[1]**. Place a small dot of epoxy resin on a clean surface and mix it with a dissecting needle **[2]**.

2.3.1. Talent inserting the capillaries into the FEP tubing.

2.3.2. Talent placing a drop of resin on a surface.

2.4. Use the same needle to quickly place a small drop of the resin at the end of the infusing capillaries where they connect with the FEP tubing **[1]**. Allow the resin to dry, outlet side up, for a few minutes **[2]**.

2.4.1. Talent placing a drop of resin on the capillaries.

2.4.2. Capillaries and tubing drying.

2.5. Meanwhile, cut a new 250-micrometer i.d. capillary, which will become the outlet capillary of the flow system **[1]**. Once the resin has dried, insert the new capillary through the FEP tubing outlet end **[2]**. The inside ends of the outlet capillary and the two infusing capillaries should be flush against each other, creating the mixing-T **[3]**. *Videographer: This step is difficult!*

2.5.1. *Use 2.2.1.*

2.5.2. Talent inserting the new capillary.

2.5.3. Assembled mixing T. *Video Editor: Insert Figure 1 B as an inset here.*

2.6. Bind the capillary and FEP tubing with fresh epoxy resin as previously described **[1]** and allow the flow system to dry overnight **[2]**. *Videographer: This step is difficult!*

2.6.1. Talent binding the capillary to the FEP tubing.

2.6.2. Flow system drying.

3. Microfluidic Flow System for in vivo FPOP

3.1. Insert 4 magnetic stirrers inside one 5-milliliter syringe, which will prevent worms from settling in this syringe during in vivo fast photochemical oxidation of proteins, or in vivo FPOP *(pronounce 'F-pop')* **[1]**. Fill this and an additional 5-milliliter syringe with M9, making sure to avoid creating air bubbles **[2]**.

3.1.1. Talent inserting stir bars into a syringe.
3.1.2. Talent filling two syringes with M9.

3.2. Connect a Luer adapter to each syringe, making sure that they are finger tight and secured in place **[1]**. Then, attach each syringe to the middle port of a single 3-2 valve **[2]**. Secure each syringe to the dual syringe pump **[3]** and adjust the mechanical collar to prevent over pressure from the pusher block **[4]**.

3.2.1. Talent connecting a Luer adapter to a syringe and making sure that it is on tight.
3.2.2. Talent attaching the syringe to the valve.
3.2.3. Talent securing syringes to the dual pump.
3.2.4. Talent adjusting the mechanical collar.

3.3. Use a super flangeless nut, FEP sleeve, and super flangeless ferrule to attach each infusing capillary end of the previously made microfluidic system to the top port of each 3-2 valve **[1]**. Finally, attach a 10-centimeter, 450-micrometer i.d. capillary to the bottom port of the valve, which will serve as the withdrawing sample capillary **[2]**.

3.3.1. Talent attaching the microfluidic system to the valve.
3.3.2. Talent attaching a capillary to the bottom port of the valve.

3.4. Start the pump flow and visually inspect all connections for leaks **[1]**. Flow at least 3 syringe volumes using the experimental flow rate **[2]**. The flow path is marked by the arrows on the 3-2 valve handle **[3]** and each syringe can be refilled manually by moving the valve handle from expelling to withdrawing position **[4]**. *Videographer: This step is important!*

3.4.1. Talent inspecting connections for leaks.] w.t. 3.4.1
3.4.2. Shot of completely assembled microfluidic system.
3.4.3. Valve handle with the flow path marked. s l c t e d 3.4.2
3.4.4. Talent moving the valve handle from expelling to withdrawing.

3.5. After inspecting the microfluidic flow system, move it to the experimental bench **[1]** and secure the outlet capillary to the radiating stage with a stainless-steel union **[2]**. Use a long-reaching lighter to burn the coating of the fused silica at the laser irradiation window **[3]** and clean the burned coating with lint tissue and methanol **[4-TXT]**. *Videographer: This step is important!*

3.5.1. Talent moving the system to the bench.
3.5.2. Talent securing the outlet capillary to the radiating stage.
3.5.3. Talent burning the coating of the fused silica.

3.5.4. Talent cleaning the burned coating. **TEXT: Alternate between burning and cleaning cycles**

with 3.5.3

3.6. Position the magnetic stirrer block above the syringe with the magnetic stirrers **[1]** and adjust the speed **[2]** so that the stirrers are rotating slowly and constantly **[3]**.

3.6.1. Talent positioning the magnetic stirrer.
3.6.2. Talent adjusting the speed.
3.6.3. Stirrers rotating at the appropriate speed.

4. In vivo FPOP

4.1. Turn on the krypton fluoride excimer laser and allow the thyratron to warm up **[1-TXT]**. Measure the laser energy at a frequency of 50 hertz for at least 100 pulses by placing the optical sensor at the beam exit window **[2]**. *Videographer: This step is important!*

4.1.1. Talent turning on laser. **TEXT: CAUTION: Wear proper eye protection!**
4.1.2. Talent placing the optical sensor at the beam window.

4.2. Manually withdraw approximately 10,000 worms in a 500-microliter volume into the sample syringe **[1]**, then fill it with 2.5 milliliters of M9 buffer, making sure to avoid air bubbles **[2]**.

4.2.1. Talent withdrawing the worms.
4.2.2. Talent filling the syringe with M9.

4.3. **Jessica A. Espino**: It is important to have a sample size of at least ten-thousand, a smaller sample size with result in low protein yield for downstream proteomic analysis.

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

4.4. Fill the second syringe with 3 milliliters of 200 millimolar hydrogen peroxide **[1]**.

4.4.1. Talent filling the second syringe.

4.5. At the end of the outlet capillary, place a 15-milliliter conical tube with 6 milliliters of 40 millimolar DMTU, 40 millimolar PBN, and 1% dimethyl sulfoxide **[1]**. Start the excimer laser from the software window, wait for the first pulse, and start the sample flow from the dual syringe **[2-TXT]**.

4.5.1. Talent placing the conical tube.
4.5.2. Talent starting the laser. + other angles

4.6. Collect the entire sample in the 15-milliliter tube while actively monitoring the sample flow for any visual leaks **[1]**. After in vivo FPOP *(pronounce 'F-pop')*, pellet the worms by centrifugation at 805 *x g* for 2 minutes **[2]**. Aspirate the quench solution and add 250 microliters of lysis buffer **[3]**.

4.6.1. Sample collecting in the tube. ↳ 4.5.2
4.6.2. Talent putting the tube in the centrifuge and closing the lid.
4.6.3. Talent aspirating the supernatant and adding lysis buffer.

4.7. Transfer the sample to a clean microcentrifuge tube **[1]**, flash freeze it **[2]**, and store it at negative 80 degrees Celsius until sample digestion **[3]**.

4.7.1. Talent transferring the sample.
4.7.2. Talent flash freezing the sample.
4.7.3. Talent putting the tube in the freezer.