

Submission ID #: 62686

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Project Page Link: <https://www.jove.com/account/file-uploader?src=19119278>

Title: Methane Hydrate Crystallization on Sessile Water Droplets

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Author Questionnaire

1. **Microscopy:** Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**
2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes**
3. **Interview statements:** Considering the COVID-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one.**

☒ Interviewees wear masks until videographer steps away (≥ 6 ft/2 m) and begins filming, then the interviewee removes the mask for line delivery only. When take is captured, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

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

Current Protocol Length

Number of Steps: 26

Number of Shots: 62 (14 SC)

Introduction

1. Introductory Interview Statements

REQUIRED:

- 1.1. **Abigail Johnson:** The effect of various additives on gas hydrate morphology and on pressure-temperature stability can be tested but the main interest lies in finding out how biomolecules may interact and affect gas hydrates *in situ* [1].
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.4.2.*
- 1.2. **Abigail Johnson:** The main advantage of this protocol is that one can reproducibly form a hydrate shell on a sessile droplet safely [1].
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 5.2.*

OPTIONAL:

- 1.3. **Abigail Johnson:** Leveling the droplet stage is challenging and needs practice, as the droplet slides on an uneven stage. Also, familiarize with Swagelok connections and safety regarding high pressure flammable gases [1].
 - 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.3.2., 2.1., and 2.4.3.*

Protocol

2. Preparing the Equipment

- 2.1. Begin by connecting the methane cylinder regulator to the pump with the 1-by-4-inch copper pipe using a new nut and ferrule set [1-TXT].
 - 2.1.1. Talent connecting the methane cylinder regulator with copper pipe. **CAUTION: Ensure gas cylinder is closed**
- 2.2. Glue a flexible tip of IV tubing, cut at an angle, to the end of the cannula to help direct the droplet toward the sapphire window [1]. Attach a 1-milliliter syringe to the cannula [2] and pull in the desired volume of deionized water [3-TXT].
 - 2.2.1. Talent gluing the flexible tip of IV tubing to the cannula. **NOTE: Shot with 2.2.2.**
 - 2.2.2. Talent attaching a syringe to the cannula. **NOTE: Shot with 2.2.1.**
 - 2.2.3. Talent pulling deionized water in the syringe. **TEXT: ~50–300 μ L**
- 2.3. Without the needle valve or sapphire window attached, insert the end of the cannula into the top port [1] and practice expelling the droplet onto the center stage [2].
 - 2.3.1. Talent inserting the end of cannula into the top port.
 - 2.3.2. Talent practicing expelling the droplet onto the center stage.
- 2.4. Reattach the sapphire window and washers with M8 screws and attach top pressure cell valve [1]. Connect the braided stainless-steel hose from the pressure pump to the pressure cell [2] and double-check that all connections from the gas cylinder to the pressure cell are tight [3].
 - 2.4.1. Talent reattaching sapphire window and washers with M8 screws.
2.4.1b. Added shot: Top pressure cell valve attached.
 - 2.4.2. Talent connecting the braided stainless-steel hose from pressure pump to cell.
 - 2.4.3. Talent checking all the connections.
- 2.5. Open the pressure cell inlet valve [1] and set the pressure cell in the aquarium [2], then insert a fiber optic light source cable into the pressure cell illumination port [3].
 - 2.5.1. Talent opening the pressure cell inlet valve. **NOTE: Shot with 2.5.2.**
 - 2.5.2. Talent setting the pressure cell in the aquarium. **NOTE: Shot with 2.5.1.**

2.5.3. Talent inserting a fiber optic light source into the pressure cell illumination port.

2.6. In an aquarium filled with a 50-by-50 ratio of ethanol and water, add more ethanol as the solution level falls in the following weeks until it is level with the top of the pressure cell, just below the light source connection [1-TXT].

2.6.1. Talent adding more ethanol in the aquarium to make up the volume. **TEXT:**
Replace solution monthly

2.7. Set the chiller to the temperature that will achieve approximately 0 to 3 degrees Celsius inside the cell and start circulating through coils [1]. Turn on the airflow to the front of the aquarium to prevent condensation on the aquarium surface [2].

2.7.1. Talent setting the chiller temperature.

2.7.2. Talent turning on the air flow to the front of aquarium.

2.8. Start a temperature log in the data logger software [1]. Set the scanning interval to 30 seconds and wait until the temperature inside the pressure cell is stable at 2 degrees Celsius [2].

2.8.1. SCREEN: 62686_Screenshot 1.mp4. 00:01-00:29.

2.8.2. SCREEN: 62686_Screenshot 1.mp4. 00:29-00:52.

3. Adding Water Droplet into the Pressure Cell Using Camera View on the Laptop

3.1. After the camera is placed, turn on the light source to approximately 80% [1] and open the camera software [2]. In live view, focus the camera lens at the cell's inner chamber and adjust the light source for best imaging [3]. Start a new temperature log with a 1 second scanning interval [4].

3.1.1. Talent turning on the light source.

3.1.2. SCREEN: 62686_Screenshot 2.mp4. 00:01-00:18. *Videographer: Obtain a few shots of talent clicking the mouse and typing on the keyboard to use as b-roll throughout the video.*

3.1.3. Adjustment of camera lens and light source.

3.1.4. SCREEN: 62686_Screenshot 3.mp4. 00:01-00:17.

3.2. If attached, detach the outlet needle valve in the top port of the pressure cell [1]. Attach a 1-milliliter syringe to the cannula [2] and pull in the desired volume of deionized water [3-TXT].

3.2.1. Talent detaching the outlet needle valve.

3.2.2. Talent attaching syringe to the cannula. **NOTE: Use 2.2.2.**

3.2.3. Talent pulling in deionized water in syringe. **TEXT: 200 μ L** **NOTE: Use 2.2.3.**

3.3. Insert the cannula through the top port until the tip is visible in the camera software in live view mode [1]. Expel the fluid droplet from the syringe over the central thermocouple [2], then reattach the needle valve [3].

3.3.1. SCREEN: 62686_screenshot 2.mp4. 1:21 to 1:31.

3.3.2. SCREEN: 62686_screenshot 2.mp4. 1:34-1:38.

3.3.3. Talent reattaching the needle valve.

3.4. Focus the camera on the droplet in the pressure cell [1]. Begin time-lapse imaging every 60 seconds [2].

3.4.1. SCREEN: 62686_Screenshot 4.mp4. 00:01-00:15.

3.4.2. SCREEN: 62686_Screenshot 5.mp4. 00:05-00:18.

3.5. Open the pressure transducer software on the laptop [1]. Start collecting data on the chart and the data log at a scanning interval of 1 second and wait until the droplet temperature is stable between 0 to 3 degrees Celsius [2].

3.5.1. SCREEN: 62686_Screenshot 6.mp4. 00:01-00:04.

3.5.2. SCREEN: 62686_Screenshot 6.mp4. 00:04-00:20.

4. Pressurizing the Pressure Cell

4.1. Turn on the pump and the controller [1]. Close the pressure pump's inlet valve [2]. Open the pump's outlet valve and the pressure cell's valves [3].

4.1.1. Talent turning on the pump and the controller.

4.1.2. Talent closing the pressure pumps inlet valve.

4.1.3. Talent opening pump's outlet valve and pressure cell valves.

- 4.2. Tare the pump pressure by pressing **Zero** on the pressure pump controller [1]. Select **Pump A** on the pressure pump controller to monitor the pressure [2].
 - 4.2.1. Pressing Zero on the pressure pump controller. **NOTE: Shot with 4.2.2**
 - 4.2.2. Selecting Pump A. **NOTE: Shot with 4.2.1.**
- 4.3. Ensure that the pressure pump is empty if a different fluid other than methane gas was present in the pump. Do this by setting the max flow and **Const Flow to 100 milliliters per minute** and pressing **Run**. Leave it running until the pump is empty [1]. Close the pump outlet valve [2] and open the pump inlet valve [3].
 - 4.3.1. Setting the flow to ensure the pressure pump is empty.
4.3.1.b Added shot: Pressure cell outlet valve closed.
 - 4.3.2. Talent closing the pump outlet valve.
 - 4.3.3. Talent opening the pump inlet valve.
- 4.4. Open the gas cylinder [1] and set the gas cylinder regulator to 1,000 kilopascals [2]. Press **Refill** on the pressure pump controller [3]. When the pump is full and near 1,000 kilopascals, close the pump inlet valve and the gas cylinder [4].
 - 4.4.1. Talent opening the gas cylinder.
 - 4.4.2. Talent setting the gas cylinder regulator.
 - 4.4.3. Talent pressing refill on the pump controller.
 - 4.4.4. Talent closing the pump inlet valve and gas cylinder.
- 4.5. Slightly open the pump outlet valve to the cell [1]. Monitor the pressure cell pressure in the pressure transducer software as it may decrease due to the relatively lower temperature in the pressure cell [2].
 - 4.5.1. Talent opening the pump outlet valve.
 - 4.5.2. Talent monitoring the pressure cell pressure.
- 4.6. Set the max flow to **10 milliliter per minute**, max pressure to **5,000 kilopascals**, and constant pressure to **1,000 kilopascals** on the pressure pump controller as described in the text manuscript [1].
 - 4.6.1. Setting the max flow, max pressure, and constant pressure.

- 4.7. Press **Run**. When 1,000 kilopascals is reached, press **Stop** on the pump controller [1] and close the pump's outlet valve [2]. Monitor the pressure in the pressure cell to ensure there are no leaks. If the pressure drops, use the liquid leak detector to find the leak at the connections [3] and carefully tighten the leaking components [4].
 - 4.7.1. Talent pressing stop for stopping the pressure controller. **NOTE: Add a video of talent pressing the run button.**
 - 4.7.2. Talent closing the outlet valve.
 - 4.7.3. Talent observing leaks with liquid leak detector.
 - 4.7.4. Talent tightening the leaking components.

- 4.8. If the cell is stable, open the pump outlet [1] and set the **Constant Pressure** gradually to **2,000, 3000, 4000, and 5000 kilopascals**, monitoring the cell stability by pressing **Stop** after each setting [2]. If the pressure is stable, close the pump outlet and wait for around 12 to 24 hours for the gas to permeate the droplet [3].
 - 4.8.1. Talent opening the pump outlet. **NOTE: Shot with 4.8.2.**
 - 4.8.2. Setting constant pressure. **NOTE: Shot with 4.8.1.**
 - 4.8.3. Talent closing the pump outlet.

- 4.9. Switch the time-lapse to take images every 2 to 5 seconds [1]. Add dry ice to the top of the cell until the hydrate shell is seen in time-lapse. If the dry ice slides, affix tape around the top of the cell [2]. Observe the progress of the methane hydrate formation through time-lapse photos for 2 to 6 hours [3].
 - 4.9.1. SCREEN: 62686_Screenshot 7.mp4. 00:05-00:19.
 - 4.9.2. Talent adding dry ice to the top of the cell.
 - 4.9.3. SCREEN: 62686_Screenshot 8.mp4. 00:03-00:12.

- 4.10. Depressurize the cell to 2,000 kilopascals by opening the pump outlet and setting the **Constant Pressure** to 2,000 kilopascals, noting when melting occurs [1].
 - 4.10.1. Depressurizing the cell to 2000 kPa.

- 4.11. After 30 minutes, repressurize the pressure cell to 5,000 kilopascals to observe the memory effect [1]. Note when a hydrate shell begins to reform and allow the shell to form for 30 minutes to 2 hours [2].
 - 4.11.1. Repressurizing the cell to 5000 kPa.

4.11.2. LAB MEDIA: Video_S4_Trial 3 memory effect nucleation_10x.mp4

4.12. Depressurize the cell by opening the pump outlet and setting the **Constant Pressure** to 100 kilopascals. If there is residual pressure in the pressure cell, slightly open the pressure cell top valve by one sixteenth inches [1]. Save the pressure and temperature data as .csv files [2].

4.12.1. Depressurizing the cell to 100 kPa.

4.12.2. SCREEN: 62686_screenshot 9a.mp4. 00:02-00:25 &
62686_screenshot 9b.mp4. 00:02-00:17

4.13. Remove the droplet by removing the top pressure cell valve and extracting the droplet with the syringe, cannula, or IV tube. Follow the directions in the text manuscript if there is a concern for contamination between trials [1].

4.13.1. Talent removing the top pressure cell valve to remove the droplet.

Results

5. Representative Images of Methane Hydrate Shells and Analysis of Pressure-temperature Stability Diagram

5.1. There is a clear morphologic difference upon dry ice-forced hydrate shell formation where the water droplet transitioned from a smooth, reflective surface [1] to an opaque hydrate shell with a slightly dendritic surface [2].

5.1.1. LAB MEDIA: Figure 3A.

5.1.2. LAB MEDIA: Figure 3B.

5.2. The addition of 100 micrograms per milliliter Type I (*type-one*) antifreeze-proteins altered the hydrate morphology by inducing ridged edges along the droplet and protrusions from the top of the droplet [1].

5.2.1. LAB MEDIA: Figure 3C and 3D.

5.3. After the hydrate shell developed for around 1 hour, the cell was depressurized to 2 Megapascals [1], leading to a 0.2-to-0.5-degree Celsius drop in temperature near the P/T (*P-T*) stability curve due to exothermic hydrate dissociation [2-TXT].

5.3.1. LAB MEDIA: Supplemental Video S3.

5.3.2. LAB MEDIA: Figure 4. **TEXT: P/T- pressure by temperature.**

5.4. Hydrate dissociation was confirmed by visual melting through time-lapse imaging at the beginning of the decrease in temperature [1]. Negative controls with no droplet and with a droplet that did not form a hydrate shell showed no decrease in temperature during depressurization [2].

5.4.1. LAB MEDIA: Figure 4. *Video editor: Focus on the stars.*

5.4.2. LAB MEDIA: Figure 4. *Video editor: Focus on the trials 4 and 5.*

5.5. Because the apex of each temperature decrease was above the previously established P/T stability curve [1], a regression curve was calculated based on the apex P/T of these trials [2].

5.5.1. LAB MEDIA: Figure 4. *Video editor: Focus on the hydrate stability curve #1 shown in dark black line.*

5.5.2. LAB MEDIA: Figure 4. *Video editor: Focus on the hydrate stability curve #2 shown in dotted line.*

Conclusion

6. Conclusion Interview Statements

- 6.1. **Abigail Johnson:** Two things are important to keep in mind when performing this protocol: one, check that all connections are secured before pressurizing and two, practice expelling the droplet before attaching the sapphire window [1].
 - 6.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.4.3. and 2.3.2.*
- 6.2. **Abigail Johnson:** Following this protocol, one can calculate gas consumption as well as analyze the images to calculate hydrate shell thickness to determine the volume of hydrate formed [1].
 - 6.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Figure 3.*