FINAL SCRIPT: APPROVED FOR FILMING

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Title: A Multimodal Imaging Framework to Advance Phenotyping of Living Label-Free Breast Cancer Cells

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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, all done**

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

Current Protocol Length
Number of Steps: 29
Number of Shots: 59

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

1.1. **Leonardo Bianchi:** We present multimodal imaging using confocal Raman micro spectroscopy and tomographic phase microscopy for rapid and unbiased morpho chemical phenotyping of live breast cancer cells in their native environment.

1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.
Video editor: Please include the affiliation of the author as given:
Leonardo Bianchi
Massachusetts Institute of Technology and Politecnico di Milano

What advantage does your protocol offer compared to other techniques?

1.2. **Leonardo Bianchi:** Combining Raman spectroscopy and tomographic phase microscopy enables label-free, physiological-condition imaging, offering detailed biochemical and morphological cell data while avoiding perturbations from fluorescence labeling or chemical fixation.

1.2.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B.roll:6.4*

How will your findings advance research in your field?

1.3. **Leonardo Bianchi:** Our multimodal approach enables detailed morpho-chemical profiling of cancer cells. Its versatility, reproducibility, and non-invasiveness support broad biomedical applications, from basic cell biology to diagnostic research.

1.3.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B.roll:6.3*

What research questions will your laboratory focus on in the future?

1.4. **Jeon Woong Kang:** We aim to create an atlas of optical biomarkers for non-invasive embryonic quality control, detecting cellular senescence, and drug screening in organoid models to advance regenerative medicine and disease research.

1.4.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.
Video editor: Please include the affiliation of the author as given:
Jeon Woong Kang
Massachusetts Institute of Technology

Videographer: Obtain headshots for all authors available at the filming location.

Protocol

2. Raman Imaging of MDA-MB-231 Cells

Demonstrator: Leonardo Bianchi

2.1. To begin, place the MDA-MB-231 *(M-D-A-M-B-Two-Three-One)* human breast cancer cells sample into the onstage incubator **[1-TXT]**. Add water for the water immersion objective **[2].**

2.1.1. WIDE: Talent placing the biological sample into the onstage incubator. **TXT: Incubation: 37 °C, Humidity > 95%, 5% CO_2**

Added shot: Talent pipetting water for the water immersion objective.

2.2. Switch on the automated water-immersion feeder to supply water to the objective lens **[1]**. Regularly check the water levels to avoid evaporation and ensure consistent imaging conditions throughout the measurements **[2]**.

2.2.1. Talent pressing the power button on the water-immersion feeder.

2.2.2. Talent checking and refilling the water reservoir.

2.3. Now, turn on the pump laser and adjust its output to attain a laser power of 75 milliwatts at the sample plane **[1]**. Open the **Micro-manager** *(Micro-manager)* microscope control software **[2]**.

2.3.1. Talent turning on the pump laser and adjusting power output dial.

2.3.2. SCREEN: 68498_screenshot_1.mp4 00:00-00:08.

2.4. In the Configuration settings, select **BF** *(B-F)* and click **Live** on the left side of the software window **[1]**. Choose the desired single cell, then click **Stop** to end the bright-field visualization **[2]**.

2.4.1. SCREEN: 68498_screenshot_2.mp4. 00:00-00:06

2.4.2. SCREEN: 68498_screenshot_2.mp4. 00:07-00:14

2.5. Open the CCD camera control software **[1]**. In the Experiment Setting, set the **Exposure Time** to 1.5 seconds **[2]**. Click **Run** on the Experiment toolbar **[3]** and turn on the laser beam **[4]**.

2.5.1. SCREEN: 68498_screenshot_3.mp4 00:00-00:08

2.5.2. SCREEN: 68498_screenshot_3.mp4 00:10-00:18

2.5.3. SCREEN: 68498_screenshot_3.mp4 00:19-00:22

2.5.4. Talent manually turning the laser on.
AND

2.5.5. *Added shot: SCREEN: 68498_screenshot_4.mp4. 00:00-00:11*
TXT: Laser ON
Video editor: Author requests to overlay text as a short fade, appearing for 2 seconds at 00:06 before fading out. Also play both shots side by side

2.6. Next, adjust the Z position to maximize the signal counts in the fingerprint region from 600 to 1800 inverse centimeters **[1]**. Then turn off the laser **[2]**, click **Stop** on the **Experiment** toolbar, and exit the software **[3-TXT]**.

2.6.1. Talent manually adjusting Z-axis to optimize signal peaks.

Added shot: 2.6.1b Close up on the microscope controller used to adjust the Z-axis.
Added shot: 2.6.1c SCREEN: 68498_screenshot_5.mp4 00:00-00:25
Video editor: Please play 2.6.1 a to 2.1.c side by side

2.6.2. Talent manually turning the laser off.

2.6.3. SCREEN: 68498_screenshot_6.mp4 00:05-00:15
TXT: Laser OFF
Video editor: Author requests to overlay text as a short fade at 00:06, appearing for 2 seconds before fading out.

2.7. Start the data acquisition process using the MATLAB script, which automatically manages Raman channels in multi-dimensional measurements and saves the data **[1]**.

2.7.1. SCREEN: 68498_screenshot_7.mp4 00:00-00:11

2.8. Set the field of view to 50 by 50-micrometer square, with a resolution of 40 by 40 pixels, each pixel covering 1.25 by 1.25-micrometer square **[1]**. Set the exposure time for each Raman spectrum to 1.5 seconds and run the MATLAB script **[2]**.

2.8.1. SCREEN: 68498_screenshot_8.mp4 00:00-00:16

2.8.2. SCREEN: 68498_screenshot_8.mp4 00:17-00:26

3. Raman Spectroscopy Data Analysis

3.1. For the post-processing of Raman hyperspectral maps, launch the RamApp *(Rahm-App)* web based tool **[1].** Import the hyperspectral data by clicking on **New** in the **My Analyses** tab, and upload the .mat *(dot-mat)* file containing the spatial and spectral data variables along with the x-axis variable **[2]**.

3.1.1. SCREEN: 68498_screenshot_9.mp4 00:00-00:05

3.1.2. SCREEN: 68498_screenshot_9.mp4 00:06-00:37

3.2. To truncate the spectrum, in the **Crop and rotate** menu, choose **Spectral crop [1].** Define the inferior and superior borders of the Raman shift region as 600 and 1800 inverse centimeters respectively **[2]**.

3.2.1. SCREEN: 68498_screenshot_10.mp4 00:00-00:06

3.2.2. SCREEN: 68498_screenshot_10.mp4 00:07-00:23

3.3. To correct for cosmic rays, go to **Denoising**, click **Despike**, select **Z-score** as Method, and set Threshold to 8 **[1]**. Check the options **Use first difference** and **Correct spikes [2]**. Then click **Run** to apply the correction **[3]**.

3.3.1. SCREEN: 68498_screenshot_11.mp4 00:00-00:08

3.3.2. SCREEN: 68498_screenshot_11.mp4 00:15-00:17

3.3.3. SCREEN: 68498_screenshot_11.mp4 00:18-00:22

3.4. To remove noise from the maps, click **Smooth Map** , choose the **Median filter**, set the size of the square to 3 pixels, then click **Run [1]**.

3.4.1. SCREEN: 68498_screenshot_12.mp4 00:00-00:12

3.5. To smooth the spectrum of each pixel, ~~click on **Smooth spectrum** under **Denoising,**~~ choose the **Savitzky-Golay** *(sævɪtski ˈgoʊleɪ)* **filter [1].** Set filter window length to 7 and polynomial order to 2, then click **Run [2]**.

3.5.1. SCREEN: 68498_screenshot_13.mp4 00:00-00:06

3.5.2. SCREEN: 68498_screenshot_13.mp4 00:07-00:15

3.6. To subtract the average background signal, open **Optical substrate removal** and click **Identify substrate [1].** Set **k-means** as the **Method** with 2 clusters, then click on **advanced options [2].** Choose **Morphological cleaning** to remove small blobs from the foreground then click **Run [3]**.

3.6.1. SCREEN: 68498_screenshot_14.mp4 00:00-00:05

3.6.2. SCREEN: 68498_screenshot_14.mp4. 00:06-00:09

3.6.3. SCREEN: 68498_screenshot_14.mp4. 00:10-00:20

3.7. To remove the average background signal, click on **Remove substrate**, then choose **Global average (mean of the central 95% of values)** *(mean-of-the-central-95-percent-of-values)* method and click **Run [1]**.

3.7.1. SCREEN: 68498_screenshot_15.mp4 00:00-00:09

3.8. To correct for baseline fluorescence, open the **Baseline** panel, click on **Correct baseline**, select **Adaptive Smoothness PLS** *(P-L-S)* then set lambda to 5,000,000, and click **Run [1]**. For the normalization of each Raman hyperspectral map, go to **Miscellaneous**, select **Normalize**, choose **Frobenius** method, and click **Run [2]**.

3.8.1. SCREEN: 68498_screenshot_16.mp4 00:00-00:16

3.8.2. SCREEN: 68498_screenshot_16.mp4 00:17-00:27

3.9. To generate a false-color image showing subcellular distribution, on **Images** panel, click the **Open menu** symbol under **Intensity Image**, then click **Edit image [1]**. On the editing panel, select **Single band** and define the spectral range **[2].** Now choose **Double color** on the **Color map** then define the color, intensity threshold, and opacity, before clicking **Confirm [3]**.

3.9.1. SCREEN: 68498_screenshot_17.mp4 00:00-00:04

3.9.2. SCREEN: 68498_screenshot_17.mp4 00:05-00:19

3.9.3. SCREEN: 68498_screenshot_17.mp4 00:20-00:40

3.10. For the download of a single-point spectrum, choose a pixel of interest, then on the **Spectral legend** panel, click **Download spectrum [1-TXT]**.

3.10.1. SCREEN: 68498_screenshot_18.mp4 00:00-00:11

TXT: Repeat as needed for other regions

3.11. Finally, to export foreground and substrate spectra, navigate to **Spectral legend** then click **Download spectrum** for both **Foreground** and **Substrate** sections **[1]**.

3.11.1. SCREEN: 68498_screenshot_19.mp4 00:00-00:12

4. Tomographic Phase Microscopy (TPM) Measurement Protocol

4.1. Place a drop of distilled water onto the objective lens of the microscope **[1]**. Position the sample on the microscope's translation stage **[2]**. Then adjust the sample position to align it with the objective lens **[3]**.

4.1.1. Talent pipetting distilled water onto the microscope objective.

4.1.2. Talent placing the sample onto the translation stage.

4.1.3. Talent fine-tuning the position to align the sample with the objective.

4.2. Turn on the microscope and launch the imaging software **[1]**. Click the microscope icon on the toolbar **[2]**. After initialization, click **Configuration**, select **LiveCell** in the **Job** panel, and **PBS** as the **Medium [3]**.

4.2.1. SCREEN: 68498_screenshot_20.mp4. 00:00-00:06

4.2.2. SCREEN: 68498_screenshot_20.mp4. 00:07-00:14

4.2.3. SCREEN68498_screenshot_20.mp4. 00:27-00:40

4.3. Next, access the **Calibration** tab in the control panel of the imaging software **[1]**. Set the axial positions of the objective and condenser lenses by selecting **Focus** and **Surface**, respectively **[2]**.

4.3.1. SCREEN: 68498_screenshot_21.mp4 00:00-00:03

4.3.2. SCREEN: 68498_screenshot_21.mp4 00:04-00:17

4.4. Now click **Scanning mode** to manually adjust the lenses and ensure the illumination

patterns are centered and nearly static. Choose **Normal mode [1]**, then adjust the translation stage to bring the cell into the field of view and focus on the sample **[2]**.

4.4.1. SCREEN: 68498_screenshot_22.mp4 00:00-00:30

4.4.2. SCREEN: 68498_screenshot_23.mp4 00:00-00:14

4.5. Adjust the translation stage to locate a region without cells **[1]**. Choose **Calibrate** to capture multiple 2D holograms at varying illumination angles **[2]**.

4.5.1. Talent moving the stage to an acellular region.

4.5.1b Close-up on the stage controller.

4.5.2. SCREEN: 68498_screenshot_24.mp4 00:00-00:10

4.6. Adjust the translation stage to center the cell ~~within the field of view~~ . Then navigate to the **Acquisition** tab and select **3D Snapshot** to capture the tomogram of the cell **[1]**.

~~4.6.1.Talent repositioning stage to center the cell~~.

NOTE: Shot deleted at author's request

4.6.2 SCREEN: 68498_screenshot_25.mp4 00:00-00:18

5. Tomographic Phase Microscopy Data Analysis

5.1 To visualize the holographic tomograms, select the data on the **Data Navigation** panel, right-click and click **Open [1]**. On the **Data Manager** panel, click on **RI** *(R-I)* **Tomogram [2]**.

5.1.1. SCREEN: 68498_screenshot_26.mp4 00:00-00:11

5.1.2. SCREEN: 68498_screenshot_26.mp4 00:12-00:15

5.2. In the **Volume Visualization** panel, select **RI** and draw four rectangular color boxes within the RI canvas **[1]**. Set the minimum and maximum values of the **RI range** for each color box then associate an opacity and color with each box **[2]**. Click **Save** to store the defined RI Ranges and color boxes **[3]**.

5.2.1. SCREEN: 68498_screenshot_27.mp4 00:00-00:11

5.2.2. SCREEN: 68498_screenshot_27.mp4 00:12-00:34

5.2.3. SCREEN: 68498_screenshot_27.mp4 03:41-03:54

5.3. To attain quantitative descriptors of cell morphology, utilize the **Analysis** interface **[1]**. Select **Manual** under the **Segmentation** panel then set 1.3450 as the RI Threshold, and click **Apply [2]**. Click **Save** to save the calculated morphological indexes **[3]**.

5.3.1. SCREEN:68498_screenshot_28.mp4. 00:00-00:04

5.3.2. SCREEN: 68498_screenshot_28.mp4 00:08-00:16,00:35-00:37

5.3.3. SCREEN: 68498_screenshot_28.mp4 00:41-00:53

Results

6. Results

6.1. Raman spectral mapping revealed the spatial distribution of cytoplasm **[1]**, nucleus **[2]**, phenylalanine **[3]**, and lipids within individual MDA-MB-231 cells **[4]**.

6.1.1. LAB MEDIA: Figure 2A. *Video editor: Highlight the green-labeled image under "CYTOPLASM" showing bright green color in the cytoplasmic region.*

6.1.2. LAB MEDIA: Figure 2A. *Video editor: Highlight the blue-labeled image under "NUCLEUS" showing bright blue signal localized in the nuclear region.*

6.1.3. LAB MEDIA: Figure 2A. *Video editor: Highlight the red-labeled image under "PHENYLALANINE" showing red fluorescence in specific intracellular zones*.

6.1.4. LAB MEDIA: Figure 2A. *Video editor: Highlight the brown-labeled image under "LIPIDS" showing golden-brown intensity in the lipid-rich areas.*

6.2. Distinct Raman spectral peaks corresponding to cytoplasm **[1]**, nucleus **[2]**, phenylalanine **[3]**, and lipids were confirmed in the fingerprint region between 600 and 1800 inverse centimeters **[4]**.

6.2.1. LAB MEDIA: Figure 2B. *Video editor: Highlight the green vertical band at 715–725 on the x-axis representing cytoplasm peak.*

6.2.2. LAB MEDIA: Figure 2B. *Video editor: Highlight the blue vertical band at 780–790 on the x-axis representing nuclear peak.*

6.2.3. LAB MEDIA: Figure 2B. *Video editor: Highlight the narrow brown vertical band at 1004 on the x-axis indicating phenylalanine signal.*

6.2.4. LAB MEDIA: Figure 2B. *Video editor: Highlight the orange vertical band at 1440–1450 on the x-axis indicating lipid peak.*

6.3. Refractive index heat maps of single cells revealed the internal distribution of subcellular components in the axial XY plane **[1]**, sagittal YZ plane **[2]**, and coronal XZ planes **[3]**.

6.3.1. LAB MEDIA: Figure 4. *Video editor: Show the leftmost XY PLANE heatmap.*

6.3.2. LAB MEDIA: Figure 4. *Video editor: Highlight the YZ PLANE middle panel*

6.3.3. LAB MEDIA: Figure 4. *Video editor: Highlight the XZ PLANE rightmost panel*

6.4. A 3D tomographic reconstruction identified regions of volumetric refractive index corresponding to internal substructures **[1]**. Quantitative morphological measurements of a single MDA-MB-231 cell showed different descriptors of cell morphology, with a total volume of 4024.189 cubic micrometers and surface area of 2383.707 square micrometers **[2]**, with a mean refractive index of 1.356 **[3]**.

6.4.1. LAB MEDIA: Figure 5A.

6.4.2. LAB MEDIA: Figure 5B. *Video editor: Highlight the values for "VOLUME" and "SURFACE AREA" on the right panel.*

6.4.3. LAB MEDIA: Figure 5B. *Video editor: Highlight the "MEAN RI" value in the list on the right.*

Pronunciation Guide:

1. Confocal

- **Pronunciation link**: https://www.merriam-webster.com/dictionary/confocal
- **IPA**: /kənˈfoʊkəl/
- **Phonetic Spelling**: kuhn-FOH-kuhlmerriam-webster.com+6merriam-webster.com+6merriam-webster.com+6

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2. Raman

- **Pronunciation link**: https://www.merriam-webster.com/dictionary/Raman
- **IPA**: /ˈrɑːmən/
- **Phonetic Spelling**: RAH-muhnmerriam-webster.commerriam-webster.com+1merriam-webster.com+1

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3. Spectroscopy

- **Pronunciation link**: https://www.merriam-webster.com/dictionary/spectroscopy
- **IPA**: /spɛkˈtrɑːskəpi/
- **Phonetic Spelling**: spek-TRAH-skuh-peemerriam-webster.com+14merriam-webster.com+14merriam-webster.com+14

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4. Tomographic

- **Pronunciation link**: https://www.merriam-webster.com/dictionary/tomography
- **IPA**: /ˌtoʊməˈgræfɪk/
- **Phonetic Spelling**: TOH-muh-GRAF-ikmerriam-webster.com

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5. Microscopy

- **Pronunciation link**: https://www.merriam-webster.com/dictionary/microscopy
- **IPA**: /maɪˈkrɑːskəpi/
- **Phonetic Spelling**: my-KRAH-skuh-peemerriam-webster.com+15merriam-webster.com+15merriam-webster.com+15

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6. Phenylalanine

- **Pronunciation link**: https://www.merriam-webster.com/dictionary/phenylalanine
- **IPA**: /ˌfɛnɪlˈæləniːn/
- **Phonetic Spelling**: FEN-il-AL-uh-neen

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7. Hyperspectral

- **Pronunciation link**: https://www.merriam-webster.com/dictionary/hyperspectral
- **IPA**: /ˌhaɪpərˈspɛktrəl/
- **Phonetic Spelling**: HY-per-SPEK-truhl

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8. Savitzky-Golay

- **Pronunciation link**: https://www.howtopronounce.com/savitzky-golay

- **IPA**: /ˈsævɪtski ˈgoʊleɪ/
- **Phonetic Spelling**: SAV-its-kee GOH-lay

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9. Frobenius

- **Pronunciation link**: https://www.howtopronounce.com/frobenius
- **IPA**: /froʊˈbiːniəs/
- **Phonetic Spelling**: froh-BEE-nee-uhsmerriam-webster.com+1merriam-webster.com+1

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10. Morphological

- **Pronunciation link**: https://www.merriam-webster.com/dictionary/morphological
- **IPA**: /ˌmɔːrfəˈlɑːdʒɪkəl/
- **Phonetic Spelling**: mor-fuh-LOJ-ih-kuhl