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Title: Probing the Ovarian Microenvironment: Methods for Isolation, 2D Culture, and Organoid Culture of Mouse Ovarian Somatic 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? **Yes**

If **Yes**, can you record movies/images using your own microscope camera? **No**

SCOPE shots: **2.1.3., 2.2.2., 2.2.3., 2.3.3.**

Note: All SCOPE shots were filmed by the videographer. Microscope shots have a verbal slate (and files are named for shot number), as per the videographer.

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**

4. Testimonials (optional): Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **No**

Current Protocol Length

Number of Steps: 16

Number of Shots: 48

Interviews

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

INTRODUCTION:

~~REQUIRED: Summarize your research focus and the main questions it aims to answer.~~

- 1.1. **Shweta Dipali:** Our research focuses on isolating the stroma-enriched fraction of murine ovarian somatic cells to generate ovarian somatic organoids.
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. **Videographer's Note: Interview 1.1 - Use 2nd take - No slate**

~~Describe the research contexts or systems in which this protocol can be applied.~~

- 1.2. **Aubrey Converse:** This protocol enables the study of both physiological and pathological processes within the mammalian ovarian stromal compartment.
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. **Videographer's Note: Interview 2.1 - Use 2nd take**

CONCLUSION:

~~What does this protocol allow researchers to study or measure?~~

- 1.3. **Shweta Dipali:** This protocol allows researchers to evaluate ovarian organoid morphology, gene and protein expression profiles, secretory or metabolic activity, and cellular interactions.
 - 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. **Videographer's Note: Interview 3.1 - Use 2nd take**

~~Describe the most important consideration or challenge when performing this protocol.~~

- 1.4. **Aubrey Converse:** Optimizing enzymatic incubation is critical, as under- or over-digestion of ovarian tissue severely compromises cell yield and viability.
 - 1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. **Videographer's Note: Interview 4.1 - Use 2nd take**

~~How can future studies build on this work? Describe the questions or research directions that can be investigated next.~~

- 1.5. **Shweta Dipali:** Future studies will focus on optimizing the generation and characterization of these organoids across other mammalian species, including humans.

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

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

Ethics Title Card

This research has been approved by the Institutional Animal Care and Use Committee (IACUC) at Northwestern University

Protocol

***Videographer:** Please always record an audio slate while filming each shot mentioned in the shotlist.*

Note: Shot descriptions modified by the authors are provided with a note for the video editor's reference. Please ignore if not needed.

Note: All SCOPE shots were filmed by the videographer.

Videographer's Note: Microscope shots have a verbal slate (and files are named for shot number)

2. Isolation of Primary Somatic Cells from Mouse Ovarian Tissue

Demonstrator: Aubrey Converse

2.1. To begin, dissect the mouse ovaries placed in a 35-millimeter Petri dish [1] containing 3 milliliters of dissection media, or DM (*D-M*), from surrounding tissue [2]. Use fine-tipped forceps or microscissors to separate the ovaries from the attached adipose tissue, oviduct, and ovarian bursa under a dissecting microscope [3].

2.1.1. WIDE: Talent ~~placing the dissected~~ dissecting mouse ovaries ~~and~~ from surrounding bursa, adipose tissue, and oviduct into a 35-millimeter Petri dish on the laboratory bench. ***Videographer:** Please obtain CU shot as well for this shot.*

Note: The authors modified this shot description; the narration was updated to match it.

2.1.2. TEXT ON A PLAIN BACKGROUND

Dissection Media

Leibovitz's L-15 medium	15 mL
Fetal Bovine Serum (FBS)	1 %
Penicillin-Streptomycin (PS)	0.5 %

2.1.3. SCOPE: Show the separation of surrounding adipose tissue, oviduct, and ovarian bursa from the ovaries using fine-tipped forceps and microscissors.

2.2. Transfer the dissected ovaries to a 45-millimeter watch glass containing 2 to 3 milliliters of DM [1]. Anchor the ovaries using 28-gauge insulin needle with the nondominant hand [2]. Puncture all ovarian areas with a 28-gauge insulin needle for approximately 10 minutes, rotating frequently to ensure complete tissue disruption [3].

2.2.1. Talent transferring dissected ovaries into a 45-millimeter watch glass containing DM.

2.2.2. SCOPE: Show anchoring the ovarian tissue with a 28-gauge insulin needle. **Note:**

Shots 2.2.2 and 2.2.3 were combined.

- 2.2.3. SCOPE: Show puncturing the ovarian tissue with another 28-gauge insulin needle while rotating the tissue for complete disruption
- 2.3. Use fine-tipped forceps to transfer the stroma-enriched mouse ovaries to a Petri dish [1] containing equilibrated enzymatic media, or EM (*E-M*) [2], then quickly tear them into approximately 2 to 3-millimeter pieces to minimize temperature and pH changes to the EM [3].
- 2.3.1. Talent transferring of stroma-enriched ovaries using fine-tipped forceps into a Petri dish containing equilibrated EM.
- 2.3.2. TEXT ON A PLAIN BACKGROUND
- Enzymatic Media**
- | | |
|-------------------------|--|
| α MEM | 10 mL |
| FBS | 1 % |
| Penicillin-Streptomycin | 0.5% |
| Collagenase IV | 82 enzymatic units/mL |
| 0.2 mg/mL DNase I | ≥ 400 enzymatic activity units/mg |
- 2.3.3. SCOPE: Show tearing the ovarian tissue into small pieces using fine-tipped forceps.
- 2.4. Hold the Petri dish at an angle [1]. Use a P1000 (*P-one thousand*) pipette set to 1 milliliter to triturate EM containing ovarian tissue pieces 100 times or for 3 minutes [2], swirling as necessary to ensure that all tissue pieces are mechanically dissociated [3].
- 2.4.1. Talent tilting the Petri dish at an angle while handling the tissue suspension.
Note: Shots 2.4.1, 2.4.2, and 2.4.3 were combined.
- 2.4.2. Talent repeatedly pipetting the EM and ovarian tissue mixture using a p1000 pipette.
- 2.4.3. Talent swirling the Petri dish to redistribute tissue pieces.
- 2.5. For tissue digestion, incubate the Petri dish containing EM and the disrupted ovarian tissue at 37 degrees Celsius with 5 percent carbon dioxide for a total of 30 minutes [1]. Remove the dish from the incubator at 15-minute and 30-minute timepoints [2], to triturate the tissue suspension 100 times or for 3 minutes to ensure complete digestion [3].
- 2.5.1. Talent placing the Petri dish into a tissue culture incubator.
- 2.5.2. Talent removing the dish after 15 minutes.
- 2.5.3. Talent repeatedly pipetting the tissue suspension.

- 2.6. Next, add 20 milliliters of preequilibrated quenching media, or QM (*Q-M*) [1] to dilute the enzymes and slow tissue digestion [2]. Pass the cell suspension through a 40-micrometer cell strainer placed over a 50-milliliter conical tube to remove undigested tissue fragments [3]. Centrifuge the filtrate at 300 *g* for 5 minutes at room temperature to pellet the cells [4].

2.6.1. Talent adding preequilibrated QM to the ovarian tissue suspension.

2.6.2. TEXT ON A PLAIN BACKGROUND

Quenching Media

α MEM	20 mL
FBS	10 %
Penicillin-Streptomycin	0.5 %

2.6.3. Talent filtering the cell suspension through a 40 μ m cell strainer into a 50 mL conical tube.

2.6.4. Talent placing the conical tube into a centrifuge.

- 2.7. After aspirating the supernatant, wash the cells [1] by resuspending in 1 milliliter of preequilibrated plating media, or PM (*P-M*) [2]. Transfer the cell suspension to a 1.5 milliliter microcentrifuge tube [3]. Centrifuge the suspension at 300 *g* for 5 minutes at room temperature to pellet the cells again [4].

2.7.1. Talent resuspending the cell pellet in plating media.

2.7.2. TEXT ON A PLAIN BACKGROUND

Plating Media

RPMI 1640 (containing 25 mM HEPES and 2 mM L-Glutamine)	30 mL
FBS	10 %
Penicillin-Streptomycin	1 %

2.7.3. Talent transferring the cell suspension into a 1.5 milliliter microcentrifuge tube.

Videographer's Note: Use 2nd take.

2.7.4. Talent placing the microcentrifuge tube into a centrifuge.

- 2.8. After discarding the supernatant, resuspend the cell pellet in 1 milliliter of PM [1]. Stain a 10-microliter aliquot of cell suspension with trypan blue [2] and use a hemocytometer to count the cells to assess cell viability [3].

2.8.1. Talent resuspending the cell pellet in PM.

2.8.2. Talent mixing a cell aliquot with trypan blue solution and adding it to a hemacytometer. **Note:** The shot description "and adding it to a hemacytometer" was added by the author.

Videographer's Note: Use 2nd take.

- 2.8.3. Representative shot of talent counting the cells.
- 2.9. Plate cells at approximately 3×10^6 onto a tissue culture-treated 60-millimeter dish [1].
 - 2.9.1. Talent dispensing cells into a tissue culture-treated 60 mm dish.

3. Generation and Culture of Organoids

Demonstrator: Shweta Dipali

- 3.1. Use Graefe (*GRAY-fuh*) forceps to carefully transfer the pre-prepared agarose micromolds into the wells of a new 24-well plate [1]. Add 500 microliters of ovarian organoid media to each well, ensuring complete immersion of the agarose micromold [2]. Equilibrate the micromolds and media for at least 30 minutes in an incubator maintained at 37 degrees Celsius with 5 percent carbon dioxide [3].
 - 3.1.1. Talent transferring agarose micromolds into a 24-well plate using Graefe forceps.
 - 3.1.2. Talent adding ovarian organoid media into each well.
 - 3.1.3. Talent placing the 24-well plate into an incubator.
- 3.2. Next, wash the adherent ovarian somatic cells with magnesium and calcium-free PBS [1]. Add 1 milliliter of 0.05 percent trypsin to harvest the cells from 2D culture [2] and incubate for 2 to 3 minutes at 37 degrees Celsius [3].
 - 3.2.1. Talent washing the cells with Mg and Ca-free PBS. **Videographer's Note:** Use 2nd take.
 - 3.2.2. Talent adding trypsin solution to the culture vessel.
 - 3.2.3. Talent placing the culture vessel into an incubator.
- 3.3. After confirming cell detachment under a microscope, add 2 milliliters of PM to inactivate the trypsin [1]. Transfer the cell suspension to a 15-milliliter conical tube [2] and centrifuge at 300 *g* for 5 minutes at room temperature [3].
 - 3.3.1. Talent adding warmed PM to neutralize the trypsin solution. **Videographer's Note:** Use 2nd take.
 - 3.3.2. Talent transferring the cell suspension into a 15-milliliter conical tube.
 - 3.3.3. Talent placing the conical tube into a centrifuge.
- 3.4. Resuspend the cells in 1 milliliter of warmed PM [1] and transfer them to a microcentrifuge tube for cell counting [2] and washing [3].
 - 3.4.1. Talent resuspending the cell pellet in warmed PM and transferring it to a microcentrifuge tube. **Note:** The author's shot description, starting from "and

transferring it to a microcentrifuge tube,” is incorporated into the narration.

- 3.4.2. Representative shot of talent counting the cells.
- 3.4.3. Talent placing the ~~conical~~ microcentrifuge tube into a centrifuge.
- 3.5. After washing with PM, resuspend the cell pellet in ovarian organoid media to a density of 3.33×10^6 live cells per milliliter [1]. Remove the equilibrated agarose micromolds from the incubator [2].
 - 3.5.1. Talent resuspending the cell pellet in ovarian organoid media.
 - 3.5.2. Talent removing the equilibrated agarose micromolds from the incubator.
- 3.6. After verifying the upward orientation of the cell seeding chamber [1], use Graefe forceps to lift and tilt the micromolds to remove media from the cell seeding chambers [2]. Alternatively, gently hover a pipette tip over the corner of the cell seeding chamber to remove media without contacting the agarose micromold [3]. Dispense 75 μ L of cell suspension containing 2.5×10^5 cells into each micromold seeding chamber, avoiding bubble formation [4]. Return the 24-well plate to the incubator [5].
 - ~~3.6.1. Talent showing the proper upward orientation of the cell seeding chamber in the micromold.~~ **Note: This shot was removed.**
 - 3.6.2. Talent lifting and tilting micromolds using Graefe forceps to remove media.
 - 3.6.3. Talent carefully pipetting media from the corner of the seeding chamber without touching the agarose surface. **Videographer's Note: Use 2nd take - This shot has a verbal slate**
 - 3.6.4. Talent dispensing cell suspension into the agarose micromold seeding chambers. **Videographer's Note: Use 5th take - This shot has a verbal slate**
 - 3.6.5. Talent placing the 24-well plate back into the incubator.
- 3.7. One to 2 hours following cell seeding, add an additional 250 microliters of warmed ovarian organoid media around the outside of the micromolds without disturbing the seeding chambers [1]. After organoid formation, proceed to downstream applications, including histology and molecular biology analysis [2].
 - 3.7.1. Talent adding warmed ovarian organoid media around the outer edge of the agarose micromolds.
 - 3.7.2. Talent imaging aggregated organoids

Results

4. Results

- 4.1. After 6 days of culture, organoids contained vimentin-positive fibroblasts [1], F4/80 (*EFF-four-eighty*)-positive macrophages [2], Foxl2 (*fox-ELL-two*)-positive granulosa and granulosa-lutein cells [3], and 3 beta-hydroxysteroid dehydrogenase, or 3 β -HSD (*Three-beta-H-S-D*)-positive theca cells, theca-lutein cells, and steroidogenic fibroblasts [4].
 - 4.1.1. LAB MEDIA: Figure 6B. *Video editor: Please highlight the vimentin panel in the organoids row.*
 - 4.1.2. LAB MEDIA: Figure 6B. *Video editor: Please highlight the F4/80 panel in the organoids row.*
 - 4.1.3. LAB MEDIA: Figure 6B. *Video editor: Please highlight the Foxl2 panel in the organoids row.*
 - 4.1.4. LAB MEDIA: Figure 6B. *Video editor: Please highlight the 3 β -HSD panel in the organoids row.*
- 4.2. F4/80-positive macrophages were restricted to the perimeter of organoids [1], while 3 β -HSD-positive steroidogenic cells were depleted from the core of organoids, indicating cellular heterogeneity and distinct spatial organization of cell types over 6 days of organoid culture. [2].
 - 4.2.1. LAB MEDIA: Figure 6B. *Video editor: Please highlight the outer brown edge in the F4/80 panel in the organoids row.*
 - 4.2.2. LAB MEDIA: Figure 6B. *Video editor: Please highlight the outer green region in the 3 β -HSD panel in the organoids row.*
- 4.3. Organoid area increased between days 1 and 13 of culture [1], with the most rapid growth between days 7 and 13 [2]. Organoid growth plateaued between days 13 and 21 [3].
 - 4.3.1. LAB MEDIA: Figure 8A. *Video editor: Please highlight the panels D1,D7, D13 panels sequentially.*
 - 4.3.2. LAB MEDIA: Figure 8B. *Video editor: Please emphasize the rising line in the graph between Day 7 and Day 13.*
 - 4.3.3. LAB MEDIA: Figure 8B. *Video editor: Please emphasize the horizontal line in the graph between Day 13 and Day 21.*
- 4.4. Hematoxylin and eosin, or H&E (*H and E*) staining showed that organoids were solid structures after 1 and 3 weeks of culture [1]. The organoids contained very few cleaved caspase-3-positive apoptotic cells [2]. Additionally, PicroSirius Red staining of organoids

to examine extracellular matrix production showed that organoids began to secrete a collagen matrix within 1 week of culture [3] and, by 3 weeks, an abundant extracellular space with a fibrous collagen matrix was visible [4].

- 4.4.1. LAB MEDIA: Figure 8C. *Video editor: Please emphasize the dense coloring in H&E column for Day 7 and Day 21.*
- 4.4.2. LAB MEDIA: Figure 8C. *Video editor: Please highlight the brown spots in the CC3 column for Day 7 and Day 21.*
- 4.4.3. LAB MEDIA: PSR Results for filming. *Video editor: Highlight the dark red region annotated by the arrow in the PSR image in Day 7 row*
- 4.4.4. LAB MEDIA: PSR Results for filming. *Video editor: Highlight the bright red line annotated by the arrow in the PSR image in Day 21 row*

Pronunciation Guide:

1. Somatic

Pronunciation link: <https://www.merriam-webster.com/dictionary/somatic>

IPA: /soʊˈmætɪk/

Phonetic Spelling: soh·MAT·ik

2. Ovarian

Pronunciation link: <https://www.merriam-webster.com/dictionary/ovarian>

IPA: /oʊˈveriən/

Phonetic Spelling: oh·VAIR·ee·uhn

3. Leibovitz

Pronunciation link: No confirmed link found

IPA: /ˈlaɪbəvɪts/

Phonetic Spelling: LYE·buh·vits

4. Penicillin-Streptomycin

Pronunciation link: <https://www.merriam-webster.com/dictionary/penicillin>

IPA: /ˌpɛnəˈsɪlɪn/; /ˌstrɛptəˈmaɪsɪn/

Phonetic Spelling: pen·uh·SIL·in; strep·tuh·MY·sin

5. Adipose

Pronunciation link: <https://www.merriam-webster.com/dictionary/adipose>

IPA: /ˈædəˌpoʊs/

Phonetic Spelling: AD·uh·pohs

6. Oviduct

Pronunciation link: <https://www.merriam-webster.com/dictionary/oviduct>

IPA: /ˈoʊvəˌdʌkt/

Phonetic Spelling: OH·vuh·dukt

7. Bursa

Pronunciation link: <https://www.merriam-webster.com/dictionary/bursa>

IPA: /'bɜːsə/

Phonetic Spelling: BUR·suh

8. **Dissecting**

Pronunciation link: <https://www.merriam-webster.com/dictionary/dissect>

IPA: /daɪ'sektɪŋ/

Phonetic Spelling: dye·SEKT·ing

9. **Microscissors**

Pronunciation link: No confirmed link found

IPA: /,maɪkroʊ'sɪzərz/

Phonetic Spelling: MY·kroh·SIZ·erz

10. **Stroma-enriched**

Pronunciation link: <https://www.merriam-webster.com/dictionary/stroma>

IPA: /'stroʊmə/

Phonetic Spelling: STROH·muh

11. **Equilibrated**

Pronunciation link: <https://www.merriam-webster.com/dictionary/equilibrate>

IPA: /,iːkwə'laɪbreɪtɪd/

Phonetic Spelling: ee·kwuh·LY·bray·tuhd

12. **αMEM (Alpha MEM)**

Pronunciation link: No confirmed link found

IPA: /'ælfə ,ɛm iː 'ɛm/

Phonetic Spelling: AL·fuh EM EE EM

13. **Collagenase**

Pronunciation link: No confirmed link found

IPA: /kə'lædʒən,eɪs/

Phonetic Spelling: kuh·LAJ·uh·nays

14. **DNase**

Pronunciation link: No confirmed link found

IPA: /,diːɛn'eɪs/

Phonetic Spelling: DEE·EN·ace

15. **Triturate**

Pronunciation link: <https://www.merriam-webster.com/dictionary/triturate>

IPA: /'trɪtʃə ,reɪt/

Phonetic Spelling: TRICH·uh·rayt

16. **Dissociated**

Pronunciation link: <https://www.merriam-webster.com/dictionary/dissociate>

IPA: /dɪ'soʊʃi ,eɪtɪd/

Phonetic Spelling: dih·SOH·shee·ay·tuhd

17. **Incubate**

Pronunciation link: <https://www.merriam-webster.com/dictionary/incubate>

IPA: /'ɪŋkjə ,beɪt/

Phonetic Spelling: ING·kyuh·bayt

18. Preequilibrated

Pronunciation link: No confirmed link found

IPA: /ˌpriːɪˈkwɪləˌbreɪtɪd/

Phonetic Spelling: pree-ee-KWIL-uh-bray-tuhd

19. Micrometer

Pronunciation link: <https://www.merriam-webster.com/dictionary/micrometer>

IPA: /maɪˈkrɑːmɪtər/

Phonetic Spelling: my-KROM-uh-ter

20. Conical

Pronunciation link: <https://www.merriam-webster.com/dictionary/conical>

IPA: /ˈkɑːnɪkəl/

Phonetic Spelling: KON-ih-kuhl

21. Centrifuge

Pronunciation link: <https://www.merriam-webster.com/dictionary/centrifuge>

IPA: /ˈsentrəˌfjuːdʒ/

Phonetic Spelling: SEN-truh-fyooj

22. Supernatant

Pronunciation link: <https://www.merriam-webster.com/dictionary/supernatant>

IPA: /ˌsuːpərˈnɛtənt/

Phonetic Spelling: SOO-per-NAY-tuhnt

23. Resuspending

Pronunciation link: <https://www.merriam-webster.com/dictionary/resuspend>

IPA: /ˌriːsəˈspɛndɪŋ/

Phonetic Spelling: ree-suh-SPEND-ing

24. RPMI

Pronunciation link: No confirmed link found

IPA: /ɑːr piː ɛm ˈaɪ/

Phonetic Spelling: AR PEE EM EYE

25. HEPES

Pronunciation link: No confirmed link found

IPA: /ˈhɛpɛs/

Phonetic Spelling: HEP-ess

26. L-Glutamine

Pronunciation link: <https://www.merriam-webster.com/dictionary/glutamine>

IPA: /ˈgluːtəˌmiːn/

Phonetic Spelling: GLOO-tuh-meen

27. Trypan

Pronunciation link: <https://www.merriam-webster.com/medical/trypan%20blue>

IPA: /ˈtraɪpæn/

Phonetic Spelling: TRY-pan

28. Hemocytometer

Pronunciation link: <https://www.merriam-webster.com/medical/hemocytometer>

IPA: /hi:məsai'tə:mətər/

Phonetic Spelling: HEE·moh·sy·TOM·uh·ter

29. Organoids

Pronunciation link: <https://www.merriam-webster.com/medical/organoid>

IPA: /'ɔ:rgəˌnɔɪd/

Phonetic Spelling: OR·guh·noyd

30. Graefe

Pronunciation link: No confirmed link found

IPA: /'greɪfə/

Phonetic Spelling: GRAY·fuh

31. Agarose

Pronunciation link: <https://www.merriam-webster.com/medical/agarose>

IPA: /'ægəˌroʊs/

Phonetic Spelling: AG·uh·rohs

32. Micromolds

Pronunciation link: No confirmed link found

IPA: /'maɪkroʊˌmoʊldz/

Phonetic Spelling: MY·kroh·mohldz

33. Magnesium

Pronunciation link: <https://www.merriam-webster.com/dictionary/magnesium>

IPA: /mæg'ni:ziəm/

Phonetic Spelling: mag·NEE·zee·uhm

34. Trypsin

Pronunciation link: <https://www.merriam-webster.com/medical/trypsin>

IPA: /'trɪpsɪn/

Phonetic Spelling: TRIP·sin

35. Neutralize

Pronunciation link: <https://www.merriam-webster.com/dictionary/neutralize>

IPA: /'nu:trələˌzaɪz/

Phonetic Spelling: NOO·truh·luh·zyz

36. Micromold

Pronunciation link: No confirmed link found

IPA: /'maɪkroʊˌmoʊld/

Phonetic Spelling: MY·kroh·mohld

37. Histology

Pronunciation link: <https://www.merriam-webster.com/dictionary/histology>

IPA: /hɪ'stɔ:lədʒi/

Phonetic Spelling: hiss·TOL·uh·jee

38. Molecular

Pronunciation link: <https://www.merriam-webster.com/dictionary/molecular>

IPA: /mə'lekjələr/

Phonetic Spelling: muh·LEK·yuh·ler