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Monitoring *Caenorhabditis elegans* molting in a conventional luminometer

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ABSTRACT

Molting is an essential developmental process in *Caenorhabditis elegans*. However, the study of molting in the worm has been limited by the lack of automated techniques that allow monitoring the process in a simple way. In 2015, Olmedo *et al*. published an automated method to monitor the timing of each larval stage and molt in *C. elegans* using bioluminescence. This new method has greatly contributed to the study of molting in this organism but requires the use of a high-sensitivity luminometer, which many laboratories do not have. We have adapted the method to a conventional luminometer, so that it can be used by most laboratories that work with *C. elegans* and do not have high-sensitivity equipment.

- A customization of a method to study molting in *C. elegans* using a conventional luminometer instead of a high-sensitivity one.
- This adaptation allows most laboratories to use their routine luminometers to study molting in *C. elegans*.
- Although the use of a high-sensitivity luminometer, as proposed by Olmedo *et al*., remains the gold standard for studying molting, this adaptation is suitable for studying significant differences in molting and the duration of larval stages between different strains of *C. elegans*.

Specifications table

Subject area:	Agricultural and Biological Sciences
More specific subject area:	*Caenorhabditis elegans* molting
Name of your method:	*C. elegans* molting in a conventional luminometer
Name and reference of original method:	M. Olmedo, M. Geibel, M. Artal-Sanz, M. Merrow, A High-Throughput Method for the Analysis of Larval Developmental Phenotypes in *Caenorhabditis elegans*, Genetics. 201 (2) (2015) 443–448.
Resource availability:	Included in the manuscript

Method details

Olmedo's original method [1] aims to provide a simple way to monitor *C. elegans* molting. To achieve this, single worms expressing the luciferase protein are grown on liquid media with D-luciferin in 96-well plates, and the signal is monitored throughout the development using a high-sensitivity luminometer (Berthold Centro XS3, Berthold Technologies GmbH & Co. KG, Bad Wildbad,

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Germany). This method is based on the fact that when the worms molt, they enter a fasting period during which they stop consuming D-luciferin, and thus, no signal is emitted until the molt is complete. Unfortunately, the ATP detection limits of the conventional luminometers in laboratories show much lower sensitivity than that of the Berthold Centro XS3 (below 1.8 amol ATP/well). In our laboratory, we use a Thermo Fluoroskan Ascent FL (ThermoFisher Scientific Oy, Vantaa, Finland) to measure luminescence, which has a sensitivity of 40 amol ATP/well using flash reaction in a white 384-well plate. Additionally, this luminometer, like others, only measures the center of the well of a 96-well plate, making it difficult to measure luminescence in a living worm in constant motion. Both issues resulted in no signal when attemping to reproduce Olmedo's method in our conventional luminometer. To adapt this method to a less sensitive luminometer, we tested different types of plates, increased the number of worms per well, and the number of samples required to obtain experimental triplicates. Although performing this experiment in a high-sensitivity luminometer is still the gold standard, this adaptation seems suitable for studying differences in the duration of molts and larval stages of different strains of *C. elegans* using the same setup time. These modifications will enable researchers to study *C. elegans* molting using their routine luminometers and avoid the need to purchase high-sensitivity equipment specifically for the performance of this experiment.

Preparation of worms

Strain and maintenance

The PE255 strain (*feIs5* [*sur-5p*::*luc*+::*gfp; rol-6* (*su1006*)] X) was obtained from the *Caenorhabditis Genetic Center* (CGC, University of Minnesota, Minneapolis, MN). Nematodes were maintained at 20 ˚C on NGM agar plates seeded with ampicillin-resistant *E. coli* strain OP50 following standard protocols.

Generation of synchronized L1 larvae population

Animals were age-synchronized by standard treatment with sodium hypochlorite to obtain eggs that were allowed to hatch in M9 medium at 20 ˚C for 48 h with gentle shaking [2].

1. Collect gravid hermaphrodites from NGM plates into a 15 mL tube by washing the plates twice with PBST (0.01% Triton X-100 -Merck-Millipore, Billerica, MA- in phosphate-buffered saline -Gibco, Thermo Fisher Scientific Inc., Paisley, UK-). To dislodge the worms, gently swirl the plate.
2. Centrifuge the tube at 400 *g* for 4 min and remove the supernatant until 0.5 mL of volume remains.
3. Dilute the sodium hypochlorite solution EMPLURA® (Merck-Millipore, Billerica, MA) in water to a concentration of 12% v/v.
4. Add 10 mL of 12% sodium hypochlorite solution to the tube and vigorously shake for 2 min. After that, place the tube on ice for 2 min and shake again for 2 min.
5. Immediately centrifuge the tube at 400 *g* for 2 min and carefully remove the supernatant.
6. Add 10 mL of PBST to the tube and mix well. Then, centrifuge the tube at 400 *g* for 4 min and remove the supernatant until 2 mL of volume remain. Repeat this step at least once more.
7. Add approximately 10 mL of freshly prepared M9 buffer (3 g/L KH_2PO_4, 6 g/L Na_2HPO_4, 5 g/L NaCl and 1 mM $MgSO_4$) and shake to resuspend the pellet.
8. Transfer the contents of the tube to a new 15 mL tube to avoid bleach residues.
9. Allow the eggs to hatch at 20 ˚C for 48 h with gentle shaking.

Counting the worms

Once a synchronized population of L1 larvae was obtained, we counted the number of worms contained in the tube.

1. Centrifuge the tube at 400 *g* for 4 min to pellet the worms and remove the supernatant until a volume of 0.5 mL remains.
2. Mix the pellet well and pipette 10 µL. To do this, make a small cut in the tip to avoid damaging the larvae.
3. Place the 10 µL drop on a slide and count the number of worms using a dissecting microscope.
4. Finally, calculate the volume you would need to pipette to catch 10 worms.

Preparation of plates

Preparation of liquid culture medium with D-luciferin

During the assay, worms were grown in liquid culture medium (S-medium) containing 100 µM D-luciferin. Although the solutions for making the S-medium can be prepared in advance, the S-medium must be made on the day of the experiment.

1. Prepare S-basal solution: add 2.93 g NaCl, 0.66 g $K_2HPO_4 \cdot 3H_2O$ and 3 g KH_2PO_4 to a 500 mL volumetric flask and dilute with deionized water. Then autoclave and add 500 µL cholesterol (5 mg/mL in ethanol) under sterile conditions. Store at 4 ˚C until use.
2. Prepare a 1 M pH 6.0 potassium citrate solution: add 146.75 g tripotassium citrate monohydrate, 10 g citric acid monohydrate, and 400 mL deionized water to a 500 mL volumetric flask. Adjust the pH to 6.0 using 10 N KOH and bring to 500 mL. Autoclave and store at RT.

3. Prepare a trace metals solution: add 1.86 g disodium EDTA, 0.69 g $FeSO_4 \cdot 7H_2O$, 0.2 g $MnCl_2 \cdot 4H_2O$, 0.29 g $ZnSO_4 \cdot 7H_2O$, and 0.025 g $CuSO_4 \cdot 5H_2O$ to a 1 L volumetric flask and dilute with deionized water. Then autoclave and store in dark at RT until use.
4. Prepare a 1 M $MgSO_4$ solution, a 1 M $CaCl_2$ solution, and a 50 mg/mL ampicillin solution diluted in deionized water.
5. Prepare complete S-medium: mix 20 mL S-basal, 200 µL 1 M pH 6.0 potassium citrate, 200 µL trace metals solution, 60 µL 1 M $MgSO_4$, 60 µL 1 M $CaCl_2$, 20 µL 50 mg/mL ampicillin, and 200 µL 10,000 units/mL nystatin suspension (Sigma-Aldrich Co., St. Louis, MO).
6. Prepare 10 mM D-luciferin: dilute D-luciferin free acid (Biothema, Handen, Sweden) in 10 mM Tris(hydroxymethyl)aminomethane to a concentration of 10 mM and then store in the dark at -80 ˚C until use.
7. Divide the S-medium into two 15 mL tubes (~ 10 mL each) and add 100 µL 10 mM D-luciferin to each one. Keep in the dark at RT until the start of the assay.

Preparation of concentrated bacteria suspension

Ampicillin-resistant *E. coli* OP50 was added to the liquid culture medium with D-luciferin as food for the worms at a concentration of 0.02 g/mL.

1. Pick up a colony of ampicillin-resistant *E. coli* OP50 from an LB agar plate with ampicillin and allow it to grow in liquid LB with ampicillin at 37 ˚C with gentle shaking overnight.
2. The next day, prepare 5 tubes of 50 mL containing 40 mL of liquid LB and 40 µL of 50 mg/mL ampicillin and add 800 µL of the prepared liquid culture to each tube. Allow the bacteria to grow at 37 ˚C with gentle shaking overnight.
3. Centrifuge the tubes at 1800 *g* for 20 min to pellet the bacteria and remove the supernatant until a volume of ~ 3 mL remains.
4. Weigh an empty 50 mL tube.
5. Using the remaining supernatant, transfer all the pellets with a pipette to the weighed 50 mL tube.
6. Centrifuge the tube at 1800 *g* for 10 min to pellet the bacteria and remove the supernatant.
7. Weigh the tube again and remove the necessary amount of bacteria to leave 0.2 g of bacteria in the tube.
8. Transfer the 0.2 grams of bacteria to one of the 15 mL tubes containing S-medium and D-luciferin. Keep in the dark at RT until the start of the assay.

Assay procedure

Given the low sensitivity of the luminometer used (Thermo Fluoroskan Ascent FL; 40 amol ATP/well using flash reaction, 384-well white plate) and the fact that it only measures luminescence in the center of the well, at least 12 wells per strain were needed to obtain experimental triplicates, as insufficient signal was observed in some of the wells. In addition, at least 10 worms per well were required to obtain sufficient signal to distinguish molts. The assay was performed in a 96-well white Cliniplate™ microplate (Thermo Fisher Scientific Inc., Paisley, UK) since white plates reflect light and maximize the light output signal.

1. Add 100 µL of S-medium containing 100 µM D-luciferin to each well.
2. Pipette the required volume to add at least 10 L1 larvae per well.
3. Add 100 µL of S-medium containing 100 µM D-luciferin and 0.02 g/mL *E. coli* OP50 to each well.
4. Seal the plate and place it inside the luminometer.
5. Program the luminometer to take a 1 s measurement every 10 min for 96 h (577 measurements). Since the temperature inside the instrument is approximately + 3 ˚C above the ambient temperature, it is important to place the device about three degrees below the worms' developmental temperature.

Data analysis

After four days of measurement, the data is collected and analyzed using Microsoft Excel.

1. Copy all the data provided by the luminometer into a spreadsheet.
2. Plot the luminescence value against time for each well (Fig. 1).
3. Select at least three wells in which the four molts can be clearly distinguished. Each of these three wells will constitute an experimental replicate.
4. Using the graph and the data, determine at what time the luminescence begins to decrease before each molt and at what time it returns to the same value at which the decrease began (Fig. 1). These two times will mark the start and end of each molt. Use this method to study when each molt occurs and average the triplicates for these time points to obtain a final approximation of when each molt begins and ends.
5. To obtain the final graphs, once these time points have been identified, average the luminescence values obtained in the periods between each molt, which would also correspond to the L1, L2, L3, and L4 larval stages. Then divide each luminescence value by the mean of its period and keep the original luminescence values given by the luminometer for the molts. Representing these final values (Fig. 2) will allow for graphs similar to those originally presented by Olmedo *et al.* [1].

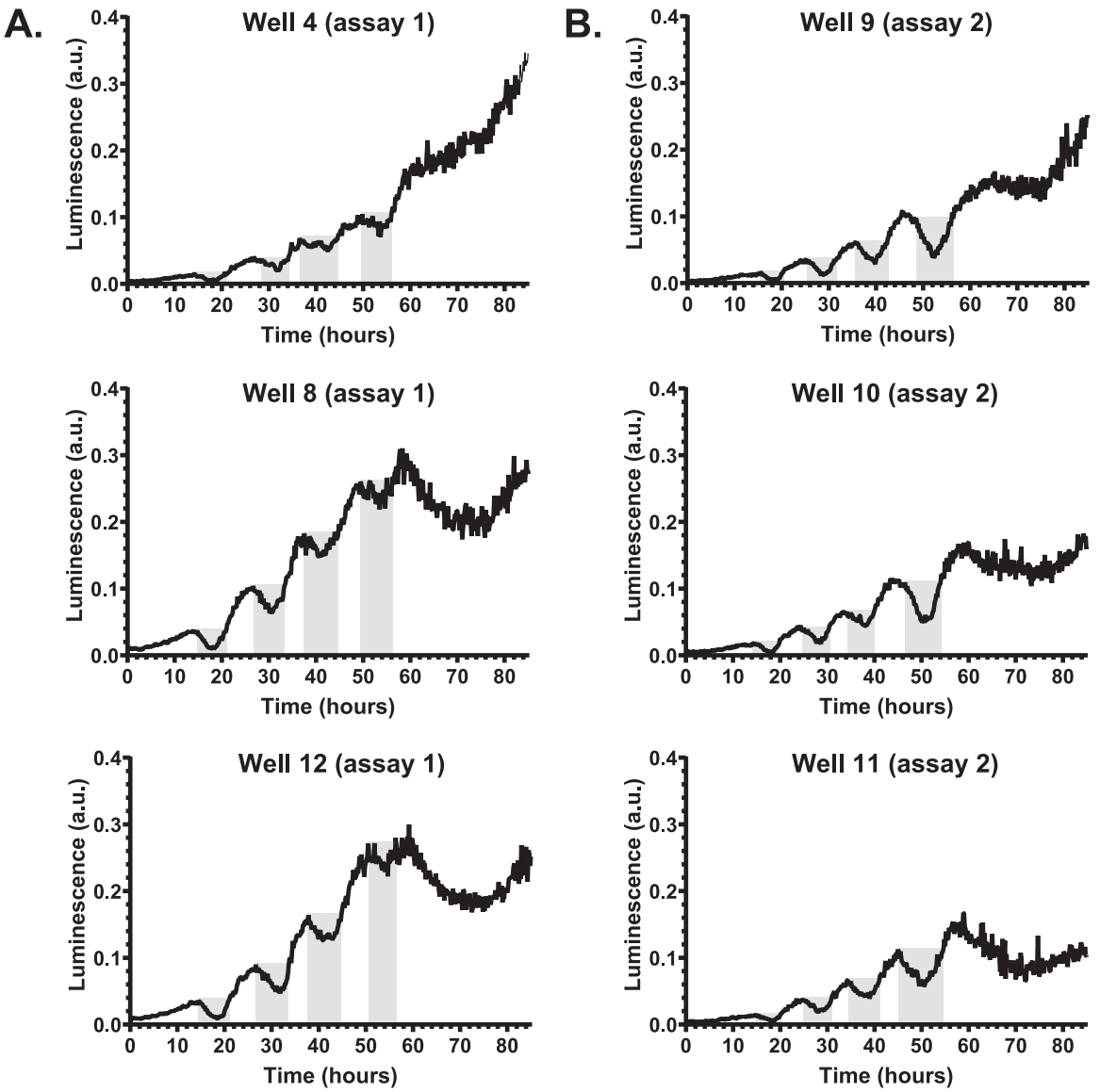

Fig. 1. Raw luminescence data (a.u., arbitrary units) from ten PE255 worms during 85 h at 20 ˚C as they develop from L1 to adult when performing the described protocol. **A**. Results obtained for each of the three wells of the first biological replicate. **B**. Results obtained for each of the three wells of the second biological replicate.

Method validation

To validate the method, we repeated the described protocol twice at 20 ˚C using the PE255 strain (*feIs5* [*sur-5p*::*luc*+::*gfp; rol-6* (*su1006*)] X). The results were consistent among experimental triplicates and between the two biological replicates (assay 1 and assay 2 in Tables 1 and 2; Figs. 1 and 2). However, it should be emphasized that even though we adapted the method to a conventional luminometer, conducting this experiment on a high-sensitivity luminometer is still the gold standard due to the higher sensitivity that allows the study of the molt of a single worm. Nevertheless, we found that this adaptation is suitable for studying important differences in molts and in the duration of larval stages of different *C. elegans* strains.

Supplementary material and/or additional information

Raw data and failed methods available in the supplementary material. Results of the first biological replicate.xlsx: this Excel file contains a table with the raw data obtained in the first biological replicate and the results of itsanalysis following the described method; Results of the second biological replicate.xlsx: this Excel file contains a table with the raw data obtained in the second

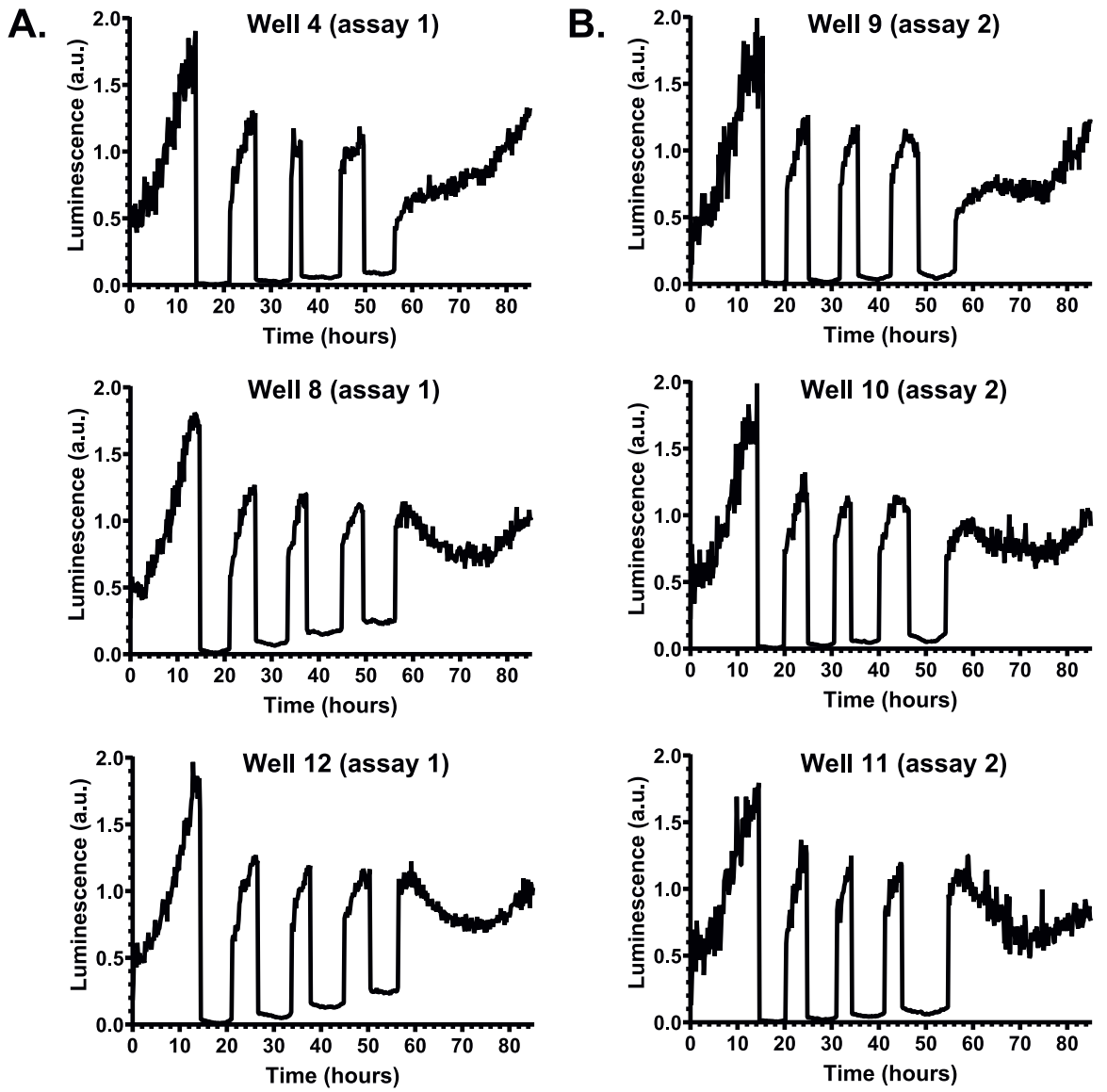

Fig. 2. Trend-corrected luminescence data (a.u., arbitrary units) from ten PE255 worms during 85 h at 20 °C as they develop from L1 to adult. **A**. Results obtained for each of the three wells of the first biological replicate. **B**. Results obtained for each of the three wells of the second biological replicate.

Table 1
Time points at which the molt starts and ends when performing the described protocol in the PE255 strain in two independent experiments.

		M1		M2		M3		M4	
		Start (h)	End (h)	Start (h)	End (h)	Start (h)	End (h)	Start (h)	End (h)
First experiment (assay 1)	Well 4	14.17	21.17	26.83	34.17	36.50	44.67	49.83	56.17
	Well 8	14.83	21.00	26.67	33.33	37.50	44.83	49.50	56.17
	Well 12	14.50	21.00	26.67	33.67	37.83	44.83	50.50	56.33
	Mean	14.50	21.06	26.72	33.72	37.28	44.78	49.94	56.22
	SD	0.33	0.10	0.09	0.42	0.69	0.09	0.51	0.09
Second experiment (assay 2)	Well 9	15.50	20.33	25.17	31.67	35.67	42.50	48.50	56.17
	Well 10	14.33	19.83	25.00	30.50	34.17	40.00	46.50	54.17
	Well 11	14.67	20.17	24.83	31.00	34.33	41.17	45.00	54.67
	Mean	14.83	20.11	25.00	31.06	34.72	41.22	46.67	55.00
	SD	0.60	0.26	0.17	0.59	0.82	1.25	1.76	1.04

Table 2
Duration of each molt and larvae stage when performing the described protocol in the PE255 strain in two independent experiments.

		L1 (h)	M1 (h)	L2 (h)	M2 (h)	L3 (h)	M3 (h)	L4 (h)	M4 (h)
First experiment (assay 1)	Well 4	14.17	7.00	5.66	7.34	2.33	8.17	5.16	6.34
	Well 8	14.83	6.17	5.67	6.66	4.17	7.33	4.67	6.67
	Well 12	14.50	6.50	5.67	7.00	4.16	7.00	5.67	5.83
	Mean	14.50	6.56	5.67	7.00	3.55	7.50	5.17	6.28
	SD	0.33	0.42	0.01	0.34	1.06	0.60	0.50	0.42
Second experiment (assay 2)	Well 9	15.50	4.83	4.84	6.50	4.00	6.83	6.00	7.67
	Well 10	14.33	5.50	5.17	5.50	3.67	5.83	6.50	7.67
	Well 11	14.67	5.50	4.66	6.17	3.33	6.84	3.83	9.67
	Mean	14.83	5.28	4.89	6.06	3.67	6.50	5.44	8.34
	SD	0.60	0.39	0.26	0.51	0.34	0.58	1.42	1.15

biological replicate and the results of its analysis following the described method; Failed methods.pdf: description of the adjustments to the method that did not work.

Ethics statements

Not applicable.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Ana Guijarro-Hernández: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Writing – original draft, Visualization. **Cristina Hurtado:** Investigation. **Juan José Martínez-Irujo:** Conceptualization, Methodology, Resources, Writing – review & editing. **José Luis Vizmanos:** Conceptualization, Software, Formal analysis, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Data availability

Data is available in supplementary material

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.mex.2023.102235.

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