This protocol describes how inductively coupled plasma mass spectrometry (ICP-MS) can quantify metals, sulfur, and phosphorus present in biological specimens. The high sensitivity of ICP-MS enables detection of these elements at very low concentrations, and absolute quantification is achieved with standard curves. Sulfur or phosphorus standardization reduces variability that arises due to slight differences in sample composition. This protocol bypasses challenges due to limited sample amounts and facilitates studies examining the biological roles of metals in health and disease.
Protocol

Sulfur- and phosphorus-standardized metal quantification of biological specimens using inductively coupled plasma mass spectrometry

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SUMMARY

This protocol describes how inductively coupled plasma mass spectrometry (ICP-MS) can quantify metals, sulfur, and phosphorus present in biological specimens. The high sensitivity of ICP-MS enables detection of these elements at very low concentrations, and absolute quantification is achieved with standard curves. Sulfur or phosphorus standardization reduces variability that arises because of slight differences in sample composition. This protocol bypasses challenges because of limited sample amounts and facilitates studies examining the biological roles of metals in health and disease.

For complete details on the use and execution of this protocol, please refer to Hartwig et al. (2020).

BEFORE YOU BEGIN

This protocol was adapted from the recent publication from Hartwig et al. (2020) (based on McAllum et al. (2020) and Ganio et al. (2016)), where we quantified total larval copper content in several Drosophila melanogaster strains harboring different transgenes to modulate expression of the copper transporter ATP7, the homolog to human ATP7A and ATP7B. We confirm here that systemic overexpression of ATP7 does not significantly modify larval copper content.

The innovation of the present protocol is a streamlined oxygen-based detection method to quantify endogenous metals, phosphorus, and sulfur using a triple quadrupole ICP-MS instrument. This protocol streamlines the measurement of sulfur, phosphorus, and target elements to a single analysis. (This is in contrast to running the sample twice to obtain measurements of sulfur or phosphorus and then target elements, which requires more sample and instrument time.) The measurement of sulfur or phosphorus provides an internal standard that is proportional to protein, phospholipid, and nucleic acid concentration in cellular and larval samples. The sulfur and/or phosphorus content can be used as equivalent internal normalizing factors that are less susceptible to error as compared to measuring cell number, cellular protein, cellular DNA, or larval weight in equivalent yet independent samples where metals are measured.

Note: Room temperature in this protocol ranges from 20°C–25°C.
Note: Abbreviations: ICP-MS, inductively plasma-coupled mass spectrometry; PBS, phosphate buffered saline; EDTA, ethylenediaminetetraacetic acid; BCS, bathocuproinedisulfonic acid.

Culture cell lines

© Timing: 1–3 h (24 h for cell attachment and growth)

Note: This protocol describes the steps for preparing SH-SY5Y neuroblastoma cell samples as in Figure 1. Steps are similar for other cell types, but certain parameters (e.g., centrifugation speed and time) may need to be optimized based on cell type and/or genotype. These areas of optimization are noted in the relevant sections of the protocol. Minor specific parameters which deviate from this standard protocol are described in the figure legends as appropriate. We recommend cells passage number 30 or less.

Note: Depending on the scale and purpose of your experiment, plan for total number of cells required, number of replicates, and various controls. This may require optimization depending on the cells and conditions you are using and the metals you plan to quantify. In our experiments, we aim to use 3–5 technical replicates per experimental condition. All replicates and conditions contain the same number of cells at the time of plating. The Biorad Cell Counter is used to measure cell counts. For accuracy, cell count measurements were made in duplicates.

Note: One million cells are more than sufficient for simultaneous metal, sulfur, and phosphorus detection, so we ensure a minimum of 1 million cells per replicate are plated on 10 cm plates or in total on a 15 cm plate (i.e., 1 million cells each across five 10 cm plates or 5 million cells on a single 15 cm plate). This number may vary depending on the cell type or line used, as cell sizes may differ. Here, we list the steps to use a 15 cm plate to prepare 5 replicates per condition. This protocol can be easily modified to utilize one 10 cm plate per replicate.

Note: Example: For SH-SY5Y cells, we seeded 6 million cells on a 15 cm dish. 48 h after seeding, the cells were at 60%–80% confluence. One 15 cm plate of SH-SY5Y cells at 80% confluency yields at least $10 \times 10^6$ cells (approximately 5 mg total protein lysate), which is sufficient to prepare 5 technical replicates (each with $2 \times 10^6$ cells or approximately 1 mg total protein).

1. On Day 1, seed a minimum of 5 million cells on a 15 cm plate.
2. On Day 2, confirm your cells at the desired level of confluency (we recommend at least 80% confluency).
   a. If you are not treating your cells, simply change media the day before you intend to freeze your samples.
   b. If cells are too sparse, change media and wait for 24 h.

Prepare for *D. melanogaster* sample collection

© Timing: 30 min (optional 24 h)

3. Prior to the experiment, prepare the 1× PBS to be used for larval collection.

Optional: Make SYLGARD™-filled petri dishes and allow to cure a minimum of 24 h. (Here, we used SYLGARD™-filled petri dishes as this helps maintain the surface tension of the PBS pool,
which keeps larvae in a smaller field of view and easier to localize under the microscope. Empty petri dishes may also be used.

**Prepare buffers for ICP-MS**

♀ Timing: 1–2 h

4. Prior to the experiment, prepare the chemicals and buffers to be used for ICP-MS, listed below. These can be used within 2 months.
   a. 2% nitric acid.
   b. Internal Standard for ICP-MS.
   c. Sulfur Calibration Standard for ICP-MS (100 mg/mL).
   d. Multielement Calibration Standard for ICP-MS (1,000 μg*L⁻¹ and 100 μg*L⁻¹).

⚠ CRITICAL: Nitric acid (HNO₃) is highly corrosive, and exposure to liquid and fume form can irritate the eyes, skin, and mucous membrane among other harmful outcomes. All work with concentrated nitric acid should be done with appropriate work practice controls and personal protective equipment (PPE), i.e., working with small volumes while using a chemical fume hood, lab coat, protective eyewear, and gloves. Nitric acid bottles can build pressure inside and should be vented monthly. Only plastic pipettes should be used with 70% nitric acid.

⚠ CRITICAL: You should prepare blank controls with no sample for all ICP-MS experiments. These blank controls that are free of any biological materials will enable the quantification of any elements derived from the tubes and reagents used.

⚠ CRITICAL: Use only analytical grade reagents to avoid contamination with trace metals. (See troubleshooting 2).

**KEY RESOURCES TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Chemicals, peptides, and recombinant proteins | | |
| ICP Internal Standard | AccuStandard | Cat#AG-INT2-ASL-5 |
| Multi-element Calibration Standard #2A | AccuStandard | Cat#AG-MECAL2A-ASL-5 |
| Sulfur Standard | Inorganic Ventures | Cat#AAAS1 |
| Phosphorus ICP Standard | AccuStandard | #IPP-41W-1, CAS: 7723-14-0 |
| ICP-MS iCAP TQ Tune Solution | Thermo Fisher Scientific | Cat#555611.AP |
| Trace Elements Serum L-2 | Seronorm | Cat#203113 |
| Trace Elements Serum L-1 | Seronorm | Cat#201413 |
| ~Ultra-high purity oxygen (99.999%) | Select Gases | Cat#UN1072 |
| **~Copper(II) chloride | Sigma-Aldrich | Cat#203149; CAS: 7447-39-4 |
| **~Bathocuproinedisulfonic acid (BCS) | Sigma-Aldrich | Cat#B1125; CAS: 52698-84-7 |
| ~Phosphate Buffered Saline (PBS) | Corning | 21-040-CV |
| *~Trypsin Ethylenediaminetetraacetic Acid (EDTA) 0.25% | Corning | 25-053-CI |
| *~Ethylenediaminetetraacetic Acid (EDTA) Disodium Salt Solution | Sigma-Aldrich | E7889-100mL |
| *~DMEM [+1] 4.5 g/L glucose, L-glutamine, sodium pyruvate media | Corning | 10-013-CV |

**Experimental models: Cell lines**

Human: SH-SYSY Cells (Passage 20) | ATCC | Cat#CRL-2266; RRID: CVCL_0019 |

(Continued on next page)
## MATERIALS AND EQUIPMENT

**Final buffer/chemical concentrations and volumes.**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Human: HAP1 Cells** (Passage 20) | Horizon Discovery | Cat#C631; RRID: CVCL_Y019 |
| **D. melanogaster: wildtype: w1118** (female, 3rd instar) | Bloomington Drosophila Stock Center (NIH P40OD018537) | Cat#5905; RRID: BDSC_5905 |
| **D. melanogaster: Actin5C-GAL4,UAS-GFP** (female, 3rd instar) | Bloomington Drosophila Stock Center (NIH P40OD018537) | Cat#42713; RRID: BDSC_42713 |
| **D. melanogaster: UAS-ATP7-wt** (female, 3rd instar) | Richard Burke, Monash University, Australia | N/A |

**Software and algorithms**

| Qtegra | Thermo Fisher Scientific | Cat#IQLAAEGABSFAOVMBCE |

**Other**

| Item | Source | Identifier |
|------|--------|------------|
| Bioreactor | NuAire | NU-425-400, Option A-0 |
| CO2 Incubator | Thermo Fisher Scientific | 3110 |
| Refrigerated Microcentrifuge | Eppendorf | 5425 R |
| Refrigerated Centrifuge | Eppendorf | 5910 R |
| Automated Cell Counter | Bio-Rad | 1450102 |
| Automated Cell Counter slides | Bio-Rad | 1450101 |
| Drosophila units | SHELL LAB | SR020P |
| Drosophila Incubator, IB-15G | Lab Companion | AAH21176U |
| Drosophila Vials | Genesee Scientific | 32-110 |
| Dissecting Microscope | Zeiss Sterni | 2000 |
| Analytical Balance | OHAUS | PA114 |
| Dumont #2 Laminctomy Forceps | Fine Science Tools | 11223-20 |
| Petri dishes (35 x 10 mm) | Genesee | 32-103 |
| SYLGARD™ 184 Silicone Elastomer Kit | Dow | 2646340 |
| Chemical Fume Hood | N/A | N/A |
| Microcentrifuge | Eppendorf | 5430 |
| Dry Bath Heater | Coming | 6895-FB |
| iCAP TQ ICP-MS | Thermo Fisher Scientific | 731546 |
| 2DX prepFAST M5 | Elemental Scientific | 2DXF-73A |
| MASTERBLOCK 96-Well Storage Plate, Polypropylene, 1.2 mL, Chimney Style, Round Bottom, Clear | VWR | 780201 |
| X-Pierce Sealing Films for Robotics & High-Throughput Processing | Southern Labware | XP-100 |
| Milli-Q® ultra pure water | N/A | N/A |
| Adjustable Tip Spacing Multichannel Pipette 125 μL | Integra Biosciences | 4722 |
| Adjustable Tip Spacing Multichannel Pipette 125 μL Tips for 96-Well Plates | Integra Biosciences | 4422 |
| Repeater Pipette | Eppendorf | 4982000322 |
| 0.5 mL tips for Repeater Pipette | Eppendorf | 0030089456 |
| 5 mL tips for Repeater Pipette | Eppendorf | 0030089421 |
| Microcentrifuge tubes | Sarstedt | 72.607 |
| Microcentrifuge tube screw caps | Sarstedt | 65.716.999 |
| 15 mL tubes | VWR | 352097 |
| Kimwipes | Kimberly-Clark Professional | 34155 |
| Dry ice | N/A | N/A |

**Note:** Symbols indicate the following: ~ this item or an equivalent; * only necessary for cell culture experiments; # only necessary for D. melanogaster experiments; ^ optional but recommended or experiment-specific.
### PBS-Trypsin

| Reagent          | Final concentration     | Volume to 44 mL |
|------------------|-------------------------|----------------|
| Trypsin EDTA 0.25% | 10:1 v/v (0.02%)        | 4 mL           |
| PBS              | 40 mL                   |                |

Store at 4°C. Use within 1 month.

### PBS-EDTA

| Reagent | Final concentration | Stock concentration | Volume to 50 mL |
|---------|---------------------|---------------------|-----------------|
| EDTA    | 10 mM               | 0.5 M               | 1 mL            |
| PBS     |                      |                     | 49 mL           |

Store at 4°C. Use within 1 month.

### Media with copper(II) chloride for SH-SYSY cells (300 μM)

| Reagent                  | Final concentration | Stock concentration     | Volume to 50 mL |
|--------------------------|---------------------|--------------------------|----------------|
| Copper(II) chloride      | 300 μM              | 30 mM                    | 500 μL         |
| DMEM or media of choice  |                     |                          | 49.5 mL        |

Store at 4°C. Prepare same day.

### Media with copper(II) chloride for HAP1 cells (400 μM)

| Reagent                  | Final concentration | Stock concentration     | Volume to 50 mL |
|--------------------------|---------------------|--------------------------|----------------|
| Copper(II) chloride      | 400 μM              | 30 mM                    | 667 μL         |
| IMDM or media of choice  |                     |                          | 49.4 mL        |

Store at 4°C. Prepare same day.

### Media with BCS for HAP1 cells (400 μM)

| Reagent | Final concentration | Stock concentration | Volume to 50 mL |
|---------|---------------------|---------------------|-----------------|
| BCS     | 400 μM              | 400 mM              | 50 μL           |
| IMDM or media of choice |                     |                     | 50 mL           |

Store at 4°C. Prepare same day.

### Standard fly flood

| Reagent                  | Final concentration | Volume to 1 L | Mass   |
|--------------------------|---------------------|---------------|--------|
| active dry yeast         | 4.8% (w/v)          |               | 48 g   |
| cornmeal                 | 12% (w/v)           |               | 120 g  |
| agar                     | 9% (w/v)            |               | 9 g    |
| molasses                 | 12% (w/v)           |               | 120 g  |
| tegosept                 | 0.24% (w/v)         |               | 2.4 g  |
| propionic acid           | 0.9% (w/v)          | 9 mL          |        |
| Milli-Q water            |                     | 900 mL        |        |

Store at 4°C. Use within 3 weeks. (See Hartwig et al. (2020) and Gokhale et al. (2016) for more information).

### Nitric acid (2%)

| Reagent | Final concentration | Stock concentration | Volume to 1 L |
|---------|---------------------|---------------------|---------------|
| Nitric acid | 2%                 | 70%                 | 28.6 mL       |
| Milli-Q water |                   |                     | 971.4 mL      |

Store at room temperature. Use within 2 months.
Preparing the standard solution by weight rather than by volume is recommended, as this method is more precise (0.02% precision for 50 g on a balance with 0.01 g accuracy). Additionally, it is easier to weigh larger volumes and there is reduced risk of contamination as fewer containers are required.

CRITICAL: For safety information while working with nitric acid (HNO₃), please refer to Prepare buffers for ICP-MS in the before you begin section.

Alternatives: We recommend using an adjustable tip spacing multichannel pipette and the 2DX prepFAST MS system to prepare and run samples and standards for ICP-MS experiments in order to save time and reduce the likelihood of pipetting error, particularly when working with large numbers of samples. However, users can also prepare and load samples without this equipment (see notes throughout the protocol where applicable).

### STEP-BY-STEP METHOD DETAILS

#### Cell sample preparation

**Cell growth and drug treatment**

© Timing: 1–2 h

Confirm that your cells will be ready the following day and change media.

1. If your experiment involves drug treatments:
   a. When cells reach approximately 80% or desired level of confluency, change media and replace with media containing vehicle or your drug of interest.
   b. Treat for 24 h.
Note: Here, we used 300 μM copper(II) chloride in SH-SYSY cells. We used 400 μM copper(II) chloride and 400 μM BCS in HAP1 cells.

Note: Drug and metal concentrations should be selected based on the cell type studied and the goals of the experiment (see troubleshooting 3). If a metal or drug dose that may cause toxicity is being tested for ICP-MS, cell survival in increasing concentrations of the metal or drug should be performed first. For example, the IC50 for copper can for copper can be determined by quantifying cell survival using colorimetric assays like crystal violet (Comstra et al., 2017) or fluorescence assays such as Resazurin, and primary cultured fibroblasts can be treated with 50 μM without overt toxicity (Morgan et al., 2019). Thus, we recommend treating cells with 50–400 μM copper(II) chloride.

2. If you are not treating your cells:
   a. Simply change media the day before you intend to freeze your samples.

**Preparation of cell pellets**

© Timing: 2–3 h

**Detach your cells and snap freeze them for storage at –80°C.**

**Note:** You can detach cells using PBS with either trypsin or EDTA (referred to here as PBS-Trypsin and PBS-EDTA). We describe both methods here, as trypsin may be preferred if speed of dissociation is a priority, whereas EDTA may be preferred for some experiments as it better maintains cell integrity. Additionally, some highly adherent cell types (e.g., fibroblasts) may be difficult to detach using PBS-EDTA. In the data shown here, we used PBS-Trypsin.

**Note:** The concentration of trypsin used to detach cells may differ depending on cell type. In SH-SYSY cells, the 10:1 v/v dilution of 0.25% trypsin-EDTA in PBS described here is sufficient to detach cells without cell damage.

**Note:** Cell pellets can be prepared up to a month before the ICP-MS analysis.

3. Preparation on the day of the experiment:
   a. For each 15 cm plate, label 1 × 15 mL tube and 5 × microcentrifuge tubes.
   b. Label an additional 15 mL tube and 5 × microcentrifuge tubes for blank controls.
   c. If using PBS-Trypsin:
      i. Warm PBS and PBS-Trypsin to 37°C.
      ii. Place additional PBS on ice.
      iii. Fill each 15 mL tube with 3 mL of media. (This will neutralize the trypsin).
   d. If using EDTA:
      i. Place PBS and PBS-EDTA on ice.
   e. Get dry ice.
   f. Cool centrifuge and microcentrifuge to 4°C.

**Dissociation with trypsin**

**Note:** Skip to step 11 if using PBS-EDTA.

4. Wash cells once with 10 mL of warm PBS.
5. Add 3 mL PBS-Trypsin and incubate for 2 min at 37°C until cells begin to detach.

**Note:** This may take longer depending on the cell type.
**Note:** Do not treat cells too long with trypsin, as this will cause cell rupture and alter cytoplasmic pools of metals and other elements.

6. Detach cells from the plate with gentle aspiration using a pipette.
   a. Transfer cell suspension to a 15 mL tube and dilute with 6 mL of warm PBS.
   b. For blank controls, add 3 mL of PBS with trypsin and 6 mL of PBS to the corresponding 15 mL tube.
7. Centrifuge at 130 × g (800 rpm) for 5 min at 4°C to pellet the cells.
8. Remove supernatant.
   a. Resuspend in 5 mL of ice-cold PBS.
   b. Mix well and add 1 mL of your cell suspension to each microcentrifuge tube.

**Note:** If you wish to prepare samples with known cell number, replace step 8 with steps 19 and 20.

9. Centrifuge at 210 × g (1,500 rpm) for 5 min at 4°C to pellet the cells.
   a. Remove supernatant (taking care to remove as much PBS as possible).
   b. Immediately transfer tubes to dry ice to snap freeze your samples.
10. Leave samples on dry ice for 15–30 min.
    a. Transfer to –80°C for long-term storage.

**Pause point:** Cell pellets can be prepared up to a month before the ICP-MS analysis.

### Dissociation with EDTA

**Note:** Skip to step 19 if using PBS-Trypsin.

11. Wash each plate twice with 10 mL ice-cold PBS.
12. Add 10 mL PBS-EDTA to each plate and place on ice for 10 min.

**Note:** This may take longer depending on the cell type.

13. Detach cells from the plate with gentle aspiration using a pipette.
    a. Place the suspension in a 15 mL tube.
    b. For blank controls, add 10 mL of PBS-EDTA to a tube.
14. Centrifuge at 130 × g (800 rpm) for 5 min at 4°C to pellet the cells.
15. Remove the supernatant.
    a. Resuspend the pellet in 5 mL of ice-cold PBS.
    b. Transfer to labeled screw cap tubes.

**Note:** If you wish to prepare samples with known cell number, replace step 15 with steps 19 and 20.

16. Centrifuge this cell suspension at 210 × g (1,500 rpm) for 5 min at 4°C.
17. Remove the supernatant (taking care to remove as much PBS as possible).
    a. Immediately transfer tubes to dry ice to snap freeze your samples.
18. Leave samples on dry ice for 15–30 min before transferring to –80°C for long-term storage.

**Pause point:** Cell pellets can be prepared up to a month before the ICP-MS analysis.

### Normalization of sulfur and phosphorus content to cell count

**Note:** As sulfur and/or phosphorus content were used to standardize measurements across replicates, it is not necessary to count cells before preparing cell pellets. Phosphorus and
sulfur are present in multiple biomolecules and represent 1.1% and 0.2% of the mass of a human body respectively (see ID#101913 in BioNumbers (Milo et al., 2010; Rumble, 2021)). These facts make sulfur and phosphorus good standards likely to be stable under diverse biological natural and experiments conditions. Sulfur is present in cysteine and methionine in proteins and in sugars, nucleic acids, lipids, vitamin cofactors and metabolites, thus acting as a proxy for cell mass that can replace cell number counts or protein determinations. A compendium of biomolecules that contain sulfur can be found in Raab and Feldmann (2019). Phosphorus is an abundant element present in nucleic acids, nucleotides, and phospholipids, making it another good proxy for abundant biomolecules (Walsh, 2021).

Note: If you wish to prepare samples with known cell number as we did here, incorporate these additional steps as you prepare the cell pellets.

19. If preparing samples with known cell number (otherwise, disregard):
   a. After dissociating your cells and transferring the cell suspension to a 15 mL tube (step 6 or 13):
      i. Mix well and count cells.
      ii. Calculate the volume of PBS needed to aliquot 1 mL of your cell suspension per tube with your desired cell number.
      iii. (Example: For $1 \times 10^6$ cells per tube, if you count $8 \times 10^6$ cells, resuspend in 8 mL PBS).

20. If preparing samples with known cell number (otherwise, disregard):
   a. After the first centrifugation (step 7 or 14):
      i. Resuspend in approximately half of the cold PBS needed to obtain your desired cell concentration.
      ii. Mix well and count cells again.
      iii. Dilute the cell solution with cold PBS to get desired cell concentration.
      iv. Mix well and add 1 mL of your cell suspension to each microcentrifuge tube.

Note: As cells can be lost during centrifugation, not adding the full volume of PBS initially ensures that cells are not over-diluted and final counts are accurate.

Note: For our samples, we generated cell number curves in SH-SY5Y cells (Figure 1A) and HAP1 cells treated with copper(II) chloride or BCS (Figures 2A and 2B). Example for SH-SY5Y cells: We first resuspended cells to $2 \times 10^6$ cells/mL to prepare samples with $1 \times 10^6$, $2 \times 10^6$, or $3 \times 10^6$ cells (0.5 mL, 1 mL, or 1.5 mL of cell suspension per tube, respectively). We diluted the remaining cells by 4 in PBS (500,000 cells/mL) to prepare samples with 250,000 or 500,000 cells (0.5 mL or 1 mL of cell suspension per tube, respectively).

Note: The number of cells per sample should be optimized for each mass spectrometry system and selected based on the expected outcomes, i.e., more cells may be needed to detect smaller differences between groups. Our results suggest high sensitivity for detection of as few as 250,000 cells, with similar correlations between sulfur or phosphorus and metal content for all groups (Figures 1C and 1E, see r² values). (See the limitations section for additional discussion of the sensitivity of measurements of ultra-trace elements like Co.) Based on extrapolation from the cell counts and standard curves used here, we anticipate that our ICP-MS system can accurately quantify Fe, Cu, and Zn from samples with as few as 1,000 cells, though we prepare larger samples when cells can easily be collected in bulk.

D. melanogaster larvae sample preparation

Larvae collection

© Timing: 1–2 h

Collect larvae from each genotype and wash in PBS to remove food particles.
**Note:** *D. melanogaster* crosses utilized the UAS system ([Brand and Perrimon, 1993](#)) and were raised in polystyrene vials containing 10 mL of standard fly food. Vials were kept at 24°C in a humidified incubator with a 12 h:12 h light:dark cycle. Females from the Actin5C-GAL4,UAS-GFP driver line were crossed to either male w1118 animals or male UAS-ATP7-wt. This allowed comparison between controls and animals overexpressing ATP7 (later referred to as Con and ATP7-OE, respectively). ([See Hartwig et al. (2020)](#) for more information).

**Note:** Complete the steps below for each genotype separately. After collecting, weighing, and freezing all the larvae for one genotype, rinse both petri dishes and refill with fresh PBS before collecting larvae from an additional genotype.

21. Preparation on the day of:
   a. Place small puddles of 1 x PBS in each of two SYLGARD™-filled petri dishes or empty petri dishes.
   b. Get tweezers, Kimwipes, analytical balance, and dry ice.

   **Note:** The PBS is being used to rinse the larvae, so the quantity does not need to be precise. A smaller pool of PBS in the petri dish where larvae are initially collected will make it easier to keep within the field of view under the microscope.

22. Use forceps to collect 3–5 wandering, third-instar larvae, and place them in the pool of 1 x PBS in one of the petri dishes.

   **Note:** The initial pool of PBS will become cloudy with food residue, so it is best to collect larvae from the vial in small batches.

23. With aid of a dissecting microscope, select female larvae for analysis and remove male larvae.

   **Note:** Male larvae can be distinguished by the presence of gonads under the cuticle in the posterior ventral segment of the animal ([Karabasheva and Smyth, 2020](#)).

24. Lightly grasp an individual larva with the forceps and gently jostle it side-to-side within the PBS to remove the food residue.
   a. If any food residue is still visible after this process, use the side of one forceps tip to gently brush along the larval body to dislodge the food residue.

25. Once cleaned, move female larva to the pool of PBS in the second petri dish.

   **Note:** If the larvae are cleaned sufficiently prior to being moved to the second petri dish, this PBS should remain clear and will not need to be replaced as additional larvae of the same genotype are collected. If food particles are visible in the second petri dish, repeat steps 24 and 25 using a third petri dish.

26. If additional animals are needed, clean the petri dish used in step 22, add fresh PBS, and repeat steps 23–25.

**Weighing and freezing larvae**

*© Timing: 1–2 h*

After all animals of a single genotype have been collected, individually weigh and freeze each larva.

27. Use forceps to remove an individual larva from the petri dish. Place it on a Kimwipe to remove PBS.
28. Use forceps to pick up larva from the Kimwipe and place it gently in a microcentrifuge tube. Screw on the cap and label the tube.
   a. For blank controls, prepare empty microcentrifuge tubes.

   **Note:** If you wish to prepare samples with known larval weight, first determine the baseline weight of the microcentrifuge tube and screw cap using an analytical balance. Record this value. Reweigh the microcentrifuge tube with the larva and cap and record this value. The baseline value can be subtracted from this post-weight to determine the weight of individual larva. See steps 31 and 32 for pooling samples.

29. Transfer tube to dry ice to snap freeze the larva.
30. Repeat steps 27–29 for each larva collected.

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### Pause point:

Samples can be stored at −80°C for up to a month before the ICP-MS analysis.

### Normalization of sulfur or phosphorus content to fly number or weight

© Timing: 1–2 h

Pool larvae together to prepare samples that can be normalized to fly number and/or weight.

**Note:** As sulfur content is used as a proxy for fly mass to standardize measurements across replicates (see above: “Normalization of Sulfur Content to Cell Count”), it is not necessary to weigh flies before preparing samples. If you wish to prepare samples with known fly weight and/or pool samples from multiple larvae with similar total weight as we did here, incorporate these additional steps.

31. If preparing samples with known larvae weight (otherwise, disregard):
   a. To pool larvae, assign larvae by weight to groups for analysis so that the average fly weight is similar across samples.

32. If preparing samples with known larvae weight (otherwise, disregard):
   a. Gather all microcentrifuge tubes with individual larva and place on dry ice.
   b. Combine all larvae for a given group in a single tube.
      i. This can be done using forceps or by gently tapping the microcentrifuge tube containing the larva to be transferred over the top of the destination tube.
      ii. If the larva is stuck to the side of the tube, gently flick the tube or tap against the bench to dislodge it before transferring.

**Note:** For our samples, we analyzed between 2–3 independent biological replicates of groups consisting of 1, 2, 4, and 8 individual larvae for each genotype (Figure 1F). This data was pooled to give a total of 12 independent replicates for WT flies and 10 independent replicates for ATP7-OE flies (Figures 1F and 1G).

**Note:** The number of larvae per sample should be optimized for each mass spectrometry system and selected based on the expected outcomes, i.e., more flies may be needed to detect smaller differences between groups. (See troubleshooting 3.) Our results suggest high sensitivity for detection even for single larva, with similar correlations between sulfur and metal content for all groups (Figure 1G, see $r^2$ values).

### ICP mass spectrometry

© Timing: 1–12 h depending on the number of samples
Sample preparation

**Timing:** 1–3 h depending on the number of samples

**Dissolve samples in 70% nitric acid.**

33. Turn on heat block and set to 95°C.
34. Rinse a 25 mL or 50 mL tube with MilliQ water.
   a. Add a small volume 70% nitric acid (approximately 2 mL) to the tube.
35. Add 20 μL 70% nitric acid to each sample using the repeating pipettor. Take care to ensure that the same volume is added to each tube.
   a. Briefly centrifuge to collect the nitric acid and the sample at the bottom of the tube if required.

*Note:* Use as little nitric acid as possible to dissolve your samples to maximize the metal content loaded into the ICP-MS. 20 μL is the minimum volume we used. Larger volumes can be used if needed, but it is recommended that you start from 20 μL nitric acid and only add more acid if the sample does not fully dissolve. We increased the volume of acid used for samples with 1 million cells or more to be able to re-run samples if necessary (see volumes listed in Table 1). We scaled the volume of acid based on cell or larvae number; however, this is not necessary. We would recommend increasing the amount of acid only if necessary to yield a homogeneous solution clear of precipitates for each sample.

36. Incubate samples in the heat block at 95°C for 10 min.

*Note:* Fully dissolved samples should be fully homogeneous without any particulates and are typically yellow in color. For samples with a particularly high lipid content, like brain tissue, follow the incubation with nitric acid with equal volume of concentrated trace element grade hydrogen peroxide. For further details, see McAllum et al. (2020) and troubleshooting 1.

**Pause point:** Samples in 70% nitric acid can be immediately diluted with 2% nitric acid for analysis or stored at room temperature for 1–3 months. (Typically, we leave samples at room temperature for 24 h before diluting and running the samples. See troubleshooting 5 if samples are stored long-term and undergo evaporation).

### Table 1. Nitric acid dilutions used in this study

| Sample type                          | Number cells or larvae | Volume nitric acid used (μL) |
|--------------------------------------|------------------------|-----------------------------|
| SH-SY5Y Cells                        | 250,000                | 20                          |
| SH-SY5Y Cells                        | 500,000                | 20                          |
| SH-SY5Y Cells                        | 1 million              | 50                          |
| SH-SY5Y Cells                        | 2 million              | 50                          |
| SH-SY5Y Cells, treated with vehicle or copper (Figure 1) | Between 1–2 million | 50                          |
| HAP1 Cells, treated with vehicle, copper, or BCS (Figure 2) | Between 0.5–3 million | 50                          |
| D. melanogaster                      | 1                      | 20                          |
| D. melanogaster                      | 2                      | 40                          |
| D. melanogaster                      | 4                      | 80                          |
| D. melanogaster                      | 8                      | 80                          |
| Blank                                | –                      | 20                          |
Sample dilution
Dilute samples in 2% nitric acid and load them into the 96 well plate.

37. Dilute samples 1:40 in 2% nitric acid (add 780 μL to each tube containing 20 μL of sample).
   a. Transfer each of your samples to a deep 96 well plate (minimum volume 400 μL).

   Note: Alternatively, you can transfer the samples in 70% acid directly to the deep 96 well plate
   and dilute in 2% nitric acid in the well plate.

   Note: We used adjustable tip spacing multichannel pipettes to load our samples in order to
   save time and reduce the likelihood of pipetting error. This is not necessary but is suggested
   when working with large numbers of samples.

   Note: We recommend arranging your samples in a tube rack as you load them into the 96 well
   plate, then take a picture of the layout. This will help ensure that the plate layout can be
   matched to each sample.

Pause point: If required, seal the 96 well plate with sealing tape and store at room temper-
ature for up to 1 month; otherwise, analyze immediately. (Evaporation is more easily
controlled with concentrated nitric acid; thus, it is typically recommended to stop after step
36 if necessary).

Instrument set-up, calibration, and running samples

Note: Operating procedures may vary between instruments and models. Contact the
instrument technical specialist to learn more about how to configure the ICP-MS being
used.

Note: We recommend the use of the 2DX prepFAST MS system. Alternatively, a user can
manually prepare the standard curve through standard dilution practices.

Note: Store all standards in polyethylene bottles washed using 1% HNO₃ in MilliQ-H₂O prior
to use.

Note: The wash, sample dilution, and sample carrier solutions are 2% nitric acid in 18.2 Milli-Ω
water.

Note: Here, quantitation of trace elements in larvae or cultured cells were determined with a
Thermo iCAP-TQ series inductively coupled plasma triple quadrupole mass spectrometer
(ICP-MS). A triple quadrupole ICP-MS is required for this method. The instrument was oper-
ated in oxygen reaction mode with detection of elements of interest with the third quadrupole
(see Table 2). This method removes polyatomic interferences by mass shifting the target
element by 16Da after reaction with oxygen. The oxygen mass shift and MS/MS detection us-
ing a triple quadrupole instrument are critical to measure sulfur and phosphorus. The mea-
urement of S⁺ and P⁺ with standard single quad ICP-MS using oxygen mass shift suffers
from the inability to distinguish 48SO⁺ & 47PO⁺ from 47Ti⁺, 48Ca⁺, 36Ar12C⁺ and 48Ti⁺ spectral
interferences. The implementation of a triple quadrupole allows for the first quad to mass filter
for m/z ion at 31 and 32 which filters out the Ti and Ca interference ions. The reaction of S⁺
and P⁺ with O₂ in the octopole then mass shifts them by one oxygen. Because the 48Ti⁺, 47Ti⁺, and
48Ca⁺, and spectral interferences were filtered out by the first quadrupole, the detection at
m/z 47 and 48 of 48SO⁺ & 47PO⁺ after the third quadrupole occurs without poly atomic or
ion Ca and Ti interference (Balcaen et al., 2013; Diez Fernández et al., 2012). Together, the
relatively recent introduction of the triple quad ICP-MS instruments improves the selectivity and sensitivity for detection of some biological samples.

38. Prepare a nine-point external calibration curve to quantify iron (Fe), copper (Cu), manganese (Mn), cobalt (Co), and zinc (Zn).
   a. Prepare 50 mL stock solutions of Multielement Calibration Standard for ICP-MS in 2% nitric acid (1,000 and 100 μg*L⁻¹; see prepare buffers for ICP-MS and materials and equipment).
   b. Prepare solutions for the calibration curve. The calibration curve values should range from 0.5 to 1,000 μg*L⁻¹ and increase by 2× at each point (0, 0.5, 1, 5, 10, 50, 100, 500 and 1,000 μg*L⁻¹).
      i. Dilutions can be prepared using the 2DX prepFAST M5 system (Elemental Scientific) or manually through standard dilution practices.
39. Control and monitor for potential instrument drift by automatic dilution of the sample with the Internal Standard for ICP-MS during sample introduction using the 2Dx prepFAST M5 system.

   Note: The Internal Standard for ICP-MS is a reference element solution containing 10 μg*L⁻¹ of Li-6, Sc, Y, In, Tb, and Bi (see prepare buffers for ICP-MS and materials and equipment). The 2Dx prepFAST M5 system automatically dilutes the sample with the Internal Standard during sample introduction. Commonly, the Internal Standard solution is introduced with the sample via a ‘T’ piece using a peristaltic pump. Alternatively, the Internal Standard can be added manually immediately before analysis is performed; however, this is exceedingly rare.

40. Prepare a four-point standard curve for S.
   a. Dilute stock solutions of the Sulfur Calibration Standard for ICP-MS, a 100 mg*L⁻¹ solution (0.5, 1, 5, 10 mg*L⁻¹), in 2% nitric acid. Prepare 50 mL of each concentration.
   b. Dilutions can be prepared using the 2DX prepFAST M5 system (Elemental Scientific) or manually through standard dilution practices.

   Note: Here, we did not use a phosphorus standard, so all data in Figure 2 are presented as counts per second (cps). However, we plan to perform quantitation of P similarly to S in the future using a certified P standard. (See the key resources table for catalog information for the Sulfur and Phosphorus Standards).

41. Warm-up and tune the instrument as recommended by the manufacturer. (Typically requires 20–30 min).

   Note: ICP-MS iCAP TQ Tune Solution is the tuning solution for instrument optimization (see the key resources table for catalog information). It contains 1 μg L⁻¹ of cerium (Ce), cobalt (Co), lithium (Li), thallium (Tl), and Y in 2% nitric acid.
Table 3. Thermo iCAP-TQ-ICP-MS acquisition parameters

| Element   | Q3 analyte | Internal standard | Dwell time (s) | Q1 resolution |
|-----------|------------|-------------------|----------------|---------------|
| Phosphorus| 31P.16O    | 45Sc.16O          | 0.05           | High          |
| Sulfur    | 32S.16O    | 45Sc.16O          | 0.1            | High          |
| Scandium  | 45Sc.16O   | –                 | 0.02           | Normal        |
| Manganese | 55Mn       | 45Sc.16O          | 0.05           | Normal        |
| Iron      | 56Fe.16O   | 45Sc.16O          | 0.1            | Normal        |
| Cobalt    | 59Co       | 45Sc.16O          | 0.05           | Normal        |
| Copper    | 63Cu       | 45Sc.16O          | 0.05           | Normal        |
| Zinc      | 66Zn       | 45Sc.16O          | 0.05           | Normal        |
| Selenium  | 80Se.16O   | 89Y.16O           | 0.1            | Normal        |
| Yttrium   | 89Y.16O    | –                 | 0.02           | Normal        |

*Q3 resolution was set to normal for all of the elements. The list can be expanded to include other elements of interest. Each element was measured 4 times.

Note: Table 3 contains the acquisition parameters used. The $r^2$ for calibration curves was > 0.9998. The prepFast system was set up with a 0.25 mm ID sample probe and a 250 μL sample loop. The 0.25 mL Precision Method used was provided as a prebuilt program by the manufacturer (Elemental Scientific) that is standard with any PrepFAST system and automates sample uptake, dilution, internal standard introduction, and sample introduction.

42. Make up Seronorm Trace Elements Serum L-1 and L-2 standard reference material according to the manufacturer’s instructions.

Note: Seronorm™ Trace Elements Serum L-1 and L-2 are used to externally assess analytical performance.

43. Replace any sealing tape previously added with X-Pierce Sealing Film for robotics.

44. Add samples and Seronorm Trace Elements Serum L-1 and L-2 to the sample deck and build the worklist.

45. Run the Seronorm Trace Elements Serum L-1 and L-2 (800 μL of a 1:40 dilution in 2% nitric acid) and verify measurement of elements are within +/-20% of the accepted values (see troubleshooting 2).

46. Continue with measurement of samples (see troubleshooting 3 and troubleshooting 4).
   a. Intersperse a Seronorm measurement every 20–30 samples.

47. Conduct data analysis within the Qtegra software.
   a. Export values from Qtegra.
   b. Statistical analysis can be conducted with Excel, GraphPad Prism, or any other relevant statistical analysis software.

EXPECTED OUTCOMES

This protocol describes how inductively coupled plasma mass spectrometry (ICP-MS) can be used to quantify metals, sulfur, and phosphorus present in biological specimens. Classically, ICP-MS and trace analysis are optimized for geological and environmental samples. These sample types do not have the same constraints as biological samples, which have limited amounts available for analysis. With current ICP-MS analytical technology, the measurement of trace elements in the biological sciences is readily achievable, yet many core ICP-MS facilities require large mL to gram quantities. Here we describe the application of ICP-MS to biological samples. The main adaptations that are required relate to sample introduction and sample preparation. One challenge in measuring biological samples that have unknown quantities and/or contain μg of sample (rather than gram) is normalization. When conducting an analysis of trace-elements, it is not possible to normalize...
with housekeeping proteins or total protein in each sample as they are destroyed in the ionization process. Normalization to cell number is possible but can be time-consuming and can have low accuracy, as determined by the coefficient of variation (see Table 5). However, ICP-MS offers a similar internal normalization approach. Here, we apply the measurement of sulfur or phosphorus as a surrogate marker of total biological content. Measurement of sulfur and phosphorus is made possible by the recent commercialization of the triple quad ICP-MS combined with the use of oxygen as a reaction gas. This is particularly useful when care is taken to prevent contaminations from common buffers and reductants (e.g., dithiol threitol, sodium dodecyl sulfate, ammonium sulfate, or drugs that contain sulfur in their structure).

Here, we illustrate how this protocol can be used to quantify metals in cultured cells and D. melanogaster (Figure 1). We compared the performance of sulfur or phosphorus as surrogate standards with similar outcomes (Figure 2). Both standardization methods similarly detect cellular conditions where copper content has been increased by copper chloride incubation (Figures 1D and 2, blue symbols) or decreased by the addition of the copper chelator BCS (Figure 2, black symbols). The sensitivity of the standardization method can be observed in Figure 2A, where incubation with BCS increases the amount of sulfur per cell due to the presence of 2 mols of sulfur per mol of BCS (C26H20N2O6S2, p < 0.0001, Figure 2A, compare BCS and control). This shift is abrogated when normalization is performed with phosphorus (Figure 2A). Sulfur content is highly correlated with cell number and fly number (Figures 1A, 1B, and 1F) and is a more accurate normalizing factor with a lower coefficient of variation than cell number (see Table 5). Similar results are obtained when cell number is correlated with phosphorus content (Figure 2A, sulfur r2 > 0.86; phosphorus r2 > 0.74). We compared the correlations between cell numbers with either sulfur or phosphorus content and noticed no differences by Fisher r-to-z transformation statistics (Figure 2A). There is a strong fit of sulfur content to cell number correlation with a linear model, as depicted in Figure 1B showing a predicted vs actual plot for each replicate with r2 values > 0.9 calculated using Prism v9.2.0 (283). Our results are highly consistent across replicates, with accurate quantification of biologically relevant metals even in single larva or with as few as 250,000 cells.

This method is robust and can be used to compare groups based on genotype or drug treatment. For example, we illustrate a dramatic increase in copper content in cells following 24 h treatment irrespective of whether we use sulfur or phosphorus to normalize data (Figures 1D, 2B, and 2C). It is important to emphasize that expected outcomes of copper cellular content were achieved when cells were incubated with copper or the copper chelator BCS (Figures 2B and 2C). The effect of these additions to cultured cells was selective for copper without affecting other metals.

Table 4. Average metal content per cell in SH-SY5Y cells plus or minus SD

| Metal content (g)   | Sulfur 4.7E-15 | Copper 2.6E-15 | Zinc 4.5E-14 | Manganese 1.6E-17 | Iron 4.2E-17 | Cobalt 4.2E-17 |
|--------------------|----------------|----------------|--------------|------------------|--------------|----------------|
| SD (g)             | 4.2E-17        | 1.8E-15        | 7.3E-14      | 1.4E-15          | 1.6E-14      | 2.7E-17        |
| Metal content (fg) | 8700           | 47             | 140          | 2.6              | 45           | 0.042          |
| SD (fg)            | 4500           | 1.8            | 73           | 1.4              | 16           | 0.027          |

1Data in this table correspond to Figure 1.

Table 5. Coefficient of variation in metal content in SH-SY5Y cells normalized to cell number or sulfur content

| Normalized to cell number | Copper 42% | Zinc 61% | Manganese 75% | Iron 61%   | Cobalt 106% |
|---------------------------|------------|----------|---------------|------------|-------------|
| Normalized to sulfur      | 23%        | 41%      | 51%           | 42%        | 81%         |

1Data in this table correspond to Figure 1.
Further, this protocol can be easily adapted for new purposes, including metal quantification in specific cellular compartments. For example, we have used it to quantify metals in fractions enriched in mitochondria. Metal quantification can also be performed in single cells by infusing the cell suspension into the instrument, although some other modifications are required for single cell analysis. Alternatively, we can calculate the metal content per cell from analyses such as those presented in Figures 1 and 2. For example, we estimate a cellular content of 4.7 fentograms of copper per cell (Table 4). This is in the range of what has been previously determined in a single yeast cell (2 fentograms/mammalian nucleated cell equivalent) or red blood cell (6 fentograms/mammalian nucleated cell equivalent) after adjusting by nucleated cell volume. We used a HeLa cell volume of ~3,000 cubic microns to normalize other cell volume values (Cao et al., 2020; Puck et al., 1956; Rae et al., 1999).
LIMITATIONS

This method is broadly applicable to all samples of biological origin. However, normalization based on sulfur content cannot be done if buffers and detergents containing sulfur are used. Commonly used sulfur containing compounds used in biological research include, dithiothreitol, sodium dodecyl sulfate (SDS), HEPES (4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid) buffer and ammonium sulfate. Similarly, if phosphorus is used for data normalization then the use of phosphate-based buffer saline solutions should be replaced with saline buffered with sulfur- and phosphate-free buffers such as TRIS (Tris[Hydroxymethyl]aminomethane). There may also be limitations with the measurement of ultra-trace elements like Co where potential imprecision at the sub 1ppb level requires a larger number of cells than would be needed for Fe, Cu and Zn (see Figure 1G). In both larval and cell counts above ~500,000, there is a better linear response for Co (Figure 1G).

Figure 2. Comparison of sulfur and phosphorus standardized metal quantification in HAP1 cells using ICP-MS

(A) Metal content in increasing numbers of WT HAP1 cells control (gray symbols) or incubated in the presence of either copper chloride (400 μM, blue symbols) or BCS (400 μM, white symbols) for 24 h. r² for sulfur correlations = 0.8924, 0.8787, and 0.8619 for control, copper chloride-, and BCS-treated cells. r² for phosphorus correlations = 0.8610, 0.7429, and 0.8317 for control, copper chloride-, and BCS-treated cells.

(B) 32S and 31P correlation with copper content in control, copper chloride-, and BCS-treated cells. r² for sulfur correlations = 0.9014, 0.9464, and 0.6233 for control-, copper chloride-, and BCS-treated cells. r² for phosphorus correlations = 0.9012, 0.9082, and 0.6174 for control, copper chloride-, and BCS-treated cells. In (A and B), best fit correlation and 95% confidence interval are depicted.

(C) 63Cu, 66Zn, 55Mn, 56Fe, and 56Co normalized to 32S and 31P content. Symbols colored as in (A). Two-tailed Anova with Bonferroni multiple corrections reveal p<0.001 for copper measurements using both sulfur and phosphorus normalization. All other metal determinations were not significantly different. (Note: Cells were prepared according to this protocol with the following changes: steps 3–7: Cells were collected in 2 mL PBS-trypsin, neutralized with 1 mL media, and diluted with 7 mL PBS. Step 9: Cells were microcentrifuged at 3,000 g for 2 min).
TROUBLESHOOTING

Problem 1
The sample is not breaking down in 70% nitric acid (step 36).

Potential solution
Start with 20 μL of 70% nitric acid per sample. If the sample does not dissolve, add more acid and return samples to the heat block for another 10 min. Inspect tube for particulates and repeat as necessary. (Example: We prepared samples with 3 million HAP1 cells, not shown here. These samples dissolved once we added 100 μL of acid).

Samples with large amounts of lipids (such as brain tissue) may need to be further broken down with hydrogen peroxide. In this case, hydrogen peroxide (analytical grade) should be added 1:1 with the volume of 70% nitric acid. Incubate at 95°C for 10 min. In addition, some samples (e.g., tissue) may require homogenization. In this case, samples can be homogenized by manual Dounce or sonication for 5 min using Tris buffered saline (50 mM Tris pH 8.0, 150 mM sodium chloride + EDTA free protease inhibitors; avoid buffers with detergents). Clarify homogenates by centrifugation at 16,000 × g for 5 min. Collect the resulting supernatant and keep samples at 4°C. (Note: All samples must be centrifuged prior to loading into the deep well plate.) See Ganio et al. (2016); Hare et al. (2013); Lothian and Roberts (2016); McAllum et al. (2020).

Problem 2
Blanks produce background noise (step 45).

Potential solution
It is important to use only analytical grade chemicals, materials, and reagents that have minimal levels of trace metals. We highly recommend conducting preparation blanks (see “prepare buffers for ICP-MS section” for more information) before doing any biological experiments to validate that the buffers, solvents, and plasticware being used are not contaminated. Similarly, all bottles and beakers used should be rinsed with MilliQ water. If you need to lyse your sample, you can use RIPA buffer (0.5% Nonidet P-40, 50 mM Tris pH 8, 0.5% sodium deoxycholate, 2 mM EDTA, 150 mM NaCl). Triton X and tween are also acceptable detergents to use.

Problem 3
Measurements for one or more metals are below detection limits (step 46).

Potential solution
Usually the lower abundant elements (including Cu, Co, and Mn) are the limiting factors in dilution of the sample. To better detect these metals, increase the number of cells/larvae in each sample. You can also treat with a solution containing the metal if desired (see step 1).

Problem 4
Variation is seen in sulfur or phosphorus content between replicates (step 46).

Potential solution
It is possible that different amounts of each sample were loaded into the ICP-MS. This could arise due to the accuracy of the cell counting method and preparation of cell samples (i.e., loading different numbers of cells) or differing larval weights across replicates. If sulfur or phosphorus content for each replicate is only slightly variable and is well correlated with other metals, no additional action needs to be taken, though additional attention can be directed toward preparing samples with precision.

If needed, additional methods can be used to normalize metal content. For example, you can normalize to total protein. This requires you to measure protein after either preparing samples in
RIPA buffer and sonicating as described above. For flies, you can normalize to fly weight rather than fly number and/or pool larvae to prepare samples of similar weights.

It is possible that some conditions may affect sulfur or phosphorus content (such as treatment with BCS in Figure 2A). Figure 2A clearly illustrates the benefit of quantifying both sulfur and phosphorus and using both as normalization factors, as BCS-treated cells have increased levels of sulfur per cells but phosphorus remains unchanged.

**Problem 5**
The liquid evaporates from samples stored in 70% nitric acid (step 36).

**Potential solution**
When samples are stored for an extended period of time, it is not uncommon to have complete evaporation of the small amount of nitric present. You can avoid this by adding 70% nitric acid to your samples and running them in the same week. If samples have been stored long-term and some or all of the acid has evaporated, bring each sample to 800 μL with 2% nitric acid. When absolute concentration beyond 1%–2% accuracy is required, we suggest fully drying the samples in the heat block and resuspending with 800 μL of 2% nitric acid. Alternatively, you can estimate the volume remaining using a p20 pipette and bring the volume of each sample to 800 μL or simply add 800 μL 2% nitric acid to each sample. The important variable is to treat all the samples the same. (The error in having 5 μL vs 20 μL when diluting to a total volume of 800 μL is less than 1.5%, which does not significantly impact the experiment due to the large biological variation observed).

**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contacts, Blaine Roberts (blaine.roberts@emory.edu) and Victor Faundez (vfaunde@emory.edu).

**Materials availability**
This study did not generate new unique reagents.

**Data and code availability**
The study did not generate any unique datasets or code.

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**AUTHOR CONTRIBUTIONS**
Conceptualization, V.F., B.R., and A.L.; Methodology, B.R. and A.L.; Validation, B.R.; Formal Analysis, V.F.; Investigation, A.G., A.F., A.L., A.R., E.W., and B.R.; Resources, V.F. and B.R.; Writing – Original Draft, V.F., A.G., A.F., A.L. and B.R.; Writing – Review & Editing, V.F., A.L., and B.R.; Visualization, V.F. and A.L.; Supervision, V.F. and B.R.; Funding Acquisition, V.F.

**DECLARATION OF INTERESTS**
The authors declare no competing interests.

**INSTITUTIONAL PERMISSIONS**
No institutional permissions were necessary for these experiments. If adapting this protocol for use in vertebrates or higher invertebrates, acquire permissions from the relevant institution(s) and ensure...
that all experiments are performed in accordance with relevant institutional and national guidelines and regulations.

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