Chapter 3

Multi-macromolecular Extraction from Endosymbiotic Anthozoans

Anderson B. Mayfield

Abstract

Obligately symbiotic associations between reef-building corals (anthozoan cnidarians) and photosynthetically active dinoflagellates of the family Symbiodiniaceae comprise the functional basis of all coral reef ecosystems. Given the existential threats of global climate change toward these thermo-sensitive entities, there is an urgent need to better understand the physiological implications of changes in the abiotic milieu of scleractinian corals and their mutualistic algal endosymbionts. Although initially slow to leverage the immense breakthroughs in molecular biotechnology that have benefited humankind, coral biologists are making up for lost time in exploiting an array of ever-advancing molecular tools for answering key questions pertaining to the survival of corals in an ever-changing world. In order to comprehensively characterize the multi-omic landscape of the coral holobiont—the cnidarian host, its intracellular dinoflagellates, and a plethora of other microbial constituents—I introduce a series of protocols herein that yield large quantities of high-quality RNA, DNA, protein, lipids, and polar metabolites from a diverse array of reef corals and endosymbiotic sea anemones. Although numerous published articles in the invertebrate zoology field feature protocols that lead to sufficiently high yield of intact host coral macromolecules, through using the approach outlined herein one may simultaneously acquire a rich, multi-compartmental biochemical pool that truly reflects the complex and dynamic nature of these animal–plant chimeras.

Key words Coral reefs, Gene expression, Mass spectrometry, Multi-omics, Proteomics, Symbiosis

1 Introduction

1.1 Safety Considerations

High-efficiency extraction of RNAs, DNAs, proteins, lipids, and polar metabolites from endosymbiotic anthozoans warrants the use of toxic, corrosive chemicals, namely, an acid–guanidinium+phenol-based solvent (e.g., TRIzol®, Thermo-Fisher Scientific [TFS]) and chloroform. All work should be undertaken while wearing nitrile gloves, a laboratory coat, safety goggles, and closed-toe shoes. All steps should be performed in a fume hood except for those associated with the RNA/DNA spin column protocols, which can be performed on a standard laboratory benchtop. Dispose of all organic solvent waste as recommended by your local

Sanjoy K. Bhattacharya (ed.), *Lipidomics: Methods and Protocols*,
Methods in Molecular Biology, vol. 2625, https://doi.org/10.1007/978-1-0716-2966-6_3,

Occupational Health and Safety Administration (OSHA). I recommend that even plastics and glassware that have come into contact with the aforementioned chemicals be treated as hazardous material.

1.2 Drawbacks to Current Approaches

The extraction and subsequent purification of the major cellular macromolecular species—DNAs, RNAs, proteins, lipids, and other (polar) metabolites—from reef-building corals (Fig. 1), endosymbiotic sea anemones, and their dinoflagellate endosymbionts (Fig. 2) is still in its infancy, despite publications on these topics dating back into the 1980s (e.g., [1, 2]). This statement would appear to be at odds with the fact that many hundreds of peer-reviewed articles have been produced on coral gene expression alone (e.g., [3–6]). Has our field reached the state-of-the-art level of analysis that was once only realized by those working on model organisms, such as mice? Unfortunately, this is not yet the case. For one, over 10 years ago, Mayfield et al. [7] revealed that the vast majority of reef coral and endosymbiotic sea anemone (which can serve as a model system for the more-difficult-to-study corals [8, 9]) articles had either completely failed to extract macromolecules from the dinoflagellate endosymbionts (family Symbiodiniaceae) or, if they *were* extracted, they were left unanalyzed (or treated as contamination). The failure to either extract dinoflagellate biological material or include Symbiodiniaceae data in molecularly focused publications of that era (e.g., [10]) potentially left many to conclude that these algae, which are obligate, photosynthetic mutualists without which reef-building corals would perish from

Fig. 1 An Indo-Pacific coral reef featuring a high abundance of a branching coral species (*Acropora* sp.). (Photo by the author)

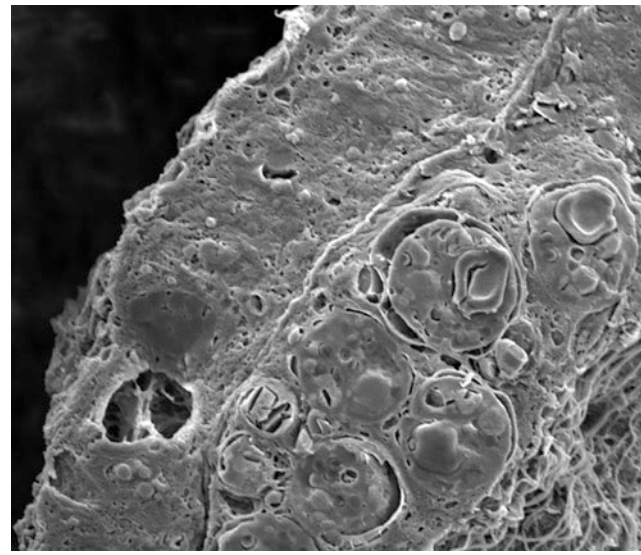

Fig. 2 A scanning electron micrograph of decalcified, freeze-fractured reef coral (*Pocillopora* sp.) tissues. The spherical objects (~10 μm in diameter) in the gastrodermal (right-most) tissue layer represent the dinoflagellate endosymbionts (family Symbiodiniaceae)

starvation [11], are "background players" with respect to their contribution to the macromolecular pool emerging from coral tissue biopsies; this uni-compartmental focus remains regrettably prevalent to this day (Table 1), and very few studies have made an effort to profile macromolecular species from multiple members of the coral "holobiont" (the collective name for the association of coral, dinoflagellates, and other eukaryotic and bacterial microbes that associate with the anthozoan host). My primary goal in this chapter is to challenge marine biologists to attempt to more comprehensively characterize the complex molecular landscapes of their target species; it may result in a more exhausting day in the laboratory, but I hope to convince you that it is worth the extra effort.

Although it is certain that the intracellular dinoflagellate populations do not contribute *more* biomass to the holobiont than the host anthozoans in which they reside [12, 13], when using proper, robust extraction techniques, they can actually yield as much, if not more, of the target macromolecules of interest. Perhaps this is unsurprising. After all, dinoflagellates have amongst the largest genomes ever characterized [14], meaning that, even if there were, for instance, five times more host tissue material than dinoflagellate, if the latters' genomes are truly five times larger than those of the average coral (a good current estimate), a similar amount of host coral and Symbiodiniaceae DNA might very well be extracted from the same biopsy; in fact, this does indeed appear to be the case [15].

Table 1
Simple-random-sample of 20 endosymbiotic anthozoan molecular biology articles

Reference	Molecules characterized	Extraction approach	Considered anthozoan host?	Considered dino-flagellates?	Host/symbiont molecular ratio	Molecular integrity reported?
Mayfield [40]	RNA, DNA, protein	TRIzol® + mortar and pestle	Yes	Yes	~2:1	Yes
Mayfield & Dempsey [45]	RNA, DNA	TRIzol® + mortar and pestle	Yes	Yes	~2:1	Yes
Rubin et al. [46]	RNA	NucleoSpin® TriPrep+mortar and pestle+bead mill	Yes	Yes	~1:2	No
McRae et al. [47][a]	Protein	TRIzol® + mortar and pestle	Yes	Yes	~14:1	Yes
Tisthammer et al. [48]	Protein	Urea+tissue shearer	Yes	No	NA	No
Sproles et al. [49][b]	Protein, DNA	LN2 + mechanical homogenization	Yes	No	NR	No
Rocker et al. [50]	RNA, lipid	Aurum™ Total RNA minikit, 2:1 dichloromethane:methanol	Yes	No	NA	No
Dimos et al. [51]	RNA	RNAqueous®	Yes	No	NA	Yes
Lohr et al. [52]	Metabolites	Methanol	Yes	No	NA	No
Wright et al. [53]	RNA	RNAqueous® + bead mill	Yes	Yes	~9:1	No
Seveso et al. [54]	Protein	SDS buffer+mortar and pestle[c]	Yes	No	NA	No
Chen et al. [55]	Lipid, protein	Bligh and Dyer [56]	Yes	Yes	NR	No

Ricaurte et al. [57]	Protein	Rehydration buffer+mortar and pestle	Yes	No	NA	No[d]
Vidal-Dupiol et al. [58]	RNA	TRIzol® (details not reported)	Yes	No	NA	No
Barshis et al. [59]	RNA	TRIzol® + bead mill	Yes	No	NA	No
Putnam et al. [60]	RNA, DNA, protein	TRIzol® + mortar and pestle	Yes	Yes	~2:1	Yes
Mayfield et al. [16]	RNA, DNA, protein	TRIzol® + mortar and pestle	Yes	Yes	~2:1	Yes
Kenkel et al. [61]	RNA	RNAqueous® + razor blade	Yes	No	NA	Yes
Mayfield et al. [15]	RNA, DNA	TRIzol® + mortar and pestle	Yes	Yes	~2:1	Yes
Mayfield et al. [7]	RNA, DNA	TRIzol® + mini-pestle	Yes	Yes	Variable[b,e]	Yes

Unless otherwise noted, all studies were undertaken with adult scleractinian corals. All trademarked reagents and kits are from Thermo-Fisher Scientific except NucleoSpin® (Machery-Nagel) and Aurum™ Total RNA minikit (Bio-Rad)

NA not applicable, *NR* not reported

[a]Analyzed both adult and larval corals

[b]Used endosymbiotic sea anemones

[c]Erroneously claims to be a dinoflagellate-free preparation method (not demonstrated)

[d]Proteins appear degraded on the gel images presented

[e]Depended on infection status of anemones

While genome size is static within a living cell, it is more difficult to quantify or model a "typical" host coral or Symbiodiniaceae transcriptome, proteome, lipidome, or metabolome because the respective molecules are in a constant state of flux due to the metabolic needs of the cells. In the few instances in which the relative host coral/Symbiodiniaceae mRNA ratios were reported (e.g., [16]; Table 1), it is not uncommon for the dinoflagellates to contribute over 1/3 of all extracted RNA in a healthy reef coral. Those looking to model the health of a coral, then, would be wise to include the dinoflagellate contigs in their bioinformatic analyses [17], though to this date, the more common reductionistic approach is taken; researchers interested in host coral gene expression omit the Symbiodiniaceae genes, while others exclusively focused on the endosymbionts omit those genes of their hosts (Table 1). Although this approach is sensible for certain laboratory experiments, I argue that, for coral biologists seeking to predict the ultimate fates of reef-building corals (e.g., [18–21]), such a simplistic approach is decidedly sophomoric. Instead, those interested in characterizing the health and stress tolerance of corals using molecular approaches (*sensu* [22]) would do far better by considering all constituents of the coral holobiont or at least the coral host and endosymbiotic dinoflagellates at a minimum.

The overall tendency to exclude Symbiodiniaceae molecules from omics analyses, however, may not actually stem from a scientific desire to focus exclusively on one member of the association. I surmise herein that it actually follows just as much from poor-quality extraction techniques, or, potentially more likely, the use of extraction protocols originally optimized for use with model organisms such as *E. coli*. Were the latter to be the case, one might actually expect a host coral-dominated macromolecular extract. To understand why this is the case, a more detailed understanding of coral-dinoflagellate biology is warranted. Firstly, reef-building corals and endosymbiotic sea anemones (e.g., *Exaiptasia*) house the Symbiodiniaceae dinoflagellates in only half of their cells, those of the gastrodermal tissue layer [23]. In essence then, the coral/endosymbiotic anemone is an animal–plant chimera. The anthozoan host cells feature a cell membrane that entirely ensheaths the endosymbionts (Fig. 2); the endosymbionts are not swimming freely within an open space, as are the bacterial microbes in our guts, but are effectively trapped in what is known as a "symbiosome" [24]. From an extraction standpoint, this does not pose an issue because the anthozoan cell membranes are decidedly flimsy and are easily disrupted by manual or chemical agitation [25]. In fact, were one to immerse a coral polyp in freshwater, many anthozoan cells would lyse due to osmotic stress alone [26], without any need for corrosive solvents, bead mills, mortars and pestles, etc.

Where technique becomes more critical, though, is in the lysing of the dinoflagellate cells, which are surrounded by some of the strongest cell walls ever before studied [27]. In the hypothetical example above of freshwater lysis, it is doubtful that any Symbiodiniaceae cells would burst. This is not to say that they are resistant to osmotic stress, only that their hardy cell walls would prevent lysis. Extraction buffers featuring even guanidinium-based salts (found in virtually all commercially available spin column kits) and beta-mercaptoethanol may even be insufficient to thoroughly lyse the majority of Symbiodiniaceae cells in a coral tissue biopsy and are only useful when employed with vigorous mechanical agitation (e.g., a bead mill with acid-washed sand; [28]). For those researchers aiming to characterize only host anthozoan biomolecular material, this is actually advantageous; one could realistically expect to extract *only* host macromolecules using these "gentler" extraction approaches. Within seconds of immersion in guanidinium-based lysis buffers (or sodium dodecyl sulfate [SDS] at 2% or greater), the vast majority of anthozoan cells will lyse, freeing the cellular macromolecules into solution to be concentrated, purified, and characterized as outlined by the protocols described herein.

However, those like myself who seek to also analyze the macromolecules encumbered within the dinoflagellate cells must unfortunately use a more time-consuming, rigorous extraction approach to ensure that the incredibly resistant algal cell walls are destroyed. In other words, the extraction approach outlined in this chapter is best suited for researchers seeking to characterize RNA, DNA, proteins, lipids, and metabolites from host corals (or anemones), endosymbiotic dinoflagellates, and the myriad other microbes that call the polyp, coenosarc, or colony home (Fig. 3). A far simpler and

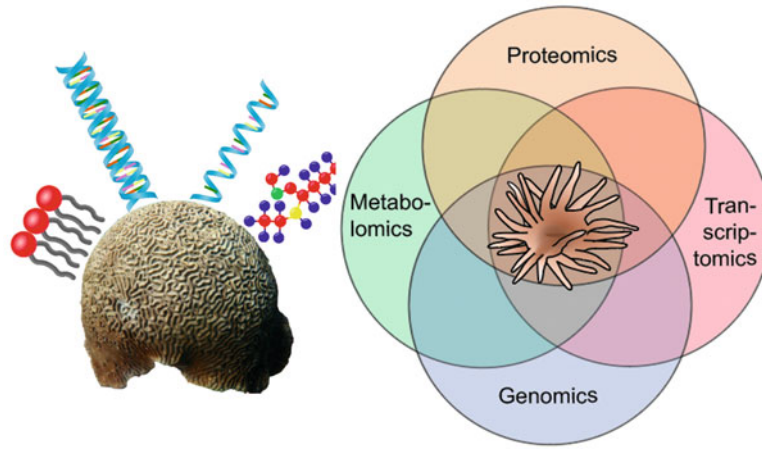

Fig. 3 A schematic featuring a brain coral (~50 cm in diameter) and a depiction of the multi-omic approach presented herein. Note that, in the Venn diagram on the right, "metabolomics" includes both polar metabolites and lipids

safer approach featuring commercially available spin columns would be better suited for biologists with an interest in host anthozoan biological material only (not described herein).

1.3 Justification for a Multi-omic Approach

Although several exceptions abound (Table 1), another general drawback of many endosymbiotic anthozoan studies, and likely nearly all biological disciplines for that matter, is the extraction of only a singular macromolecular type from each biopsy. One might ask, "I have plentiful tissue with which to work; can I not simply extract RNA from biopsy A, DNA from biopsy B, lipids from biopsy C, etc?" For certain experiments and tissues types, this might very well be the most practical solution. Certainly, if one is interested in gene expression alone, for instance, why bother to extract the other cellular macromolecules? However, for reef-building corals, a multi-omic approach is recommended for at least two reasons. First, numerous reef-building corals associated with multiple dinoflagellate endosymbiont types [29], not to mention a plethora of other microbes [30]. Even if one were only interested in functionally profiling lipids, metabolites, mRNAs, or proteins, there is still good reason to co-extract, at minimum, the DNAs such that these complex microbial communities could be analyzed in tandem. Indeed, this is exactly what was done in the first multi-omic analysis of a reef coral [16].

Second, although gene expression research is far more popular these days given growing dataset sizes and diminishing sequencing costs, there is no correlation between anthozoan or Symbiodiniaceae mRNA levels and concentrations of the proteins they encode in the few studies in which such congruency has been experimentally tested [31–34]. As such, those looking to understand the physiological implications of climate change or other environmental changes on reef corals and their diverse microbial communities should at least (1) profile protein concentrations and (2) characterize the identity of the holobiont from genetic or even metagenomic co-analysis of the DNAs. More generally, then, reef coral biologists are among the most justified in employing a multi-omic approach in their research projects.

1.4 Protocol Overview and Rationale

Analysis of complex suites of macromolecules—namely, RNAs, DNAs, proteins, lipids, and polar metabolites—of biological specimens involves the following steps (Fig. 4): (1) preservation of the biological specimen (i.e., biopsy), (2) extraction of target macromolecules from the cells, (3) separation of the macromolecules from one another, (4) concentration of the macromolecules, (5) washing of the macromolecules, and (6) solubilization of the macromolecules. Although myriad options abound, I recommend and will consequently describe two over-arching approaches:

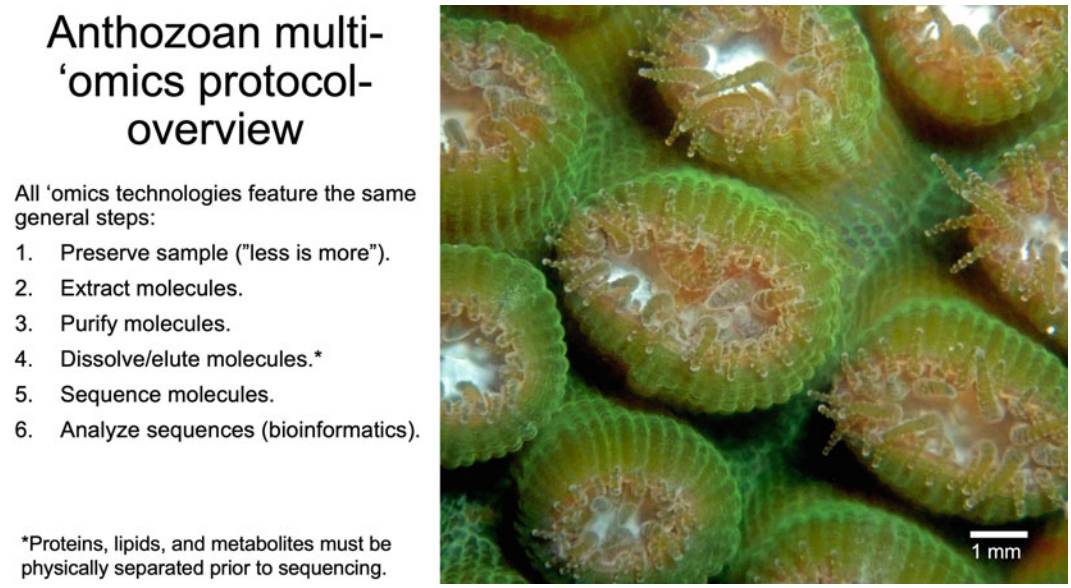

Fig. 4 An overview of the multi-omic extraction and representative coral sample material. For scleractinian corals with large corallites (e.g., those of the *Montastraea cavernosa* colony in this figure), a single polyp will yield sufficient RNA, DNA, protein, lipid, and metabolite quantities for all manner of omic analyses. Small-polyped corals (~1 mm, e.g., pocilloporids) may instead necessitate 15–30 polyps to yield sufficient protein, in particular

Option A (Fig. 5) and Option B (Fig. 6). The former is superior in that the proteins are far higher in quantity relative to Option B. The major disadvantage of Option A is that polar metabolites are co-isolated with RNAs and DNAs and must be subsequently pooled, then separated from one another, concentrated, and purified. This results in a large number of steps, as well as many dozens of microcentrifuge tubes to track. In contrast, lipids and polar metabolites are easily partitioned with a modified Bligh and Dyer extraction in Option B, which is derived from a seminal work by Podechard et al. [35]. However, in this approach, the proteins form a lens between the lipids and polar metabolites, and it is difficult to re-dissolve these irreversibly denatured species. They may, then, only be suitable for normalizing lipid and metabolite levels to total protein and not for explicit proteomic analyses; in fact, I have never used the proteins from Option B for shotgun or label-based proteomics (both described below).

With the exception of the proteins of Option B, these protocols consistently yield high-quantity and high-quality macromolecules from a diverse array of marine organisms, though they have only ever been thoroughly optimized for reef-building corals (both larvae lacking in skeletal material and adult corals featuring large quantities of calcium carbonate skeleton) and model anthozoan–dinoflagellate endosymbioses, namely, *Exaiptasia* spp. Differing sample types may require slight adjustments.

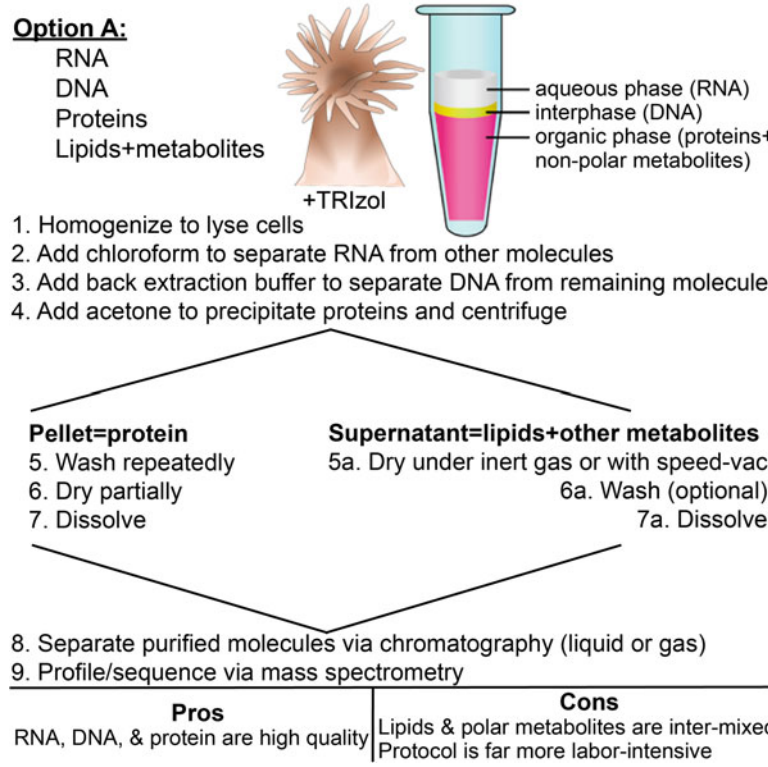

Fig. 5 Endosymbiotic anthozoan multi-omic extraction approach-Option A. Please note that the cartoon coral polyp has been magnified approximately ten-fold relative to the adjacent cartoon of the microcentrifuge tube. Also note that it is possible that a new solvent system could be exploited to better separate lipids from polar metabolites (between **steps 4** and **5a**)

2 Materials

Those familiar with more traditional phenol–chloroform DNA extractions will recognize the vast majority of reagents and materials required for successful extraction of macromolecules from reef-building corals and endosymbiotic sea anemones. Please take note of the safety concerns outlined previously, particularly with the handling of TRIzol (TFS) and chloroform. With four exceptions (denoted by asterisks), all chemicals found in the list below can be purchased directly from your preferred vendor. For solvents, I have noted the preferred quality grade in parentheses (when applicable), and for less common chemicals, I have mentioned a representative manufacturer; never use ACS or "reagent-grade" chemicals. When a chemical/reagent name has been abbreviated from hence forth, the abbreviation is shown as the first word within the parentheses.

Option B
RNA
DNA
Protein
Lipids
Polar matabolites

- Same as Option A except:
- Instead of adding acetone to proteins, carry out a Bligh and Dyer* extraction using the organic phase containing proteins & lipids.
- **Pros**: better separates lipids from other metabolites.
- **Cons**: the protein is far lower quality and quantity.

*Methanol+chloroform+water

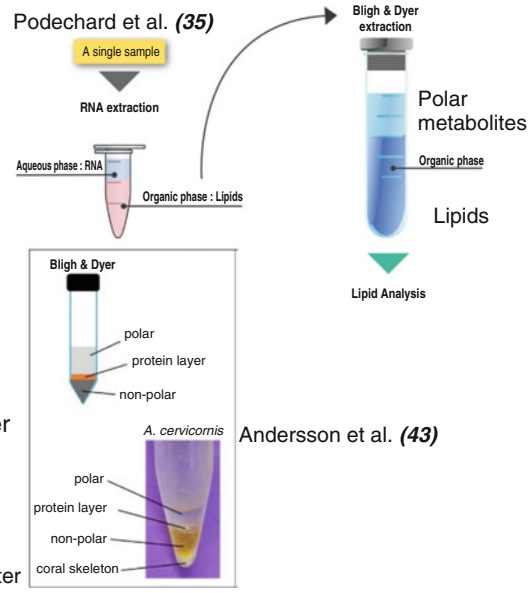

Fig. 6 Endosymbiotic anthozoan multi-omic extraction approach-Option B. Please note that these images have been modified from the respective publications (both of which are open access and cited in the reference list [35, 43]). *A. cervicornis* = *Acropora cervicornis* (a reef-building coral that is the primary focus of reef restoration efforts in South Florida, USA)

1. Acetone (molecular-grade; 100%; numerous vendors).
2. Acetonitrile (ACN; 100%; HPLC-grade; numerous vendors).
3. Agarose (powder; molecular-grade; numerous vendors).
4. Ammonium bicarbonate (AB; e.g., Sigma-Aldrich). Only buy in powdered form as solutions are unstable.
5. Autoclave.
6. Back extraction buffer* (BEB): 1 M Tris base, 4 M guanidinium (iso)thiocyanate, and 50 mM sodium citrate (Na citrate):
   (a) This near-saturated buffer is, in my experience, the only means of co-extracting high molecular weight DNA from samples stored in TRIzol; using TFS' DNA extraction protocol instead leads to recovery of DNA of far lower molecular weight.
   (b) Guanidinium thiocyanate is unstable in solution, even when kept in the dark; make only enough for several weeks' worth of sample processing (you will need 0.5 mL/sample.).
   (c) Place a stir bar in the glass bottle you will use and add a small amount of deionized+distilled water (ddH_2O).
   (d) Weigh out the appropriate quantity of guanidinium thiocyanate and add to bottle.

(e) Add a small amount of water to coat the guanidinium thiocyanate in the bottle.

(f) Weigh out additional solutes (Tris base and Na citrate) and add to bottle.

(g) Normally, you will not need to add much excess water; the guanidinium thiocyanate accounts for the majority of the BEB volume.

(h) For this reason, do not add a large amount of water to the bottle first, or you will far surpass your target volume upon addition of the guanidinium thiocyanate.

(i) Given the large amount of solutes, several hours on a stir plate may be required to fully solubilize all components; make the BEB the day prior to starting your extractions.

(j) Wrap bottle in aluminum foil and store in the dark.

(k) Periodically check coloration; once solution has turned yellow, discard (as hazardous material) and make a new batch.

7. Balance with a precision of at least 1 mg.
8. Beads (steel or ceramic) for bead mill (optional; numerous vendors).
9. Bead mill (optional; e.g., MP Biomedical's FastPrep™ series).
10. Beta-mercaptoethanol (100% solution; hazardous; numerous vendors).
11. Bovine serum albumin (BSA; numerous vendors). Can buy in lyophilized form or pre-solubilized (in which it is important that a preservative has been added).
12. Butylated hydroxytoluene (BHT; numerous vendors).
13. C18 spin tips (optional; Pierce).
14. Centrifuge capable of speeds up to 12,000 × *g* for 1–2 mL microcentrifuge tubes and 1500 × *g* for 15 mL tubes.
15. Chloroform (molecular-grade; amylene-free; hazardous; numerous vendors).
16. Culture tubes–glass with Teflon-coated plastic lids (15 mL; e.g., Pyrex®).
17. Culture tube racks.
18. Diethyl pyrocarbonate (DEPC; numerous vendors):
    (a) DEPC is highly carcinogenic.
    (b) Those with the means to do so should consider purchasing nuclear-free water, rather than performing in-house DEPC treatments (not discussed herein).
19. Dithiothreitol (DTT; note that this is a poison with a noxious smell; numerous vendors).

20. DNA gel stain: SYBR® Red or SYBR® Gold (TFS)—avoid using ethidium bromide.
21. DNA molecular weight ladder (numerous vendors, e.g., Bio-Rad).
22. DNA spin column kit (optional, e.g., Qiagen).
23. Ethanol (100%; molecular-grade [nuclease-free]; numerous vendors).
24. Ethylenediaminetetraacetic acid (EDTA; e.g., Sigma-Aldrich).
25. Formic acid (numerous vendors).
26. Fume hood.
27. Gel doc+power supply for agarose gel electrophoresis.
28. Gel doc+power supply for SDS-PAGE (e.g., Phastgel®).
29. Glycerol (100% stock; nuclease-free; numerous vendors).
30. Graphite spin columns (optional; Pierce).
31. Guanidine hydrochloride (HCl; hazardous; numerous vendors).
32. Guanidinium thiocyanate (aka isothiocyanate; hazardous; numerous vendors).
33. High salt solution* (HSS): 0.8 M Na citrate and 1.2 M sodium chloride (NaCl) in DEPC-treated water:
    (a) Unlike the BEB, the HSS is non-toxic, easy-to-make, and the solutes dissolve fully within several minutes.
    (b) HSS is stable for many weeks at room temperature, though precipitation of salts in this near-saturated solution is inevitable:
        (i) Consider making small quantities (5–10 mL; enough for 20–40 samples at 0.25 mL/sample).
        (ii) Limit opening of the bottle, which can allow RNases to enter.
    (c) Weigh out appropriate quantities of NaCl and Na citrate and add to DEPC-treated or otherwise nuclease-free water.
    (d) Vortex vigorously and allow several minutes for salts to go into solution.
34. Hydrochloric acid (HCl; hazardous; numerous vendors).
35. Inert gas (argon or nitrogen).
36. Iodoacetamide (powder; numerous vendors).
37. Isobaric tags for relative and absolute (protein) quantification (iTRAQ; Sciex).
38. Isopropanol (100%; molecular-grade [nuclease-free]; numerous vendors).

39. Kim® Wipes (for drying samples in inverted microcentrifuge tubes on benchtop).
40. Laboratory coat.
41. Laemmli sample buffer: 2% sodium dodecyl sulfate, 2% beta-mercaptoethanol, 10% glycerol, and 0.0625 M Tris-HCl (pH 6.8).
42. Liquid chromatography (LC) column (e.g., Acclaim™ PepMap™ RSLC [75 μm × 15 cm] nanoViper column [TFS]).
43. Lipid standards (optional; e.g., Avanti SPLASH® LIPIDOMIX®).
44. Liquid nitrogen (LN_2; optional).
45. Liquid nitrogen vapor (i.e., "dry") shipper (optional).
46. Mass spectrometer (MS; e.g., Orbitrap/Q Exactive [TFS]).
47. Metabolite standards (optional; e.g., IROA's suite of pre-mixed standards).
48. Methanol (HPLC-grade or higher; numerous vendors).
49. Microcentrifuge tubes: 0.5, 1.5, and 2 mL (all nuclease-free; numerous vendors).
50. Microcentrifuge tubes with screw caps for bead mill (optional): 2 mL (nuclease-free; e.g., MP Biomedical).
51. Microcentrifuge tube racks (plastic; numerous vendors).
52. Mixing apparatus: shaker table or dedicated mixing device (e.g., ELMI's Intelli-Mixer®).
53. Mortar and pestle (pre-sterilized).
54. Nitrile gloves (do not use latex or vinyl).
55. Pasteur pipette (glass and with bulb).
56. Pellet Paint-NF™ (optional; Millepore).
57. Pipets: 1–20 μL, 20–200 μL, and 200–1000 μL (e.g., Eppendorf or Rainin).
58. Pipet tips-filtered only: 20, 200, and 1000 μL (do not use non-filtered tips with this protocol).
59. Protein gel stain (e.g., Invitrogen's SimplyBlue™ Safe Stain).
60. Protein molecular weight ladder pre-stained (numerous vendors).
61. Protein quantification kit: BCA (numerous vendors) or Amersham's 2D-Quant™.
62. Protein wash I* (PWI): 0.3 M guanidine HCl in 95% ethanol with 2.5% glycerol:
    (a) This solution features a much lower concentration of chaotropic salt than the BEB.
    (b) The guanidine HCl should normally fully dissolve into the ethanol in several minutes.

(c) Protect solution from light and remake fresh every few weeks.

63. Protein wash II* (PWII): 95% ethanol with 2.5% glycerol.
64. RNAse Away® or comparable product for eliminating RNAses from labware.
65. RNA spin column kit (optional; e.g., Qiagen).
66. Safety goggles.
67. Scalpel or razor blade (optional).
68. Sodium acetate (powder or as a 3 M solution in water [pH 5.2]; numerous vendors).
69. Sodium chloride (NaCl; numerous vendors).
70. Sodium citrate (Na citrate; numerous vendors).
71. Sodium dodecyl sulfate (SDS; numerous vendors).
72. Sonicator bath.
73. Spatulas.
74. Spectrophotometer: Nanodrop-like unit for nucleic acids and plate-reading unit for proteins.
75. Speed vacuum (speed vac) or lyophilizer (numerous vendors).
76. Tandem mass tags (TMT; TFS; this protocol is not discussed herein but should be considered as an alternative to iTRAQ).
77. Triethyl ammonium bicarbonate (TEAB; e.g., TFS).
78. Trifluoroacetic acid (TFA; numerous vendors).
79. Tris-2-carboxyethyl-phosphine (TCEP; 100%; numerous vendors).
80. Tris base (powder; numerous vendors).
81. Tris–HCl (pH 6.8): can make a higher concentration (~1 M) and dilute as necessary.
82. Tris–borate–EDTA (TBE; purchase as concentrated stock [e.g., 10×] or make in-house).
83. Tris–EDTA (TE; purchase as concentrated stock or make in-house).
84. TRIzol (TFS; similar products [e.g., TRI-Reagent™] can be substituted, but DNA quality may suffer).
85. Trypsin (sequencing grade [aka "modified"]; e.g., Promega; cat. V5111).
86. Tubing for inert gas tanks (optional).
87. Urea (powder; numerous vendors).
88. Vortex Genie™ or comparable (numerous vendors).

89. Water (ddH_2O; ensure that it is nuclease-free either through DEPC treatment or via purchasing pre-sterilized water from your preferred vendor).
90. Weighing paper or weigh boats.

3 Methods

3.1 Sample Collection

Tip: Alcohols and strong acids are not effective preservatives for RNAs or proteins and should be avoided.

1. A diverse array of methods can be used to sample adult corals, though two have been consistently shown to *under*perform the others: tissue "blasting" using compressed air or water picks and tissue "scraping" with razor blades or scalpels.
   (a) One issue with the former is that the tissue removal process itself is so lengthy (several minutes) that macromolecular changes can occur.
   (b) Side-by-side comparisons of tissue+skeleton extractions (from simply cutting adult corals with bone-cutting pliers, i.e., the approach advocated herein) vs. extracting RNAs, DNAs, and proteins from water-picked coral tissues [36] highlight that the RNAs in particular suffer with the latter approach.
   (c) Although scraping tissues off of coral skeletons with sterile razor blades or scalpels could be effective if done quickly, there are two issues:
      (i) The intensive handling of the fragment/biopsy (or colony) needed to take the sample elicits substantial mucus generation from the coral polyps.
      (ii) This mucus can then function as a barrier to the fixative/preservative in which the tissue will be stored, meaning metabolic activity may not be halted until the samples are fully frozen.
   (d) Instead, cutting corals with bone-cutting pliers, drills, or other means, in which skeleton and tissue are collected in tandem, will, without exception, lead to higher yield and quality of macromolecules.
   (e) A good starting point in terms of amount of starting material is a single 1 cm diameter polyp (e.g., *Montastraea cavernosa*) or 10–20 polyps of 1 mm diameter (e.g., pocilloporids).
   (f) Coral larvae or small anemones (e.g., *Exaiptasia*) can simply be aliquoted into tubes containing TRIzol or flash-frozen in LN_2.

(g) If there is scientific justification to avoid the tissue–skeleton interface or the skeleton in its entirety, then the two aforementioned skeleton-free approaches (i.e., tissue blasting and tissue scraping) will likely be the only options since the following two approaches lead to far too rampant molecular degradation to be suitable for sequencing-based analysies:

(i) Removing tissues chemically (HCl or bleach).

(ii) Dissolving the skeleton with EDTA (as is used for histological preparation).

2. Freeze samples in LN_2 (or charged dry shipper at −150 °C) and store at −80 °C (or in the dry shipper itself if the extraction will be done within several weeks of sampling).

(a) Alternatively, samples can be immersed directly in TRIzol or similar acid phenol–guanidinium solvent and stored frozen (−80 °C) until the time of extraction (macromolecules are stable when frozen in TRIzol for many months).

(b) When preserving large pieces of adult coral, such as entire branches or "plugs" (i.e., mini-cores) made from pneumatic drills, LN_2 immersion is almost certainly the preservation method of choice. The use of corrosive preservatives would necessitate bringing them to the diving vessel or aquarium wet lab in large quantities (since the biopsies must be fully immersed).

(c) Care should be taken to avoid prolonged periods between harvesting of the biopsies and immersion in preservative (be it a solvent or LN_2). Gene expression and protein concentrations can change within minutes of the collection process itself (be it via hammer and chisel, pneumatic drill, bone-cutting pliers, scalpels, or razor blades for adult corals or by mere pipetting for coral larvae or small sea anemones).

(d) Although superior to water or alcohols, RNALater® (TFS) is a poor preservative for adult corals in particular due to the near-immediate precipitation of the constituent salts upon contact with marine osmoconformers.

(i) A newer generation of "Shield" reagents from Zymo are said to yield high-quality RNA from adult reef corals, though this has yet to be demonstrated in the peer-reviewed literature as of November 2022.

(ii) Were this to be later proven, this could represent an attractive alternative since these reagents are far less hazardous than TRIzol and much less cumbersome to transport than a LN_2 dry shipper or dewar.

3.2 RNA Extraction

Tip#1: Attempt only 8–12 extractions/day.

Tip#2: See safety recommendations above.

Tip#3: These steps are identical between Option A (Fig. 5) and B (Fig. 6).

1. *See* Subheading 3.1 ("Sample Collection") above and consult Appendix Sheet 1 for a summary.
2. Remove samples from freezer or dry shipper, and, if necessary, quickly break off a small (~50 mg) piece from the frozen biopsy with sterile bone-cutting pliers.
   (a) Skip this step if samples were previously minced and then frozen in TRIzol or equivalent.
   (b) If samples were frozen in TRIzol or equivalent, thaw sample + TRIzol and transfer into mortar (Option 1) or 2 mL bead mill tube (Option 2).
3. Option 1: Quickly cover coral fragment, larvae, or small anemones with additional TRIzol (or similar phenol–guanidinium-based solution) to 1.5 mL total volume, and homogenize for several minutes with a mortar and pestle (until the solution becomes a translucent pink) in a laboratory fume hood.
4. Option 2: Transfer sample to 2 mL tube compatible with bead mill (e.g., MP Biomedical's FastPrep™ series), quickly overlay with TRIzol to ~1.5 mL total volume, and add several large steel or numerous, small steel (or ceramic) ball bearings (depending on dimensions of biopsy).
   (a) Apply upward of 5, 30, or 60 s runs of the bead mill at ~4–6 m/s.
   (b) Place tubes on ice for 5 min after each run to allow samples to cool (heat builds up during bead beating).
5. Transfer 1 mL of homogenized tissues in TRIzol to a new 1.5 mL microcentrifuge tube, vortex, and incubate samples at room temperature [14] for 5 min:
   (a) Samples can alternatively be stored in TRIzol for several weeks at −20 °C.
   (b) Long-term storage of tissues homogenized in TRIzol at −80 °C is also suitable for proteomic analyses.
   (c) Residual TRIzol+tissue can be transferred to a separate tube to be used as a backup and frozen at −80 °C.
6. Add 200 μL of molecular-grade chloroform (without amylenes) to each sample and incubate at room temperature (RT) for 2–10 min.

7. Centrifuge (hereafter "spin") samples at 12,000 × *g* for 15 min at 4 °C.
8. Remove aqueous phase (~600 μL) and add to new 1.5 mL microcentrifuge tube containing 250 μL of isopropanol.
9. Add 250 μL of HSS (*see* Subheading 2), vortex, and incubate at RT for 10 min or for longer periods (several hours to months) at −20 °C or colder.
   (a) If you do not plan to undertake RNA-based analyses in the coming days, stop here and store precipitated samples in the −80 °C freezer.
   (b) Otherwise, proceed with **step 10**.
10. Spin at 12,000 × *g* for 10 min at 4 °C.
11. Decant supernatant (~1 mL) containing salts, isopropanol, and some polar metabolites into a 15 mL glass culture tube with Teflon-coated lid and conical bottom (e.g., Pyrex® culture tubes) capable of being spun at high speeds (e.g., in an Eppendorf 5810R centrifuge run at 1500 × *g*), and store at −80 °C.
12. Resuspend the RNA pellet in the minimum volume of lysis buffer recommended by your preferred RNA clean-up (spin column) kit:
   (a) This is typically 350–500 μL.
   (b) Axygen, Zymo, Qiagen, Protech, and numerous other kits have been used successfully with this protocol.
   (c) You may need to use the pipet tip to completely break apart and resuspend the pellet.
13. After breaking apart the pellet via pipetting or vigorous vortexing, follow the kit manufacturer's recommendations. This typically involves adding an equal volume of 100% ethanol to the RNA pellet in lysis buffer prior to loading the spin column.
14. Wash and DNase treat RNA on-column (15–20 min) as recommended by the manufacturer; the DNase treatment is critical since no current RNA extraction approach yields completely DNA-free RNA.
15. After decanting the final wash, incubate spin column in a new microcentrifuge tube in a 60 °C oven for 5–10 min to evaporate residual ethanol in the column.
16. Elute RNA in 30–50 μL of DEPC-treated or nuclease-free water after letting the water incubate on the column for 5 min prior to the final spin.
17. Assess RNA quantity on a mini-spectrophotometer (e.g., NanoDrop; duplicate readings of 2 μL/reading), bioanalyzer, Qubit, or other means (see Appendix Sheet 2).

(a) DNA-free RNA using this protocol is typically between 30 and 300 ng/μL (1–10 μg of total RNA) when using a 50–100 mg piece of adult coral (including skeleton):

(i) RNA concentrations <20 ng/μL likely signify poor extraction efficiency *unless*

(ii) Very small biological material was used (e.g., <10 larvae or a bleached anemone <5 mm in diameter)

(iii) For adult corals, extraction should be re-performed if concentrations below 20 ng/μL are obtained (assuming backup material remains)

(b) The 260/280 should be between 1.8 and 2.2:

(i) If it is too low, there may be protein contamination.

(ii) If it is too high, the RNA is likely degraded.

(c) The 260/230 should be >1. If it is far lower, alcohol contamination is likely.

18. Electrophorese ~5 μL of RNA on a 0.8% TBE-agarose gel stained with SYBR® Gold or SYBR® Red (avoid highly mutagenic stains like ethidium bromide):

(a) Alternatively, a formaldehyde (denaturing) gel may be used to better estimate the size of 28 and 18s bands; mRNA should be a 0.5–12 kb smear.

(b) The best option for RNA integrity analysis is a bioanalyzer (e.g., Agilent). Many sequencing cores now bundle this with their sequencing costs.

19. Dilute RNA to desired concentration (e.g., 20 ng/μL) with DEPC-treated water and store at −80 °C.

3.3 DNA Extraction

Tip#1. This protocol can be performed simultaneously with the final steps of the RNA extraction.

Tip#2. This protocol is identical between Options A (Fig. 5) and B (Fig. 6).

1. Remove remaining RNA aqueous phase (*see* **step 8** of Subheading 3.2).
2. Add 500 μL of BEB (*see* Subheading 2).
3. Vortex and place tubes on shaker table or, preferably, a dedicated tube mixer (e.g., ELMI's RM2 Intelli-mixer) for 10–20 min.

(a) DNA can be left on shaker table/mixer for 1–2 h, but no longer.

(b) Samples should *not* be frozen at this stage.

(c) If solution becomes blue, the extraction may fail.

4. Spin samples at 12,000 × *g* for 15 min at 4 °C.

5. Transfer aqueous phase (~600 μL) to a new 1.5 mL microcentrifuge tube.
6. Precipitate DNA with 2 μL Pellet Paint-NF™ (Millepore; only necessary for adult tissue samples <50 mg, <20 larvae, or a single *Exaiptasia* anemone), 60 μL of Na acetate (3 M, pH 5.2), and 600 μL (1 vol) of isopropanol at RT for 10 min or at −20 °C for longer periods (up to several months).
   (a) Isopropanol alone can be used instead.
   (b) If you have no immediate need for the DNA, stop here and precipitate at −20 °C or −80 °C (DNA is stable in this medium for many years).
   (c) Otherwise, proceed with **step 7**.
7. Spin at 12,000 × *g* for 10 min at 4 °C.
8. Decant supernatant containing salts, isopropanol, and some polar metabolites (~1 mL) into the 15 mL glass tube containing polar metabolites in isopropanol from **step 11** of the RNA extraction (Subheading 3.2).
9. Resuspend DNA pellet in minimum recommended lysis buffer volume from preferred DNA cleanup spin column kit (normally at least 100 μL).
   (a) PCR and DNA clean-up kits from Qiagen, Zymo, Axygen, Protech, and other vendors have been used successfully with this protocol.
   (b) Use pipet tip to break apart DNA pellet and vortex until it has completely disintegrated.
10. Load spin column with entire volume of lysis buffer+DNA, and spin as recommended by the manufacturer.
11. Carry out remaining spins and washes as recommended by the manufacturer.
12. Put the "eluent" (normally Tris–HCl [pH 8.5] or TE) in a 60 °C oven for 5–10 min before adding it to the spin columns (this will help the DNA to dissolve).
13. After the second spin column wash with the kit's final buffer, place the empty columns/tubes into the centrifuge, and spin at 12,000 × *g* for 3 min to remove excess ethanol.
14. Transfer spin columns to new 1.5 mL microcentrifuge tubes, and place in 60 °C oven for 5 min. This will further help to remove residual ethanol.
15. Add desired volume (~30–50 μL) of pre-warmed eluent to each column and wait for 5 min:
   (a) DNA will dissolve poorly in unbuffered water using this protocol.

(b) Ensure that the eluent is either Tris–HCl (pH 8.5) or, preferably, diluted TE (1/10 dilution).

16. Perform final spin for 1 min at 12,000 × *g* and discard spin column.
17. Quantify 2 μL of DNA × 2 times on a spectrophotometer (e.g., Nanodrop).
    (a) The DNA quantity from a 50 mg biopsy of adult coral +skeleton should be in the range of 3–30 μg (mean = ~10 μg or 50 μL of 200 ng/μL).
        (i) Larval samples (10–20 larvae) and small anemones (<5 mm in diameter) may yield concentrations of only 10–20 ng/μL.
        (ii) Discard samples with concentrations <10 ng/μL unless DNA will be used for PCR-based analyses only (in which very low concentrations can still be amplified successfully).
    (b) The 260/280 ratio with this protocol should be 1.7–2.
    (c) The 260/230 ratio with this protocol should be >1, preferably much higher.
18. Electrophorese 5 μL on an 0.7–0.8% TBE–agarose gel (with 1× SYBR Gold or SYBR Red) at 100 V for 30–45 min.
    (a) The DNA may be of such high molecular weight that it will barely move from the well.
    (b) A long smear may also be seen if the DNA has degraded somewhat.
19. Dilute DNA to desired concentration (e.g., 1–10 ng/μL).
20. DNA can be stored at 4 °C in TE, though storage at −20 °C is also possible if freeze–thaws are kept to a minimum.

3.4 Protein, Lipid, and Metabolite Extractions

3.4.1 Option A (Fig. 5)

Option A, Phase I: Proteins (Fig. 5)

Tip#1. Purification of lipids and polar metabolites using this protocol can be found in subheading "Option A, Phase II: Lipids and Polar Metabolites (Fig. 5)" *("phase II").*

Tip#2. If desiring lipids and polar metabolites that are better (and more easily) separated from one another (albeit with lower quality proteins), see Subheading 3.4.2 ("Option B;" Fig. 6).

Tip#3. Remember that some polar metabolites have already been set aside in 15 mL glass culture tubes in the freezer (see respective steps in previous protocols).

1. After removing the DNA aqueous phase (**step 5** of Subheading 3.3), transfer remaining ~500 μL of protein+lipids+metabolites in phenol to a new 2 mL microcentrifuge tube containing 1.5 mL of acetone.
2. Precipitate proteins at −20 °C or colder while you finish the RNA and DNA extractions:

(a) Proteins can stay in acetone at −20 °C for several months or −80 °C for several years.

(b) At minimum, precipitate at RT for 10 min.

(c) If not immediately conducting proteomics, stop here and leave precipitated proteins in acetone at −80 °C.

(d) Otherwise, proceed with **step 3**.

3. Spin samples at 12,000 × *g* for 10 min at 4 °C and decant acetone supernatant (~ 2 mL; which contains most of the lipids and some polar metabolites) into the 15 mL glass tube containing co-extracted polar metabolites from the RNA and DNA precipitation steps described above (as reiterated in Tip#3 above).

(a) A large protein pellet should be visible in the original 2 mL tube.

(b) Store lipids+remaining polar metabolites in acetone (~4 mL in total) at −80 °C for later purification (described in more detail in subheading "Option A, Phase II: Lipids and Polar Metabolites (Fig. 5)" ["phase II"]).

4. Add 1 mL of PWI (*see* Subheading 2), and dislodge the pellet from the bottom of the tube with a pipet tip.

5. Sonicate for 10–30 min on ice until pellets are completely broken:

(a) Longer periods may be needed (~1–2 h):

(i) Properly disrupted proteins will create a "snow-like" appearance in the tubes.

(ii) This is the most important step of the protocol since improperly disrupted pellets will never be effectively washed nor solubilized.

(b) Ensure that water temperature remains cool in the sonicator bath by periodically adding ice.

6. Spin samples at 8000 × *g* for 5 min at 4 °C and decant supernatant; treat as hazardous material since it contains guanidine HCl.

7. Perform two additional washes with 1 mL of PWI.

(a) Sonicate on ice until protein pellets dissociate completely.

(b) Subsequent sonication steps should require less time to fully break apart pellets.

(c) Proteins in PWI can be stored at −20 °C for several months.

8. After decanting the supernatant from the third wash, add 1 mL of PWII (*see* Subheading 2), and sonicate on ice until pellets are broken.

(a) Optional step: upon full disruption of the pellet, transfer 500 μL of proteins in PWII to a new 2 mL microcentrifuge tube.

(b) Freeze at −80 °C to serve as a backup.

9. Spin at 8000 × *g* for 5 min at 4 °C and decant supernatant.
10. Dry proteins inverted on benchtop for 5–15 min; do not over-dry pellets, as this will make them harder to dissolve.
11. The optimal solubilization buffer will depend on the application:
    (a) For western blots, add ~150 μL of Laemmli sample buffer (*see* Subheading 2).
    (b) For shotgun proteomics (described in Subheading 3.5), dissolve proteins in 100–200 μL of AB (50 mM).
    (c) For iTRAQ or TMT (i.e., label-based proteomics; the former is described in Subheading 3.7), dissolve proteins in 0.1–0.5 M TEAB.
    (d) For 2-D gels (*sensu* [32]), dissolve protein pellets in rehydration buffer (with thiourea); please note that, in most cases, a "next-generation" proteomic approach (i.e., TMT or iTRAQ) will yield superior data with lower effort.
12. Sonicate on ice for 5–10 min.
13. Spin at 12,000 × *g* for 5–15 min at 4 °C. If performing western blots, boil at 100 °C for 5 min before this spin.
14. If there is still a large protein pellet present, that signifies that proteins did not dissolve entirely.
    (a) If so, add SDS (2–10% solution in water or buffer) to a final concentration of 0.067%.
    (b) Vortex vigorously on Vortex Genie or via passing the pellet through the pipette tip and re-perform **step 13**.
15. Transfer supernatant in equal volumes to four different 1.5 mL microcentrifuge tubes (to reduce potential for freeze–thawing):
    (a) To a fifth 1.5 mL tube, add a ~30 μL aliquot for quantification and quality analysis (QC).
    (b) This sample can be left on ice if quantifying on the same day.
    (c) Freeze remaining tubes at −80 °C.
16. Quantify 20–25 μL of protein:
    (a) If performing western blots or 2D gels, use the 2D-Quant™ kit (Amersham Biosciences):
        (i) This kit can quantify proteins in buffers containing strong denaturants and detergents (e.g., urea).
        (ii) A Qubit kit can also be used.

(b) If performing proteomics, BCA or Bradford assays can be used.

(i) Dilute sample ~ten-fold beforehand.

(ii) Both SDS and TEAB can interfere with the absorbances at higher concentrations.

(c) When starting with 50 mg of coral tissue+skeleton and eluting in 150 μL of any of the aforementioned buffers, this protocol yields protein concentrations of ~0.5–15 μg/μL (~75–2,250 μg of protein).

17. Regardless of proteomic approach taken, mix a 1–2-μL aliquot of protein with 2× Laemmli sample buffer and boil at 95% for 5 min.

18. Load into a PhastGel® gradient 4–15 polyacrylamide gel (GE Healthcare):

(a) Please note that this system has been discontinued.

(b) Until a similar 3D-printed gel system amenable with small protein quantities is developed, it is unclear how protein quality can be assessed prior to proteomics without sacrificing the vast majority of the extracted protein (as would be required for standard SDS-PAGE, in which >100 μg may be needed to readily visualize protein profiles).

19. Load the gel into the Phast System® (GE Healthcare) after inserting two PhastGel SDS buffer strips.

20. Run proteins (1–3 μL in 1× [final] Laemmli sample buffer) alongside 1 μL of 1 μg/μL BSA standard and 1 μL of Plus2® Pre-stained protein standard (TFS) or other such pre-stained protein standard under the Phast System's separation method 3.

21. After ~2–3 h, wash the gel three times with water on shaker table.

22. Stain with 10–20 mL of SimplyBlue™ Safe Stain (Invitrogen) for 1 h at RT; more common Coomassie stains can be used though require first fixing the gels.

23. Pour off stain (which can be reused):

(a) Wash gel repeatedly with water until bands can be seen with the naked eye.

(b) For best results, destain overnight.

24. Take a picture of the gel against a white backdrop or on an illuminated light tray.

(a) You should see an array of bands of many different molecular weights (*sensu* Fig. 7).

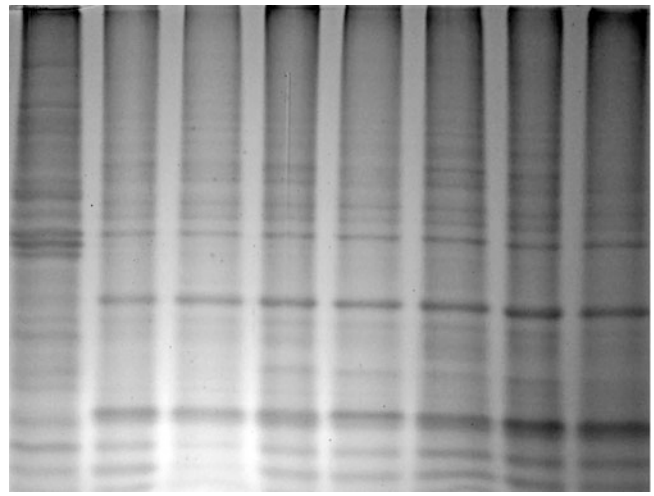

Fig. 7 Proteins (~100 μg) from the reef-building coral *Seriatopora hystrix* and its dinoflagellate endosymbionts on a 4–15% gradient SDS-PAGE gel stained with SYPRO® Ruby. Although a molecular weight ladder is not shown, the proteins range in size from ~5 kDa (bottom of gel) to 300 kDa (top). Degraded proteins are not evident

(b) What you do *not* want to see is a low molecular weight blob at the bottom of the gel (signifying degraded proteins).

Option A, Phase II: Lipids and Polar Metabolites (Fig. 5)

Tip#1: This protocol has not been vetted to the same extent as those described elsewhere in this chapter; further optimization may be required.

1. Remove ~4 mL of lipids+polar metabolites in acetone+phenol +isopropanol+water from prior steps from the −80 °C freezer and vortex.
2. Add 4 mL of methanol, 4 mL of chloroform, 3 mL of ddH_20, and 30 μL of Avanti SPLASH® LIPIDOMIX® lipid standards.
   (a) The latter aids in quantification and helps correct for differential lipid extraction efficiency across samples.
   (b) If funding permits, I recommend spiking in non-lipid metabolite standards, as well.
   (c) IROA Technologies is currently at the forefront of this initiative, though I have not yet used their metabolite standards myself.
3. Vortex vigorously and spin at 1500 × *g* for 2 min.
4. Transfer bottom, organic phase containing lipids with a glass Pasteur pipet to a new, 15 mL glass culture tube with a Teflon-coated cap.

(a) If lipids will not be analyzed immediately, add BHT to a final concentration of 10 μM, flush with argon or nitrogen gas, and store at −80 °C.

(b) If there is substantial insoluble material evident in the original tube upon removing the lipids, transfer the polar metabolite aqueous phase to a new, suitably sized glass tube.

5. If lipids and polar metabolites will be analyzed on the same day, dry both to completion via inert gas evaporation (argon or nitrogen) in a fume hood or through using a speed vac.

(a) The latter option may require several hours for the ~7–7.5 mL of lipids and ~7–7.5 mL of polar metabolites to fully evaporate.

(b) Note that, because acetone and isopropanol both dissolve in water and chloroform, the exact volumes of each fraction may vary.

(c) Long-term storage of metabolites is uncommon; empirical testing may be required to determine whether storage in liquid form at −80 °C (methanol will not freeze at this temperature) is superior to freeze-drying and/or storing dried metabolites at −80 °C.

6. Give tubes with dried lipids and metabolites to MS core facility.

7. Normalize resulting concentrations of lipid and metabolite species to internal standards and then to total protein (calculated from the protein extraction protocol presented above); the latter is critical since it is not possible to standardize loading of biological material in an adult coral extraction.

3.4.2 Option B (Fig. 6)

Tip#1: For those interested only in proteins, lipids, and polar metabolites (and not *RNA or DNA), simpler approaches could be employed* (e.g., [37]*). However, ensure that a robust enough homogenization approach is used to lyse the Symbiodiniaceae cells; simple immersion in chloroform + methanol + water is insufficient.*

1. After removing the DNA aqueous phase (**step 5** of Subheading 3.3), transfer ~0.5 mL of the organic phase containing proteins, lipids, and polar metabolites to a 15 mL glass tube with a Teflon-coated lid and conical bottom capable of being centrifuged at high speeds (e.g., Pyrex) and mix with 2.5 mL of methanol.

2. Add ~1 mL of polar metabolites in isopropanol from **step 11** of Subheading 3.2 (RNA extraction).

3. Add 2.5 mL of chloroform, 2 mL of water, and 10 μL of Avanti SPLASH LIPIDOMIX polar lipid standard cocktail to serve as an (1) internal standard and (2) extraction efficiency control:

   (a) A metabolite standard cocktail (e.g., IROA's family of metabolite standards) should also be used as these will result in a more truly quantitative dataset.
   (b) Normalization to metabolite standards is an emerging field, and so details have generally been omitted herein.
4. Vortex and spin at 1500 × *g* for 2 min.
5. Remove the lower organic phase featuring lipids in chloroform with a glass Pasteur pipet and transfer the entire volume (~3.5–4 mL) to a new glass tube.
6. Add BHT to 10 μM to limit oxidation if samples will not be analyzed on the same day.
7. Regardless of date of MS analysis, dry entire lipid volume to completion using inert gas or a speed vac and remove all air via argon gas flushing.
8. For the polar metabolites in methanol remaining in the original 15 mL glass tube, transfer entire amount via pipet to a new 5–10 mL glass tube and either dry as for the lipids or store at −80 °C (methanol will not freeze at this temperature).
9. Add 2 mL of acetone to the remaining protein "plug" (*see* Fig. 6) in the original 15 mL tube, vortex vigorously, and transfer entire volume of protein+acetone to new 2 mL microcentrifuge tube.
10. Precipitate protein and wash as described in subheading "Option A, Phase I: Proteins (Fig. 5)":
   (a) As mentioned above, this protein is of lower quality than that derived from Option A; it is likely suitable only for quantification (i.e., "total protein") rather than for proteomics.
   (b) Normalize lipid and metabolite species concentrations first to the respective standard and then to the total protein of the respective sample to control for differing amounts of biomass among samples (a veritable surety for adult reef coral samples).
11. Consult the MS core facility you will use for optimal solubilization buffer and volume for both lipids and metabolites, though most will accept dried samples provided there is at least a rough estimate of quantity.

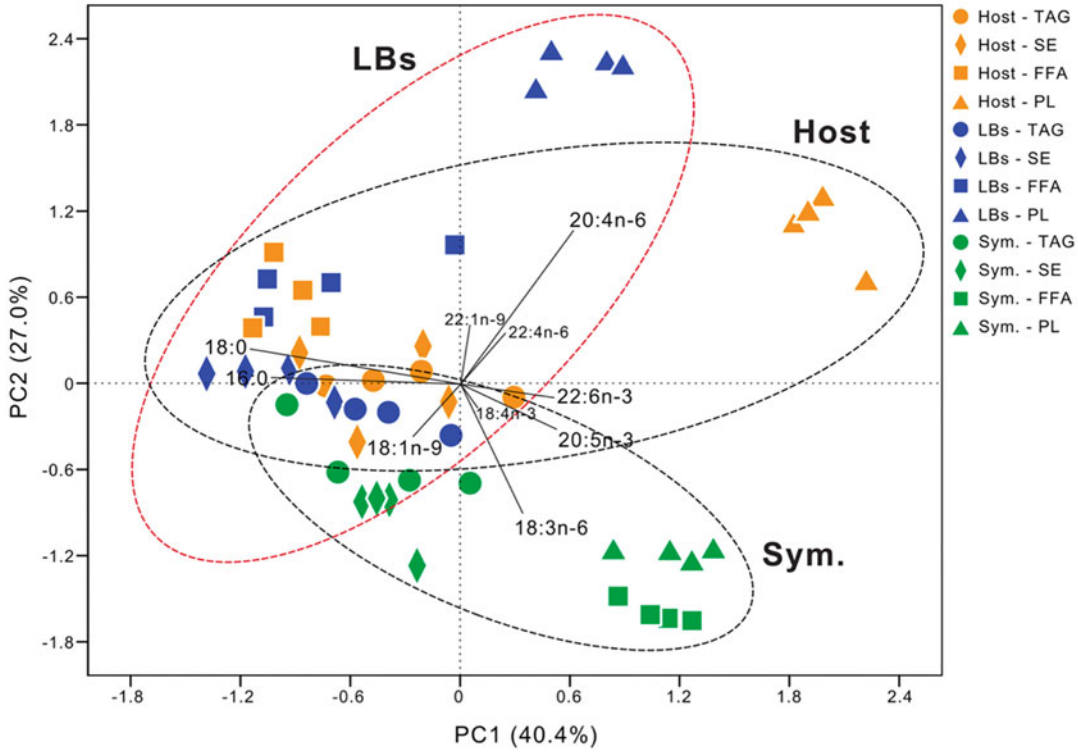

Fig. 8 A principal component analysis biplot depicting lipidomic data from an Indo-Pacific reef coral (from Chen et al. [44, 61]). Lipids were extracted using the protocol described herein from three compartments of the reef coral holobiont that were separated (pre-extraction) via centrifugation: the coral host ("Host"), the Symbiodiniaceae dinoflagellates ("Sym."), and the lipid bodies (LBs) that serve as lipid trafficking intermediaries within the holobiont. Certain fatty acid [3] species are depicted as biplot rays, though in general the lipids were grouped into one of the four primary types: triacylglycerols (TAG, i.e., triacylglycerides), sterol esters (SE), free fatty acids (FFA), and phospholipids (PL)

(a) Unlike for nucleic acids and proteins, in which quantification prior to sequencing is an integral part of the workflow, total lipid and metabolite levels are not generally known until after MS (and even then the total quantity loaded may be hard to discern).

(b) For representative data, please *see* Fig. 8.

3.5 Shotgun Proteomics

Tip#1: This protocol was first published by Mayfield et al. [38]).

Tip#2: Unlike for the label-based approaches discussed below, shotgun proteomics yields only presence-absence data.

1. After quantifying proteins and ensuring that they were not degraded on the mini-SDS-PAGE gels (i.e., PhastGels; subheading "Option A, Phase I: Proteins (Fig. 5)"), take out desired protein quantity from the freezer:
   (a) 10–100 μg of protein will suffice, though if pH fractionation will be carried out (not discussed herein but recommended to those with larger budgets and with an increased need for maximum peptide characterization), a larger quantity may be necessary.
   (b) It is not uncommon to analyze 100% of the proteins extracted (i.e., no backup material will remain).
2. Dry proteins down to 8 μL and add 15 μL of 10 M urea in 50 mM AB to denature the proteins.
3. To reduce the proteins, add 2 μL of 125 mM DTT in 50 mM AB to the sample, and incubate them at RT for 1 h.
4. Alkylate the proteins by adding 5 μL of freshly prepared iodoacetamide (90 mM; no more than 2–3 days old) in 50 mM AB and incubate in the dark for 30 min.
5. Quench the alkylation reaction in the dark by adding 3.33 μL of 125 mM DTT in 50 mM AB (1 h at RT).
6. Dilute the urea to 1 M by adding 116.7 μL of 50 mM AB (total volume = 150 μL).
7. Digest peptides by adding 5 μL of 0.1 μg/μL sequencing grade modified trypsin (e.g., Promega; cat. V5111) for 18 h at 37 °C (1:30 [w:w] enzyme/protein).
8. Stop the digestion reaction by adding 7.75 μL of 50% formic acid.
9. Add 40.6 μL of 2.5% TFA to the samples (final concentration = 0.5%) and check to ensure pH is <4.
10. Place a C18 spin tip (Pierce) in a 1.5 mL microcentrifuge tube and wet by adding 20 μL of 0.1% TFA in 80% ACN:
    (a) If using large quantities of protein (>50 μg) or if insoluble material is evident, instead use Pierce graphite spin columns (following manufacturer's protocol).
    (b) Otherwise, C18 tips will clog, making it difficult to recover proteins.
11. Spin tubes+tips at 1000 × *g* for 1 min and discard the flow-through.
12. Equilibrate tips with 20 μL of 0.1% TFA and spin at 1000 × *g* for 1 min.
13. Discard flow-through, add 50 μL of protein sample to the spin tip, and spin as above.

14. Repeat this step until all proteins have been passed through the spin tip.
15. Wash the protein-laden tip twice with 20 μL of 0.1% TFA and transfer to a new tube.
16. Elute proteins twice in 20 μL of 0.1% TFA in 80% ACN.
17. Speed vac proteins to near-complete dryness.
18. Resuspend proteins in 20 μL of 2% ACN with 0.1% formic acid prior to nano-LC, which is typically performed by a dedicated technician at a core facility; however, pertinent details can be found in Subheading 3.6.

3.6 Liquid Chromatography/Mass Spectrometry

As an example of how a shotgun proteomic approach could be taken using proteins extracted and prepared as in the previous section, I have paraphrased from Mayfield et al. [38] as follows:

1. Separate proteins in 2% ACN with 0.1% formic acid via passage through an Acclaim™ PepMap™ RSLC (75 μm × 15 cm) nanoViper column (TFS) using a Nanospray Flex ion source (TFS) on an Easy Nano LC™ 1000 (TFS).
2. Run peptide eluates from a 2–98% ACN gradient (84 min) on a Q Exactive™ Orbitrap MS (TFS) as in Musada et al. [39].
3. Operate the Q Exactive (Fourier transform) MS in MS2 mode with "higher-energy collisional dissociation" (HCD) activation (28 eV; the mass range scan is commonly 150–1600 m/z).
4. Details of the data analysis pipeline and bioinformatics can be found in Mayfield et al. [38–40] for shotgun proteomics and Mayfield [41] for iTRAQ (discussed in Subheading 3.7).

3.7 iTRAQ

iTRAQ is a label-based approach in which digested peptides are given an isobaric tag (i.e., label) that is detected by the MS and used as a proxy for concentration (analogous to the more popular RNA-Seq used in gene expression analyses). In the first pass of the MS, the mass peaks of the peptides are obtained. Then, in the second pass, the iTRAQ labels are analyzed (hence why you sometimes see "MS/MS" in the literature); eight samples can be analyzed in parallel. Below I have included a protocol that, while satisfactory in yielding quantitative data for several hundred–thousand proteins in a single sample, is nevertheless characterized by poor labeling efficiency (5–20% of peptides receive an iTRAQ label.); whether this is an issue inherent with the labels themselves or the protocol cannot yet be known. This approach has been used to analyze reef coral protein profiles since at least 2020 [41, 42]; please *see* Fig. 9 for representative data:

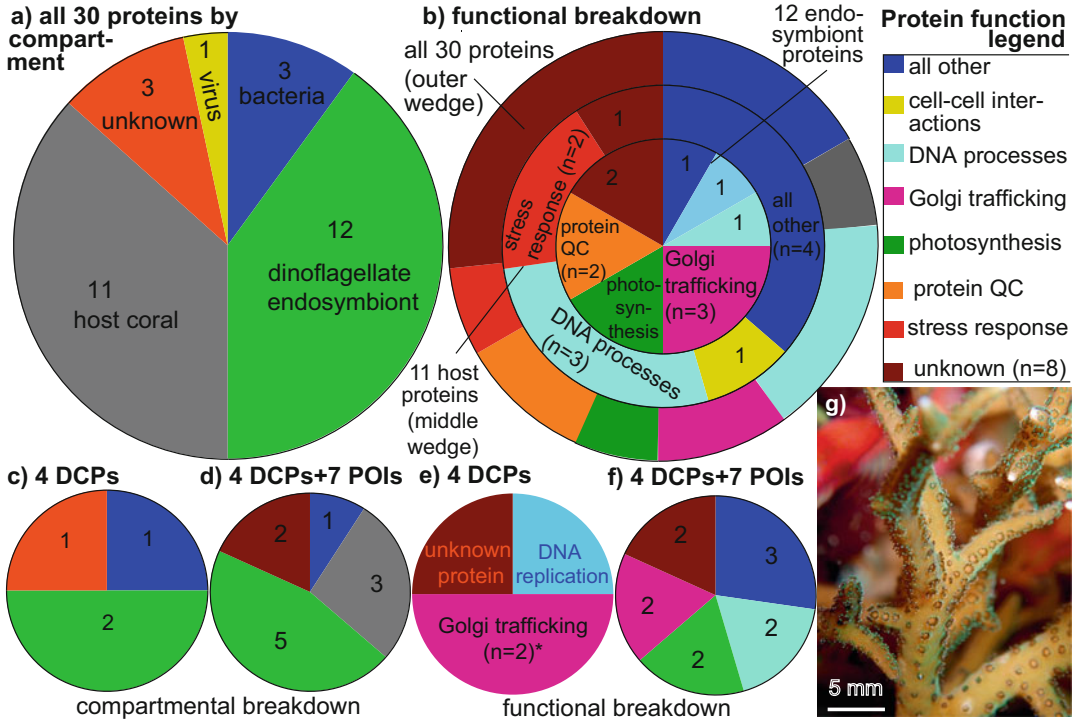

Fig. 9 Representative proteomic data obtained using the protein extraction and iTRAQ protocols outlined in this chapter (adapted from Mayfield [41]). Please note that the 30 proteins (11, 12, and 7 from the host coral, Symbiodiniaceae dinoflagellates, and other microbial constituents of the coral holobiont, respectively) represent a highly reduced subset of the initial number obtained from the mass spectrometer since several stringent quality control (QC) steps were enacted; for details on the associated bioinformatics and post-peptide identification QC, please consult Mayfield [41]. DCP = differentially concentrated protein. POI = protein of interest (a term used to distinguish proteins that were useful in predictive model building but not necessarily differentially concentrated across treatments)

1. Dilute all proteins in 0.5 M TEAB to the same concentration in a 30 μL volume:
   (a) A good target amount is 50–150 μg in 30 μL of 0.5 M TEAB (i.e., 1.5–5 μg/μL).
   (b) AB is incompatible with this protocol because of how the labeling step is actualized.
   (c) A speed vac will almost surely need to be used to achieve these high concentrations.
2. Add SDS to a final concentration of 0.067% (e.g., 1 μL of 2% SDS to 30 μL of protein in TEAB).
3. Add 1 μL of TCEP (*see* Subheading 2) to reduce the dissolved proteins' disulfide bonds.

4. Vortex and spin samples at 15,000 RPM for 5 min (hereafter referred to as "spun").
5. Incubate at 60 °C for 1 h and spin samples again.
6. Alkylate samples with 1 μL of freshly prepared 84 mM iodoacetamide in water or 0.5 M TEAB.
7. Vortex, spin, and incubate in the dark (aluminum foil or dark box) at RT for 30 min.
8. Spin samples again and mix with 10 μL of 0.1 μg/μL sequencing grade modified trypsin (e.g., Promega cat. V5111) for 3 h at 37 °C.
9. Add an additional 1 μL of trypsin and complete digestion overnight at 37 °C.
10. After spinning, dry samples (~43 μL) to 30 μL in the speed vac.
11. Resuspend dried proteins in 0.5 M TEAB (without SDS).
12. Mix proteins with 50 μL of isopropanol and 17–22 μL of the appropriate iTRAQ reagent (Sciex iTRAQ Reagent 8-plex 25 U kit) according to the manufacturer's recommendations (exact volumes vary by lot.).
13. Vortex samples, spin, and incubate at RT for 2 h.
14. Quench reactions with 100 μL of water for 30 min and dry to 10–20 μL in the speed vac.
15. Combine all eight samples of same batch into one tube and dry to completion.
16. Resuspend in 100 μL of water, vortex, spin, and dry to completion.
17. Repeat **step 16** two more times.
18. After the last wash, dry to 30 μL in the speed vac, and mix with 30 μL of 2.5% TFA.
19. Purify acidified proteins with Pierce graphite columns (for samples with >100 μg protein in total) according to the manufacturer's protocol to remove residual buffers, salts, and/or insoluble material.
20. Resuspend purified and labeled peptides in 2% ACN with 0.1% formic acid prior to nano-LC on an Easy Nano LC 1000:
    (a) The resuspension volume will normally be established by the MS core, but is likely in the range of 10–50 μL.
    (b) Subsequent nano-LC/MS analyses are normally performed by a core MS facility (see abbreviated protocol in Subheading 3.6).
21. Run peptide eluates from a 2–98% ACN gradient individually on a Q Exactive Orbitrap LTQ MS (TFS).

(a) This protocol has not been attempted using non-Orbitrap instruments.

(b) Please see references [38–41] for a detailed treatise on the bioinformatics associated with the raw MS data generated by the Orbitrap.

Appendices

Appendix Sheet 1 Printable protocols for RNA, DNA, and protein extractions. Note that the blank spaces are to be used to either place check marks (to demonstrate completion of the respective step) or to fill in pertinent details, namely with respect to temperatures (temp.), times, and volumes. Alternative RNA and DNA spin column kits can be substituted.

RNA Extraction **Date:** _____

Homogenization and Phase Separation

1. Homogenized in: LN_2_____ TRIzol®_____ TRI-Reagent®_____ Other_____
   (a) w/: mortar and pestle___ micro-pestle___ tissue lyser__ bead mill__ other___
2. Incubated at _____(temp.) for _____(time) after vigorous vortexing.
   (a) w/: shaker table_____ tissue lyser_____
3. Added 200 μL of chloroform and incubated at RT for _____(time). w/: new tube_____
4. Spun at 12,000 × *g* for 15 min at 4 °C and transferred aqueous phase to new tube_____.

Precipitation

5. Precipitated w/ 250 μL of isopropanol and 250 μL of HSS at ____(temp.) for _____(time) and spun at 12,000 × *g* for 10 min at 4 °C.
   (a) w/: Pellet Paint™ _____

Purification

6. Resuspended pellet in ____ μL of lysis buffer A and ____ μL of 100% ethanol.
7. Added ____ μL to _____(manufacturer's name) RNA kit spin column.

8. Followed manufacturer's recommendations and incubated columns in 60 °C oven.
   (a) On-column DNase digestion_____(yes or no)
9. Eluted into _____ μL of DEPC-treated water.

DNA Extraction

Phase Separation

1. Added 500 μL of BEB and incubated on shaker table for _____(time).
2. Spun at 12,000 × *g* for 10 min at 4 °C and transferred aqueous phase to new tube_____.

Precipitation

3. Precipitated DNA w/ 60 μL of 3 M NA acetate and 600 μL of isopropanol at _____(temp.) for ____(time).
   (a) w/: 2 μL of Pellet Paint™_____(yes or no).
4. Spun at 12,000 × *g* for 10 min at 4 °C_____.

Purification

5. Resuspended pellet in a minimum volume of 100 μL of the first buffer of the preferred DNA clean-up kit (e.g., PCR-A buffer from Axygen's PCR clean-up kit)_____.
6. Carried out spins and washes as recommended by manufacturer_____.
7. After evaporating residual ethanol in 60 °C oven, eluted DNA into ___μL of pre-warmed (to 60 °C) "eluent" (must include Tris at a pH of at least 8 for best DNA elution).

Protein Extraction

8. Precipitated protein in 1.5 mL of acetone at _____(temp.) for _____(time).
9. Spun at 12,000 × *g* for 10 min at 4 °C____.
10. Washed pellet 3× w/ PWI and 1× w/ PWII at 8000 × *g* for 5 min at 4 °C, and dried on benchtop for _____ min.
11. Resuspended protein in _____μL of_____ buffer, sonicated for _____ min, boiled for ____ min at 100 °C, and transferred _____μL to each of ____tubes.
12. Quantified ____μL with the 2-D Quant kit, ____ μL with a Bradford assay, ___ μL with a BCA assay, or ___ μL with the Qubit protein assay kit.

Appendix Sheet 2 Printable sheets for RNA, DNA, and protein quantification and quality control analyses. A spectrophotometer

or, preferably, a bioanalyzer can be used to generate these (or comparable) data. Subscripts correspond to the technical replicate number. BSA = bovine serum albumin (the most common standard used in protein quantification assays). QC = quality control.

RNA									
Sample name	**$[RNA]_1$**	**$260/280_1$**	**$260/230_1$**	**$[RNA]_2$**	**$260/280_2$**	**$260/230_2$**	**$[RNA]_{avg}$**	**$260/280_{avg}$**	**$260/230_{avg}$**
DNA									
Sample name	**$[DNA]_1$**	**$260/280_1$**	**$260/230_1$**	**$[DNA]_2$**	**$260/280_2$**	**$260/230_2$**	**$[DNA]_{avg}$**	**$260/280_{avg}$**	**$260/230_{avg}$**

PROTEIN					
Sample	Absorbance @ ___ nM (depends on assay)	ng/assay volume	[protein] ng/µl	Total protein	Total protein-post QC
0 BSA					
10 BSA					
20 BSA					
30 BSA					
40 BSA					
50 BSA					
Average					

References

1. Schoenberg DA, Trench RK, Smith DC (1980) Genetic variation in *Symbiodinium* (=*Gymnodinium*) *microadriaticum* Freudenthal, and specificity in its symbiosis with marine invertebrates. II. Morphological variation in *Symbiodinium microadriaticum*. Proc R Soc London Ser B 207:429–444. https://doi.org/10.1098/rspb.1980.0032
2. Schoenberg DA, Trench RK, Smith DC (1980) Genetic variation in *Symbiodinium* (*Gymnodinium*) *microadriaticum* Freudenthal, and specificity in its symbiosis with marine invertebrates. I. Isoenzyme and soluble protein patterns of axenic cultures of *Symbiodinium microadriaticum*. Proc R Soc London Ser B 207:405–427. https://doi.org/10.1098/rspb.1980.0031
3. Mayfield AB, Chan PH, Putnam HM et al (2012) The effects of a variable temperature regime on the physiology of the reef-building coral *Seriatopora hystrix*: results from a laboratory-based reciprocal transplant. J Exp Biol 215:4183–4195. https://doi.org/10.1242/jeb.071688
4. Mayfield AB, Fan TY, Chen CS (2013) Real-time PCR-based gene expression analysis in the

model reef-building coral *Pocillopora damicornis*: insight from a salinity stress study. Platax 10:1–29, Taiwan

5. Mayfield AB, Chen CS, Liu PJ (2014) Decreased green fluorescent protein-like chromoprotein gene expression in specimens of the reef-building coral *Pocillopora damicornis* undergoing high temperature-induced bleaching. Platax 11:1–23, Taiwan
6. Mayfield AB, Chen YH, Dai CF, Chen CS (2014) The effects of temperature on gene expression in the Indo-Pacific reef-building coral *Seriatopora hystrix*: insight from aquarium studies in Southern Taiwan. Int J Marine Sci 4: 1–23. https://doi.org/10.5376/ijms.2014.04.0050
7. Mayfield AB, Hirst MB, Gates RD (2009) Gene expression normalization in a dual-compartment system: a real-time quantitative polymerase chain reaction protocol for symbiotic anthozoans. Mol Ecol Resour 9:462–470. https://doi.org/10.1111/j.1755-0998.2008.02349.x
8. Mayfield AB, Hsiao YY, Chen HK et al (2014) Rubisco expression in the dinoflagellate *Symbiodinium* sp. is influenced by both photoperiod and endosymbiotic lifestyle. Mar Biotechnol 16:371–384. https://doi.org/10.1007/s10126-014-9558-z
9. Chen WN, Hsiao YJ, Mayfield AB et al (2016) Transmission of a heterologous clade C *Symbiodinium* in a model anemone infection system via asexual reproduction. PeerJ 4:e2358. https://doi.org/10.7717/peerj.2358
10. Moya A, Tambutté S, Béranger G et al (2008) Cloning and use of a coral 36B4 gene to study the differential expression of coral genes between light and dark conditions. Mar Biotechnol 10:653–663. https://doi.org/10.1007/s10126-008-9101-1
11. Muscatine L, Cernichiari E (1969) Assimilation of photosynthetic products of zooxanthellae by a reef coral. Biol Bull 137:506–523. https://doi.org/10.2307/1540172
12. Mayfield AB, Dempsey AC, Chen C-S (2019) Modeling environmentally-mediated variation in reef coral physiology. J Sea Res 145:44–54. https://doi.org/10.1016/j.seares.2019.01.003
13. Mayfield AB, Tsai S, Lin C (2019) The Coral Hospital. Biopreserv Biobank 17:355–369. https://doi.org/10.1089/bio.2018.0137
14. LaJeunesse TC, Lambert G, Andersen RA et al (2005) *Symbiodinium* (Pyrrhophyta) genome sizes (DNA content) are smallest among dinoflagellates. J Phycol 41:880–886. https://doi.org/10.1111/j.0022-3646.2005.04231.x
15. Mayfield AB, Hsiao Y-Y, Fan T-Y et al (2010) Evaluating the temporal stability of stress-activated protein kinase and cytoskeleton gene expression in the Pacific reef corals *Pocillopora damicornis* and *Seriatopora hystrix*. J Exp Mar Biol Ecol 395:215–222. https://doi.org/10.1016/j.jembe.2010.09.007
16. Mayfield AB, Wang LH, Tang PC et al (2011) Assessing the impacts of experimentally elevated temperature on the biological composition and molecular chaperone gene expression of a reef coral. PLoS One 6:e26529. https://doi.org/10.1371/journal.pone.0026529
17. Mayfield AB, Wang YB, Chen CS et al (2014) Compartment-specific transcriptomics in a reef-building coral exposed to elevated temperatures. Mol Ecol 23:5816–5830. https://doi.org/10.1111/mec.12982
18. Mayfield AB, Chen CS, Dempsey AC (2017) Biomarker profiling in reef corals of Tonga's Ha'apai and Vava'u archipelagos. PLoS One 12:e0185857. https://doi.org/10.1371/journal.pone.0185857
19. Mayfield AB, Chen CS, Dempsey AC (2017) Identifying corals displaying aberrant behavior in Fiji's Lau Archipelago. PLoS One 12: e0177267. https://doi.org/10.1371/journal.pone.0177267
20. Mayfield AB, Chen CS, Dempsey AC (2017) The molecular ecophysiology of closely related pocilloporid corals of New Caledonia. Platax 14:1–45, Taiwan
21. Mayfield AB, Dempsey AC, Inamdar J, Chen CS (2018) A statistical platform for assessing coral health in an era of changing global climate-I: a case study from Fiji's Lau Archipelago. Platax 15:1–35, Taiwan
22. Mayfield AB (2016) Uncovering spatio-temporal and treatment-derived differences in the molecular physiology of a model coral-dinoflagellate mutualism with multivariate statistical approaches. J Marine Sci Eng 4. https://doi.org/10.3390/jmse4030063
23. Peng SE, Chen WN, Chen HK et al (2011) Lipid bodies in coral-dinoflagellate endosymbiosis: proteomic and ultrastructural studies. Proteomics 11:3540–3555. https://doi.org/10.1002/pmic.201000552
24. Mayfield A, Fan TY, Chen C-S (2013) Physiological acclimation to elevated temperature in a reef-building coral from an upwelling environment. Coral Reefs 32:909–921. https://doi.org/10.1007/s00338-013-1067-4
25. Mayfield AB, Hsiao YY, Fan TY, Chen CS (2012) Temporal variation in RNA/DNA and protein/DNA ratios in four anthozoan-dinoflagellate endosymbioses of the Indo-

Pacific: implications for molecular diagnostics. Platax 9:1–24, Taiwan

26. Mayfield AB, Gates RD (2007) Osmoregulation in anthozoan-dinoflagellate symbiosis. Comp Biochem Physiol A Mol Integr Physiol 147:1–10. https://doi.org/10.1016/j.cbpa.2006.12.042
27. Lin KL, Wang JT, Fang LS (2000) Participation of glycoproteins on zooxanthellal cell walls in the establishment of a symbiotic relationship with the sea anemone, *Aiptasia pulchella*. Zool Stud 39:172–178, Taiwan
28. Wang LH, Lee HH, Fang LS et al (2013) Fatty acid and phospholipid syntheses are prerequisites for the cell cycle of *Symbiodinium* and their endosymbiosis within sea anemones. PLoS One 8. https://doi.org/10.1371/journal.pone.0072486
29. Mayfield AB, Chen CS, Dempsey AC, Bruckner AW (2016) The molecular ecophysiology of closely related pocilloporids from the South Pacific: a case study from the Austral and Cook Islands. Platax 13:1–25, Taiwan
30. Wegley L, Edwards R, Rodriguez-Brito B et al (2007) Metagenomic analysis of the microbial community associated with the coral *Porites astreoides*. Environ Microbiol 9:2707–2719. https://doi.org/10.1111/j.1462-2920.2007.01383.x
31. Mayfield AB, Chen YJ, Lu CY, Chen CS (2016) Proteins responsive to variable temperature exposure in the reef-building coral Seriatopora hystrix. In: Ortiz S (ed) Coral reefs: ecosystems, environmental impact and current threats. New York, NOVA Publishing
32. Mayfield AB, Wang YB, Chen CS et al (2016) Dual-compartmental transcriptomic + proteomic analysis of a marine endosymbiosis exposed to environmental change. Mol Ecol 25:5944–5958. https://doi.org/10.1111/mec.13896
33. Mayfield AB, Chen YJ, Lu CY, Chen CS (2018) Exploring the environmental physiology of the Indo-Pacific reef coral *Seriatopora hystrix* using differential proteomics. Open J Marine Sci 8:223–252. https://doi.org/10.4236/ojms.2018.82012
34. Mayfield AB, Chen YJ, Lu CY et al (2018) The proteomic response of the reef coral *Pocillopora acuta* to experimentally elevated temperatures. PLoS One 13:e0192001. https://doi.org/10.1371/journal.pone.0192001
35. Podechard N, Ducheix S, Polizzi A et al (2018) Dual extraction of mRNA and lipids from a single biological sample. Sci Rep 8:7019. https://doi.org/10.1038/s41598-018-25332-9
36. Mayfield AB, Chen MN, Meng PJ et al (2013) The physiological response of the reef coral *Pocillopora damicornis* to elevated temperature: results from coral reef mesocosm experiments in southern Taiwan. Mar Environ Res 86:1–11. https://doi.org/10.1016/j.marenvres.2013.01.004
37. Chen WNU, Kang HJ, Weis VM et al (2012) Diel rhythmicity of lipid-body formation in a coral-*Symbiodinium* endosymbiosis. Coral Reefs 31:521–534. https://doi.org/10.1007/s00338-011-0868-6
38. Mayfield AB, Aguilar C, Kolodziej G et al (2021) Shotgun proteomic analysis of thermally challenged reef corals. Front Mar Sci 8. https://doi.org/10.3389/fmars.2021.660153
39. Musada GR, Dvoriantchikova G, Myer C et al (2020) The effect of extrinsic Wnt/β-catenin signaling in Muller glia on retinal ganglion cell neurite growth. Dev Neurobiol 80:98–110. https://doi.org/10.1002/dneu.22741
40. Mayfield AB (2022) Machine-learning-based proteomic predictive modeling with thermally-challenged Caribbean reef corals. Diversity 14. https://doi.org/10.3390/d14010033
41. Mayfield AB (2020) Proteomic signatures of corals from thermodynamic reefs. Microorganisms 8. https://doi.org/10.3390/microorganisms8081171
42. Reynolds DA, Yoo MJ, Dixson DL et al (2020) Exposure to the Florida red tide dinoflagellate, *Karenia brevis*, and its associated brevetoxins induces ecophysiological and proteomic alterations in *Porites astreoides*. PLoS One 15:e0228414. https://doi.org/10.1371/journal.pone.0228414
43. Andersson ER, Day RD, Loewenstein JM et al (2019) Evaluation of sample preparation methods for the analysis of reef-building corals using ^{1}H-NMR-based metabolomics. Meta 9. https://doi.org/10.3390/metabo9020032
44. Chen HK, Song SN, Wang LH et al (2015) A compartmental comparison of major lipid species in a coral-*Symbiodinium* endosymbiosis: evidence that the coral host regulates lipogenesis of its cytosolic lipid bodies. PLoS One 10. https://doi.org/10.1371/journal.pone.0132519
45. Mayfield AB, Dempsey AC (2022) Environmentally-driven variation in the physiology of a new Caledonian reef coral. Oceans 3:15–29. https://doi.org/10.3390/oceans3010002
46. Rubin ET, Enochs IC, Foord C et al (2021) Molecular mechanisms of coral persistence within highly urbanized locations in the Port of Miami, Florida. Front Mar Sci 8. https://doi.org/10.3389/fmars.2021.695236

47. McRae CJ, Mayfield AB, Huang W-B et al (2021) Contrasting proteomic responses of adult and larval coral to high temperatures. Front Mar Sci 8. https://doi.org/10.3389/fmars.2021.716124
48. Tisthammer KH, Timmins-Schiffman E, Seneca FO et al (2021) Physiological and molecular responses of lobe coral indicate nearshore adaptations to anthropogenic stressors. Sci Rep 11:3423. https://doi.org/10.1038/s41598-021-82569-7
49. Sproles AE, Oakley CA, Matthews JL et al (2019) Proteomics quantifies protein expression changes in a model cnidarian colonised by a thermally tolerant but suboptimal symbiont. ISME J 13:2334–2345. https://doi.org/10.1038/s41396-019-0437-5
50. Rocker MM, Kenkel CD, Francis DS et al (2019) Plasticity in gene expression and fatty acid profiles of *Acropora tenuis* reciprocally transplanted between two water quality regimes in the central Great Barrier Reef, Australia. J Exp Mar Biol Ecol 511:40–53. https://doi.org/10.1016/j.jembe.2018.11.004
51. Dimos BA, Mahmud SA, Fuess LE et al (2019) Uncovering a mitochondrial unfolded protein response in corals and its role in adapting to a changing world. Proc R Soc London Ser B 286:20190470. https://doi.org/10.1098/rspb.2019.0470
52. Lohr KE, Khattri RB, Guingab-Cagmat J et al (2019) Metabolomic profiles differ among unique genotypes of a threatened Caribbean coral. Sci Rep 9. https://doi.org/10.1038/s41598-019-42434-0
53. Wright RM, Correa AMS, Quigley LA et al (2019) Gene expression of endangered coral (*Orbicella* spp.) in Flower Garden Banks National Marine Sanctuary after Hurricane Harvey. Front Mar Sci 6. https://doi.org/10.3389/fmars.2019.00672
54. Seveso D, Montano S, Reggente MA et al (2017) The cellular stress response of the scleractinian coral *Goniopora columna* during the progression of the black band disease. Cell Stress Chaperones 22:225–236. https://doi.org/10.1007/s12192-016-0756-7
55. Chen HK, Wang LH, Chen WU et al (2017) Coral lipid bodies as the relay center interconnecting diel-dependent lipidomic changes in different cellular compartments. Sci Rep 7:3244. https://doi.org/10.1038/s41598-017-02722-z
56. Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37:911–917. https://doi.org/10.1139/o59-099
57. Ricaurte M, Schizas NV, Ciborowski P et al (2016) Proteomic analysis of bleached and unbleached *Acropora palmata*, a threatened coral species of the Caribbean. Mar Pollut Bull 107:224–232. https://doi.org/10.1016/j.marpolbul.2016.03.068
58. Vidal-Dupiol J, Zoccola D, Tambutté E et al (2013) Genes related to ion-transport and energy production are upregulated in response to CO2-driven pH decrease in corals: new insights from transcriptome analysis. PLoS One 8:e58652. https://doi.org/10.1371/journal.pone.0058652
59. Barshis DJ, Ladner JT, Oliver TA et al (2013) Genomic basis for coral resilience to climate change. Proc Natl Acad Sci 110:1387–1392. https://doi.org/10.1073/pnas.1210224110
60. Putnam H, Mayfield AB, Fan TY et al (2012) The physiological and molecular responses of larvae from the reef-building coral *Pocillopora damicornis* exposed to near-future increases in temperature and *p*CO2. Mar Biol 160:2157–2173. https://doi.org/10.1007/s00227-012-2129-9
61. Kenkel CD, Aglyamova G, Alamaru A et al (2011) Development of gene expression markers of acute heat-light stress in reef-building corals of the genus Porites. PLoS One 6. https://doi.org/10.1371/journal.pone.0026914