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TITLE:

Fluorescence-Activated Cell Sorting for the Isolation of Scleractinian Cell Populations

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SUMMARY:

Corals create biodiverse ecosystems important for both humans and marine organisms. However, we still do not understand the full potential and function of many coral cells. Here, we present a protocol developed for the isolation, labeling, and separation of stony coral cell populations.

ABSTRACT:

Coral reefs are under threat due to anthropogenic stressors. The biological response of coral to these stressors may occur at a cellular level, but the mechanisms are not well understood. To investigate coral response to stressors, we need tools for analyzing cellular responses. In particular, we need tools that facilitate the application of functional assays to better understand how cell populations are reacting to stress. In the current study, we use fluorescence-activated cell sorting (FACS) to isolate and separate different cell populations in stony corals. This protocol includes: (1) the separation of coral tissues from the skeleton, (2) creation of a single cell suspension, (3) labeling the coral cells using various markers for flow cytometry, and (4) gating and cell sorting strategies. This method will enable researchers to work on corals at the cellular level for analysis, functional assays, and gene expression studies of different cell populations.

INTRODUCTION:

Coral reefs are one of the most important ecosystems on Earth. They facilitate biodiversity by providing critical habitats for fish and invertebrates and are crucial for sustaining anthropogenic communities by providing food and economic livelihood through tourism[1]. As the key builder of coral reefs, the coral animal (Phylum: Cnidaria) also aids coastal communities by creating large calcium carbonate frameworks that mitigate wave and storm damage[2].

Corals as adults are sessile animals that host a wide array of endosymbiotic partners, including viruses, archaea, bacteria, protists, fungi, and most notably, members of the algal dinoflagellate family Symbiodiniaceae[3]. Changes in the environment can cause imbalances in this community, often leading to disease outbreaks and coral bleaching in which the symbiotic Symbiodiniaceae are expelled from the coral colony, thus eliminating the major source of nutrition for the coral. Both of these scenarios often cause death of the coral host[4-6]. Effects of anthropogenic-induced stressors, such as rapid climate change, are accelerating mass coral death events, leading to a global decline of coral reefs[7].

Recently, many different methods have been developed to help mitigate coral reef loss. These methods include outplanting of corals on existing reefs, genetic crossing using thermally tolerant genotypes, and cellular manipulation of the microbial and symbiotic communities hosted within the coral[8,9]. Despite these efforts, much remains unknown about coral cell diversity and cell function[10-13]. A thorough understanding of coral cell type diversity and cell function is necessary to understand how the coral organism behaves under normative and stressful conditions. Efforts to maximize restoration and preservation efficiency will benefit from an enhanced understanding of how cell diversity and gene function are coupled.

Previous work on cell diversity and function has primarily focused on histological studies and whole-tissue RNA sampling[14-17]. To obtain greater detail on specific cell type function in corals, there need to be methods for the isolation of specific populations of live coral cells. This has been done successfully in nonclassical model organisms by means of fluorescence-activated cell sorting (FACS) flow cytometry technology[18]. FACS utilizes a combination of lasers tuned to varying wavelengths to measure different endogenous cellular properties at the single cell level such as relative cell size, cell granularity, and autofluorescence. Additionally, the cells may be marked by fluorescently labeled compounds to measure specific, desired properties[18,19].

Thus far, the application of flow cytometry to coral cells has mainly been for the analysis of symbiotic Symbiodiniaceae and other bacterial populations by utilizing their strong, natural autofluorescence[20-22]. FACS has also been used to estimate coral genome size by using fluorescent DNA marker signal compared against reference model organism cells[23,24]. The efficient application of FACS provides three distinct tools that are useful for cell biology studies: 1) morphological and functional description of single cells; 2) identification, separation, and isolation of specific cell populations for downstream studies; and 3) the analysis of functional assays at the single cell level.

The development and application of various exogenous fluorescent markers for the study of coral cells remains almost unexplored. Such markers may include tagged proteins, tagged substrates for enzymes, or fluorescent responses to other compounds. These markers can be used to identify cell types that have unique properties, such as highlighting cells that produce varying amounts of a specific cellular compartment feature, like lysosomes. An additional example is the use of fluorescently labeled beads to functionally identify cells competent for phagocytosis, or the engulfment of a targeted pathogen[25]. Populations of cells active in immunity responses can be easily identified by FACS after engulfment of these exogenously applied beads. While traditional histological methods require preserved tissue and many hours to approximate the percentage of cells positive for bead engulfment, a FACS-based functional assay for pathogen engulfment can be performed relatively quickly on isolated live cells. In addition to studying cell-specific responses to stress, this technology has the potential to clarify gene-specific expression and illuminate the evolutionary and developmental history of cell types entirely unique to cnidarians, such as calicoblasts and cnidocytes.

Recently, we performed an intensive screening of over 30 cellular markers that resulted in identifying 24 that are capable of labeling coral cells, 16 of which are useful for distinguishing unique populations[18], making them clusters of differentiation (CD). Here we describe the process of coral cell isolation in *Pocillopora damicornis* from removing cells from the calcium carbonate skeleton to the identification and isolation of specific cell populations with FACS (**Figure 1**).

PROTOCOL:

1. Dissociation of tissues from coral skeleton via airbrush and compressor

NOTE: Perform steps on ice and protect hands with gloves.

1.1. Assemble the airbrush kit by connecting the air compressor, hose, and airbrush (**Figure 2**). Set the pressure gauge between 276−483 kPa.

NOTE: The recommended compressor and air hose used for this study was preset to a maximum pressure of 393 kPa. Use outside of this 276–483 kPa range may result in either inadequate cell removal from the skeleton or rupture of the cell membranes. This range may differ for each species and may require a viability test. A viability test may be performed with a subset of the cell slurry with a simple hemocytometer and a 4’,6-diamidino-2-phenylindole (DAPI) dye exclusion assay, visualized on a compound microscope.

1.2. Prepare 100 mL of cell staining media in an autoclaved, sterile glass container by adding 33 mL of 10x phosphate-buffered saline (PBS; without calcium and magnesium) to a final concentration of 3.3x, 2 mL of fetal calf serum (FCS; heat inactivated at 56 °C for 30 min) to 2% of total volume, and 2 mL of 1 M HEPES buffer to a final concentration of 20 mM. Then top off at 100 mL with sterile water.

NOTE: The 3.3x PBS concentration was chosen to mimic the salinity of seawater, as demonstrated

in Rosental et al.[18]. This concentration needs to be adjusted for species found in other environments to mimic their salinity.

1.3. Transfer 10 mL of cell staining media to an airbrush bottle (labeled "F" in **Figure 2**) and attach the adaptor lid for the airbrush connector.

NOTE: Larger fragments and species with thicker tissue layers may require more media for adequate tissue removal.

1.4. For the branching coral species demonstrated here, use a bone cutter to clip or cut a coral fragment approximately 3–5 cm in length or 4 cm^2 in area.

NOTE: The fragment must be clear of macroalgae and other multicellular organisms because these may not be removed from the sample downstream and will contaminate the sample during the FACS analysis and sorting processes. A 1 cm fragment of *P. damicornis* gives approximately 2−10 x 10^6 cells after filtering, staining, and washing.

1.5. Place the coral fragment inside a plastic collection bag and use the airbrush to spray the cell staining media onto the coral (**Figure 2**). Continue this process until most of the skeleton is exposed. Remove the remaining skeleton from the collection bag.

2. Dissociation of cells from coral tissue

NOTE: Perform all steps on ice and protect hands with gloves.

2.1. Filter the cell slurry through a 40 µm nylon cell strainer into a 50 mL centrifuge tube in order to obtain a single cell suspension.

NOTE: Different cell strainer mesh sizes may be more appropriate for different species. However, larger sizes may result in inadequate tissue dissociation due to the passage of debris and cell clumps.

2.1.1. For specimens with thicker mucus layers, use the sterilized plunger of a 1 mL plastic syringe and gently grind it against the filter to break up clumps and help the cells pass through the strainer. Rinse the strainer and plunger with cell staining media into the centrifuge tube.

2.2. Pellet the cells by centrifugation at 4 °C at 450 x *g* for 5 min. Remove the supernatant and resuspend the cells in 1 mL of cell staining media.

3. Cell staining

NOTE: Perform all steps on ice and protect hands with gloves. Stains featured in this protocol are for representation purposes. Alternative stains will require different concentrations and incubation times.

3.1. Transfer 500 µL of resuspended cells to a 5 mL round-bottom tube and set aside as a control sample. Do not add stain to this aliquot.

3.2. To the remaining cell suspension, add 0.42 µL of 12 mM DAPI viability dye (**Table of Materials**), 1 µL of 5 µM reactive oxygen species (ROS) stain (**Table of Materials**), and 0.1 µL of 0.2 µM lysosome stain (**Table of Materials**). Pipette well to mix and incubate on ice for 30 min in the dark to prevent photobleaching.

NOTE: DAPI is used in this example to work as a DNA stain and viability dye for the differential gating of dead cells on the FACS machine. This assumes that DAPI penetrates dying cells due to membrane integrity. The ROS stain emits light in the 520 nm range (green), while the lysosomal marker emits light at approximately 668 nm (red).

3.3. Pellet 500 µL of the stained cells by centrifugation at 4 °C at 450 x *g* for 5 min. Remove the supernatant and resuspend the pellet in 500 µL of cell staining media. Transfer to a new 5 mL round-bottom tube and store on ice.

4. FACS startup

NOTE: The steps may vary according to the make and model of the cytometer due to differences in the lasers and channels. For this protocol, a cytometer with 405, 488, 535, and 640 nm wavelength lasers was used. Filters featured in this protocol are for representation purposes. Alternative cell stains may require a different set of filters and lasers.

4.1. Begin creating a new project template on the sorter software and choose the laser panel. For the represented combination of stains and natural fluorescence, select all four lasers (405, 488, 535, and 640 nm).

4.2. Select the appropriate filters for each expected color represented in the experiment.

4.2.1. To detect the DAPI emission and aid in the separation of live and dead cells, use a filter with a bandpass (BP) of 450/50 (425−475 nm range) such as a DAPI, Hoeschst, or Pacific Blue filter.

4.2.2. For the detection of light emitted by the green ROS signal, use a filter with a BP of 530/30 (wavelength range of 515–545 nm) such as a fluorescein isothiocyanate (FITC) or green fluorescent protein (GFP) filter. This filter will measure the concentration of ROS in each cell.

4.2.3. Add an additional filter with a BP of 670/30 (655−685 nm range) such as an allophycocyanin (APC) filter for the detection of the lysosomal stain emission.

NOTE: The APC channel will allow for the measurement of phagocytic activity. This channel will also detect the autofluorescence generated by the coral symbiotic algae from the family

Symbiodiniaceae. This signal can be separated using an additional channel that has a longer wavelength than the APC filter, however.

4.2.4. Include a filter that has a BP of 780/60 (750–810 nm range), such as the most commonly used phycoerythrin, cyanine filter (APC-Cy7), which detects the emission of far-red autofluorescence from Symbiodiniaceae and aids in the isolation of aposymbiotic cells.

5. FACS gating setup

NOTE: Steps may vary according to make and model of the cytometer and the acquisition program coupled with the cytometer.

5.1. On the project experimental screen of the cytometry software, create a scatter plot and select forward scatter (FCS) as the metric for the X-axis and side scatter (SSC) for the Y-axis.

NOTE: FCS correlates with the size of the cell and SSC correlates with granularity. This will allow for the removal of most debris, as most will be smaller in size than intact cells.

5.2. Set the axes to either logarithmic or biexponential scales, as cell sizes and granularity may vary by several orders of magnitude (**Figure 3A**), particularly in corals.

6. FACS analysis and cell isolation

NOTE: Steps may vary according to the make and model of the cytometer and the coupled acquisition program.

6.1. Place the control (i.e., the unstained cell subset) in the sample chamber of the cytometer and start the reading process. When the computer starts to receive data from each cell, or event, dots will begin to appear on the scatter plot. To clear any debris left in the cytometer chambers from previous experiments, allow approximately 30 s to pass before starting the analysis.

6.2. In the scatter plot, adjust the photomultiplier tube (PMT) voltage in order to center the points.

NOTE: The PMT voltage is used to amplify the signal strength of photons being measured; if the PMT voltage is too weak, fewer cells will be read, and if the PMT voltage is too strong, cells will be measured near maximum value and different cell types will be indistinguishable from one another. An adequate PMT voltage ensures that independently distinguishable events will populate the scatter plot. Event separation is easier to visualize by projecting logarithmic or biexponential scales on FSC and SSC scatter plots.

6.3. Start recording the events. To conserve the sample, pause data acquisition after approximately 15,000 events have been read. In most cases, this will be adequate to visualize the overall cell population patterns. Record more events if analyzing and isolating small, specialized

cell populations.

6.4. On the first graph of FSC and SSC, create a selection, or gate, of the cells around the 10^2 mark and higher on the FSC X-axis. Anything below this threshold is likely cellular debris. Draw rectangular gates, which is a regular option on flow cytometry software programs and good for clear, distinct cell populations. For cell populations that take a more irregular shape on the scatter plots, use a polygonal gating option.

NOTE: A minimum cutoff of 10^2 on the forward scatter should select for the smallest coral cells but may allow contamination of debris. To amend this, increase the lower limit of the gating to be more conservative.

6.5. Create a new scatter plot expressing the DAPI filter on the X-axis and the APC-Cy7 filter on the Y-axis. To do this, select the gate for intact cells created in step 6.4, right-click, and select the option to create a new scatter plot. Adjust the axes to either logarithmic or biexponential scales.

NOTE: The steps needed to create a new plot may vary with different software programs.

6.6. Now remove the control sample from the cytometer chamber and replace with the stained cells. Allow 30 s to pass before starting the analysis and recording the data. Record approximately 15,000 cells before pausing.

NOTE: The distinct population(s) on the higher end of the APC-Cy7 scale are those with high red autofluorescence from Symbiodiniaceae. No stain is needed to visualize these population breaks, and they can also be viewed on the control sample recording (**Figure 3C**). Select for the coral-only populations, those lower on the Y-axis, for further differentiation.

6.7. Create a new scatter plot of the aposymbiotic cells to visualize the ROS concentrations (FITC filter) and the cells positive to lysosomes (APC filter), again utilizing logarithmic or biexponential scales.

NOTE: If there are multiple distinct cell populations that are aposymbiotic, each one can be further distinguished in its own scatter plot (**Figure 3C**,**D**).

6.8. Compare the stained sample group against the control, unstained subset by either using the first recording of the control sample or reinserting and rerunning the control group. Gate the events that are unique to the stained sample group.

7. FACS sorting and collection

NOTE: Steps may vary according to make and model of the cytometer and the acquisition program coupled with the cytometer.

7.1. With a selection of cells chosen to collect, place microcentrifuge tubes (containing 250–500

µL of cell culture media or lysis buffer based on number of cells collected and method of storage) in the cytometer collection chamber.

NOTE: The represented flow cytometer is capable of sorting four different populations at a time, and the machine can be configured to hold a variety of collection devices, including various centrifuge tubes and 96 well plates.

7.2. Once the cytometer starts to read the sample and an appropriate amount of time has passed to flush out debris, start sorting the desired populations and collect anywhere from 20,000 cells to several millions.

7.2.1. For more than 500,000 cells, adjust the volume of cell culture media or lysis buffer, and the volume of the collection device.

7.2.2. For in vitro studies, store cells on ice until placed in an incubator or sterilized environment.

7.2.3. For molecular studies, either immediately start the DNA/RNA/protein isolation process or flash freeze and store at -80 °C until extraction.

7.3. Each time a new stain, species, or sorter is used, perform a purity check on the population of interest by sorting at least 20,000 cells of interest into 500 µL of staining media, then re-analyzing the sorted cells and confirming that the cells are being read within the gate used to initially sort (**Figure 4**).

REPRESENTATIVE RESULTS:

Overall, this protocol is useful because it facilitates the identification and collection of live coral cell populations that can be used for functional analyses. The workflow started with the mechanical separation of coral tissues from the underlying calcium carbonate skeleton (**Figure 1**). This is one of the most important initial steps because improper technique results in high cell mortality and can create large amounts of debris. Enzymatic separation is not advised because it can result in increased tissue shearing and high cell mortality. Mechanical separation using an airbrush reduces these challenges (**Figure 2**), reducing average cell mortality to ~10% (data not shown). Following airbrush-mediated mechanical separation of tissues from the skeleton, the resulting slurry was filtered to a cell suspension by filtration through a cell strainer. The cell suspension was then stained with DNA (DAPI), ROS, and lysosome markers and read by the FACS cytometer. A gating strategy was then developed for isolating specific cell populations based on the cell markers being used. Gated cell populations were then sorted into collection tubes (**Figure 1**).

When analyzing samples on the FACS cytometer, several steps were taken in order to remove debris and dead cells from the sample. Symbiodiniaceae cells could be selectively removed if desired due to their autofluorescence. First, debris and other noncellular particles that did not share the same shape or granularity as coral cells were removed from analyses by creating a gating selection on the forward (size) and side (granularity) scatter (**Figure 3A**). Next, dead cells

were excluded by comparing the unstained control cell suspension against DAPI-stained sample and far-red channel, then gating out the positive cells for high DAPI staining in the stained sample (**Figure 3B**, cell population P6). In parallel, cells hosting autofluorescent Symbiodiniaceae were removed by gating against cells with a high signal in the far-red channel (**Figure 3B**, APC-Cy7). After this iterative gating process, the remaining cells for analyses represent the intact live coral cell populations lacking Symbiodiniaceae.

These cells could then be classified into different cell subpopulations by comparing the control and stained samples by adding exogenous stains for features such as ROS activity and/or lysosome content (**Figure 3C**). For example, examining the data on the ROS- and lysosome-staining filters revealed four distinct subpopulations of coral cells. One cell population had a relatively low signal for lysosomal content (APC filter) and the other three had high signal in the lysosomal content and a range of ROS activity (FITC filter) (**Figure 3C**). Each of these subpopulations can be individually sorted for downstream analysis, or further analyzed and parsed into more discrete subpopulations by the intersection of size, granularity, autofluorescence, and/or DNA content. In this study, DNA, ROS activity, and lysosomal content were used to identify broad characteristics of coral cells. Importantly, this general protocol can be adapted to a range of fluorescent markers as well as specific antibody probes that can be used in conjunction for the isolation of specialized cell populations of interest such as stem cells.

FIGURE LEGENDS:

Figure 1: The general workflow of the coral cell sorting process. The featured protocol enables the removal of corals cells from the skeleton for staining, which are then run through the FACS cytometer, measured, analyzed, and isolated into cell populations.

Figure 2: Removal of tissue and cells from the coral skeleton. The air compressor (**A**) forces air and cell staining media (**F**) out of the airbrush (**E**) and works to blast the tissue off the coral skeleton (**C**) and creates a cell slurry in the bag (**D**). The maximum air pressure is controlled by the pressure gauge (**B**) on the air compressor (**A**).

Figure 3: Gating selection of live coral cells. (**A**) Live cells were isolated from debris using a minimum forward scatter value of 10^2. (**B**) The APC-Cy7 filter was used to identify red autofluorescence, effectively separating out the symbiont-hosted cells, while DAPI was used to identify cells highly positive for DNA staining. Those highly positive for DAPI were indicative of dead and dying cells. (**C**,**D**) Additional markers for ROS activity and lysosome content were used to further differentiate different coral cell populations. All data was later reanalyzed on FlowJo (v10) to generate these graphs. The inserted population label and percent corresponds to the microscopic photos taken of each sorted population (**E**). The percentages shown in each plot are of the total cells within that plot. Bottom right scale bar = 20 µm.

Figure 4: Purity check of the autofluorescent Symbiodiniaceae-associated cells. (**A**) Cells positive for fluorescence on the APC-Cy7 filter from the unstained control sample were sorted. (**B**) This sorted group of cells was then rerun on the cytometer and reanalyzed under the same conditions, showing 94% purity. Forward scatter (X-axis, FSC) was used for the anchoring axis,

but is interchangeable with any of the other parameters. The same process can be performed on stained samples.

DISCUSSION:

This protocol was adapted from Rosental et al.[18] and developed for the identification and isolation of *P. damicornis* cells. The methodology focuses on the process of filtering samples to remove debris, nonviable cells, and Symbiodiniaceae-hosted cells through the examination of cell intrinsic factors, including relative cell size, relative cell granularity, cell autofluorescence, and the presence of intact cellular membranes. These techniques can be applied to other coral species. However, FACS gating strategies may vary due to species-specific differences in cell population characteristics.

The utility of FACS for understanding coral health lies in its ability to use diverse cell intrinsic factors to differentially identify and isolate cells. Further, this protocol describes the additional use of exogenous cellular staining to differentiate coral cells based on viability, reactive oxygen species concentration, and lysosome content. Of the 30+ commercial markers that were formerly tested in Rosental et al.[18], 24 labelled *P. damicornis* cells, of which 16 produced differential signal, clusters of differentiation that would allow for the selection of cell subpopulations.

One of the critical steps of this protocol is proper tissue removal and filtering to ensure that tissue clumps are dissociated and do not block the laminar flow of the flow cytometer. Proper adjustment of the PMT voltage is crucial for the accurate detection and analysis of independent cell detection events. Compared to previous work[18], the process of coral cell isolation from the underlying skeleton via airbrush mechanical disruption significantly improves upon traditional scraping methods by reducing debris and maximizing cell viability (~90%, **Figure 2** and **Figure 3A**), thereby improving subsequent analyses by reducing false positives due to noise.

The methodology described in this paper facilitates the separation of coral cell populations through differential selection on an intersection of morphological and functional characteristics at a level not previously possible. With the development of FACS methodologies for coral cells, it is now possible to select specific cell subpopulations for the creation of defined cell cultures, as well as for probing transcriptomic and epigenetic profiles associated with specific cell subpopulations. The application of this fine-scale information will provide insight into cell type, behavior, and function, and may reveal characteristics associated with the evolution of cnidarian-specific cell types such as calicoblasts and cnidocytes. Additionally, the application of FACS technology to the development of improved stress assays and biomarkers may prove useful for the protection of severely threatened coral reefs.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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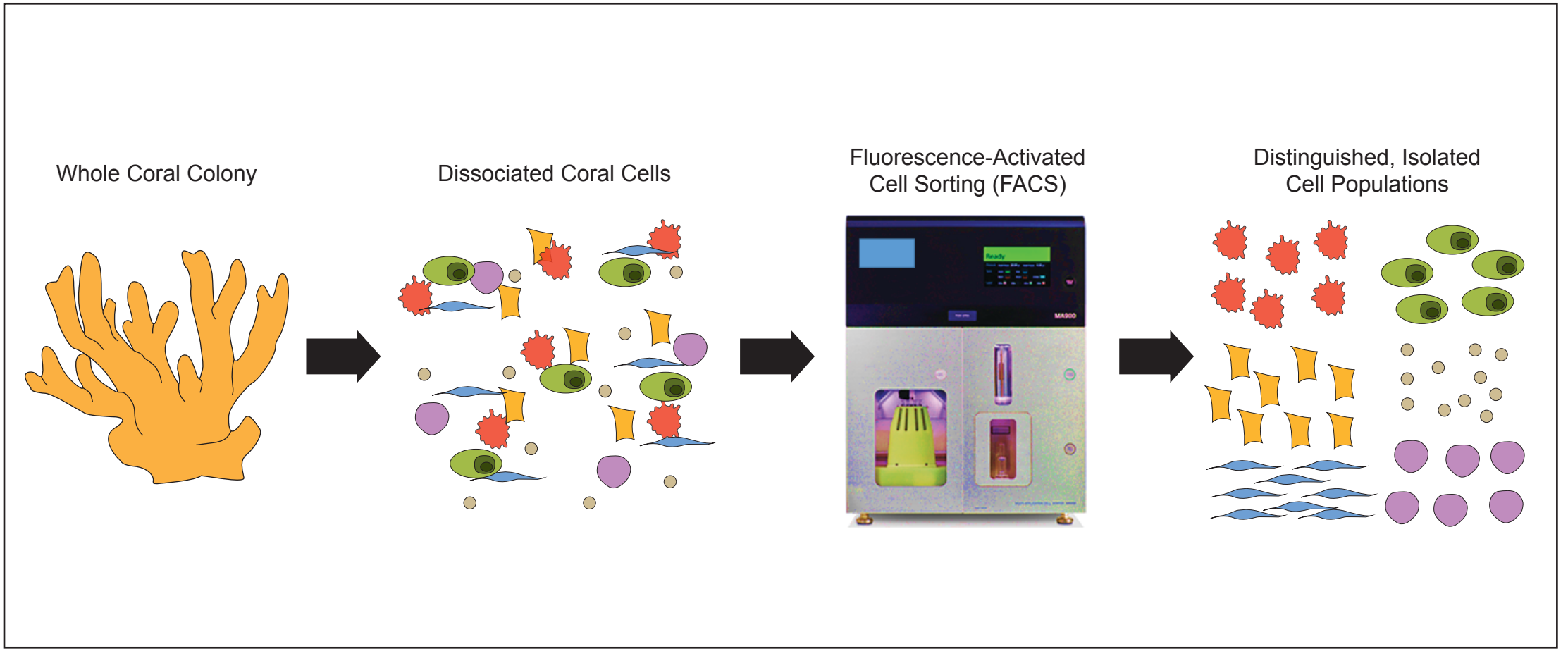


Figure 2

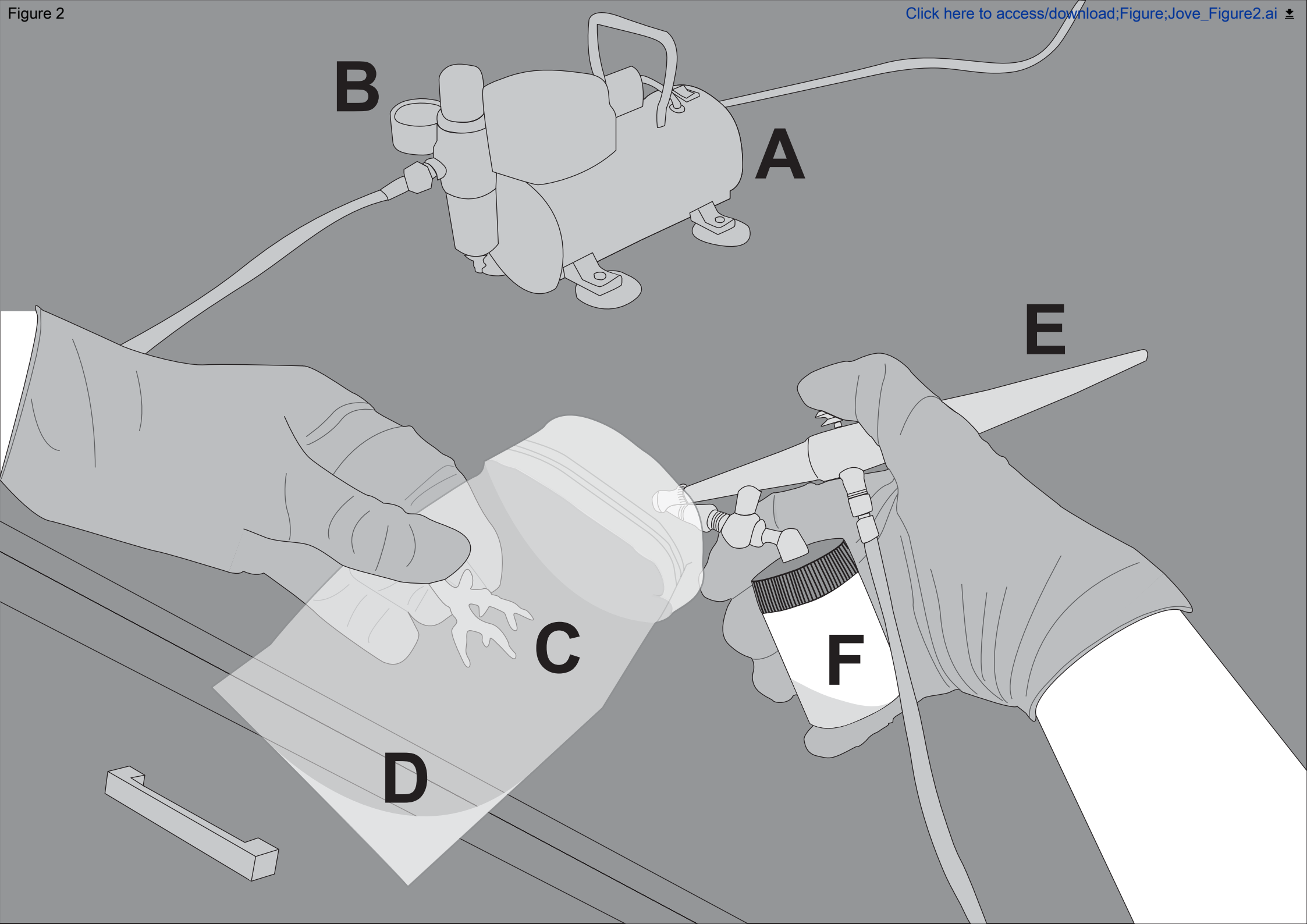


Figure 3

Click here to access/download;Figure;JoveNewFig3.ai

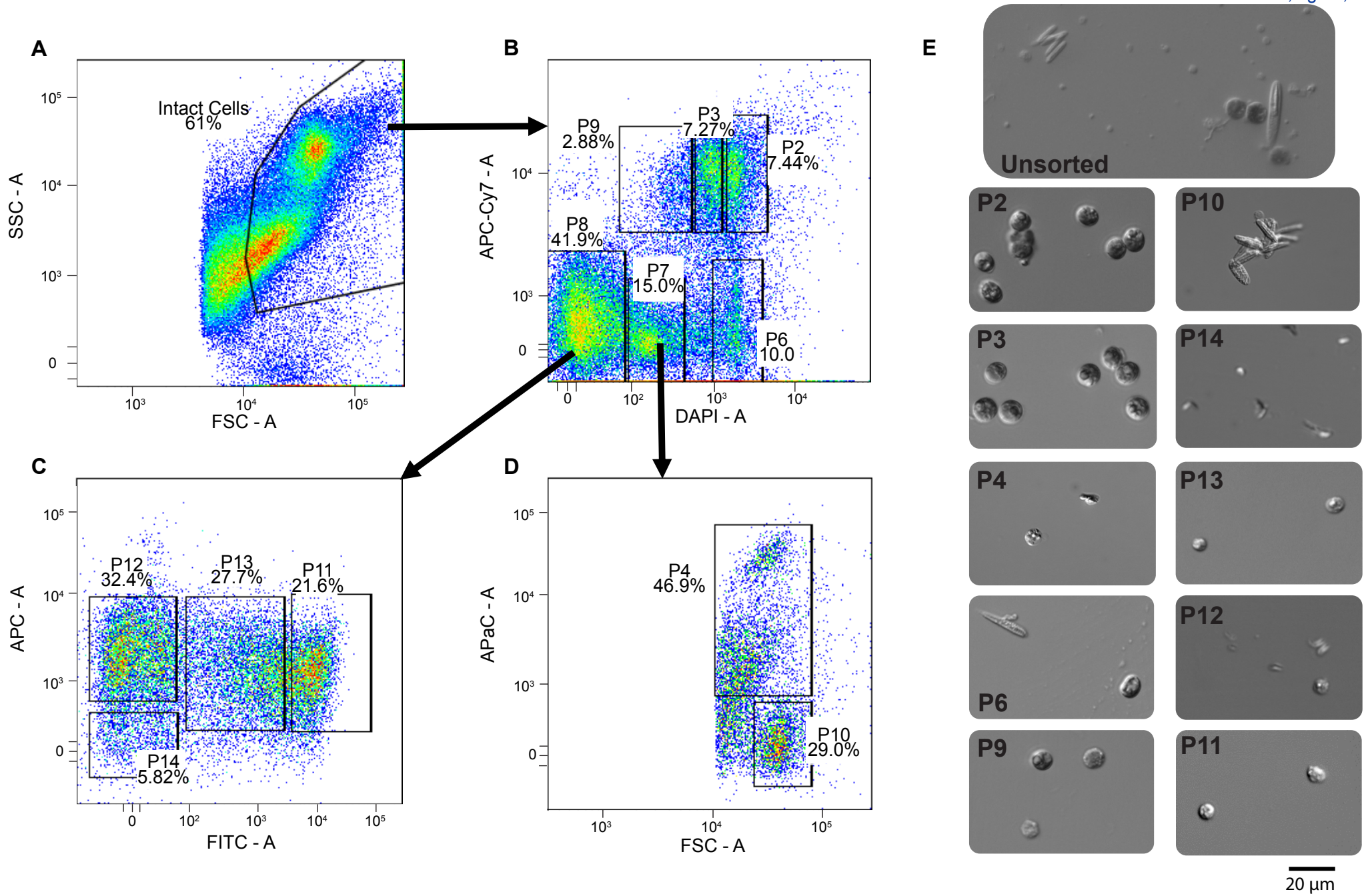


Figure 4

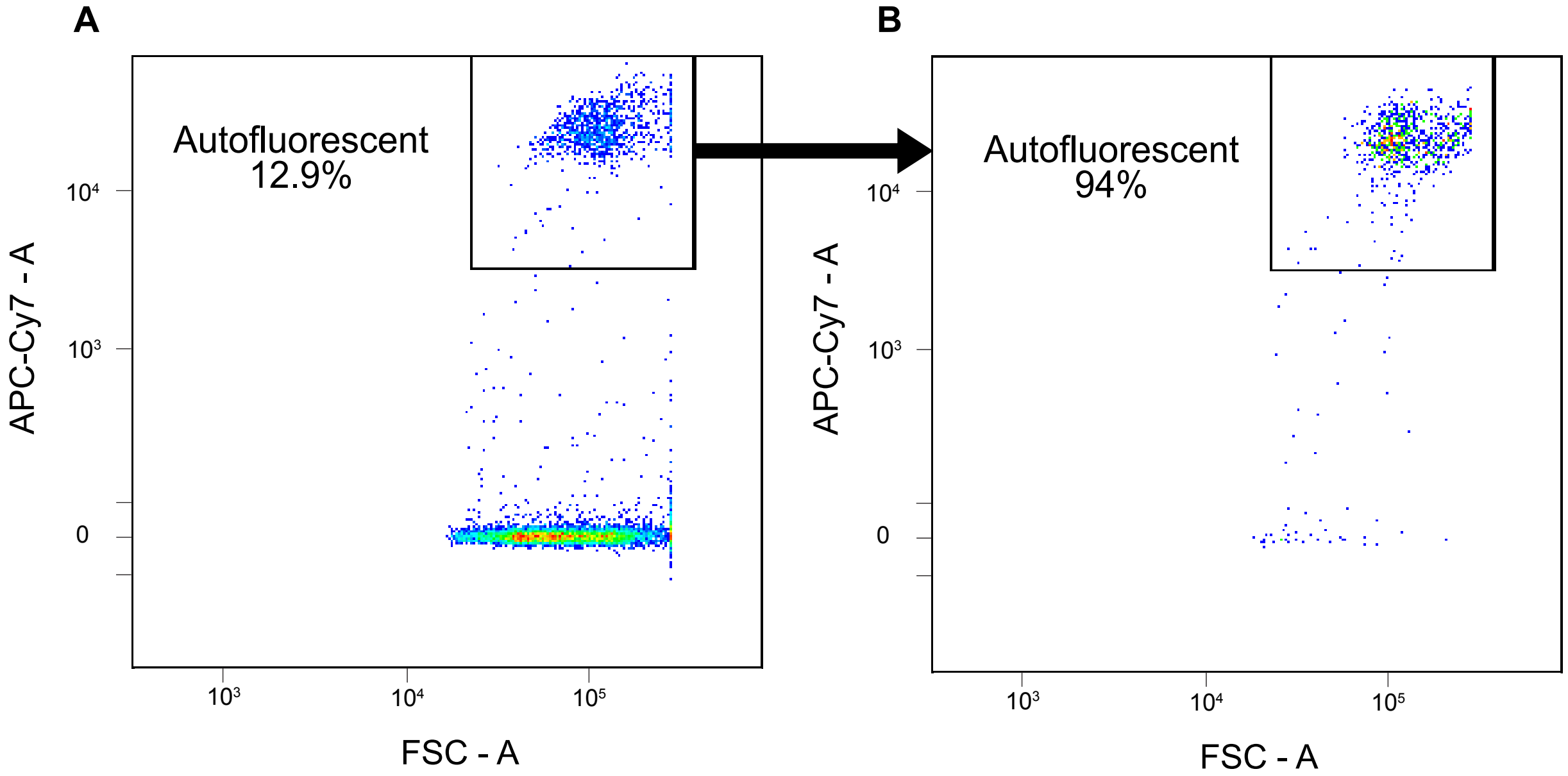


Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials.xlsx

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Airbrush Kit & Compressor	TCP Global	ABD KIT-H-SET	Paasche H Series Single-Action Siphon Feed Airbrush Kit with
BD FACSAria II	BD	644832	
Bone Cutters	Bulk Reef Supply	205357	Oceans Wonders Coral Stony Bone Cutter
Cell Strainer	Corning	352340	40 um; BD Falcon; individually wrapped; sterile; nylon
CellRox Green	Life Technologies	C10444	2.5 mM in DMSO; Excitation/Emission: 485/520 nm
Collection bag	Grainger	38UV35	Reloc Zippit 6"L x 4"W Standard Reclosable Poly Bag with Zip
DAPI	Invitrogen	D1306	10mg in H2O; Excitation/Emission: 358/461 nm
Fetal Calf Serum	Sigma-Aldrich	F2442-100ML	Heat-inactivated at 57 °C for 30 minutes
Hemacytometer	Sigma-Aldrich	Z359629	Bright-Line Hemacytometer
HEPES Buffer	Sigma-Aldrich	H0887	
LysoTracker Deep Red	Life Technologies	L12492	1mM in DMSO; Absorption/Emission: 647/668 nm
Microcentrifuge tubes	VWR	87003-294	1.7 mL
Phophate Buffered Saline (PBS)	Gibco	70011-044	pH 7.4; 10X
Round-bottom tubes	VWR	352063	5 mL Polypropylene Round-Bottom Tube
Syringe	BD	309628	1 mL BD Luer-Lok Syringe sterile, singe use polycarbonate

ı Master TC-20 Compressor & Air Hose

ɔ Seal Closure, Clear; 2 mil Thickness

Manuscript, JoVE60446,

Point by point address of the editorial and peer review comments.

To JoVE editorial board,

Thank you for considering our manuscript “Fluorescence-activated cell sorting techniques for the isolation of scleractinian cell populations”.

We thank the reviewers for their detailed suggestions and concerns and feel that the manuscript has become stronger and more complete due to their efforts.

Specifically, we have revised the text to address the comments, we have imaged the sorted cell populations (Figure 3), and we have done sorting purity analysis (Figure 4).

Please find enclosed our point-by-point response to the reviewers’ and editorial comments.

Sincerely on behalf of the authors,

Grace Snyder, William Browne, Nikki Traylor-Knowles, and Benyamin Rosental.

Point-by-point response to comments (Our responses are in Blue)

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

DONE - Thank you for your comment. We have reviewed and addressed this comment.

2. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc. Please use the micro symbol µ instead of u and abbreviate liters to L (L, mL, µL) to avoid confusion.

DONE - Thank you for your comment. We have reviewed and addressed this comment.

3. Please include a space between all numbers and the corresponding unit: 15 mL, 5 g, 7 cm, 37 °C, 60 s, 24 h, etc.

DONE - Thank you for your comment. We have reviewed and addressed this comment.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. You may use the generic term followed by "(Table of Materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: CellRox Green, LysoTracker, Sony, etc.

DONE - Thank you for your comment. We have reviewed and addressed this comment. All commercial products including reagent and cytometry brands have been specified only in the Table of Materials.

5. Please revise the Protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "NOTE." Please include all safety procedures and use of hoods, etc.

DONE - Thank you for your comment. We have reviewed and addressed this comment throughout the document.

6. 2.1.1: What volume of cell staining media is used to rinse?

DONE - Thank you for your comment. We have reviewed and addressed this comment; we stated the volume before and after centrifugation in Protocol step 3.3.

LINE 193: 3.3) Pellet 500 µL of stained cells by centrifugation at 4 ° C at 450 x g for 5 minutes. Remove the supernatant and resuspend in 500 µuL of cell staining media. Transfer to a new 5 mL round-bottom tube and store on ice.

7. Sections 4-6: For actions involving software usage, please provide all specific details (e.g., button clicks, software commands, any user inputs, etc.) needed to execute the actions.

DONE - Thank you for your comment. We have reviewed and addressed this comment throughout Protocol sections 5-7.

8. 6.1.1: How to adjust the PMT voltage?

DONE - Thank you for your comment. We have reviewed and addressed this comment in Protocol section 6.1.1.

LINE 253-259: In the scatter plot, adjust the photomultiplier voltage (PMT voltage) in order to center the points. The PMT voltage is used to amplify the signal strength of photons being measured; if the PMT voltage is too weak, fewer cells will be read, and if the PMT voltage is too strong, cells will be measured near maximum value and different cell types will be indistinguishable from one another. An adequate PMT voltage ensures that distinguishable events will populate the scatter plot. Event separation is easier to visualize by projecting logarithmic or biexponential scales on FSC and SSC scatter plots.

9. 6.2: Please describe how to create a selection.

DONE - Thank you for your comment. We have reviewed and addressed this comment in Protocol section 6.2.

LINE 267-270. Drawing rectangular gates is a regular option on flow cytometry software programs and good for clear, distinct cell populations. For cell populations that take a more irregular shape on the scatter plots, use a polygonal gating option.

10. After you have made all the recommended changes to your protocol section (listed above), please highlight in yellow up to 2.75 pages (no less than 1 page) of protocol text (including headers and spacing) to be featured in the video. Bear in mind the goal of the protocol and highlight the critical steps to be filmed. Our scriptwriters will derive the video script directly from the highlighted text.

11. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. The highlighted text must include at least one action that is written in the imperative voice per step. Notes cannot usually be filmed and should be excluded from the highlighting.

DONE. Without considering the mentioned notes, the highlighted sections fall within 2.75 pages.

12. References: Please include journal volume/issue number as well as page numbers.

DONE - Thank you for your comment. We have reviewed and addressed this comment in the References section.

13. Please remove the embedded figure(s) from the manuscript. All figures should be uploaded separately to your Editorial Manager account. Each figure must be accompanied by a title and a description after the Representative Results of the manuscript text.

DONE - Thank you for your comment. We have reviewed and addressed this comment.

14. Table of Materials: Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol.

DONE - Thank you for your comment. We have reviewed and addressed this comment.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This manuscript provides detailed instructions for FACS sorting of isolated coral cells

Major Concerns:

The authors discuss their technique solely in terms of looking at the response of coral cells to stress. However, there are many other basic science reasons that one might want to study pure populations of coral cells. Just as two examples, one might want to study how gene expression in cnidocytes differs from that in other ectodermal cells, or how gene expression in calicoblast cells differs from that in other ectodermal cells not involved in calcification. So, I think it would increase interest in their paper if they were to point out some of these other possible uses.

DONE - Thank you for your comment. We addressed this in both the introduction and last paragraph of the discussion.

LINE 104-107: In addition to studying cell-specific responses to stress, this technology has the potential to clarify gene-specific expression and illuminate the evolutionary and developmental history of cell types entirely unique to cnidarians, such as calicoblasts and cnidocytes.

LINE 429-433: The application of this fine-scale information will provide insight into cell type behavior and function and may reveal characteristics associated with the evolution of cnidarian-specific cell types such as calicoblasts and cnidocytes. Additionally, the application of

FACS technology to the development of improved stress assays and biomarkers may prove useful for the protection of severely threatened coral reefs.

Minor Concerns:

I question the logic of the first sentence of the Abstract: "Coral reefs are under threat due to anthropogenic stressors caused by climate change." Are the authors of the belief that climate change is not anthropogenic? I suggest dropping "caused by climate change".

DONE - Thank you for your comment. We addressed this in the first line of the Abstract.

LINE 38: Coral reefs are under threat due to anthropogenic stressors.

Line 83 What is the antecedent of "its"? I suggest substituting "their".

DONE - Thank you for your comment. We addressed this in the first sentence of the fifth paragraph of the Introduction.

LINE 85-87: Thus far, the application of flow cytometry to coral cells has mainly been for the analysis of symbiotic Symbiodiniaceae and other bacterial populations by utilizing their strong, natural autofluorescence [20-22].

Line 119 Can the authors suggest a suitable viability test? Perhaps add "such as DAPI, which will only stain dead and dying cells."

DONE - Thank you for your comment. We have addressed this and suggested DAPI in Protocol step 1.1.2.

LINE 128-129: A viability test may be performed to a subset of the cell slurry with a simple hemocytometer and a DAPI dye exclusion assay, visualized on a compound microscope.

Line 193 "longer" not "larger" wavelengths

DONE - Thank you for your comment. We addressed this in Protocol section 4.2.2, but the relevant sentence was ultimately removed. “Larger wavelengths” was not used anywhere else in the manuscript.

LINE 213-216: For the detection of light emitted by the green ROS signal, use a filter with a BP of 530/30 (wavelength range of 515 to 545 nm). A fluorescein isothiocyanate (FITC) or green

fluorescent protein (GFP) filter is recommended. This filter will measure the concentration of ROS in each cell.

Line 229 "which" rather than "and" will be flushed out

DONE - Thank you for your comment. We addressed this and rephrased the last sentence of Protocol section 6.1.

LINE 250-251: Allow approximately 30 seconds to pass before starting analysis to clear any debris left in the cytometer chambers from previous experiments.

Fig 3B caption or labels on the graph need to explain what solid and dotted lines are separating. DAPI only stains dead and dying (i.e. membrane compromised) cells. If that is the case then I suggest putting a label "dead cells" to the right of the dotted line, but what is the solid line at 600 telling me? Is it only to say that 89.1% of the cells are viable or is it a cut-off of some sort? If the former, then I suggest getting rid of that line, putting "89.1 % live cells" in the box to the left of the dotted line, and "dead cells" to the right.

We have revised the figure and discussed the DAPI positive population in the legend and the results.

Reviewer #2:

Manuscript Summary:

This manuscript describes a protocol for sorting coral cells via FACS. This protocol will be useful for anyone who is interested in a detailed description of how to sort different cnidarian cell populations starting with isolating coral tissue and dissociating the cells into a suspension of single cells to labeling the cells with various stains and markers and finally gating and sorting the cells.

Major Concerns:

Overall this manuscript is well written and detailed. I believe that it will be useful to any biologist studying cnidarians at a cellular level.

I have only a few major concerns to raise.

(1) The authors state that the cells are stained in 3.3X PBS solution (with FCS and HEPES). It would be helpful if the authors could state in the manuscript why this particular concentration of PBS was chosen (rather than 3X, for example) and what the salinity of 3.3X PBS (with FCS and HEPES) is. Different marine organisms are cultured in various levels of salinity and might need to adjust the PBS concentration accordingly. This could be stated explicitly. Also, if this recipe comes from another published source, please cite it.

DONE - Thank you for your comment. We have addressed this in Protocol section 1.2.1.

LINE 137-139: 3.3X PBS concentration was chosen as demonstrated in Rosental et al. 2017[18] to mimic the salinity of seawater. This concentration needs to be adjusted for species found in other environments to mimic their salinity.

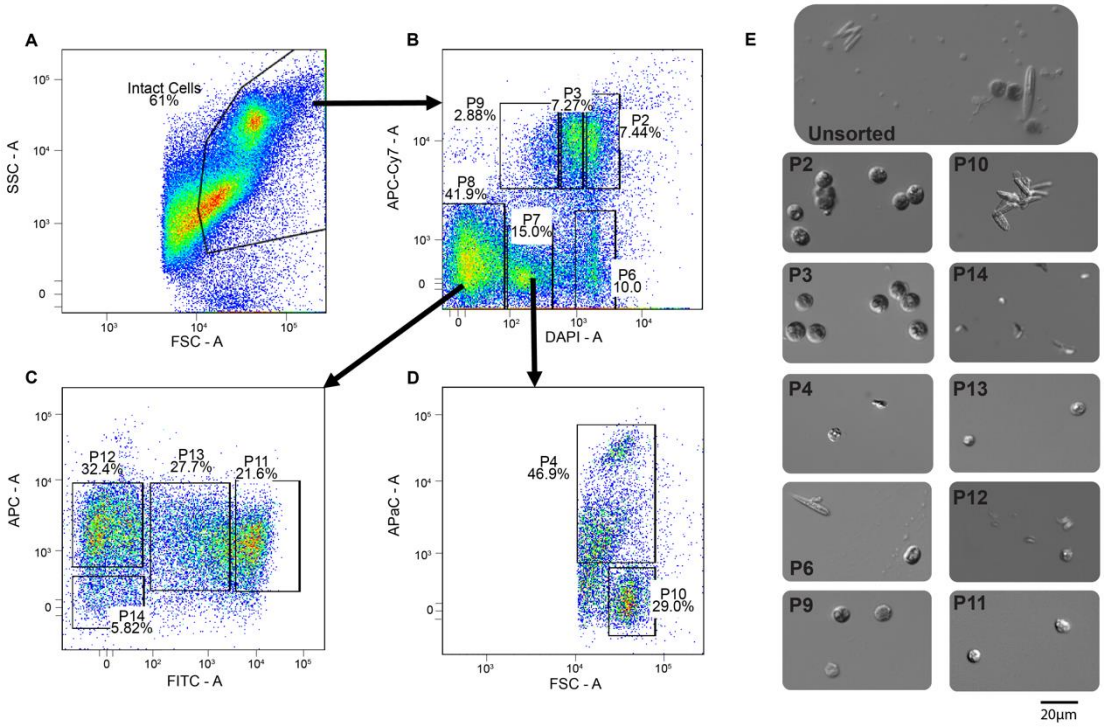
(2) I would highly recommend adding compound microscope images of the following:

a. A heterogeneous mixture of cell types in the suspension of single cells after dissociation but before sorting. These could be stained with DAPI, Trypan Blue or other viability dye to show the proportion of live vs dead cells.

b. Sorted cell types corresponding to the different gated populations in Figure 3A, C, D. This could be similar to what was done in Figure 4 of Rosental et al. 2017 BMC Cell Biology, although those images were quite tiny and it would be great if they could be bigger (even in a separate figure). It would be quite helpful to see the morphology and homogeneity of the sorted cell populations to verify what the authors are stating about the isolated coral cell populations. To show that the CellRox and LysoTracker dyes are working you could also show fluorescence micrographs of these sorted cell populations and discuss any morphological differences in the sorted populations.

Thank you for your comment. As suggested, we have revised Figure 3 and have added the images of the sorted cell populations, as well as an example of an unsorted sample:

Figure 3:



(3) One step that should be added would be a "purity check" where you take a small subsample from the sorted sample and run it back through the sorter again to make sure all cells in the sorted population fall within the target gate. This is common practice in the field of cell sorting and gives more confidence that the sorted cells are truly exclusively from the target population.

DONE - Thank you for your comment. We have addressed this by adding Figure 4 and referring to it in Protocol section 7.2.4.

7.2.4) Each time a new stain, species, or sorter is used, a purity check needs to be done on the population of interest. Do this by sorting at least 20,000 cells of interest into 500 µL staining media, and then re-analyze the sorted cells and confirm that the cells are being read within the gate used to initially sort (Figure 4).

FIGURE 4:

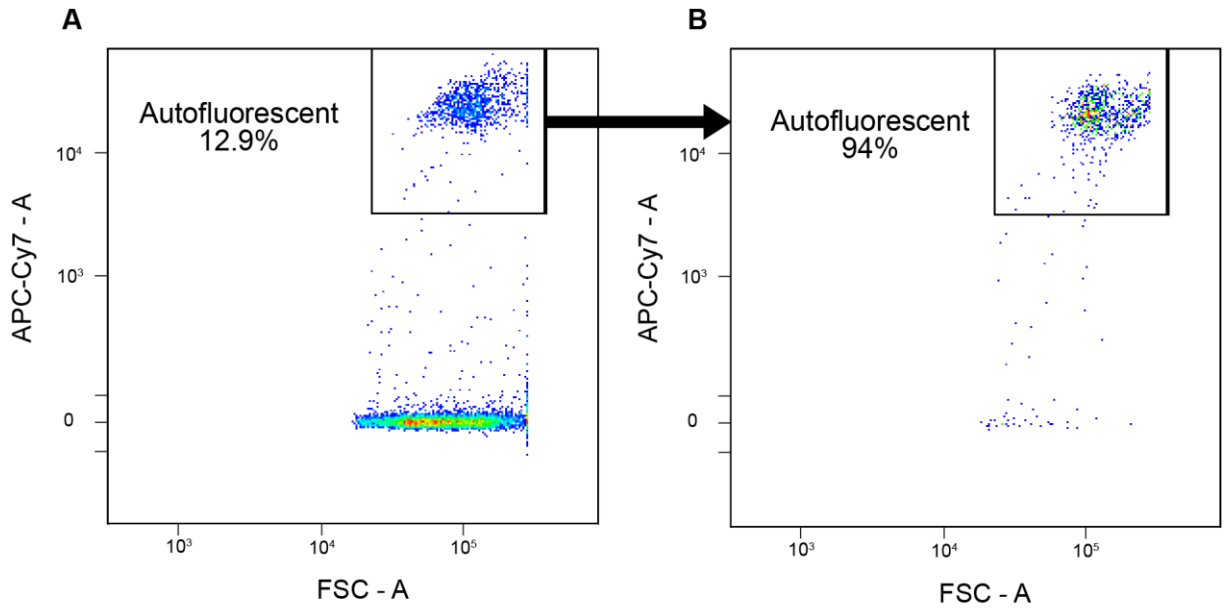


Figure 4: Purity check of the autofluorescent Symbiodiniaceae-associated cells. A) Cells positive for fluorescence on the APC-Cy7 filter from the unstained control sample were sorted. This sorted group of cells was then re-run on the cytometer and re-analyzed under the same conditions, showing 94% purity (B). Forward scatter (x-axis, FSC) was used for the anchoring axis, but is interchangeable with any of the other parameters. The same process can be performed on the stained samples.

Minor Concerns:

None.