Chapter 8

Pigment Granule Transport in Chromatophores

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Contents

Introduction

This laboratory is taught as part of an upper-division cell biology laboratory. Students have completed the core sequence for biology majors which includes an introductory cell and molecular biology course. Most of the students are juniors and seniors. It is also taught as part of a cell biology class for second semester sophomore cell biology/biochemistry majors who have also had the introductory cell and molecular biology course. This laboratory is scheduled following completion of units dealing with cell signaling and the cytoskeleton; thus students have the background knowledge to deal effectively with issues arising in this type of inquiry-based laboratory. Although we use this laboratory primarily for upper-division cell biology students, it could readily be modified for an introductory class. This could be accomplished by selecting several defined conditions under which the melanophores will be observed, and in general, taking a more directed or structured approach to the laboratory.

The laboratory works best for us if taught as a two-week experiment. During week one, the students are introduced to the system. The fish are observed under conditions where color adaptation can occur. Put one fish in a glass bowl on the black benchtop and another on a white background and proceed with your discussion. When you come back to the fish, you should be able to note a significant difference. During the pre-laboratory discussion we focus on the numerous levels at which regulation of aggregation/dispersion of the melanosomes can occur. Note is made of some of the differences among species. We have also found that it is quite helpful to view a brief video of melanocytes in action since some excellent photography of melanocytes, particularly of melanocytes in culture, is available and impressive (the video we use is described on page 12). The students then do a brief experiment to become familiar with the experimental system. Students are told to remove a scale from the fish, place it in the perfusion chamber, and perfuse it for two minutes with Ringer's solution. The pigment granules will markedly disperse. The students will quickly learn how to handle the fish scale, recognize dispersed melanophores, and hopefully even recognize that not all of the melanophores on the

scale respond in an identical manner. Once the students are familiar with the dispersed state of the melanophores, have them treat the fish scale in a way that will make the granules aggregate. Perhaps the easiest way is to simply have the students perfuse with perfusion buffer; it rapidly causes aggregation. (A note of caution: The perfusion buffer contains high quantities of divalent ion chelators. In the absence of external calcium, the granules aggregate; external calcium is required for the dispersed state. Elevated internal levels of calcium cause aggregation. This can be a point of confusion for students; it can also be a good learning experience.) The students should observe the aggregated state; the melanophores look like small back spots. Again, there may be some differences among the melanophores on the scale. Finally, ask the students to again perfuse with Ringer's solution and the dispersed state should be restored. The students should now feel comfortable with the system and know that, if properly stimulated, these cells can be coaxed into repeated rounds of aggregation/dispersion.

Once the students are familiar with the system, they can more effectively design realistic experiments of their own. At this point, students working in pairs are asked to design an experiment, or series of experiments, that they would like to conduct. I ask them to write down on a piece of paper a) a clear statement of the question being addressed, b) basic experimental design including the nature of their controls, c) any chemicals or solutions that will be required (issues such as concentration and solubility of chemicals needs to be addressed at this point), and d) how is the experimental data going to be analyzed. Most students will require a second meeting with the instructor sometime during the following week. During the second week each group of students will come into the laboratory prepared to do their own experiment. We have found that there is an almost endless number of questions that the students can pose using this experimental system. It presents students with an opportunity to design their own experiments; they generally respond quite well to that opportunity. It presents an opportunity to develop science "process" skills at a level appropriate for upper-division courses since the students possess sufficient background information to design informed experiments. It presents the instructor with numerous opportunities to teach the importance of a) experimental design, b) controls, c) critical thinking skills, d) collecting quantitative data, e) interpreting data, and f) making one's own solutions.

Notes for the Instructor

General Comments

1. If you are trying this laboratory for the first time, it might be better to restrict your experiments to compounds that act at the cell surface or are membrane-permeant. Consult Haimo and Rozdzial (1989) for an excellent discussion of permeabilization problems with fish scales. The covering epidermis may restrict permeabilization of the melanophore. The covering epidermis may be removed with collagenase, but this is a "tricky" process; I have on more than one occasion removed everything from the scale. Use of DMSO as a solvent will aid entry into the cell, but you may not wish to use DMSO, for reasons of safety, particularly if you have an introductory level biology class. I have Haimo and Rozdzial's (1989) permeabilization buffer available for lab even if students are not trying to work with permeabilized cells.
2. Early in the laboratory stress good care of the fish scales. They should be kept moist at all times. We initially anesthetize fish in a 0.2% solution of MS222 in salt water. We then sacrifice the fish by decapitation and wrap it in wet paper towels. The cycloid-type scales are removed from the dorsal truck above the lateral line using fine forceps and placed in saltwater Ringer's. Under magnification, the scale appears as concentric circles with the melanophores distributed throughout. The scales itself is slightly concave and thus will not "lie flat" – use fine adjustments as necessary.

3. If scales are permeabilized, we find that permeabilization works better in the perfusion chamber. Attempts to permeabilize scales in larger volumes of buffer often result in unresponsive melanocytes. I think the large volume of buffer leaches out necessary components from the cells.

For Week-One

1. You may wish to make the pertinent periodical literature more readily available to the students. We have two notebooks in the laboratory containing photocopied articles available for their use. One contains a copy of the review articles listed at the end of the laboratory guide. The second notebook contains articles from the primary literature that relate to chromatophores. Unfortunately, much of the primary literature is in journals many college libraries do not have, so I lend them my copy. Access to these articles may be particularly useful to students, for example, when determining what concentrations of a compound to use, or perhaps the solubility of a compound. A major problem for the students (and for the instructor) is to decide on what concentration of a chemical to use in their experiment. I try to anticipate this by looking up some of the literature myself. This, also, is where the collection of articles (second notebook mentioned previously) from the primary literature is very useful. The students, however, make up many of their own solutions. Considerable time will be spent calculating how to make up various solutions. I view this as a positive part of the lab rather than a distraction. Make sure you know, or can find out, the solubility of these compounds and the molecular weights. I also have a "crib sheet" for myself as to what might be "reasonable" concentrations (see Appendix B). On the other hand, an experimental determination of effective concentrations for some putative regulator can be a very good experiment.
2. Be available for discussion with each group. Set a time, perhaps a day or two following laboratory, when students can again talk with you about their experimental plans. When talking with them check the following:
   a) What concentrations of the experimental test solutions are you going to use?
   b) How do you make up this solution?
   c) Are you going to encounter any solubility problems?
   d) What data are you going to collect? Can it be quantitative or just qualitative?
   e) Make sure they realize that the melanocytes will initially be encountered in a dispersed state.
   f) Is your experimental design really going to give you an answer to your original question?
   g) Are you going to permeabilize the cells? If so, how will you know that they are permeabilized?
3. Encourage creative thinking on the part of the student.

For Week-Two

1. How this week proceeds is very much dependent upon the success of week one. It is particularly important to avoid a situation where 8 - 10 groups descend upon you at once saying "How do I make up this solution?" at the beginning of the week-two laboratory. Groups should already have thought about that and done their calculations or even made up the solutions. You could also assist by making up some of the basic stock solutions (see Appendix A) ahead of time. If you know that solutions will have to be made up in lab, you could have a teaching assistant in lab with you who knows how to help students make solutions. If you "get caught" spending all your time showing students how to make solutions, much of the more interesting biological discussions about melanophores cannot occur.

2. Help students systematically observe the melanophores and learn as much as they can about these interesting cells. Encourage them to record their observations in their notebook for future reference.
3. Avoid projecting the image that you know what is going to happen in their experiments and that their job is to make it work right! Observe and learn with them.
4. Encourage the students to talk with each other about their experimental question, their design and their observations.
5. Make sure students know when the laboratory report is due and what is expected in the report. We require that the results be written up in the form of a research paper with an Abstract, Introduction, Materials and Methods, Results, Discussion and Reference sections.

Materials

Have the following supplies available for use in the laboratory**.**

Pasteur pipettes
Pipette bulbs
Microscope slides (The larger 76 x 50 mm slides work well)
Note: We have found that it facilitates work in the laboratory if the instructors make the perfusion chambers before the laboratory using slides and coverslips. Permount will prevent slipping during perfusion.
Coverslips (You may wish to have several different sizes)
MS 222
Paper towels
Compound microscopes (one for each two students)
Dissecting microscopes (one or two for the laboratory)
Tweezers (one for each two students)
Eppendorf tubes for individual aliquots of solutions
Fish net
DMSO (optional)
Assorted chemicals (see list in Appendix B)

Student Outline

Pigment Granule Transport in Chromatophores

And What Do We Expect of Everyone In This Laboratory?

First of all, have some fun. This is an interesting experimental system. Probably most of you have seen fish lighten or darken their coloration depending upon their environmental surroundings. If you have not been so fortunate as to observe this, we will see it in this laboratory. But, now to the point. In this laboratory you will first become familiar with the chromatophores by carefully reading the introduction to the system that is printed below. If you need more information, go to one of the references listed at the end of the guide. Once you think you have a good understanding of the literature, meet with your laboratory group for a short planning session. You will be working in groups. Once in your group, agree upon approximately two questions that you would like to answer experimentally. Write down your questions; then design experiments to answer each of these questions. This planning session should occur at least several days before the actual laboratory. Before beginning your experimental work show one of your instructors 1) your questions and 2) the

experimental design that you have devised to answer these questions. Once you have decided upon your questions and experiments, check to make sure that the necessary chemicals are available. Please keep your experimental designs such that they are not cost prohibitive. For example, we cannot order $100/mg hormones, etc. A separate list of chemicals that are available in the laboratory will be provided to each group. Inquire about any chemicals you would like that are not on this list.

The following is your **schedule** for this two-week laboratory:

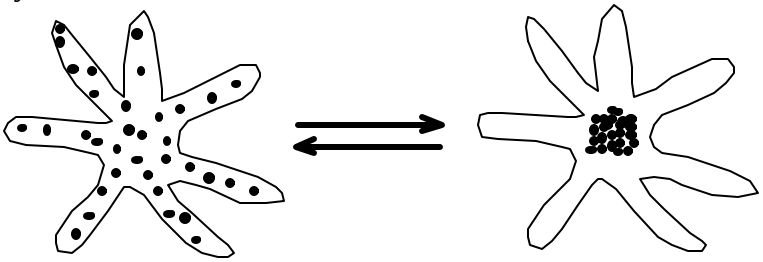
Week One: Become familiar with the melanophore, do some background reading, meet with your laboratory group and decide on the experiment(s) to be conducted. Briefly write up the experiment and turn it in to your instructor. Let your instructor know what chemicals will be required to do your experiment.

Week Two: Do the experiment, record your observations, and write up your results.

Background Reading

Introduction to the Experimental System: Fish Chromatophores

Many animals such as fish can rapidly change their coloration, an ability that offers protection from predation (or their efficient predation) through camouflage and mimicking of the background coloration. Fish possess a variety of **chromatophores**, mostly of dermal origin. These cells possess numerous granules in which pigments are stored. In some of these chromatophores, pigment granules migrate out from the cell center resulting in increased coloration of the animal; alternatively these pigment granules may aggregate in the cell center and the animal appears less colored. Whether or not these granules move, the speed at which they move, and the manner in which this movement is controlled varies from species to species. Just a few basic features of this system will be reviewed below.



The information presented below can be found in more detail in the literature cited at the end of this laboratory (Fujii, 1993; Fujii and Oshima, 1986 and Haimo and Rozdzial, 1989). To help you read the literature, let us begin with some semantics, i.e. the manner in which chromatophores are named. Chromatophores storing the black pigment, **melanin**, are called **melanophores**. **Xanthophores** store yellow pigments (carotenoids), **erythrophores** store red pigments (pteridines). **Leucophores** lack pigments. **Iridophores** store guanine or other purine crystals which reflect light giving the iridescent or shimmering appearance of many fish. Chromatophores also may be grouped by the manner in which they effect coloration of the host fish. For example, the melanophores, xanthophores, and erythrophores store pigments that absorb light. Leucophores and iridiophores, on the other hand, act via reflecting light.

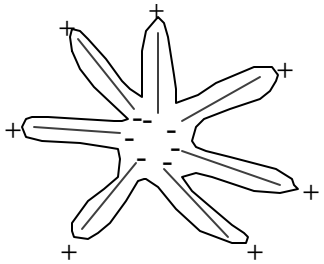
In chromatophores that possess a dendritic cell body, and in which pigment granules move out into the dendritic extensions (**dispersion** of granules) and back in (**aggregation** of granules), the time frame within which the pigment granules move is quite variable. The manner in which their movement is controlled is also quite variable. Movement may require less than a minute to about an hour. Goldfish xanthophores are reported to require about 30 min to 1 hr to completely aggregate or disperse their granules (less than 0.1 µm/sec); melanophores transport pigment granules about an order of magnitude faster, requiring about 1 min (1-2

µm/sec); and erythrophores from the squirrelfish, *Holocentrus ascensionis*, require only a few seconds for complete aggregation or dispersion (15-20 µm/sec).

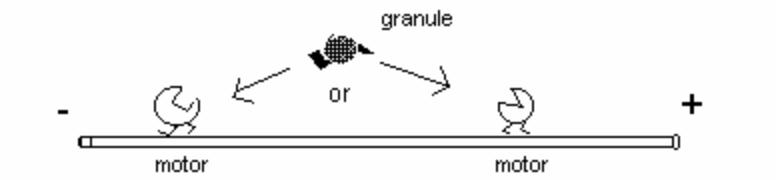
The mechanism of transport, although extensively studied in many species, involves a number of regulatory elements and still has some unanswered questions. Transport in melanophores and erythrophores generally appears to occur in association with microtubules; however some recent studies suggest that pigment granule transport in xanthophores occurs in association with intermediate filaments, and a number of older studies suggested the involvement of microfilaments (few still suggest this). Along with the suggested use of microtubules to facilitate dispersion/aggregation of the granules, the involvement of "**motors**" has been demonstrated in some systems. A motor is a nucleoside triphosphate, ATP, hydrolyzing protein that drives the displacement of the granule relative to the adjacent microtubule (or filament). Can you make any testable predictions about what might be observed if microtubules were the cytoskeletal structure used? What if microfilaments or intermediate filaments were the cytoskeletal structure involved?

Although the mechanism appears to involve motors and microtubules, the regulation of these systems is rather complex and seems to involve both **neural** and **endocrine** mechanisms. Interestingly, chromatophores are cells derived from the **neural crest**; thus they have been dubbed "paraneurons" by some investigators. Neural regulation is via the **sympathetic** component of the autonomic nervous system. Recall that this is an **efferent system** with both a pre- and post-ganglionic component.

In this laboratory we will choose to work with melanophores for a variety of practical reasons, so the rest of this discussion will be limited to this cell. What do we know about movement of **melanosomes** (pigment granules) in this cell? It is thought to involve primarily the microtubule (see references), and the microtubules are oriented with their + end toward the distal end of the dendritic extension as shown below.



What types of motors would you expect to be involved in either aggregation or dispersion (provided motors are involved). As you recall **dynein-like motors** typically facilitate movement toward the - end of a microtubule whereas another family of motors, **kinesins**, facilitates movement toward the + end of microtubules. Kinesin motors would be expected to bring about dispersion of the pigment granules, melanosomes; whereas dynein motors would be expected to bring about aggregation. In melanophores from a few limited species of fish, evidence for the existence of both types of motors has been obtained. If this is the mechanism, then the pigment granules must contain receptors for both kinesin and dynein motors (as shown below), and a regulatory system must be present that determines which motors bind both to the granule and the microtubule.



If motors are indeed involved in the aggregation and dispersion phenomena that you are going to be studying, can you make some predictions that would be experimentally testable? For example, would ATP be required? Also, for your information, there are selective inhibitors of dynein motors available such as vanadate. How might you confirm, or disprove, that microtubules are an essential element in dispersion and aggregation?

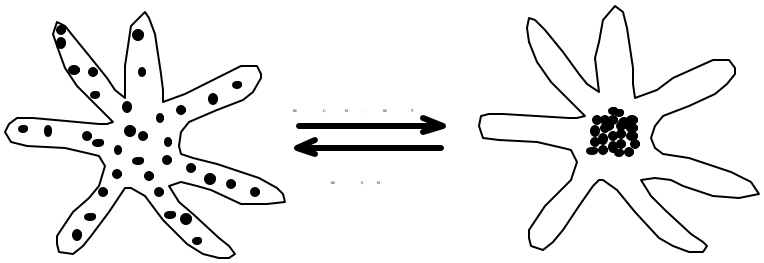
Neural Regulation:

What do we know about neural regulation of melanophores? Neural synaptic connections have been observed with the melanophore. These presynaptic regions of the synapse contain **dense-cored granules** similar to other **catecholamine** containing synapses. These synapses generally appear to be of the **alpha-adrenergic** type as evidenced by studies using adrenergic **antagonists**. Data relative to whether the receptors involved are a_1 or a_2 are inconclusive. Some investigators suggest that both are present and that stimulation of the a_1-adrenergic system results in aggregation of the pigment granules, and the resultant lightening of the fish scale coloration; whereas stimulation via the a_2-adrenergic system inhibits aggregation.

Another receptor, the **adenosine receptor**, has been shown to be present on the surface of the melanophore. The adenosine receptor antagonist, methylxanthine, for example, has been shown to inhibit dispersion of the pigment granules. Activation of this receptor is thought to promote dispersion of the melanosomes. ATP is packaged along with the catecholamine (norepinephrine or epinephrine) into the presynaptic vesicle. ATP is therefore released along with the norepinephrine. This **dual-transmitter** system is thought to function as follows: The catecholamine promotes a rapid aggregation of the granules, but this transmitter is rapidly degraded by enzymes and the stimulus ends. The ATP, however, is much more slowly degraded, thus with the rapid removal of the catecholamine, this transmitter promotes return to the darkened state of coloration.

Hormonal Regulation:

Now let us briefly comment on some of the major hormonal controls that impinge upon the melanocyte. **Melanocyte-stimulating hormone (MSH)**, produced in the intermediate lobe of the pituitary, is known to target the melanophore where it causes pigment dispersion. MSH is a peptide, and the melanocytes possess a surface receptor specific for this peptide. It has been shown that Ca^{2+} must be present in the external medium for MSH to act; thus this receptor may represent a potential calcium channel into the melanocyte (or perhaps it is required for binding of the hormone to its receptor). **Melanin concentrating hormone (MCH)**, produced in the posterior lobe of the pituitary, also targets the melanocyte, and as its name implies, causes an aggregation of the pigment granules and a lightening of the fish scale. MCH also binds to a specific receptor on the surface of the melanocyte. **Melatonin (MT)** is the third hormone known to target the melanocyte. It is produced in the pineal gland and functions to aggregate pigment granules. Concentrations of this hormone often exhibit very distinct circadian rhythms. The actions of these hormones are diagrammed below.



Intracellular second messengers:

Finally, let me comment about the lesser known processes whereby the stimulation of the receptor is translated into altered granule motility. In other words, what changes in intracellular second messenger, such as cAMP, cGMP, Ca^{2+}, or IP_3, occur upon activation of receptors associated with either neuronal or hormonal stimulation? Are G-proteins involved? Perfusion of permeabilized melanocytes with cAMP has been clearly shown in a number of systems to result in dispersion of pigment granules. Initially this was interpreted to be the result of an elevated cytosolic second messenger, cAMP. With the discovery of the adenosine receptor, this interpretation has been called into question. In other words, the question is whether the dispersion of pigment granules is the result of elevated cytosolic cAMP, or is it the result of the activation of the adenosine receptors? More work needs to be done in this area, but the bulk of evidence seems to suggest that cAMP acts as a second messenger to effect dispersion of the melanosomes. Additionally, evidence from several systems suggests that elevated cAMP levels are associated with phosphorylation of a specific protein of about 57 kD.

Can you think of a simple test with your melanocytes to distinguish between these two alternatives? Remember that the adenosine receptors are located on the outside of the cell, whereas the second messenger cAMP acts internally. Also, remember that you can use antagonists to block receptors. Can you block the production of cAMP? Remember that it is produced from ATP via the action of the adenylate cyclase enzyme. You should be able to think of several toxins or effector substances that can alter the activity of this enzyme.

Now to the questions regarding the role of calcium and the inositides. Elevated calcium levels appear to be associated with aggregation of melanosomes in many fish. Using calcium specific ionophores such as A23187, it is possible to control the intracellular concentration of calcium. Permeant fluorescent indicators such as Fura-2 are also available to directly measure intracellular calcium concentrations. Studies using both of the above techniques show that high calcium concentrations promote aggregation. The question of where the calcium comes from has been raised by many investigators, i.e. is it intracellular or extracellular? IP_3 (inositol triphosphate) is commonly thought to function in the release of Ca^{2+} from the ER, and IP_4 is commonly thought to be involved in calcium entry from the extracellular space. Additionally, binding of agonist to receptor commonly involves the generation of IP_3 and diacyl glycerol from hydrolysis of phosphatidyl inositol via phospholipase C. Diacyl glycerol is an activator of protein kinase C and, as such, it is another possible mechanism for the specific phosphorylation of proteins. The question as to the origin of the calcium that regulates pigment granule motility, as well as the extent to which IP_3 or IP_4 are involved, is still not clear in most systems.

Procedure

Working with the Fish Scale

Scales from a number of different species of fish may be chosen for study of chromatophores. For this exercise, we will use the **marine (or marsh) killifish, *Fundulus heteroclitus (or grandis)*.** Other species that may be of interest are the swordtail (*Xiphophorus helleri*), the angelfish (*Pterophyllum scalare*), the marine squirrelfish (*Holocentrus ascensionis*), or the freshwater African cichlid (*Tilapia mossambica*).

Removal of Scales

Prior to the laboratory your instructor will sacrifice the fish following use of 0.2 % MS 222 in salt water. A wet towel should be placed over the fish to prevent drying. As needed, remove scales using a fine forceps. Simply grasp a scale and pull it from the body. Place the scale in saltwater Ringer's solution.

Removal of the Epidermis from the Scale

(Note: Before beginning this procedure check with your instructor. Some fish scales can be permeabilized and observed quite readily without removing the epidermis.) If the epidermis is removed, this will leave the underlying dermis exposed so that the chromatophores can be directly observed. The procedure for doing this is detailed by Haimo and Rozdzial (1989). Do not do this unless the species of fish you are working with requires it.

Construction of a Perfusion Chamber

You will need to construct a perfusion chamber into which you can place your fish scale. On your glass slide, use small (or broken) coverslips to elevate your coverslip (this is shown below).

Your perfusion chamber will be open on two sides. Add the solutions of interest, beginning with the permeabilization buffer, to one side of the chamber and draw the solution through the chamber using a filter paper wick at the opposite side.

Permeabilization of the Chromatophores

The next step that you should do is to lyse (or open) the chromatophores so that pigment granule movement can be observed as test compounds are applied to the cell. Caution: Failure to carry out this step successfully will mean that test compounds will not gain access to the cytoplasm of the melanocytes. Several methods have been developed to accomplish this without negatively affecting the ability of the chromatophore to undergo reversible aggregation or dispersion of pigment granules. Original procedures utilized the detergent Brij 58, but recent procedures have relied primarily on the detergent digitonin (0.001%). This glycoside interacts with cholesterol, and since cholesterol is distributed primarily in the plasma membrane, digitonin will permeabilize the plasma membrane and leave the pigment granule membrane relatively intact. Digitonin causes vesiculation of the membrane and produces small holes. This allows the soluble cellular contents to slowly leak out of the cell into

the surrounding environment. The digitonin is applied in permeabilization buffer, and the permeabilization should be complete in about 1 to 2 minutes.

Testing of the Permeabilized Chromatophores

After permeabilizing the chromatophores you should be able to see the hyperdispersed state of the pigment granules. You will probably work primarily with the 10X objective. Note the appearance of the darkened portion of the chromatophore. The pigment granules have migrated out into the cytoplasmic arms of the chromatophores.

Your Plan of Study

You are now ready to begin your study of the melanocytes. A variety of test compounds will be available for use in your study. Think carefully about whether or not you wish to permeabilize your scale. When might you not want to permeabilize your chromatophores? Make sure that you are certain about your recognition of the melanocyte and its appearance in the dispersed and the aggregated state. Once you think you know what you are looking for, conduct the series of tests on your melanocyte that you and your group have planned. Feel free to digress from your plan if some interesting results are obtained. Make sure you know what solutions have been prepared for your use. You are responsible for making up any solutions not on that list. Good luck, let us see some clear thinking and good design. Keep an accurate record of your tests and their results.

Several words of caution about the interpretation of your results. Remember that both aggregation and dispersal are probably energy dependent processes, thus if ATP is depleted nothing may happen. Note: These chromatophores are reported to be able to produce ATP for 30 to 60 minutes following permeabilization. If you get no response, you may need to add ATP. Also, some cellular responses require intact cells, and the response is elicited at the cell membrane. These hormonal/neural regulators then generate internal second messenger compounds. If you wish to test one of these compounds directly, the cells must be permeabilized or the compound must be cell permeant otherwise test compounds will be unable to enter. Also, it may be wise to limit the number of tests run on a single fish scale otherwise the necessary components of the cytoplasm may be extracted due to the multiple perfusions. This could produce artifactual negative results.

Laboratory Report

Write your laboratory report in the format of a typical research paper that includes Introduction, Materials and Methods, Results, Discussion and References sections. For the Introduction, set the questions you decided to ask in a brief theoretical framework and include a background summary of the relevant literature on melanophores and their regulation. The specific questions being tested should be briefly stated at the end of your Introduction. Write a Materials and Methods section but make that somewhat “lean”. For example, don’t chase down the source of all your chemicals. It is not necessary to repeat what is described in the laboratory guide. You should, however, make sure that what you did is explicitly clear. Then present your results. Carefully discuss the interpretation of these results indicating the possible limitations in your experimental design, and if several interpretations are possible, indicate what they might be. Good luck!

Stock Solutions or Chemicals Available:

Note: We use solutions in either perfusion buffer or Ringer's solution. You may wish to use a different vehicle. Remember, however, that Ringer's alone causes dispersion whereas perfusion buffer alone causes aggregation.

1 mM cAMP.
10^{-5} M epinephrine (or norepinephrine) in perfusion buffer.
1 mM ATP in perfusion buffer
1 mM ATP and 1 mM cAMP in perfusion buffer
3 mM ATP in perfusion buffer
10 mM calcium in perfusion buffer
coffee
tea
10 mM caffeine in perfusion buffer
1 mM theophylline in perfusion buffer
1 mM adenosine in perfusion buffer
10^{-7} M melanocyte stimulating hormone in perfusion buffer
10^{-8} M melanin concentrating hormone in Ringer's solution
10^{-5} M melatonin in perfusion buffer
100 µM sodium orthovanadate in perfusion buffer
2 mM methylxanthine
0.2-0.02 ug/ml colchicine in perfusion buffer
10 mM theophylline

Acknowledgments

We wish to thank Haimo and Rozdzial (1989) for writing their review of lysed chromatophores as a system for studying organelle transport. Reading this article prompted the development of this laboratory; much of the methodology is taken from this article.

Literature Cited

Haimo, L.T., and M.M. Rozdzial. 1989. Lysed Chromatophores: A Model System for the Study of Bidirectional Organelle Transport. *Methods in Cell Biology* 31: 3-24.

Fujii, R. 1993. Cytophysiology of Fish Chromatophores. *International Review of Cytology: A Survey of Cell Biology*. 143: 191-255.

Fujii, R., and N. Oshima. 1986. Control of Chromatophore Movements in Teleost Fishes. *Zool. Sci*. 3: 17-47.

Chromatophore Video

Porter, K., M. McNiven, J. Pickett-Heaps. Pigment Translocation in Chromatophores. In: Cellular Motile Processes: Molecules and Mechanisms. Cellular Motility and the Cytoskeleton. Video Supplement 2: Microtuble-Based Motility, J.M. Sanger and J.W. Sanger Eds. Wiley-Liss. [ISBN 0-471-56170-3]

APPENDIX A
Solutions to be Prepared

Saltwater Ringer's:

134 mM NaCl
2.5 mM KCl
1 mM $MgCl_2$
0.5 mM Na_2HPO_4
15 mM $NaHCO_3$

To prepare the above solution, weigh out the following and dissolve in l liter of water.

7.83 g NaCl, MW = 58.4,
186 mg KCl, MW = 74.6
95 mg $MgCl_2$, MW = 95.2
71 mg Na_2HPO_4, MW = 142
1.26 g $NaHCO_3$, MW = 84.0

Perfusion buffer**:**

33 mM potassium acetate
30 mM HEPES
10 mM EGTA
5 mM $MgSO_4$
0.5 mM EDTA
2.5% polyethylene glycol, pH 7.4

To prepare the above solution, weigh out the following and dissolve in 1 liter of distilled water.
Adjust the pH to 7.4.

3.23 g potassium acetate (MW = 98.14)
7.15 g HEPES (MW = 238.3)
3.80 g EGTA (MW = 380)
0.19 g EDTA (MW = 380)
12.33 g $MgSO_4$ (MW = 246.5)
25 g polyethylene glycol

Permeabilization buffer:

First make up a 0.4% Digitonin Stock in 50% ethanol. Make this up by weighing out 200 mg digitonin and dissolving it in 50 ml of ethanol. This stock can be stored at room temperature for about a month.

To make the working perfusion buffer (0.001% digitonin in perfusion buffer), dilute the 0.4% digitonin stock described above (1:400).

APPENDIX B
Optional Chemicals and Solutions

The following chemicals may be very useful. I anticipate their use and obtain a stock before this lab begins.

dibutyryl cAMP
ATP
caffeine
theophylline
calcium chloride
coffee
tea
colchicine
melatonin
melanocyte stimulating hormone
melanin concentrating hormone
epinephrine
methyl xanthine
ATP
sodium orthovanadate

Instructions for making some frequently requested solutions:

Epinephrine, 0.1 mM, MW = 183.2

To get a stock solution, weigh out 36.6 mg and dissolve in 2 ml of ethanol. This will provide a 100 mM stock solution. This can be diluted 1:1000 to get a 10^{-4} working solution. Further dilutions into perfusion buffer can then be made as needed. This solution should be made up just before use and stored in a dark bottle since it is light sensitive.

Melatonin, MW = 232.3

To get a 10 mM stock solution, dissolve 23 mg in 10 ml of ethanol. Do a 1:1000 dilution into perfusion buffer to get a 10^{-5} M solution.

ATP, 1 mM, MW = 551, in perfusion buffer

Weigh out 55 mg ATP and dissolve in 100 ml of perfusion buffer. Adjust the pH as ATP is rather acidic. If a 5mM solution is desired, weigh out 275 mg ATP and dissolve this in 100 ml of perfusion buffer.

cAMP, MW = 329

To get a 1 mM solution, weigh out 32.9 mg and dissolve this in 100 ml of perfusion buffer.

Theophylline, MW = 180.2

To get a 10 mM solution, weigh out 180 mg and dissolve this in 10 ml of perfusion buffer.

Melatonin, MW = 232.3

To get a 10 mM solution, dissolve 23 mg in 10 ml of ethanol. Do a 1:1000 dilution into perfusion buffer to get a 10^{-5} working solution.

Methyl xanthine, MW = 166

To get a 10 mM solution, dissolve 16.6 mg in 10 ml of perfusion buffer.

Calcium chloride, dihydrate, MW = 147

To get a 10 mM solution, dissolve 73.5 mg in 50 ml of perfusion buffer.

ATP and cAMP, 1 mM, in perfusion buffer

To 50 ml of the above ATP solution add 16.5 mg cAMP.

ATP, 3 mM, in perfusion buffer Weigh out 82.5 mg ATP and dissolve in 50 ml of perfusion buffer.

Melanin concentrationg hormone, MW = 2354.8

Dissolve 100 ug of hormone in 42.5 ml of perfusion buffer to get a 10^{-6} M solution.

Melanocyte stimulating hormone, MW = 1664.9

To get a 10^{-4} M stock solution, dissolve 1 mg in 6.2 ml of perfusion buffer. Dilute this 1:100 in perfusion buffer to get a 10^{-6} M working solution.