Ascidian Laboratory

Woods Hole Embryology Course, 2010

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Ascidians have a long history as an experimental model system for development. Edwin Grant Conklin followed the cell lineage of the ascidian *Stylela partita* during studies here at MBL and published his results in a 1905 monograph (Conklin, 1905). Several of the plates from that monograph have been reproduced at the end of this guide to help you follow developmental stages of the various species you will observe. The laboratory is divided into several sections. The first section will cover normal embryogenesis *in Ciona intestinalis* that is perhaps one of the most widely studied tunicates. If we are able to procure the material, we will also look at embryos from *Styela clava, Molgula* sp., *Botrylloides sp.* and *Ascidiella aspersa*. The second section will provide an opportunity to examine different tissue territories using specific histochemical stains. We will also try to combine these staining techniques with microsurgery to examine differentiation in partial embryos. Lastly, we will use electroporation to introduce several different gene enhancer-reporter constructs into fertilized eggs.

This is a picture of an adult ascidian:

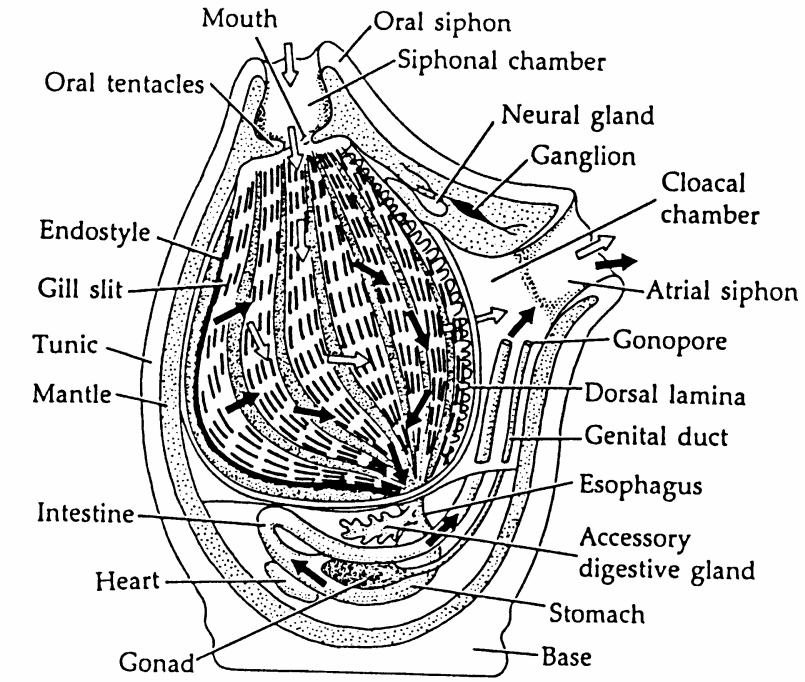

Figure 1. Adult ascidian. From Satoh, 1994.

The adult ascidian has lost the features that classify these animals as chordates. The chordate features are present in the tadpole larvae and subsequently lost during metamorphosis into the adult form.

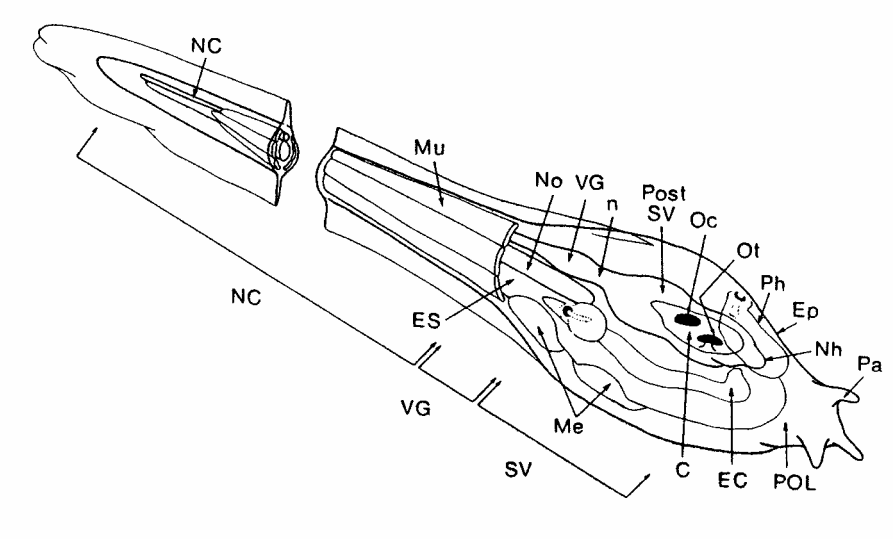

Figure 2. Typical ascidian tadpole. **C**, cavity of sensory vesicle; **n**, neck; **NC**, nerve cord; **Nh**, neurohypophysis; **Oc**, ocellus; **Ot**, otolith; **Post SV**, posterior sensory vesicle; **SV**, sensory vesicle; **VG**, visceral ganglion; **EC**, endodermal cavity; **Ep**, epithelium; **ES**, endodermal strand; **Me**, mesenchyme; **Mu**, muscle; **No**, notochord; **Pa**, papilla; **Ph**, pharynx; **POL**, pre-oral lobe (Nicol and Meinertzhagen, 1991).

The typical larva has about 2500 cells. In *Ciona*, there are 36 muscle cells and 40 notochord cells. Two prominent pigment spots are visible - the otolith and the ocellus. The otolith contains a single large pigment granule and is probably involved in geotactic responses. The ocellus contains a single pigment cell which itself contains many melanin granules. There are 3 lens cells and 15-20 sensory cells. A spinal cord runs along the length of the tail, dorsal to the notochord. The trunk region contains the gut primordium and several pockets of mesenchyme cells that will give rise to adult structures. The larva is surrounded by an epidermal layer of cells. Development of the tadpole larvae is usually very rapid - *Ciona* tadpole larvae hatch out from the chorion about 18 h post-fertilization. The next section will describe the normal development of ascidian embryos.

Normal Development

Ciona intestinalis is very widely distributed. Like many ascidian species, it has been spread around the world by hitching a ride in ship's ballast tanks. Depending on the local climate, gametes may be obtained most of the year. Ripe sperm and eggs accumulate in easily visible gonoducts (remember, these animals are hermaphrodites!). Eggs appear pink and sperm appears white. Gametes are self-sterile, therefore you must mix the gametes from two or more individuals for successful fertilization. The eggs are surrounded by a chorion and an inner layer of test cells that may occlude visualization of later cleavage stages. The unfertilized egg has several different regions of cytoplasm. Unfortunately, *Ciona* eggs do not have colored cytoplasms, however the eggs of *Styela* do. The periphery contains the "myoplasm" which mainly segregates with the muscle lineage. In *Styela*, this cytoplasm is yellow in color. Internal to the myoplasm is the endoplasm (maroon in Styela). The ectoplasm is located nearest the animal pole and is clear in *Styela*.

The eggs are arrested in prophase I of meiosis. Fertilization triggers the completion of maturation and initiates ooplasmic streaming. Two polar bodies are formed; these are generally visible if the orientation of the egg and the light source are aligned properly. The first phase of ooplasmic streaming moves the myoplasm to the vegetal pole of the egg. The ectoplasm also streams towards the vegetal pole and come to reside just above the myoplasm. The endoplasm moves into the animal half of

the egg. During the second phase of ooplasmic streaming, the two pronuclei will migrate and fuse. The myoplasm moves upwards to the future posterior region of the embryo and spreads to form the yellow myoplasmic crescent. Just prior to first cleavage, the 3 primary axes of the embryo have been established. The different cytoplasms will be distributed to different cell lineages during cleavage (Figures 3, 4). You may be able to follow the yellow myoplasm quite far in development if the *Styela* eggs are well pigmented.

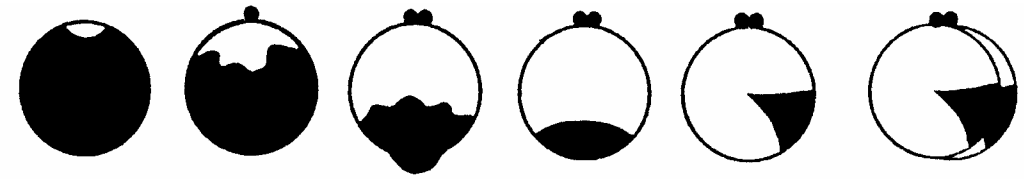

Figure 3. Myoplasm movements during the two phases of ooplasmic streaming (From Jeffery, 1992)

The first cleavage is bilaterally symmetric and bisects the embryo into right and left halves. Cleavage is determinate and occurs in a very regular pattern (see appendix with Conklin's figures). Gastrulation occurs around the 110-cell stage. It is soon followed by neurulation that begins on the vegetal pole side and proceeds in a posterior to anterior wave. The next phases are the development of the tailed embryo and hatching. Depending on the species, the larvae swim for up to a day or two and then settle onto a firm substrate and begin metamorphosis.

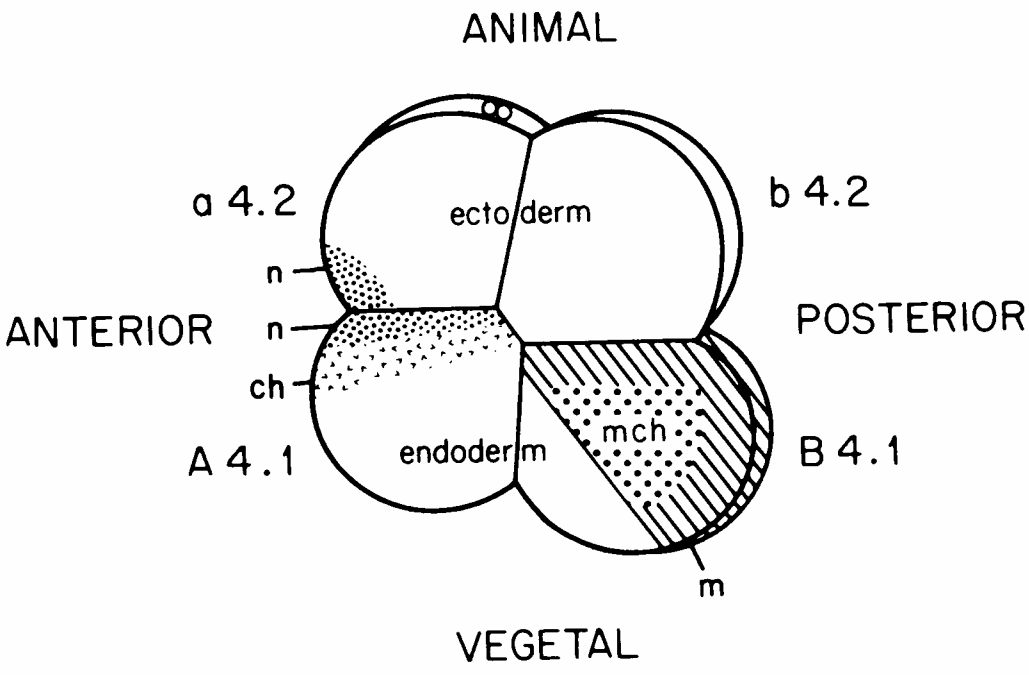

Figure 4. 8-cell embryo showing future embryonic territories. (From Whittaker, 1987).

The complete lineage of the ascidian embryo is known in detail until all but a few cells are specified. The lineage diagram may be found in the back of this handout in the appendix.

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Getting Started:

PROCEDURES AND SUGGESTIONS FOR EMBRYO OBSERVATIONS

One of the first things you should do is to fertilize *Ciona* eggs and observe normal development. A second species, *Ascidiella aspersa*, has very clear eggs and develop at the same rate as *Ciona*. There should also be some staged specimens that have been fertilized before class that you may wish to observe. Some of the major morphological changes that occur during embryogenesis are neurulation, the intercalation and extension of the notochord cells/tail and the subsequent vacuolization of the notochord cells. These are all easy events to observe under the microscope. A more detailed look at notogenesis is presented in the appendix.

Procedure for fertilizing *Ciona* and *Ascidiella* eggs:

1) Obtain two adults from the holding tank. The animals are maintained under constant light to prevent them from shedding their gametes (normally under day/night control).
2) Using a sharp pair of dissecting scissors carefully cut the excurrent ("atrial" in Figure 1) siphon back to expose the gametes.
3) Using the tip of the scissors, nick the ovoduct and disperse eggs into a clean dish of filtered seawater. Do this with both adults.
4) Nick the sperm ducts from both adults and add a SMALL amount of sperm to the eggs. Gently mix and allow to fertilize for 1-1.5 minutes.
5) Using egg baskets, rinse eggs through several changes of clean seawater and place at 18°C.

Table of normal *Ciona* development at 18°C:

Stage	Time Post Fertilization
2-cell	60 minutes
4-cell	90 minutes
8-cell	120 minutes
16-cell	160 minutes
32-cell	210 minutes
64-cell	250 minutes
Early Gastrula (110 cells)	5 hours
Neurula	7 hours
Otolith melanization	12 hours
Ocellus melanization	15 hours
Tadpole hatches	18 hours

Procedure for fertilizing *Styela* eggs:

1) Obtain 2 adults, carefully slit the test and body wall by cutting along a plane through the siphons.
2) Spread out the body halves; dissect away body tissue from gonads (testes = white, ovaries = maroon).
3) Remove gonads and place in dish of filtered seawater (be sure to add gonads from 2 adults!) and gently mix. Allow fertilization to proceed for about 5 minutes.
4) Remove fertilized eggs and place in clean dish of filtered seawater. Eggs must be removed from excess sperm for proper development. Look for eggs in which cytoplasmic streaming has begun.
5) Culture at 18°C.

Table of normal *Styela* development at 18°C:

Stage	Time Post Fertilization
2-cell	40 minutes
4-cell	70 minutes
8-cell	100 minutes
16-cell	120 minutes
32-cell	140 minutes
64-cell	160 minutes
Early Gastrula	180 minutes
Mid Gastrula	200 minutes
Neurula	5 hours
Tadpole hatches	12 hours

Procedure for obtaining *Botrylloides* embryos - metamorphosis:

Botrylloides sp. is a colonial ascidian that broods larvae. Tease apart the colonies with needles and you may release some large orange larvae. Mature larvae will undergo metamorphosis rapidly (~20-30 minutes). If you place larvae in dishes of clean seawater (before metamorphosis) you should have zooids tomorrow.

References:
Cloney, 1977; Cloney, 1979; Cloney and Cavey, 1982; Cloney, 1990

Suggestions for Experiments

The lab exercises are designed to give you a feel for the types of classical embryological experiments that have been performed on ascidian embryos. Many of these experiments can be combined with each other as well as with electroporated embryos to investigate mechanisms of cell determination and differentiation. You should try to think of some experiments that combine 2 or more of the techniques listed below. For example, you could make quarter embryos, allow them to develop and then stain for histochemical markers of differentiated cell types. You could also electroporate reporter genes into embryos, grow them for several cleavages and then cleavage arrest the embryos with cytochalasin B and look for evidence of reporter gene expression. The following list of experiments provides some specific examples of things to try during the laboratory exercises. Detailed protocols for staining and electroporation follow.

Experiment 1: Pigment cell formation in partial embryos – part A

Create twins at the two-cell stage and grow each pair of cells together, but not in contact with each other. Do both "half embryos" produce pigment cells? How many? Do the embryos preferentially produce either the otolith or the ocellus?

Experiment 1: Pigment cell formation in partial embryos – part B

At the 8-cell stage, separate animal and vegetal half embryos. Grow the halves together, but not in contact with each other. Do both "half embryos" produce pigment cells? How many? Do the embryos preferentially produce either the otolith or the ocellus?

Experiment 2: Endoderm formation in partial embryos

At the 8-cell stage, separate anterior and posterior partial embryos. Grow the halves together, but not in contact with each other. Fix and stain for alkaline phosphatase expression. Is alkaline phosphatase expressed in both the anterior and posterior partial embryos?

Experiment 3: Electroporation of ascidian eggs.

There are many transgenes for you to use to generate transgenic ascidian embryos. 20μg aliquots of each construct have been provided for individual electroporations and can be identified by the 3-letter codes shown below. **Note: be sure to obtain current list of available constructs as some of these may not be available in 2008.**

Tissue Specific Reporters:

1. Tyrosinase::CFP	TCY	pigment cells
2. Tyrosinase::H2-YFP	THY	pigment cells (nuclear)
3. Tyrosinase::mbnRFP	TMR	pigment cells (membrane localized)
4. Brachyury::RFP	BMR	notochord cells
5. Epi B::H2-CFP	EHC	epidermis (nuclear)
6. Epi B::H2-YFP	EHY	epidermis (nuclear)
7. Epi B::GFP	EBG	epidermis
8. EF::H2-YFP	EFY	ubiquitous (nuclear)
9. EF::H2-CFP	EFC	ubiquitous (nuclear)
10. EF::H2-RFP	EFR	ubiquitous (nuclear)
11. EF::H2-GFP	EFG	ubiquitous (nuclear)
12. Etr::RFP	ETR	CNS
13. β tubulin::H2-YFP	BHY	CNS & some PNS (nuclear)
14. Gelsolin::YFP	GYF	sensory neurons of PNS
15. Gelsolin::RFP	GRD	sensory neurons of PNS
16. HrM::GFP	HMG	muscle
17. Snail::H2RFP	SHR	muscle (nuclear)
18. Fkh::GFP = FoxA::GFP	FGF	notochord, endoderm, floorplate
19. Pax3/7::H2-CFP	PHC	neural plate border 6-9hr, dorsal neural tube10h-tadpole (nuclear)
20. Pax3/7::H2-YFP	PHY	neural plate border 6-9hr, dorsal neural tube10h-tadpole (nuclear)

Experimental Constructs:

21. Tyrosinase::DTA	TDT	genetic cell ablation of pigment cells
22. Forkhead::Brachyury	FBR	ectopic expression of Brachyury gene
23. Epi B::Brn3 (Pou4)-CFP	EB3	ectopic expression of Brn3-CFP fusion
24. Epi B::DnSuH-CFP	ESH	expression of dominant negative form of Suppressor of Hairless-CFP fusion
25. EF::ArtmiR-RFP	TAR	Try RNAi in ascidians, see Exp. 12
26. EF::MITF-YFP	EMY	ectopic expression of MITF, a pigment cell regulator

Electroporate constructs individually or in combination into fertilized eggs as desired. Many embryos are generated so you could coordinate and work in groups.

Constructs 21-26 will generate embryonic phenotypes. See experiments 7, 8, & 10-13 below for details on how to use these.

Experiment 4: Repeat experiment 1 with embryos electroporated with tyrosinase::C/Y/RFP.

Experiment 5: Notochord gene expression in partial embryos.

At the 8-cell stage, separate anterior and posterior partial embryos. Grow the halves together, but not in contact with each other. Observe Brachyury::RFP expression. Is RFP expressed in both anterior (primary) and posterior (secondary) lineages?

Experiment 6: CNS gene expression in partial embryos electroporated with βtubulin::H2-YFP or Etr::RFP.

At the 8-cell stage, separate animal and vegetal half embryos. Grow the halves together, but not in contact with each other. Is YFP/RFP expressed in the animal (CNS - brain) and the vegetal (dorsal neural tube) partial embryos?

Experiment 7: Genetic cell ablation of pigment cells using tyrosinase::DTA.

Co-electroporate eggs with tyrosinase::DTA and some fluorescent transgene (not tryosinase::C/Y/RFP as translation will be inhibited). Look for loss of one or both pigment cells. Is there a preference for which pigment cell is lost in cycloptic embryos?

Experiment 8: Ectopic Brachyury Expression – transfating endoderm/floor plate to notochord.

Co-electroporate Fkh::Brachyury and the Brachyury::RFP transgene. Many more cells than the normal 40 cells should express RFP. You could also perform alkaline phosphatase labeling on these embryos to see if there is a loss of alkaline phosphatase activity in the endoderm.

Experiment 9: Cleavage arrested embryos.

Use cytochalasin B (see procedure below) to cleavage arrest Ciona embryos at various stages. You could stain these embryos for alkaline phosphatase activity; observe pigment cell formation or GFP/CFP/YFP transgene expression.

Experiment 10: Ectopic expression of pro-neural transcription factors in epidermis.

Transgene #23 generates embryos with PNS phenotypes. Visualization of CFP will confirm mis-expression of the Pou4 protein. Co-electroporate with a YFP or RFP reporter like GYF and TRD (above) to examine sensory neuron or melanocyte development, respectively. (*Also try antibody staining these larvae to examine the ciliated epidermal sensory neurons using the α acetylated tubulin Ab, see procedure below)

Experiment 11: Expression of Dominant Negative SuH - transfating epidermis to sensory neurons.

Supressor of Hairless plays a role in Notch/Delta signaling and lateral inhibition, which is thought to specify sensory neurons in the PNS of *Ciona.* Interrupt lateral inhibition with this transgene (#24) and observe sensory neuron development with a reporter like GYF. Compare the number and location of sensory neurons to those of control embryos. (*Also try antibody staining these larvae to examine the ciliated epidermal sensory neurons using the α acetylated tubulin Ab, see procedure below)

Experiment 12: Ectopic expression of the pigment cell transcription factor - MITF.

Construct #26 ubiquitously expresses MITF fused to YFP. Co-electroporate with a pigment cell reporter construct of a different color to look for ectopic activation of Tyrosinase (eg. TCY or TMR).

Antibody Staining Protocol

1. Fix embryos in 2% Paraformaldehyde in sea water for 10-12 minutes (or by adding 100 µL of formalin directly to the culture dish containing ~10mL of seawater (1% formalin)).
2. Wash in PBT, REPEAT 2x
3. Wash in cold 100% Methanol
4. Wash in PBS for 10 minutes, REPEAT 3x
5. Block in 1% serum (as appropriate)/PBS for 10 minutes
6. Incubate with 1°antibody (in 1% serum/PBS) for at least 1 hr (preferably overnight) – see below
7. Wash in PBT for 10 minutes, REPEAT 3x
8. Block in 1% serum (as appropriate)/PBS for 10 minutes
9. Incubate with 2° antibody (in 100µL of 1% serum/PBS) for at least 1 hr in the dark (or overnight) – see below
10. Wash in PBT for 10 minutes, REPEAT 3x
11. Store embryos in 100µL of 50% glycerol or 50% glycerol/antifade (50:50) in the dark

1° Antibodies (working dilution 1:1000)

Mouse α-acetylated tubulin
Rabbit α-GFP (also detects YFP and CFP, but **not** RFP)

2° Antibodies (working dilution 1:200)

Goat α-rabbit (Alexa Fluor 488 & 546)
Donkey α-goat (Alexa Fluor 546)
Goat α-mouse (Alexa Fluor 546)
Donkey α-mouse (Alexa Fluor 488)

Cleavage Arrested Embryos

Cleavage arrested embryos are made by incubating embryos in seawater containing cytochalasin B at a concentration of 2 ug/ml. Cytochalasin B is a specific inhibitor of actin polymerization and will prevent microfilaments from properly forming. Under these culture conditions, cell nuclei will continue to divide and various aspects of cell differentiation can be observed. Although chorionated embryos can be cleavage arrested with this drug you will find that it is much easier to see embryos that have been dechorionated.

Microsurgical Techniques

This part takes practice! This should be similar to the sea urchin embryo dissociations you will do soon in the class. You should make very thin glass "hairs" which you can then use to dissect apart embryos. It is often helpful to place embryos in Ca^{2+} free seawater (CFSW) about 1 cleavage before the desired stage for dissection (i.e. for making quarter embryos at the 8-cell stage, place embryos into CFSW at the 4-cell stage). If you use CFSW be sure to place embryos back into normal seawater for further development. Ascidian embryos are well known for their "mosaic" development. You can take an 8-cell embryo and divide it into 4 "quarter" embryos (i.e. separate the cell pairs A4.1, a4.2, B4.1, b4.2 - see Figure 5). Allow these partial embryos to develop to about 18 hours or so. See if you can see any obvious features - e.g. notochord cells? Try using some of the staining methods below to examine muscle and/or endoderm differentiation. If you are very good, you could try microsurgery on electroporated embryos to look at notochord formation too. If you are interested in trying some of these experiments please ask about them!

Embryo Dissociations

It is possible to grow ascidian embryos in a completely dissociated state to examine cell specification mechanisms. To do this, place dechorionated embryos (protocol in electroporation section below) embryos into CFSW at least 1 cell cycle prior to the desired time (i.e. to dissociate embryos at the 8-cell stage, place into CFSW at the 4-cell stage). Gently pipet embryos several times until cells are dissociated. Continue this process at all desired cleavages. Return dissociated cells to normal seawater for continued development.

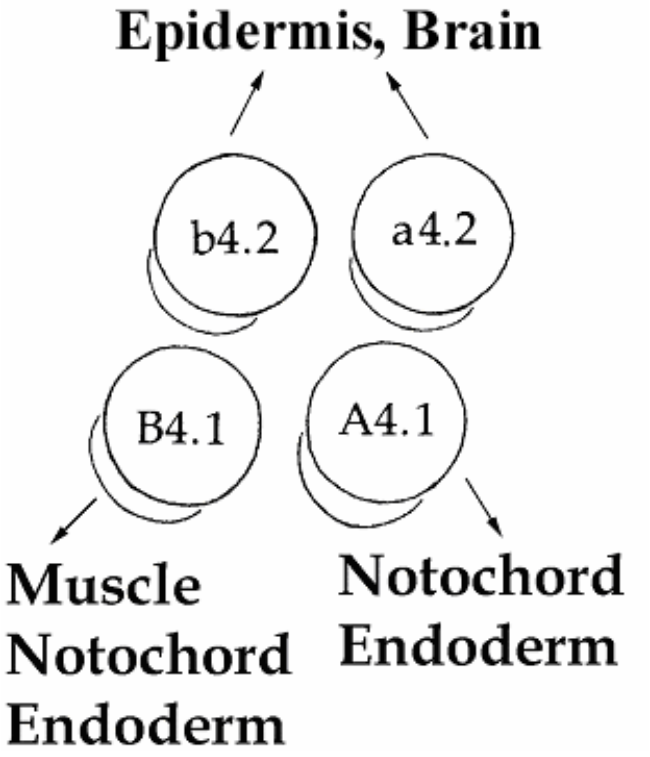


Figure 5. Major fates of quarter-cell embryos (Compiled from several sources)

Formation of Multiple Pigment Cells

It is possible to create embryos that contain many more pigment cells than the normal complement of two (the ocellus and the otolith). To do this, place fertilized (not dechorionated) embryos in CFSW at timepoints between the 16-cell stage and 110-cell gastrula stage and allow the cells to continue cleaving. Leave the embryos in CFSW throughout development or try removing half the embryos from CFSW at 110-cell gastrula through neurula stages and return them to normal seawater to see if this deminishes the effect. Grow embryos until ~18 hrs to observe pigment cell pigmentation. Try this experiment on various ascidian species for comparison.

Histochemical Staining Methods

I have listed 2 histochemical staining methods to detect endoderm and muscle differentation. This year, we will only stain for endoderm alkaline phosphatase expression. You should first try these on tadpole larvae; it works best on either dechorionated (see below) or hatched larvae. The staining methods may also be used on partial embryos and on embryos that have been arrested with cytochalasin B.

1) Alkaline phosphatase

Alkaline phosphatase will stain **endodermal** tissues in ascidian embryos. Be careful not to let the staining reaction go too far, as this will bring up background (all tissues have low levels of AP activity)

1) Grow embryos to appropriate stage, fix in 5% paraformaldehyde in seawater for 15-30 minutes on ice.
2) Rinse several times with reaction buffer (100mM Tris-HCl pH9.5, 100mM NaCl, 5mM $MgCl_2$) to remove fixative.
3) Incubate in reaction medium (scale as necessary - 1 ml reaction buffer, 2.2 ul NBT stock (75 mg/ml in 70% DMF), 1.5 ul BCIP stock (50 mg/ml in 100% DMF)).
4) Allow color to develop to desired intensity, stop reaction by rinsing specimens into PBS.

References: Whittaker, 1990.

2) Acetylcholinesterase – we are not doing this in 2007

Acetylcholinesterase will stain **muscle cells** in ascidian embryos.

1) Fix embryos in 5% paraformaldehyde in seawater for 15 minutes on ice.
2) Remove fixative and wash with clean seawater.
3) Add staining reaction mixture*, incubate 2-4 hours at 18°C or RT.
4) Stop reaction by running embryos in PBS.
5)

*Reaction mixture (Add in following order, with swirling to mix):

20 mg acetylthiocholine iodide
26 ml 100 mM sodium maleate buffer, pH 6.0
2 ml 100 mM Citrate
4 ml 30 mM Copper sulfate
4 ml distilled water
4 ml 0.5 mM potassium ferricyanide

References:
Whittaker, 1973

3) *LacZ* Staining Protocol

(We are not staining any *LacZ* constructs this year, but this is the protocol)

This is a quick protocol for staining embryos that have been labeled by *lacZ*.

1) Fix embryos by adding 3 drops of formaldehyde to the 10 plastic dish in which they are growing.
2) Make the staining buffer (1 ml):
   100 ul redox buffer
   (50mM Kferricyanide, 50mM Kferrocyanide)
   1 ul 1M MgCl2
   4 ul 1% SDS
   895 ul SW
3) Place fixed embryos in a small, agar-coated dish or well slide in the staining buffer.
4) Add 8 ul 3% Xgal per ml of staining buffer.
5) To stop staining, rinse embryos well in PBS or SW.
6) Post fix with 4% paraformadehyde and run through EtOH series to mount in resin if desired.

Electroporation of exogenous DNA into fertilized *Ciona* eggs.

This section is pretty long and the procedure, while not hard, has many steps that need to be executed fairly quickly. Please read it through and ask questions before trying to electroporate! Please DO NOT USE the electroporation device until you have been properly instructed

In the last several years we developed a technique for the efficient introduction of exogenous DNA into ascidian eggs. Until this time we had relied on microinjection techniques. While microinjection works, it is slow and you get very few normally developing embryos when you are finished after a long, hard day. On the other hand, electroporation allows one to generate hundreds or thousands of embryos in about an hour. In this section you will have the opportunity to examine the expression of different tissue-specific enhancers driving green fluorescent protein (GFP) as a reporter. First let's look at how electroporation works:

Electroporation works by applying a short, pulsed voltage to cells suspended in a DNA solution. The voltage applied across the cell causes the cell membrane to break down at which point molecules can be taken up into the cell. After the voltage pulse, the cell membrane reseals. It is still unclear exactly how the molecules get into the cell. Current theory suggests that DNA is "electrophoresed" into the cells. There is also an apparent tradeoff between survival and expression of exogenous DNA constructs. Commercial electroporation units are available but are rather expensive. We optimized BioRad's Gene Pulser for our initial experiments achieving ~50-75% survival and usually >50% of what survived expressed the construct of interest. In the course you will use the third generation ascidian electroporation device. This machine is simple to use and optimized to provide a high percentage of transgenic embryos (~85%) with ~50% mosaic expression.

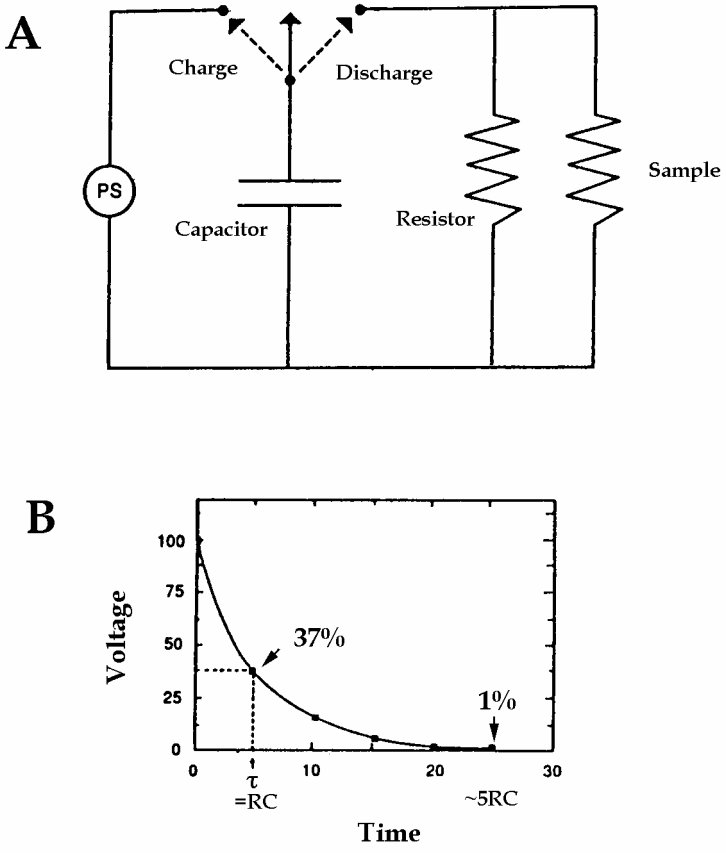


Figure 6. Electroporation theory. (**A**) Circuit diagram. DC voltage is applied to a capacitor. The charge is then applied to the sample that is in parallel with a second resistor. (**B**) Typical exponential decay of a capacitor-resistor discharge. The time constant is the time at which the applied voltage drops to about 37% of its initial value. This time is equal to the product RC where C is capacitance in farads and R is resistance in ohms. The time at which the voltage drops to about 1% of its initial value is equal to about 5RC.

A DC voltage is applied to a capacitor (Figure 6A). The capacitor "stores" this voltage until it is discharged across the sample. The critical parameter to this method seems to be the length of the pulse. As shown in Figure 6B, the discharged voltage drops at an exponential decay (typical for a capacitor-resistor circuit). The time constant has a value of RC (resistance X capacitance). If your sample has a high resistance (ours will) then the time constant will be too long for success. If you place a second resistor in parallel with the sample (Figure 6A) and your sample resistance is very large, then the total resistance will effectively be equal to the second resistor (don't worry about the electronic theory, just know that the resistor you will chose will play the major role in determining the length of the pulse). Thus you can use the second resistor to "control" the time of the pulse.

A 50 VDC input is provided by a standard electrophoresis power supply. Our sample cuvette has an electrode spacing of 0.4 cm so the electric field applied to our embryos is 125 V/cm. Please ask for the latest settings for capacitance and resistance. The basic procedure will be to set the capacitance and resistance, fill the cuvette, charge the device and discharge the device. That's pretty much how electroporation works. There are several different constructs to try – consult chalkboard.

References for some of the constructs: Corbo, 1997; Corbo *et al.*, 1997

Specific protocols for Electroporation (See Zeller, 2004, Zeller et. al. 2006):

1) Add 500 ul 0.77M mannitol to your DNA samples (25 µg supercoiled, CsCl banded).
2) Mix dechorionation solution:
   100 mg sodium thioglycolate
   10 ml sea water, 25 ul 10M NaOH.

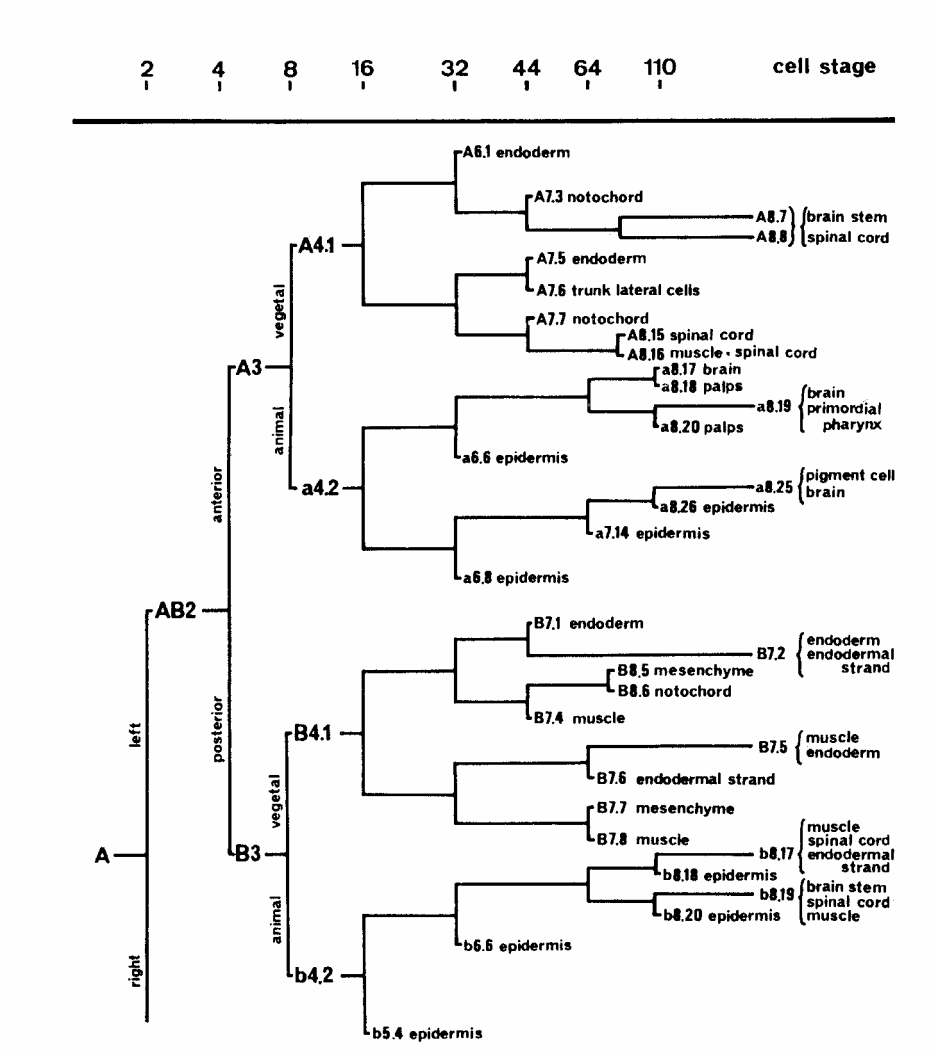
3) Fertilize eggs as you normally would, then proceed with chemical dechorionation:
   Chemical dechorionation:
   1) Concentrate fertilized eggs with egg basket; add to 35 mm dish.
   2) Exchange with 1-2 changes of dechorionation solution.
   3) Add 1 tube of protease (final conc. 0.05%), occasionally swirl/pipet eggs.
   4) After about 5-7 minutes, eggs should be dechorionated.
   5) Transfer through 3 changes of clean seawater.

4) Add ~300 ul seawater and eggs (several hundred) into DNA sample (final vol. ~800 ul).
5) Verify capacitance/resistance settings on electroporator.
6) Add eggs/DNA to cuvette.
7) Charge electroporator.
8) Discharge electroporator.
9) Remove eggs, place in dish with clean seawater and antibiotics (10 ul) at 18°C.

**Note for *Ascidiella* eggs, fertilize eggs and place 10 mls of eggs/seawater in a 15 ml conical tube. Add 100 mg thioglycolate directly to the tube followed by 32 ul of 10M NaOH. Pour contents into 60 mm dish and add 1 tube of protease (final protease concentration is 0.025%).

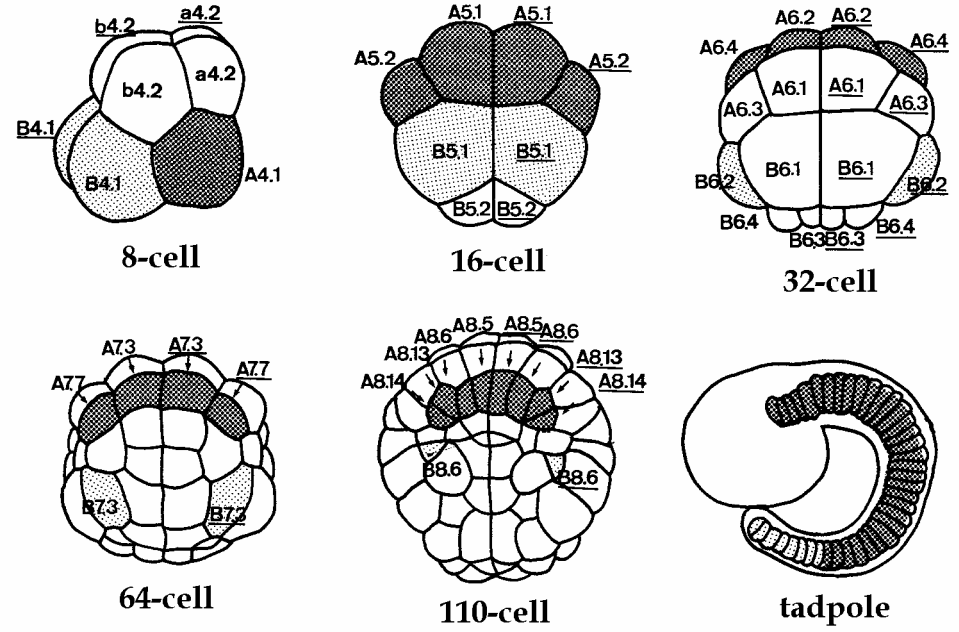
Appendix I.

Cell lineage diagram (From Satoh, 1994)



Appendix II.

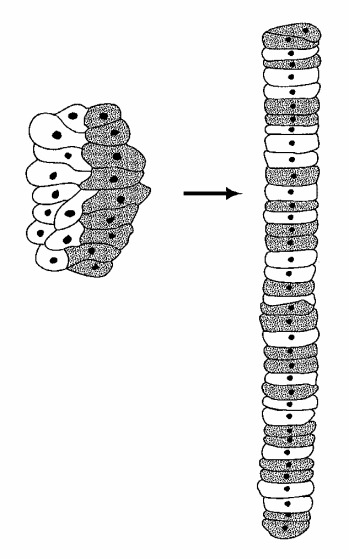
Notogenesis (diagrams from Cloney, 1990; Jeffery, 1992; Satoh, 1994)



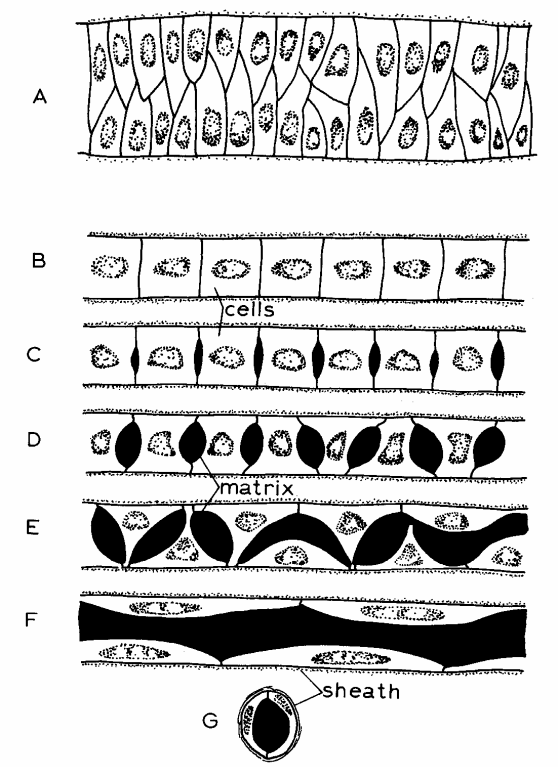
Notochord lineage. The notochord is derived from 4 founder cells formed at the 64-cell stage (the A7.3 and the A7.7 cell pairs) which will give rise to the 32 primary lineage cells and 2 founder cells formed at the 110-cell stage (the B8.6 cell pair) which will give rise the 8 secondary lineage cells. The notochord is induced at the 32-cell stage. It has been shown that A6.1 or A6.3 (endoderm precursors) can induce the precursor cells to form notochord (Nakatani and Nishida, 1994). They also showed that A6.2 pairs can form notochord and that A6.2 + A6.4 can form notochord. At the 64-cell stage, the *Brachyury* gene turns on only in the 4 cells fated to become notochord. These cells will undergo 3 more divisions until the 32 primary notochord cells are formed (dark gray in diagram). At the next division, the 110-cell stage, the B8.6 cells are fated to be the secondary notochord lineage founder cells and *Brachyury* turns on. They will undergo 2 more divisions to form the 8 secondary notochord cells (light gray in diagram). If the induction event at the 32-cells stage does not occur, notochord will not form (hint: you may wish to try the following experiment with embryos electroporated with the *Brachyury*-GFP reporter: dissociate embryos before and after the 32-cell stage with calcium-free SW and see what happens to the GFP reporter gene expression).

References:

Nishida, 1992; Nakatani and Nishida, 1994; Nakatani *et al.*, 1996; Tanaka, Chiba and Nishikata, 1996



The process of cell intercalation/extension in the ascidian embryo. Cells from each half of the embryo migrate to form a single file "stack of coins" arrangement.



This diagram shows what is believed to happen during notochord vacuolization. Initially, small vacuoles form either within or between cells. These vacuoles continue to grow in size and will eventually fuse to form the lumen of the notochord (the notochord at this point is similar to a pipe that is capped at both ends. The lumen of the notochord is like the inside of the pipe).

Appendix III

Selected Ascidian References:

Reviews:
Satoh, 1994; Satoh and Jeffery, 1995; Satoh *et al.*, 1996; Satoh, 2001; Satoh, 2003; Satoh *et al.*, 2003; Okamura, Okado and Takahashi, 1993; Nishida, 1997; Nishida, 1997; Di Gregorio and Levine, 1998; Corbo, Di Gregorio and Levine, 2001; Satoh, 2001; Di Gregorio and Levine, 2002

Cell Lineage:
Conklin, 1905; Nishida and Satoh, 1983; Nishida and Satoh, 1985; Nishida, 1987

Genomics:
Satou, Imai and Satoh, 2001; Satou *et al.*, 2002; Chiba *et al.*, 2003; Mochizuki, Satou and Satoh, 2003; Wada *et al.*, 2003; Yagi *et al.*, 2003

Genetics:
Moody *et al.*, 1999; Nakatani, Moody and Smith, 1999; Sordino *et al.*, 2001; Sasakura *et al.*, 2003

Notochord:
Cloney, 1964; Whittaker, 1973; Miyamoto and Crowther, 1985; Crowther and Whittaker, 1986; Nakatani and Nishida, 1994; Yasuo and Satoh, 1994; Yasuo, Harada and Satoh, 1995; Nakatani *et al.*, 1996; Corbo, Levine and Zeller, 1997; Nakatani and Nishida, 1997; Corbo *et al.*, 1998; Yasuo and Satoh, 1998; Di Gregorio and Levine, 1999; Hotta *et al.*, 1999; Takahashi *et al.*, 1999; Takahashi *et al.*, 1999; Hotta *et al.*, 2000; Di Gregorio, Corbo and Levine, 2001; Shimauchi, Chiba and Satoh, 2001; Shimauchi, Murakami and Satoh, 2001; Imai, Satoh and Satou, 2002; Imai, Satoh and Satou, 2002; Imai, Satou and Satoh, 2002; Takada *et al.*, 2002

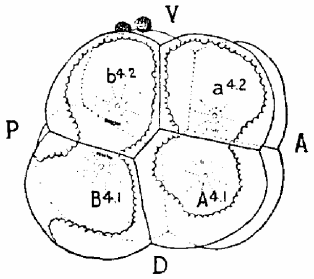
Nervous System:
Nicol and Meinertzhagen, 1988; Nicol and Meinertzhagen, 1988; Nicol and Meinertzhagen, 1991; Okado and Takahashi, 1993; Okamura, Okado and Takahashi, 1993; Crowther and Whittaker, 1994; Wada *et al.*, 1996; Corbo *et al.*, 1997; Wada *et al.*, 1997; Takamura, 1998; Wada *et al.*, 1998; Darras and Nishida, 2001; Ohtsuka, Obinata and Okamura, 2001

Muscle:
Whittaker, 1973; Whittaker, Ortolani and Farinella-Ferruzza, 1977; Whittaker, 1982; Crowther and Whittaker, 1983; Meedel, Farmer and Lee, 1997; Erives, Corbo and Levine, 1998; Kim and Nishida, 1999; Kim, Yamada and Nishida, 2000; Nishida and Sawada, 2001; Kobayashi *et al.*, 2003; Kondoh, Kobayashi and Nishida, 2003

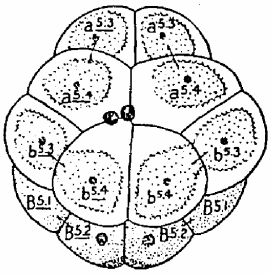
Appendix IV.

Conklin's (1905) ascidian embryo diagrams.

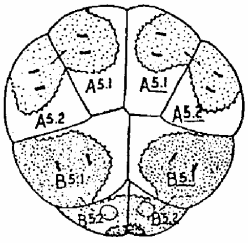
The following diagrams are presented to help you identify the blastomeres of different staged embryos. The first figure is a composite of the first few cleavages to help with orientation. The last figures are more detailed in nature.



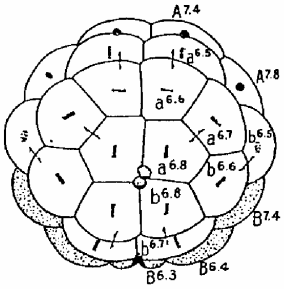
8-cell



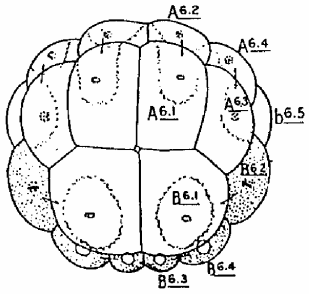
16-cell, animal



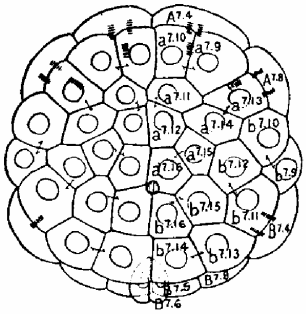
16-cell, vegetal



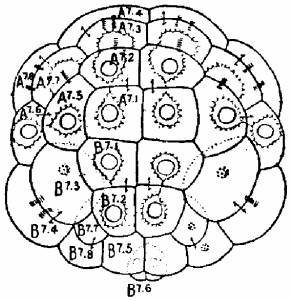
32-cell, animal



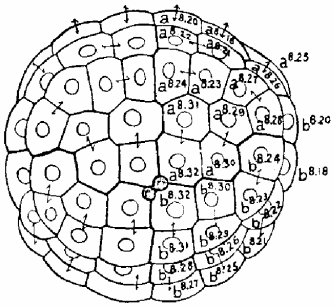
32-cell, vegetal



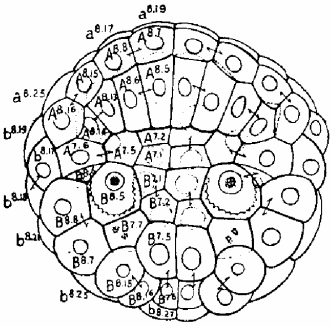
64-cell, animal



64-cell, vegetal



110-cell, animal



110-cell, vegetal

Plate VII

Sections of Eggs of Cynthia partita; Fertilization and Early Cleavages.

Fig. 88. Division of sperm aster; crescent substance at periphery on posterior side.

Fig. 89. Similar to preceding but showing egg nucleus.

Figs. 90 and 91. Sections at right angles to first cleavage plane but oblique to egg axis, showing the union of pronuclei; clear and yellow protoplasm and sperm amphiaster in posterior half of egg.

Fig. 92. Section in plane of the first cleavage (future median plane) showing the union of the germ nuclei; the clear protoplasm and crescent on the posterior side of the egg, and the polar bodies above.

Fig. 93. Early prophase of the first cleavage, in the plane of the spindle axis.

Figs. 94 and 95. Two dispermic eggs; the first showing two sperm nuclei on the posterior side of the egg with a single crescent; the second, two sperm nuclei and one egg nucleus, with the clear protoplasm and crescent about equally divided, by a tongue of yolk, between the two sperm nuclei.

Fig. 96. Section in the plane of the first cleavage and transverse to the spindle, showing a cross section of the equatorial plate near the middle of the egg, surrounded by clear protoplasm, also the crescent at the posterior border with some of the yellow spherules all around the spindle.

Fig. 97. Metaphase of the first cleavage; equatorial section at right angles to the preceding.

Figs. 98 and 99. Early and late anaphases of the first cleavage; sections in equatorial plane.

Fig. 99. Constriction of cell body; chromosomal vesicles at the ends of the nuclear spindle. In both figures the daughter centrosomes are elongating in the equatorial plane and at right angles to the first spindle axis.

Fig. 100. Telophase of the first cleavage, showing the rotation of nuclei, centrosomes and cytoplasm toward the animal pole of the egg.

Fig. 101. Equatorial section, prophase of second cleavage; crescent substance at the posterior ends.

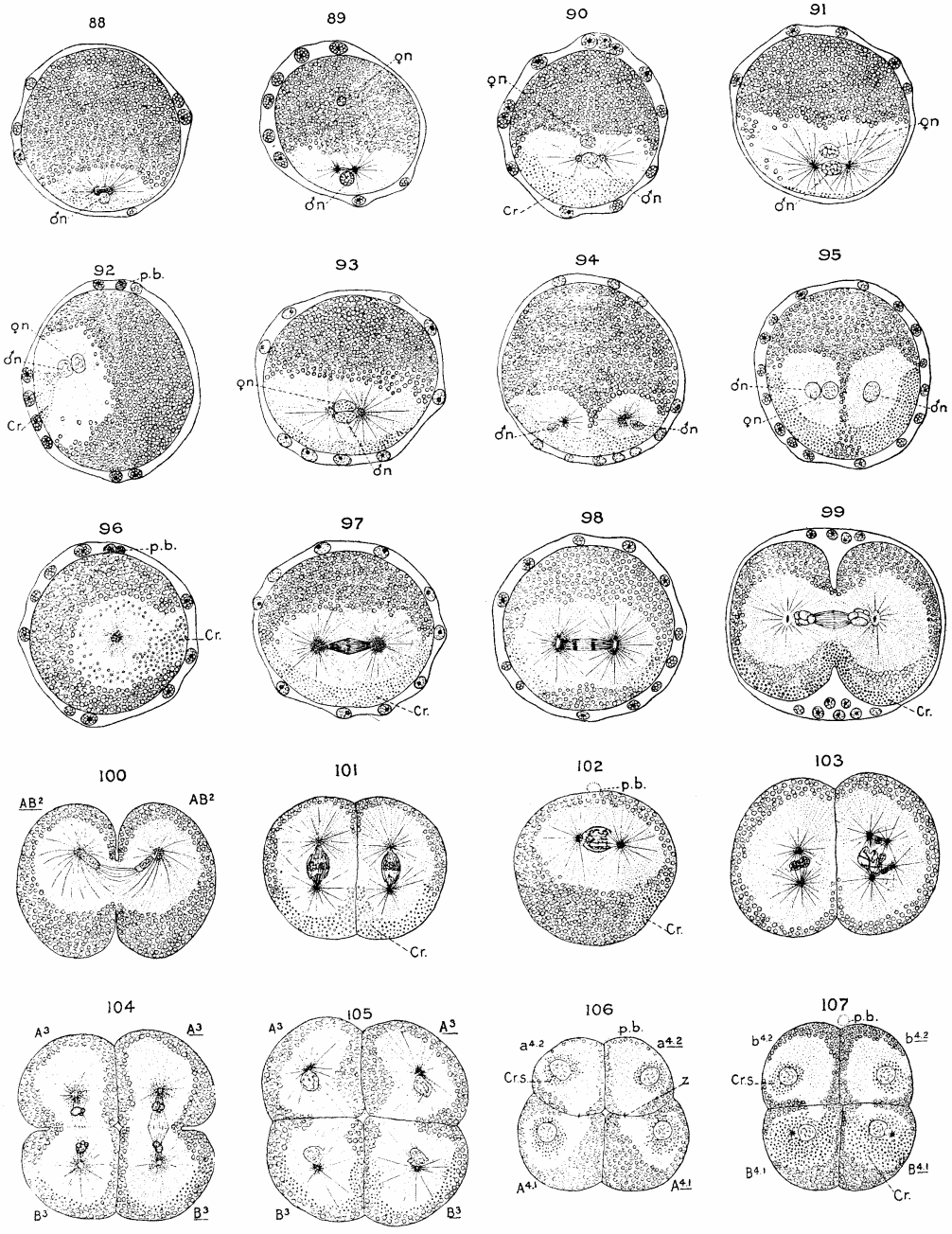
Fig. 102. Section at right angles to the preceding an through the axis of one of the spindles; nucleus and cytoplasm lie above the equator, yolk and crescent below.

Fig. 103. Equatorial section showing precocious division of the centrosomes in one of the blastomeres.

Fig. 104. Anaphase of second cleavage; equatorial section; chromosomal vesicles at ends of nuclear spindles.

Fig. 105. Telophase of second cleavage; equatorial section, showing the bending of the middle of the spindle toward the center of the egg, and a large amount of clear cytoplasm and of crescent substance in the posterior quadrants.

Figs. 106 and 107. Two sections through one and the same egg in the eight-cell stage; the first through the anterior blastomeres, the second through the posterior. The cytoplasm is most abundant in the cells at the animal pole, the crescent substance in the two posterior-vegetal cells, though it is also found around all nuclei. The polar bodies, which are shown in stippled outlines, are not in the plane of either of these sections, but in the region between them.



CONKLIN, Organization of Ascidian Egg.

Plate VIII.

Surface Views of Entire Eggs of Cynthia partita; Eight to Forty-four Cells.

Fig. 108. Eight-cell stage; left side of egg; showing spindles of third cleavage.

Fig. 109. Anterior view of 8-cell stage, showing cytoplasm most abundant in the animal pole cells, and the yolk largely collected in the anterior cell of the vegetal hemisphere.

Figs. 110, 111, 112. Stages in the fourth cleavage; figs. 110 and 112 viewed from the animal pole, fig. 111 from the vegetal pole.

Fig. 113. Telophase of fourth cleavage, vegetal pole view; caps of deeply staining protoplasm lie at the hinder borders of the small posterior cells (B5.2).

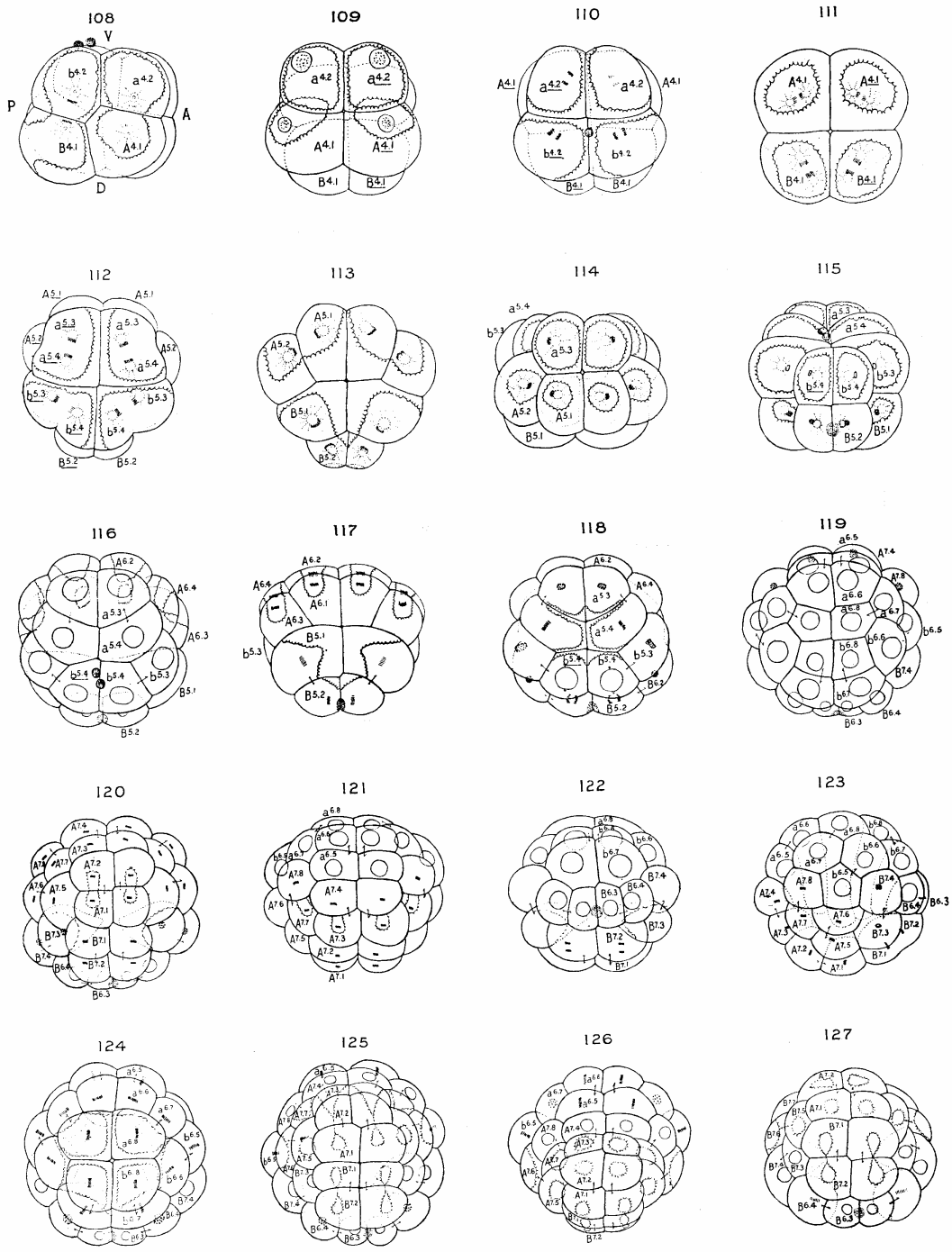
Figs. 114 and 115. Anterior and posterior views of the 16-cell stage; fig. 115 showing caps of deeply staining protoplasm at the posterior pole, which later go into the posterior mesenchyme cells (B7.6, figs. 130, 131).

Figs. 116 and 117. Ventral and dorsal views of a 20-cell stage, showing the cells at the vegetal pole dividing before those at the animal pole.

Fig. 118. Slightly older stage with some of the animal pole cells dividing.

Figs. 119 - 123. Five views of one and the same egg; fig. 119, ventral; 120, dorsal; 121, anterior; 122, posterior; 123, right side; the latter shows in dotted outlines the great elongation of the cells at the animal pole and the flattened shape of the cells at the vegetal pole; all the designations of cells in fig. 123 should be underscored; 44 cells, 16 ectoderm, 10 endoderm, 10 mesoderm, 4 chorda and 4 neural plate cells.

Figs. 124-129. Six different views of one and the same egg in the 44-cell stage showing the divisions of the ectodermal cells and the second cells of the crescent (B6.4); when these divisions are completed there will be 62 cells. Fig. 124, ventral; 125, dorsal; 126, anterior; 127, postereo-dorsal.



CONKLIN, Organization of Ascidian Egg.

Plate IX.

Surface Views of Entire Eggs of Cynthia partita; Forty-four to Two Hundred and Eighteen Cells.

Figs. 128, 129. Same egg as shown in figs. 124-127. Fig. 128 from the left side; the equator of the egg (plane of the third cleavage) is the heavy line running between A and P and separating cells designated by lower case from those designated by capital letters. Fig. 129. View from the posterior pole.

Figs. 130 and 131. Ventral and dorsal views respectively of one and the same egg; 64 cells, 32 in each hemisphere, distributed as follows: Ventral hemisphere, 26 ectoderm, 6 neural plate cells (a7.10, a7.9, a7.13); Dorsal hemisphere, 10 endoderm, 4 chorda (A7.3, A7.7), 4 neural plate (A7.4, A7.8), 10 mesenchyme (B7.6, B7.5, B7.7, B7.3, A7.6), 4 muscle (B7.8, B7.4).

Fig. 132. Postereo-dorsal view of an egg in the same stage as the preceding showing direction of division of mesenchyme cell (B7.3).

Figs. 133 and 134. Ventral and dorsal views respectively of one and the same egg; 110 cells; Ventral hemisphere 64 cells, 52 ectoderm, 12 neural plate (a8.19, a8.20, a8.17, a8.18, a8.25, a8.26);Dorsal hemisphere 46 cells, 10 endoderm, 8 chorda (A8.5, A8.6, A8.13, A8.14), 8 neural plate (A8.7, A8.8, A8.15, A8.16), 12 mesenchyme (B7.6, B7.5, B7.7, B8.6, A7.6), 8 muscle (B8.7, B8.8, B8.15, B8.16). Gastrulation has begun.

Fig. 135. Dorsal view of a slightly more advanced stage showing increasing gastrulation; 118 cells, ventral hemisphere 64 cells, dorsal hemisphere 54, *viz.*, 10 endoderm, 16 mesenchyme, 12 muscle, 8 chorda, 8 neural plate; when the divisions indicated by spindles are completed there will be 4 additional endoderm and 2 additional mesenchyme cells.

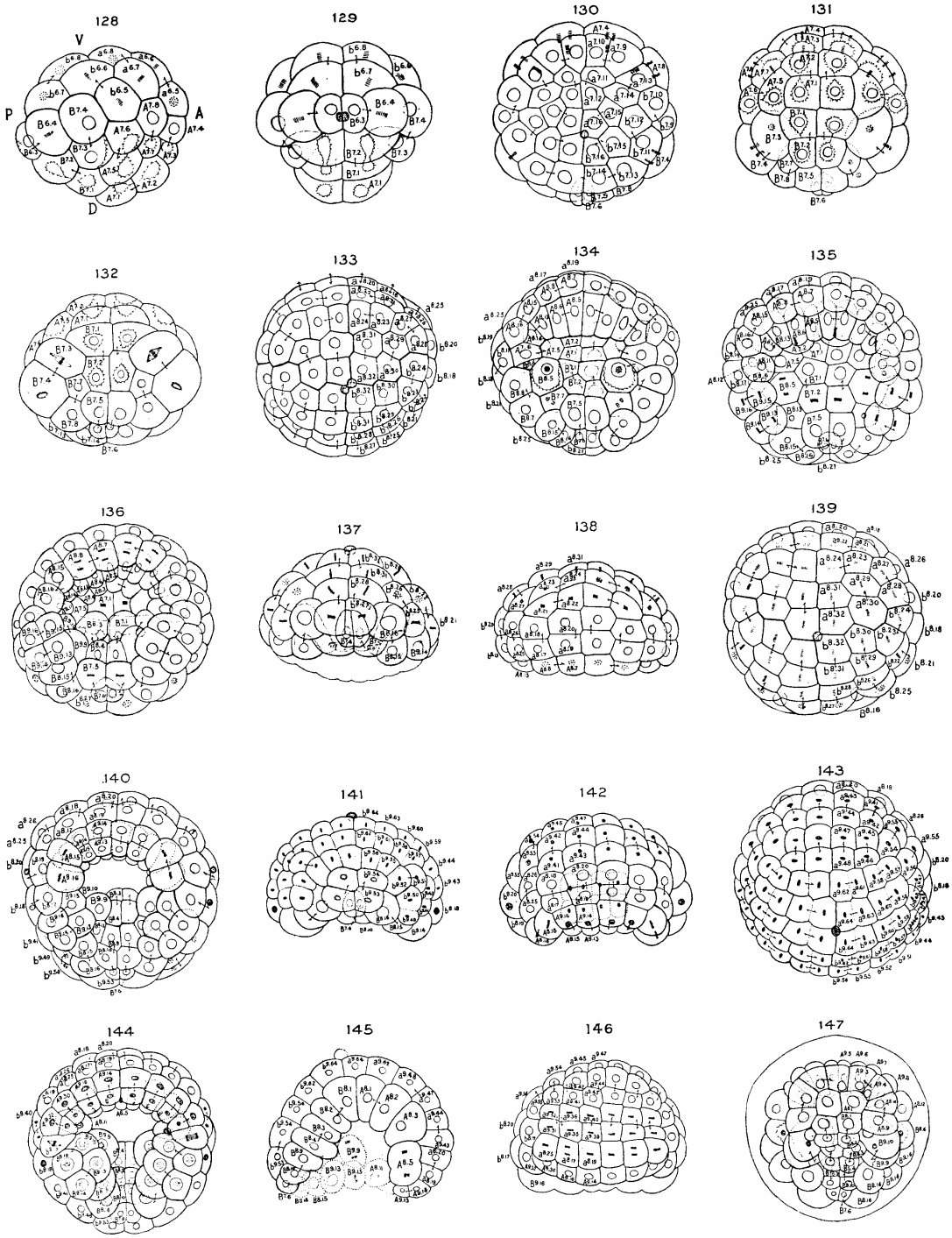
Figs. 136-139. Fours views of one and the same egg; fig. 136 dorsal, 137 posterior, 138 anterior, 139 ventral; gastrulation well advanced. 124 cells; Ventral hemisphere 64 cells, 52 ectoderm, 12 neural plate (a8.19, a8.20, a8.17, a8.18, a8.25, a8.26); Dorsal hemisphere 60 cells, 14 endoderm, 8 chorda, 8 neural plate, 18 mesenchyme, 12 muscle. Spindles are already present for divisions, which, when completed, will lead to 178 cells, viz., 96 ectoderm, 12 neural plate of ventral hemisphere, 12 neural plate of dorsal hemisphere, 8 chorda, 20 mesenchyme, 12 muscle, 18 endoderm.

Figs. 140-143. Four views of one and the same egg; 140 dorsal, 141 posterior, 142 anterior, 143 ventral. Gastrulation is here far advanced. 180 cells; Ventral hemisphere 108 cells, 96 ectoderm, 12 neural plate; Dorsal hemisphere 72 cells, 12 neural plate of dorsal hemisphere, 8 chorda, 20 mesenchyme, 12 muscle, 20 endoderm; when the divisions indicated by spindles are completed there will be 4 additional neural plate cells.

Figs. 144 and 145. Two views of the same egg; 144 dorsal, 145 median optical section in sagittal plane. In fig. 145 the polar body is not visible, but its supposed position is indicated by the dotted outline at the animal pole; the dotted outlines at the lower pole indicate the mesoderm cells which lie in the lateral lip of the blastopore and out of the plane of the section; the rolling in of the muscle cells in the lateral lip is well shown.

Fig. 146. Anterior view of an egg about the same stage as the preceding, showing the division of the 12 neural plate cells of the ventral hemisphere.

Fig 147. Ventral view of a similar stage with the ectoderm omitted in order to show the endoderm and mesoderm from the ventral side. At this stage all the ectoderm cells have passed into the 9^{th} generation, all the endoderm into the 8^{th} or 9^{th}, all the mesoderm except B7.6 into the 8^{th} or 9^{th}, all the chorda and neural plate cells into the 9^{th}. There are 218 cells; Ventral hemisphere 128 cells, 104 ectoderm, 24 neural plate of ventral hemisphere; Dorsal hemisphere 90 cells, viz., 16 neural plate, 16 chorda, 20 mesenchyme, 12 muscle, 26 endoderm.



CONKLIN, Organization of Ascidian Egg.

Plate X.

Entire Embryos of Cynthia partita; Gastrula to Tadpole.
Muscle cells shaded by vertical lines, mesenchyme by transverse lines, nerve plate and tube by fine stipples, chorda by coarse stipples.

Figs. 148-151. Four views of the same embryo; fig. 148 dorsal, 149 ventral-posterior, 150 same view but deeper level, showing mesoderm and endoderm, 151 right side. Many of the ectoderm cells are passing into the 10^{th} generation.

Figs. 152-154. Three views of one embryo; fig. 152 dorsal, showing closure of the blastopore from in front, 153 same view but deeper level showing cells beneath the ectoderm, 154 ventral view of mesoderm and endoderm below ectoderm.

Figs. 155 and 156. Two ventral views of an advanced gastrula, the first showing the superficial ectoderm, the latter the endoderm and mesoderm lying beneath the ectoderm.

Fig. 157. Left side of embryo, showing, in optical section, muscle cells, mesenchyme, chorda and nerve plate.

Figs. 158 and 159. Two optical sections of the same embryo, the former (158) through the open blastopore at the posterior end of the embryo, the latter (159) through the region anterior to the blastopore.

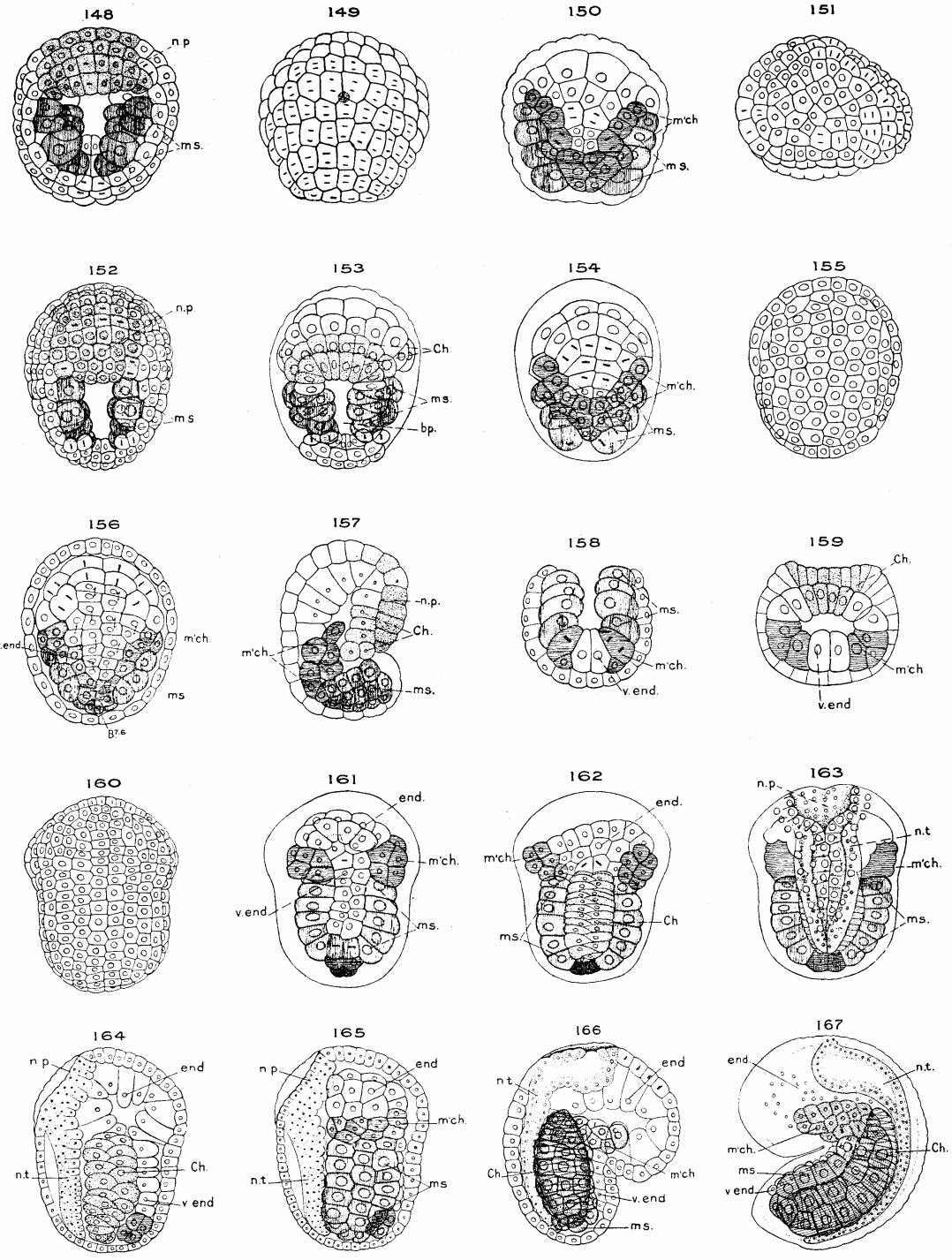
Figs. 160-162. Three views of the same embryo from the ventral side but drawn at different levels; 160 surface view showing ectoderm; 161 same view deeper level, showing mesoderm and endoderm; 162 same view still deeper level, showing chorda, mesoderm and endoderm.

Fig. 163. Dorsal view of same stage as preceding showing nerve plate and tube, mesoderm and chorda.

Figs. 164 and 165. Two views of the same embryo from right side; fig. 164 median optical section showing chorda, nerve tube and endoderm; 165 more superficial view showing muscle cells and mesenchyme.

Fig. 166. Older embryo from right side, showing in optical section nerve tube, chorda, mesoderm and endoderm.

Fig. 167. Young tadpole from left side showing in optical section nerve tube, chorda and mesoderm.



CONKLIN, Organization of Ascidian Egg.

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