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Chick-embryo culture techniques employed at Karnatak University in Dharwad, India, for studying cellular and molecular aspects of morphogenesis

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ABSTRACT The objective of the courses which this syllabus describes is to expose developmental biologists to embryo culture and embryo manipulation techniques and applications in quantitative analyses. The laboratory program complements classroom teaching by exposure to both inductive and deductive methodologies. Developmental biology teaching requires good background in cell biology, molecular biology and genetics. Developmental biology research requires computer literacy and an aptitude for quantitative methodology and graphics.

KEY WORDS: *chick embryo, culture, single-ring chick embryo culture, double-ring culture method, avian embryo technique, chick embryo laboratory exercise*

Background Information

Scholarly Interests of the Author

Major research interests include the structure of the eukaryotic chromatin, development and differentiation of the eye lens, and cell cycle regulation. More recently, I have been involved with curriculum development in biotechnology, quantitative computerized methods in biology, the development of a context-based knowledge management learning system, and English for scientists.

Representative Publications

BHARGAVA S. and MODAK, S.P. (2002) Expression of nerve growth factor during the development of nervous system in early chick embryo. *Dev. Brain Res.* 136: 43-49.

DESHMUKH, S. SONAWANE, M. and MODAK, S.P. (2001). The neural inductive response of competent chick ectoblast decreases away from the host axis and correlates with an increased proliferative activity. *Int.J.Dev.Biol.* 45: 759-766

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MODAK, S.P. (1992-98). Twenty-five 30-min video programs for the Indian Educational Television Network (Doordarshan). Discussions on cell, developmental, and molecular biology and biotechnology with guest scientists (e.g., Makoto Asashima, University of Tokyo; George Spohr, University of Geneva; Edwin Southern, Oxford University; Jim C. Smith, National Institute for Medical Research (UK); John Gurdon, Cambridge University; Ronald W. Hart, National Center for Toxicological Research (US); Jean Jacques Lawrence, Institut Albert Bonniot; M. Muramatsu, Tokyo Medical University, Japan).

MODAK, S.P., GHATPANDE, S.K., RANE, R.K. and MULHERKAR, L. (1993). Caudalization by Retinoic acid is correlated with inhibition of cell population growth and expansion of chick blastoderms cultured *in vitro*. *Int. J. Dev. Biol.* 37: 601-607.

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General Teaching Philosophy

I teach life science in sequence as follows: particles, atoms, molecules, macromolecules, organelles, cells, tissues, organs, organisms, societies, diversity, systematics and evolution. I believe one should speak with clarity and brevity and make ample use of metaphor to simplify apparently complex processes and structures. For example, the transcriptional unit with superimposed coding sequence is compared to an athletics race track, and the genetic code is taught as a language comparable to, but immensely simpler than, English. Students are allowed to ask questions at any time. If none are forthcoming, I question students one by one until a collective and composite answer is generated. All teachers of theory courses must also teach laboratory exercises. This method is applied to both undergraduater and postgraduate (*graduate*) courses I developed a summer training program that sends M.Sc. students to laboratories and universities all over India, where they spend 2 months obtaining hands-on experience in modern biology research.

General Features of the Developmental Biology Courses

An introductory course in developmental biology (25 h lecture, ten 3-h labs) is required for a degree (B.Sc. or M.Sc.) Two more specialized courses in developmental biology, Developmental Biology I and Developmental Biology II, are electives. Each has both a lecture and a laboratory component. Before taking developmental biology, students must have taken basic physical chemistry, biochemistry, and molecular biology even for the introductory course**.** For the specialization in Developmental Biology, postgraduate students must take DB I and DB II in sequence. Developmental Biology I deals with rearing and surgical and chemical manipulation of amphibian embryos (exogastrulation, transplantation of the dorsal lip of the blastopore, treatment with LiCl, parabiosis, etc.) and staging and histology of frog and chick embryos. Developmental Biology II focuses on *in vitro* methods of culturing the chick embryo and experimental manipulations, such as treatment with retinoic acid and trypan blue, and surgical operations, such as transplanting Hensen's node for neural induction, extirpating Hensen's node during and after gastrulation to examine the effect on morphogenetic movements, and marking cells with colored Sephadex beads to study morphogenetic movements. Laboratory exercises are coordinated with lecture content whenever possible. The students must also complete just one research project using a specific experimental model.

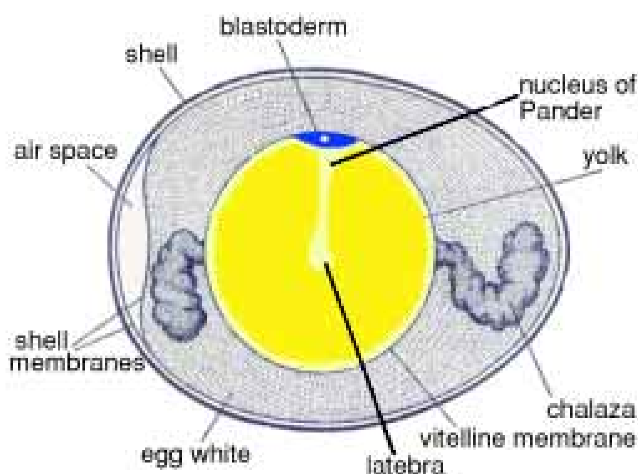

Fig. 1. Sagittal section of a hen egg showing the latebra and Pander's nucleus. *The blastoderm lies over the nucleus of Pander and beneath the vitelline membrane. The chalazae are balancers which support the yolk. The two shell membranes are separated by an air space at the blunt end of the shell (adopted from Bellairs, R.,* Developmental Processes in Higher Vertebrates, *1971).*

M.Sc. Course Outline

Developmental Biology I

1. Spermatogenesis in mammals, structure of sperm. Regulation of sperm locomotion.
2. Oogenesis in amphibia and mammals. Types of eggs. Molecular strategy: synthesis, transport and storage of yolk in amphibia. synthesis and storage of maternal gene products during amphibian oogenesis. Ribosomal gene amplification. mRNA transcription of lampbrush chromosomes.
3. Cellular and biochemical processes during fertilization and strategies for monospermy and conservation of species specificity. Acrosome reaction and signal transduction. Egg activation.
4. Types of cleavages and blastulation in *Drosophila*, *Amphioxus*, frog, and chick.
5. Early development of *Drosophila*. Morphogen gradients and role of pattern-forming genes in the establishment of body axis: anteroposterior, dorsoventral and bilateral symmetry. Hox complex in mammals and the fly.
6. Nieuwkoop center, primary organizer and mesoderm induction (Slack and Smith experiments).
7. Gene expression during early development and mid-blastula transition.
8. Gastrulation in the frog and chick: presumptive fate maps and morphogenetic cell movements. Origin of embryonic endoblast.
9. Early mammalian development till implantation.
10. Cell-cell interactions: temporal and positional specificity in neural induction and neural competence in vertebrates. Molecular signaling by inducers and hierarchy in anteroposterior polarity of the neural tube. Animal cap model and experiments in *Xenopus*.
11. Concept of growth and differential cell proliferation in shaping organ primordia.
12. Development of somites and formation of presumptive derivatives.
13. Cell differentiation: lens development, cell and molecular biology of lens fiber cell differentiation.
14. Cell dedifferentiation and tissue metaplasia in the eye lens regeneration and comparison with neoplasia.
15. Oncogenes in development.
16. Myogenesis, erythropoiesis and chondrogenesis. The concept of quantal mitosis.

Developmental Biology II

17. Nucleo-cytoplasmic interactions in ameba and *Acetabularia*.
18. Morphogenetic cell death in the interdigital regions and during closure of body tubes.
19. Limb development and regeneration.
20. Tail regeneration in amphibia.
21. Mammalian embryo fusion: allopheny.

22. Amniocentesis: karyotyping and biochemical tests for genetic counseling.
23. Genetic defects and teratogenesis.
24. Transfer by microinjection, electroporation and transfection of genes in single cells, eggs, zygotes, and blastomeres using reporter sequences.
25. *In vitro* fertilization in mammals.
26. Embryonic stem cell culture, mutagenesis and transfection using cloned genes. Knockout mouse model.
27. Nuclear transplantation experiments of Briggs and King and Gurdon in the frog. Nuclear transplantation and cloning in mammals. The concept of totipotency.
28. Genome equivalence: the *Ascaris* case (Boveri, Weissmann, and Ursprung) .
29. *Caenorhabditis* model. Tracing gene to cell lineages.
30. Organization of the oogonium, fertilization and formation of early embryo in angiosperms. Seed formation and synthesis of storage protein.
31. Germination and early plantlet formation: totipotency.
32. Hormonal regulation in plant development.
33. Somacional variation and gene transfers in plants.
34. Animal cell and tissue and embryo culture techniques.

Examinations

Format: Multiple choice (40%), short answer (40%), essay (20%)

Textbooks for Assigned Readings

Developmental Biology	by Scott Gilbert
Molecular Biology of the Cell	by Alberts *et al.*
Molecular Biology of the Gene	by Watson *et al.*
Principles of Development	by Wolpert
Genes	by Lewin

Readings are also assigned from journals and from Internet resources such as Medline (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) and BioMedNet (http://www.bmn.com/).

Chick Embryo Culture Techniques

Background Information on Culturing Vertebrate Embryos

Vertebrate embryos can be cultured and manipulated *in vitro* to make various observations on cell type-specific or organ primordium-specific interactions as well as on the behavior of cell populations. Included among the observations are cell migrations, cell-cell interactions, differential cell division, expression and depletion of maternal gene products, onset of expression of new gene batteries, shaping of organ primordia, temporal and position-specific programmed cell death, and acquisition of new phenotypes and differentiated states. For example, Vogt (1929) studied morphogenetic movements by staining amphibian embryos with vital dyes. Those studies represent some of the first nondestructive experimental manipulations of a whole embryo which is readily amenable to observation. Early embryologists conducted extensive microsurgery on embryos to understand the time- and position-dependent "cause and effect" relationships among parts of multicellular embryos. While the amphibian model historically has been studied the most by developmental biologists (especially in the classroom), one of its disadvantages was the seasonal nature of the availability of wild-caught gravid females or field-harvested eggs/embryos. Eventually, of course, maintenance and induced-breeding protocols were prepared for laboratory-raised *Xenopus,* which alleviated that constraint.

Avian embryos were first subjected to culture by Waddington (1932), who cultured chick embryos. Such embryos have always been available throughout the year, which provided a distinct advantage for both research and teaching. Since those early years, *in vitro* chick embryo culture techniques have undergone a sea change. Alternative culture methods are now available, and a choice can be made which depends on the nature of the experiment.

This report describes examples which are currently used in our classroom laboratories.

The Chick Embryo as a Traditional Research/Teaching Model

Chick embryos are flat, discoidal multicellular structures positioned on the yolk at the animal pole of the yellow of the egg which is itself enclosed in a vitelline membrane that is wrapped in sheets of thick albumen and bathed in thin albumin (Fig. 1).

Various composite salt solutions (e.g., Tyrode or Pannett-Compton) are available for manipulating chick embryos under physiological conditions of salinity, osmolarity, and pH at the physiological temperature of 37.5°C. Waddington's original technique (1932) involved detaching the embryo from the vitelline membrane of the blastodisc and then explanting it over a semi-solid substratum composed of a clot of chicken plasma and embryo extract. Under those conditions, the embryo undergoes normal morphogenesis until formation of the neural axis (which can be easily monitored). Waddington effectively used this method to demonstrate "induction of the nervous system" by transplantation of Hensen's node onto a host gastrula-stage embryo. However, the blastodisc, with its radial symmetry, has a zone of active cell proliferation along its outer rim, the "margin of outgrowth" (New, 1959; Ghatpande *et al.*, 1993; and Deshmukh *et al.*, 2001), and cells in this region invade the substratum and anchor themselves onto it, thus impeding further outward expansion. That is, they interfere with the normal morphogenetic movements of epiboly, restricting the period of development *in vitro,* and, consequently, limiting opportunities for inquiry. Nonetheless, it is still possible to carry out a marvelous series of experiments under these conditions.

A decade and half after Waddington's technique was introduced, Spratt (1947) modified it by explanting the blastodiscs onto semi-solid agar. That procedure improved the ease with which the embryo could be visualized, but unfortunately offered the same impediment as Waddington's original method. In 1955, New introduced the glass-ring culture technique in which the vitelline membrane itself serves as the substratum for the embryo culture. Early avian embryos normally adhere to the vitelline membrane and undergo area expansion by outward growth. This method was a quantum improvement over all preceding methods.

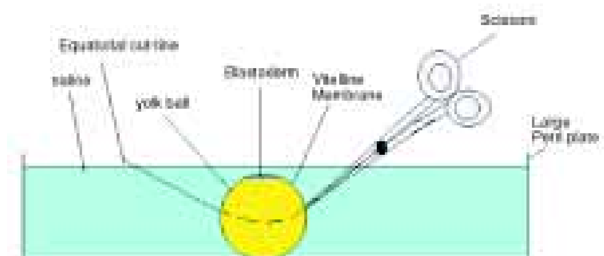

Fig. 2. Harvesting chick embryos for *in vitro* culturing.

Procedures for Classroom Manipulations of Chick Embryos

Single-ring culture method: For *in vitro* culturing, a freshly fertilized egg is incubated for 12-18 h. Then, the shell is punctured at the end, and the albumin is poured out after first chipping off small pieces of the shell and unwinding the coat of thick albumen near and around the yolk ball. The yolk ball is then transferred to a glass bowl or a large Petri dish (250 mm diameter, 25-30 mm tall) containing physiological saline solution (Fig. 2).

Excess albumen wrapped around the vitelline membrane of the yolk ball is sucked off with a wide-mouth Pasteur pipette. The vitelline membrane is then cut along the equator, with the blastoderm facing up, and gently peeled off by grasping the cut edges of the membrane with two pairs of semi-blunt forceps. We thereby harvest the vitelline membrane, along with the adhering chick gastrula blastoderm (primitive streak stages 2-4, according to the staging system of Hamburger and Hamilton, 1951). After flushing off excess yolk, the complex is transferred to a watchglass and positioned with the blastoderm facing up. A glass ring (12 mm in diameter) is placed on the vitelline membrane. That membrane is then stretched around the ring and folded over it. The watchglass with the embryo-retaining ring is transferred onto a bed of moist cotton in a Petri dish (100 mm in diameter). Next, the saline solution is drained off and replaced by a 1.5-ml layer of thin egg albumin. The dish is then covered with a lid (Fig. 3).

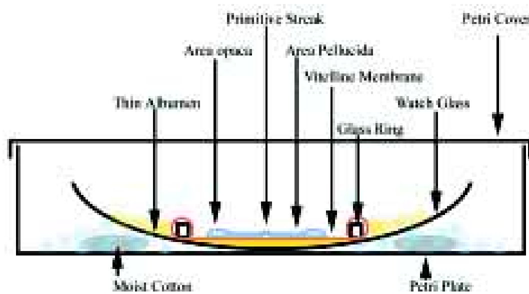

Fig. 3. The single-ring culture technique *(after New, 1955).*

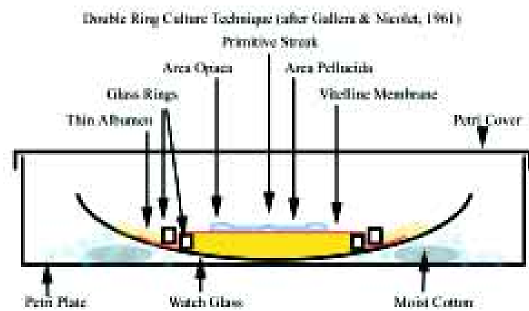

Fig. 4. The double-ring culture technique *(after Gallera and Nicolet, 1961).*

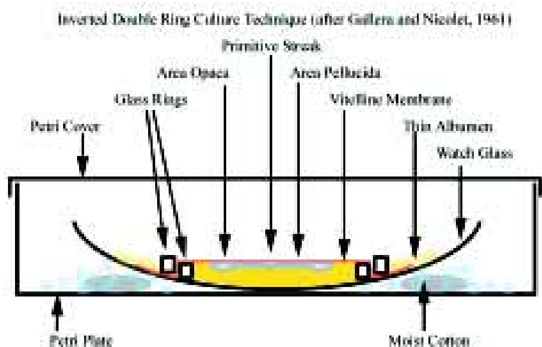

Fig. 5. The inverted double-ring culture technique *(after Gallera and Nicolet, 1961).*

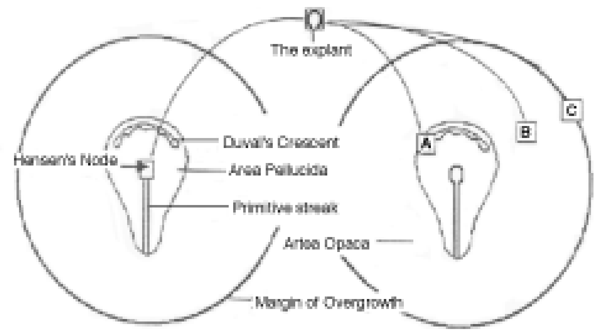

Fig. 6. Transplantation of Hensen's node.

Usually, the blastoderm outline and morphology of the embryo, together with its developmental stage, are carefully described. For some exercises, they are drawn with the aid of a camera lucida attached to a dissecting microscope. The culture is kept for incubation in a humidified incubator at 37.5°C. Under these conditions, a chick embryo undergoes morphogenesis and routinely reaches stage 14, but dies just as the anterior amniotic fold and cervical flexure begin to form. Nevertheless, these cultured embryos provide a good model for surgical operations or treatment with various teratogens, growth factors, radiation, or other agents.

Double-ring culture method: Gallera and Nicolet (1961) modified New's technique by introducing the use of a double-ring sandwich in which the vitelline membrane, with the adhering blastoderm, is clamped between two snug-fitting glass rings so that the blastoderm with the vitelline membrane is lifted up (Fig. 4) and floated over the cushion of thin albumen.

In this double-ring culture format, access to the blastoderm for microsurgery such as extirpation and transplantation of tissue pieces is dramatically improved. For example, when the entire vitelline membrane and embryonic endoblast were scraped off cultured chick blastoderms of Hamburger-Hamilton stages 2 to 4, a new endoblast was found to have formed as a pear-shaped shield under Hensen's node (Modak, 1965), which was then shown as having originated from the invagination of cells through the primitive streak. Later, Modak (1966) and Nicolet (1970) extirpated Hensen's node from ^{3}H-thymidine-labeled blastoderm and transferred it to unlabeled blastoderm from which an equivalent piece had been cut out. Those studies demonstrated that (a) the labeled transplant heals and merges completely with the unlabeled host; and (b) when endoblast in the area pellucida is peeled off, newly formed endoblast under the node contains ^{3}H-thymidine-labeled cells which then go on to form the foregut. Those experiments provided formal proof that also in the bird embryo the endoblast is formed during gastrulation by invagination through the anterior portion of the primitive streak, a position equivalent to the dorsal lip of the amphibian blastopore (Modak,

1966; Nicolet, 1971). Similar replacement experiments were performed by Rosenquist (1966) with comparable results. Furthermore, one can transplant multiple Hensen's nodes or postnodal pieces onto host gastrula blastoderms. In the double-ring format, the survival rate is improved and blastoderm expansion is superior, compared with the single-ring format. Nevertheless, embryos still die before amnios formation.

When using New's single-ring culture technique for the treatment of embryos with teratogens or growth factors, the agent (dissolved in saline) is dropped onto the blastoderm, where it becomes diluted to an unknown degree because of the presence of saline held along the edges of the ring. In the double-ring method, however, the agent (2X) is dissolved in saline and mixed in equal parts with thin albumen to obtain the desired concentration and then allowed to diffuse through the vitelline membrane during incubation, thereby ensuring its uniform distribution and concentration (Nicolet, 1965). The double-ring technique also makes it easier to transfer the culture from one watchglass to another.

Inverted double-ring culture method: Gallera and Nicolet (1961) introduced yet another innovation in the double-ring culture technique that involves clamping the vitelline membrane between two glass rings such that the adherent blastoderm faces down and is bathed directly in the thin-albumen nutrient (Fig. 5).

This operation requires both skill and patience but yields extraordinary improvement in the duration of the culture period. The embryo continues development by completing the formation of the amniotic sac, limb buds, and eye cups (with lens vesicles), and beginning the formation of the allantois. This inverted double-ring method offers for the first time a condition wherein the blastoderm is placed in a normal position with reference to the gravitational field, as it exists *in ovo*.

Yet another culture technique which is both rapid and simple has been described (Olszanska and Lassota, 1980) in which the chick blastoderm is transferred to a dry square or round filter paper support, with a hole in the center to which it adheres, and then floated over thin albumen. This technique is good for short-term treatments involving minor surgical operations.

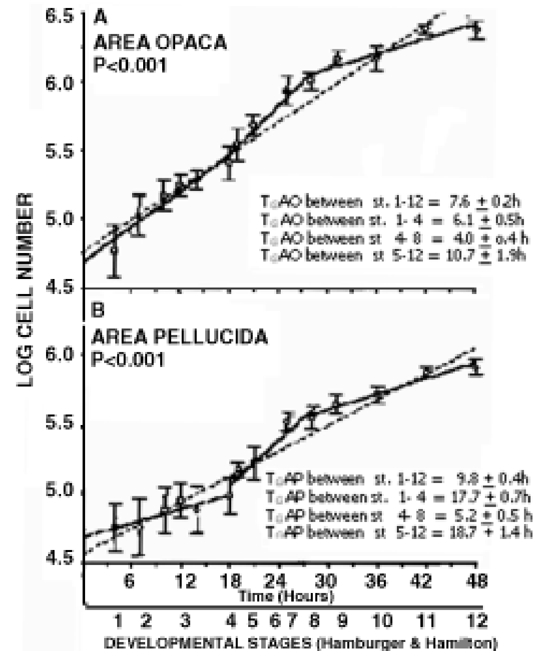

Fig. 8. Data plotted on a semi-log graph against the stage of development and the developmental age in hours. *The slope of the line is estimated by linear regression (McMaster and Modak, 1977; Ghatpande* et al., *1990, 1993; Sonawane, 2001; Joshi-Banka* et al., *2002).*

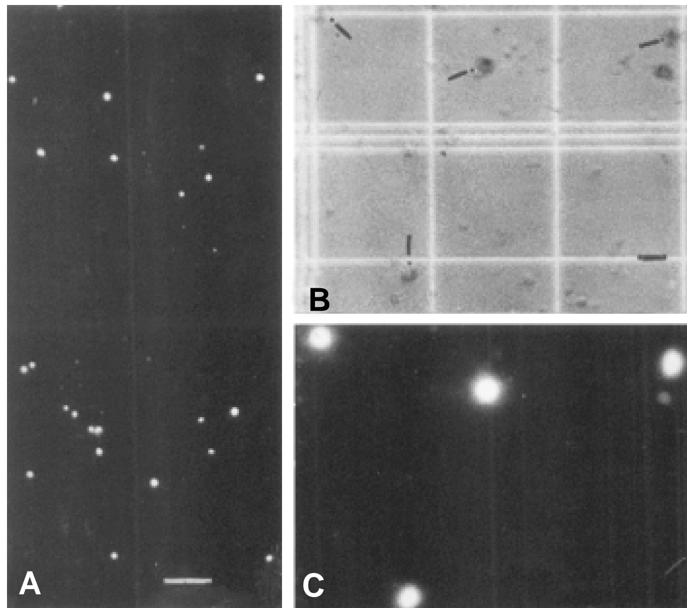

Fig. 7. Ethidium bromide-stained lysate visualized under a phase contrast and fluorescence microscope. *Fluorescent nuclei are counted per grid square (1 × 1 mm). Chick embryo cells are mononucleate, and determination of the nuclear number allows estimation of the total number of cells per sample. Homogenates of at least three independent samples are examined at each stage to estimate the cell population size.* **(A)** *Fluorescent nuclei in NP40 lysates.* **(B)** *Nuclei (indicated by bars) in Neubauer´s chamber seen under phase contrast.* **(C)** *The same field as in (B), but seen under epifluorescence revealing ethidium bromide stained nuclei for counting.*

Laboratory Exercises

These exercises accompany the Developmental Biology II course. Detailed protocols for the following experiments are available from the author.

Exercise 1: Chick Embryo Double-Ring Culture Method

This experiment demonstrates an *in vitro* culture method for chick embryos for observation of normal development. The embryos are cultured for the entire period relevant to the establishment of the primitive embryonic axis, including the neural tube regionalized into brain and spinal cord, notochord, somites, heart and basic circulatory system, and foregut. Alternatively the culture can be treated with a chemical or physical agent or surgically manipulated before proceeding to the incubation.

Exercise 2: Neural Induction by transplanted Hensen's Node

Hensen's node, when transplanted from a donor gastrula to a primitive-streak-stage host chick embryo, induces neuralization

of the host ectoblast (Fig. 6). This response is best viewed in serial sections of fixed host embryos (Gallera, 1971). The chick embryo is a powerful model for examining the time frame of the process of neural induction (Joshi-Banka *et al.*, 2001) and the discrete intracellular changes during the inductive process, and it is useful for establishing the hierarchy of specific genes expressed before induction and in response to inducers. Donor and host chick blastoderms are cultured using the double-ring technique.

Exercise 3: Determination of Cell Population Growth Parameters in Chick Embryos during Early Morphogenesis

In this experiment, chick embryos mounted on double-ring platforms are dissected to separate the area pellucida from the area opaca at Hamburger-Hamilton stages between 1 and 12 (Hamburger and Hamilton, 1951). Area pellucida circles and area opaca rings are separately homogenized in TENM2-sucrose in the presence of the nonidet detergent NP-40 and ethidium bromide-stained red fluorescent nuclei are counted against epifluorescence (Fig. 7). Data are plotted on semi-log plots, and the slopes of the fitted regression lines give the average cell population doubling times (T_G) for the area opaca and the area pellucida. Closer examination of the data reveals that in both cases the data fit three independent regression lines, indicating that growth is relatively slow until full gastrula (stage 4), accelerates to stage 8, and then slows down again. Thus, during the period of early axis development, the chick embryo cell population grows exponentially, and growth is fastest between stages 4 and 8 (Fig. 8).

This experiment can be extended to selected cell populations (e.g., ectoblast, yolk endoderm, embryonic endoblast, etc.) or to other regions of the blastoderm (e.g., margin of overgrowth) or to embryos treated with teratogens or with dorsalizing or caudalizing agents (Ghatpande *et al.*, 1993; Modak *et al.*, 1993; Joshi-Banka *et al.*, 2002)

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