Hydra: Research Methods

Edited by

Howard M. Lenhoff

University of California
Irvine, California

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Contributors

Richard S. Blanquet, Department of Biology, Georgetown University, Washington, D.C. 20057

Hans R. Bode, Developmental Biology Center and Department of Developmental and Cell Biology, University of California, Irvine, California 92717

Patricia M. Bode, Developmental Biology Center and Department of Developmental and Cell Biology, University of California, Irvine, California 92717

Richard D. Campbell, Department of Developmental and Cell Biology, University of California, Irvine, California 92717

Jean Danner, Biochemistry Section, NIOSH, Morgantown, West Virginia 26505

Charles N. David, Department of Molecular Biology, Albert Einstein College of Medicine, Bronx, New York 10461

Robert M. Day, Department of Developmental and Cell Biology, University of California, Irvine, California 92717

John F. Dunne, Developmental Biology Center, University of California, Irvine, California 92717

Kristine M. Flick, Developmental Biology Center and Department of Developmental and Cell Biology, University of California, Irvine, California 92717

Cheng-Mei Fradkin, Department of Developmental and Cell Biology, University of California, Irvine, California 92717

Cornelius J. P. Grimmelikhuijzen, Max-Planck-Institut für medizinische Forschung, Abteilung Biophysik, 6900 Heidelberg, Federal Republic of Germany

Wyrta Heagy, Department of Microbiology, University of Massachusetts, Amherst, Massachusetts 01003

David A. Hessinger, Department of Physiology and Pharmacology, School of Medicine, Loma Linda University, Loma Linda, California 92350

Jeanne Ivy, Department of Developmental and Cell Biology, University of California, Irvine California 92717

Robert K. Josephson, School of Biological Science, University of California, Irvine, California 92717

Edward S. Kline, Department of Biochemistry, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298

Howard M. Lenhoff, Department of Developmental and Cell Biology, University of California, Irvine, California 92717

Georgia E. Lesh-Laurie, Department of Biology, Cleveland State University, Cleveland, Ohio 44115

C. Lynne Littlefield, Developmental Biology Center, University of California, Irvine, California 92717

Stephen S. Macintyre, Department of Anatomy, Case Western Reserve University, Cleveland, Ohio 44106

Harry K. MacWilliams, Department of Anatomy, University of Massachusetts Medical Center, Worcester, Massachusetts 01605

Beverly A. Marcum, Department of Biological Sciences, California State University, Chico, California, 95929

L. Muscatine, Department of Biology, University of California, Los Angeles, California 90024

That T. Ngo, Department of Developmental and Cell Biology, University of California, Irvine, California 92717

Patricia Novak, Department of Developmental and Cell Biology, University of California, Irvine, California 92717

Joann J. Otto, Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907

R. L. Pardy, School of Life Sciences, University of Nebraska, Lincoln, Nebraska 68588

Donald W. Phipps, Jr., School of Life Sciences, University of Nebraska, Lincoln, Nebraska 68588

M. Rahat, Department of Zoology, The Hebrew University of Jerusalem, Israel

Vanda Reich, Department of Zoology, The Hebrew University of Jerusalem, Israel

Norman Rushforth, Department of Biology, Case Western Reserve University, Cleveland, Ohio 44106

Charles L. Rutherford, Biology Department, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061

H. Chica Schaller, Max-Planck-Institut für medizinsche Forschung, Abteilung Biophysik, 6900 Heidelberg, Federal Republic of Germany

Tobias Schmidt, Max-Planck-Institut für medizinsche Forschung, Abteilung Biophysik, 6900 Heidelberg, Federal Republic of Germany

G. Scott Smith, Developmental Biology Center and Department of Developmental and Cell Biology, University of California, Irvine, California 92717

Alan E. Stiven, Department of Biology 046A, University of North Carolina, Chapel Hill, North Carolina 27514

Tsutomu Sugiyama, National Institute of Genetics, Mishima, Shizuoka-ken 411, Japan

Joseph R. Voland, Department of Pathology, University of California, San Diego, La Jolla, California

Nancy Wanek, Developmental Biology Center and Department of Developmental and Cell Biology, University of California, Irvine, California 92717. Current address: Department of Biology and Health Science, Chapman College, Orange, California 92666.

Vaman S. Waravdekar, Microbiological Associates, Bethesda, Maryland 20016

Richard L. Wood, Department of Anatomy, University of Southern California, School of Medicine, Los Angeles, California 90007

Chapter 33

Culturing Interstitial Stem Cells in Hydra Aggregates

Charles N. David

PURPOSE

To investigate the growth and differentiation of interstitial stem cells of hydra, we have developed a method for culturing those cells within aggregates of cells taken from hydra previously treated with nitrogen mustard (NM) (David and Murphy, 1977; Sproull and David, 1979).

INTRODUCTION

When small numbers of cells are seeded in such NM aggregates, which serve as "feeder layers," individual stem cell clones can be identified and counted. In addition, the feeder layer technique is suitable for following the differentiation of interstitial cells committed to become nerve cells or nematocytes (Gierer *et al.*, 1972; Venugopal and David, 1981; Fujisawa and David, 1981; Yaross *et al.*, 1982).

GENERAL COMMENTS

The procedure for culturing stem cells in NM aggregates is outlined schematically in Fig. 1. Treat hydra with NM to destroy endogenous

Charles N. David • Department of Molecular Biology, Albert Einstein College of Medicine, Bronx, New York.

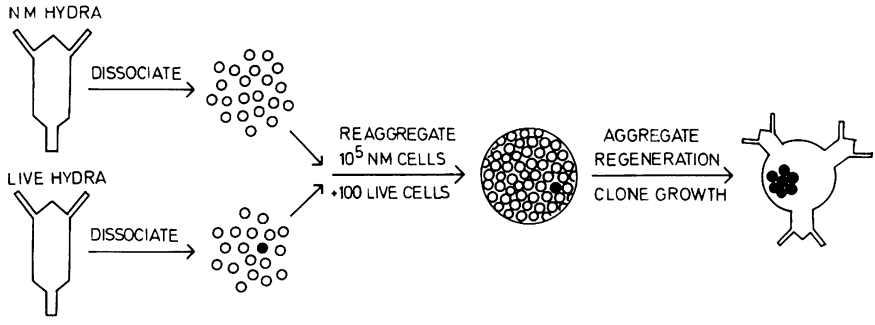

FIGURE 1. Schematic representation of method for cloning interstitial stem cells in aggregates of NM-treated cells. (●) Stem cell.

interstitial cells (see Chapter 40). Dissociate the treated hydra to yield a cell suspension (see Chapter 32). Likewise, dissociate sample of untreated hydra tissue. Mix an aliquot of the untreated cell suspension containing an appropriate number of stem cells with ~200,000 NM-treated cells and centrifuge the mixture to form a cell aggregate. During incubation of the aggregate over the next few days (see Chapter 32), stem cells from the untreated sample will proliferate to form interstitial cell clones. To visualize such clones, stain the aggregates with Toluidine Blue. Interstitial cells and differentiating nematoblasts in clones stain darkly and can be easily identified against the lightly stained epithelial cells of the host tissue.

About 0.6% of total hydra cells give rise to interstitial cell clones in NM aggregates (David and Murphy, 1977). Since about 50% of the cells used to form the aggregates are sloughed during the cloning procedure, about 2 times 0.6% or 1.2% of hydra cells are estimated to be stem cells by the cloning assay. Since the fraction of stem cells in intact hydra estimated from cell flow analysis is 4% (David and Gierer, 1974), the apparent cloning efficiency of stem cells in NM feeder layers is about 25%. This estimate is a minimum one since some stem cells differentiate in NM aggregates instead of forming clones.

MATERIALS

Polyethylene centrifuge tubes (0.4 ml) with smooth, conical bottoms and 100-ml pear-shaped centrifuge bottles with graduated tips.

Prepare cell culture medium (Chapter 4) in large batches and store it frozen. Do not sterilize the medium since the tissue used in experiments is not sterile.

"Ecology" dishes are Petri dishes in which hydra have been cultured for 2–3 days. In addition to hydra, they contain amoebae, flagellates, and other protozoa which normally coexist with hydra in our cultures. They are the best "antibiotic" available for developing aggregates.

PROCEDURES

Preparing NM-Treated Host Tissue

Treat about 2000 hydra with 0.01% NM for 10 min. Feed and wash the hydra 1 day after NM treatment and use them for cloning experiments 5–6 days later. By this time the tissue is essentially free of endogenous interstitial cells. Note that the NM treatment must be done very carefully because only one live untreated hydra, which contains 1000 stem cells, among 1000 NM-treated hydra, can contribute a significant background of stem cell clones to the NM host tissue.

Dissociating Cells

Dissociate NM-treated hydra in cell culture medium and collect the cells by centrifuging (200*g*; 6 min) them in 100-ml pear-shaped centrifuge bottles. Discard the supernatant and resuspend the cell pellet in 30 ml fresh medium per 0.2 ml cell pellet. This suspension corresponds to a cell concentration of about 5×10^5/ml; 0.4-ml aliquots, on centrifugation, yield aggregates containing 200,000 cells.

To dissociate the small amounts of tissue (5–10 hydra) required for cloning experiments, add 100–200 NM-treated hydra as carrier to the live tissue and dissociate the mixture. Typically such suspensions contain 200–500 clone-forming cells (CFU) per milliliter, so that about 2 μl of the suspension per aggregate is sufficient to yield an average of one clone per aggregate.

Preparing Aggregates

To prepare aggregates, mix an aliquot of NM host suspension with a sample of the live cell suspension such that the mixture contains ~2.5 CFUs/ml. Distribute the mixture to 0.4-ml polyethylene centrifuge tubes with a Pasteur pipet. The suspension is held in the tubes by capillarity so the tubes can be put on their side and otherwise handled rather roughly. Place 15–20 such tubes into large 50-ml tubes and centrifuge them at 100*g* for 6 min to pellet the cells.

After centrifugation, remove the small tubes from the centrifuge and stand them upside down in a 100-ml beaker. The cell pellets come off the bottom of the tubes after 5–15 min and fall to the meniscus from which they can easily be dropped into a Petri dish with fresh medium. Allowing the pellets to come off the bottom in this matter minimizes cell loss due to handling.

Dilute the cell culture medium in twofold steps over the next 18 hr (see Chapter 32) and then transfer aggregates to "ecology" dishes for further incubation. Two to three hundred aggregates can be conveniently processed in an experiment using the procedure outlined above.

After 5–6 days of clone growth, fix aggregates with ethanol, stain with 0.1% of Toluidine Blue and prepare whole mounts (Diehl and Burnett, 1964). Clones are easily identified as groups of darkly staining interstitial cells and differentiating nematoblasts. By focusing up and down through an aggregate, it is possible to identify clones throughout an aggregate.

Quantitatively Estimating Clone-Forming Cells

Before using the NM feeder layer technique to assay CFUs, it is useful to estimate the number of CFUs in the sample to be assayed. To do this, determine the total cell number in a parallel sample of tissue using maceration (Chapter 20) and multiply this number by 0.006 to obtain an estimate of the number of CFUs in the sample. Then prepare several sets of 30 NM aggregates with dilutions of the live cell suspension estimated to yield 0.5–2.0 CFUs/aggregate. Score the total number of clones in each set after 5–6 days of clone growth.

To determine the actual number of CFUs present in the sample, (1) count the total number of clones in a set of 30 aggregates. This value equals the number of CFUs in the sample used to prepare the aggregates. (2) Calculate the fraction of the aggregates which contain clones and use the Poisson distribution to estimate the average CFUs/aggregate (n): (aggregates with clones)/(total aggregates) $= 1 - e^{-n}$.

Both methods should yield comparable results. The variability between replicate assays done on the same day and equivalent assays performed on different days is $\pm 30\%$.

Rapid CFU Assay

To improve on the speed and accuracy of the cloning assay, we have developed an alternative procedure in which large numbers of CFUs are seeded in NM aggregates and the total size of the interstitial cell population is

determined by maceration after 4 days of clone growth (David and Plotnick, 1980). Since the growth rate of stem cell clones is independent of clone density up to 100 clones per aggregate (Sproull and David, 1979), the number of interstitial cells (1s+2s) on day 4 is linearly proportional to the number of CFU seeded in the aggregates on day 0. The proportionality constant is 19 $(1s+2s)_{day\ 4}/CFU_{day\ 0}$.

To use this procedure inoculate NM aggregates with 5–50 CFUs each. After 4 days of clone growth (the clones are confluent and individual clones cannot be distinguished), macerate five aggregates (Chapter 20) and count the total number of interstitial cells (1s+2s) per aggregate. Divide this number of 19 to determine the number of CFUs in the inoculum.

PRECAUTIONS

Because bacterial contamination can overtake and destroy aggregates, add streptomycin (50 μg/ml) to the medium with each daily medium change. In addition, maintain aggregates in "ecology" dishes so that the protozoa present will consume much of the contaminating bacteria.

To achieve good cloning, complete an experiment rapidly. Stem cells are not viable in culture medium for more than a few hours, and the ability of host tissue to form good aggregates also decays with time. It is best to complete an experiment from the start of dissociation to centrifugation of aggregates in less than 1 hour. For this reason the procedure as outlined above contains no cell-counting steps or other delays.

Carry out the entire procedure at 20°C. At higher temperatures cell losses are greater and aggregates survive poorly.

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