MOLECULAR NEUROSCIENCE

A LABORATORY MANUAL

EDITED BY

RUSTY LANSFORD

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Foreword

I remember sitting in my office at Stanford in the fall of 1985 and receiving the call from Susan Hockfeld asking me to start a summer course at Cold Spring Harbor Laboratory that utilized molecular biology techniques. Sue was a developmental neuroscientist who was on staff at CSHL at that time and she was in charge of the summer courses as well. In talking with Sue about the remit of the course, she indicated that the techniques of molecular biology were slowly coming into the neurosciences and she thought that a laboratory course would help speed this up. I had never attended a course at CSHL; and while Sue said that they were easy to do because of the quality of students, I really had no idea what I was in for (it is my guess that none of the directors that followed me knew exactly what they were in for, either). My colleagues, Drs. Beth Schachter (then at Mt. Sinai) and Marian Evinger (at Stony Brook), agreed to co-direct the course that first year. At that point the emphasis was on cloning of neural genes (hence the original name of the course, "Molecular Cloning of Neural Genes") and the students isolated RNA, made cDNA libraries, screened the libraries, isolated clones and sequenced them. Other methodologies that were taught were in situ hybridization, DNA isolate, restriction mapping, etc.

That first year I remember walking along Bungtown Road with Marian around three in the afternoon when none of the cDNA libraries turned out, bemoaning our failure and trying to figure out how to move forward. As anyone who has attended this course or any of the other courses knows, it is usually the failures that bring out the most discussion and the greatest opportunity to learn the nitty-gritty of the techniques that are being taught.

Over the nearly 30 years of its existence, the course directors and instructors have changed but the remit of teaching the latest molecular techniques to neuroscientists is still important and has been expanded to include more cell biological experiments. This is reflected in the transitions from the directors who were molecular biologists, including myself, Beth Schacter, Marian Evinger, Moses Chao, Robert Margolskee, Robert Darnell, and of course Jim Boulter. Boulter was director for two different stretches of time and was an exemplary "librarian." He brought Cary Lai into the fold as director, and Cary has the remarkable record for the most continuous summers of co-directing the course at 23. It was during Boulter's directorship that the course name changed to "Advanced Techniques in Molecular Neuroscience," reflecting the advances of genomics and the fact that most genes had been cloned, sequenced, and were available for use.

The course curriculum changed as molecular neuroscience evolved, as illustrated during the directorship of Thomas Hughes (Montana State) who introduced manipulation of green fluorescent protein as a major emphasis of the course. Of course, microscopy has been the bulwark of neuroscience since the time of Cajal, and its marriage with molecular biology was ably conveyed to the course students during the directorship of Rusty Lansford (editor of this manual), who made live cell imaging and ways of manipulating live cells a major emphasis of the course.

Consistent with the director's vision for the course, there were many instructors who brought their technologies to the course and were generous in their time, giving didactic lectures and providing hands-on teaching of their sections, which typically ran from a couple of days to a week or more. There were also many lecturers who came to give an evening research seminar in the course. The lecturers came from many institutions, ranging in experience from advanced post-docs to Nobel Laureates. Some of the lecturers truly reveled in the opportunity to lecture in the course, as any student will tell you of Robert Darnell's lectures, which always exceeded 3 h.

For the directors, instructors, and lecturers, the highlight of course attendance was interacting with the students. The students came from many countries and with differing levels of experience; some were grad students and others were members of the U.S. National Academies of Science. They

were united in their desire to learn the newest technologies and to be able to apply these methodologies to their own scientific questions. While it is clear that the technical details were important, the students also learned how to think about the techniques they were employing—the sensitivity of the methodologies, whether the question can be answered using these technologies, and how the data might be interpreted. These insights separate this course from many of the other courses taught around the world. It is this "ATMN/CNGEN effect" that has enabled the course participants to utilize the course in ways that significantly benefit their work, and through this, scientific advancement.

The course is still running strong each summer, teaching 16 lucky students selected to participate. Beyond these students, however, are many others who would benefit from detailed knowledge of the most current techniques. This manual is intended to provide this opportunity to the many who can't attend the course. Rusty has done an exceptional job of bringing together past directors and instructors as well as former students to provide detailed protocols for the cutting-edge science they do that reflect and expand upon the continuing goals of the course.

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Preface

The molecular genetics revolution has significantly impacted all fields of biological and medical research. The ability to manipulate and analyze DNA, RNA, and proteins permits investigations of cells, tissues, and organisms using approaches unimagined a generation ago. We are currently in the midst of another technical revolution, in which cells may be dynamically viewed and manipulated with laser-based tools; organismal genomes, transcriptomes, proteomes, and other 'omes' can be acquired overnight; and novel methods are being developed by interdisciplinary teams to quantitate the massive amounts of data emerging from such experiments. The modern neuroscientist is tasked with understanding how these interdisciplinary tools and techniques may be synergistically integrated to investigate the inner workings of normal and diseased nervous systems.

I have had the pleasure to teach and direct the Advanced Techniques in Molecular Neuroscience (ATMN) course at Cold Spring Harbor Laboratory (CSHL) for the past decade. Recently my colleagues and I decided that it would be helpful to pull together the protocols that have been taught and tested at ATMN over this period to produce a laboratory manual that would have broad use for neuroscientists beyond this course. This manual, *Molecular Neuroscience*, primarily draws from the protocols used in the various modules of the ATMN course, as well as those of its predecessor, Molecular Cloning of Neural Genes. By necessity and design, the ATMN course is constantly evolving to keep pace and teach the most advanced techniques in molecular neuroscience in order to prepare the next generation of neuroscience researchers to fearlessly and imaginatively develop the next-next generation of techniques and technologies. As the field of neuroscience changes, driven by emerging tools and technologies, so do the instructors and the protocols, as reflected here in *Molecular Neuroscience.* ATMN course instructors and lecturers, internationally recognized as experts in various fields of molecular neuroscience, wrote many of the protocols in this manual.

The ATMN course comprises three components: an extensive and up-to-date set of laboratory exercises, lectures on theoretical and practical aspects of advanced molecular biological and imaging techniques, and lectures by invited speakers that serve to showcase the ways in which these molecular techniques have been used to advance specific areas of neuroscience. The laboratory portion of each course includes the following topics: in vitro and in vivo gene delivery systems; loss-of-function approaches (e.g., RNAi) to study gene function; optogenetics to probe neural circuits within animals; the use of RNA-seq and microarray analyses to profile gene expression; methods to quantitate gene expression in single cells; and techniques to isolate, propagate, and analyze desired neuronal and glial cell populations. As many of these advanced techniques also involve the use of more standard procedures to first modify and then 'move' DNA fragments from one vector to another, the laboratory exercises also provide training in these methods, which are essential for the successful execution of the more sophisticated approaches. Advanced epifluorescence and confocal imaging microscopy and image analysis are often used to assay the various experiments, thereby educating the students in additional emerging technologies. Finally, course participants are introduced to bioinformatics analysis of microarray or high-throughput sequencing data sets.

These topics are all captured within the protocols and discussions that make up the *Molecular Neuroscience* lab manual. The book is divided into five sections. Section 1 begins with an introduction to tissue culture using sterile techniques and soon moves on to culturing primary neural cells. Tissue culture is an essential technique in neuroscience and is fundamental for many of the protocols found in the rest of the manual—for collecting and assaying adequate and intact samples of DNA (Section 2) and RNA (Section 3), for cell transfection and infection to deliver genetic payloads into cells and tissues (Section 4), and for imaging living cells and tissues (Section 5). Section 2 initiates with the basics of growing bacteria and manipulating DNA before advancing into transgenic

and ChIP techniques. Section 3 has numerous protocols to assay RNA complexity using standard and emerging approaches including microarray analysis and RNA-seq. The fluctuating transcriptome can be reliably assayed in single cells and assembled tissues, which is transforming the questions pursued by basic researchers along with how clinicians diagnose and treat diseases. Section 4 introduces and guides the reader through various gene transfer techniques in vitro and in vivo. Section 5 describes several types of microscopes increasingly being used to observe and perturb cells at unprecedented spatiotemporal resolution, drawing on Sections 1–4 to pull it off.

Each section begins with a brief introduction into the issues to be considered. The sections continue with protocols for the basic techniques that must be mastered before moving on to the more advanced techniques at the end of the sections. The advanced protocols draw heavily from the bleeding edge of science and often require multidisciplinary approaches. The protocols concisely explain required reagents and equipment before delving into the experimental steps. They include discussions and notes on troubleshooting potential problems that may arise during the experiment or with the interpretation of the data. As with any new protocol or recipe, users are strongly advised to familiarize themselves with the procedure prior to beginning.

Molecular Neuroscience is intended to provide neuroscientists at all levels with a conceptual and practical understanding of key techniques in molecular neuroscience. Based on its advanced content, scholars that range from advanced undergraduate students to laboratory heads will find it indispensable. A number of the protocols and discussions were drawn from other CSHL Press manuals and the online methods journal *Cold Spring Harbor Protocols.*

This manual owes an enormous debt of gratitude to the ATMN students who spent weeks of sleepless nights attempting the protocols. They helped the instructors to fine-tune and troubleshoot the methods, contributing to the evolution of this manual's protocols. Most of them will acknowledge that their end-of-course exhaustion was due to time very well spent—even if they did not appreciate it for 6 or so months. I think one of the most valuable aspects of the course, one that I hope will be used by the readers of this book, is the willingness to overcome the activation barriers and fear and try new protocols, to boldly go where few have gone, and to follow and develop novel protocols that bypass experimental roadblocks. ATMN is a place where the instructors and lecturers expect the students to talk science with them into the wee hours. It is a place where the students are expected to embrace and exercise their inner geek.

I am indebted to my predecessors Jim Eberwine, Moses Chao, Robert Margolskee, Jim Boulter, Bob Darnell, Thom Hughes, and currently Joe Loturco for establishing a course with high expectations and standards. Jim Eberwine's creativity and support are unparalleled and greatly appreciated. Worth special note is Cary Lai, who has been involved with the course for over 20 years as a student, teaching assistant, and instructor, for continually teaching me his patient and detailed teaching style. David Huss—my right and left hand in the lab—helped in innumerable ways. Before I even ask, I almost always find that he has done what is needed, calmly making difficult experiments work seamlessly, helping all who need help. The editorial staff at CSHL Press have been great to work with and extremely supportive and guided me with a gentle hand. Of special note are Maria Smit, Joanne McFadden, and Maryliz Dickerson. Finally, I thank my son Jack who has always very lovingly, albeit reluctantly, supported my need to leave for CSHL each summer to teach at ATMN. I always return tired and spent, fully in need of his perceptive insistence to come out and play.

Rusty Lansford
May 2014

General Safety and Hazardous Material Information

This manual should be used by laboratory personnel with experience in laboratory and chemical safety or students under the supervision of such trained personnel. The procedures, chemicals, and equipment referenced in this manual are hazardous and can cause serious injury unless performed, handled, and used with care and in a manner consistent with safe laboratory practices. Students and researchers using the procedures in this manual do so at their own risk. It is essential for your safety that you consult the appropriate Material Safety Data Sheets, the manufacturers' manuals accompanying equipment, and your institution's Environmental Health and Safety Office, as well as the General Safety and Disposal Cautions in the Appendix, for proper handling of hazardous materials in this manual. Cold Spring Harbor Laboratory makes no representations or warranties with respect to the material set forth in this manual and has no liability in connection with the use of these materials.

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SECTION 1

Working with Cells

The basic need for and principles of culturing cells and tissues can be traced back over a century to developmental neurobiologists interested in the relation of form to function. Scientists had long desired the ability to grow cells and tissues in order to analyze the individual cellular building blocks separate from the whole organism. Ross Harrison was the first to report a reliable technique for growing cells in vitro when he cultured the medullary tube from a frog embryo in a globule of clotted lymph (supplemented with the embryo's own yolk) on a coverslip and observed neurites growing from individual cell bodies.

The need to culture cells is no less pressing now. Over 400 distinct cell types are speculated to exist in the human body, and this number is sure to grow as the tools and techniques to identify and evaluate single cells improve in sensitivity and precision. Scientists must keep cells and tissues alive and happy in order to assay normal cell behavior and function, to vitally observe them under the harsh conditions of laser microscopes, to manipulate the genomes of undifferentiated stem cells to generate "knockout" animals for study, and so forth. Culturing neural cells and tissues simplifies the overwhelming complexity of the task at hand—understanding brain development and function—by permitting individual cells to be identified, isolated, and examined from the various regions of the nervous system.

"Tissue culture" is a generic phrase that embraces cell culture and tissue culture. It signifies the in vitro growth of cells or assemblies of cells derived from a multicellular organism. The ability to readily culture, manipulate, and analyze cells requires a well-defined environment; the proper temperature, gas composition, pH, and nutrients must be established for the particular cells being studied. The cells or tissues are typically cultured in a liquid, semi-solid, or solid growth medium that contains the chemical components and nutrients required for proper cell growth, differentiation, and/or function. Great care and proper training are needed in order to determine the culture conditions and then reproduce them day in and day out, all while maintaining a safe and sterile environment.

"Primary cell culture" refers to cells that have been derived directly from tissues and propagated in culture. Primary cells usually exhibit a finite life span in culture, typically dividing less than