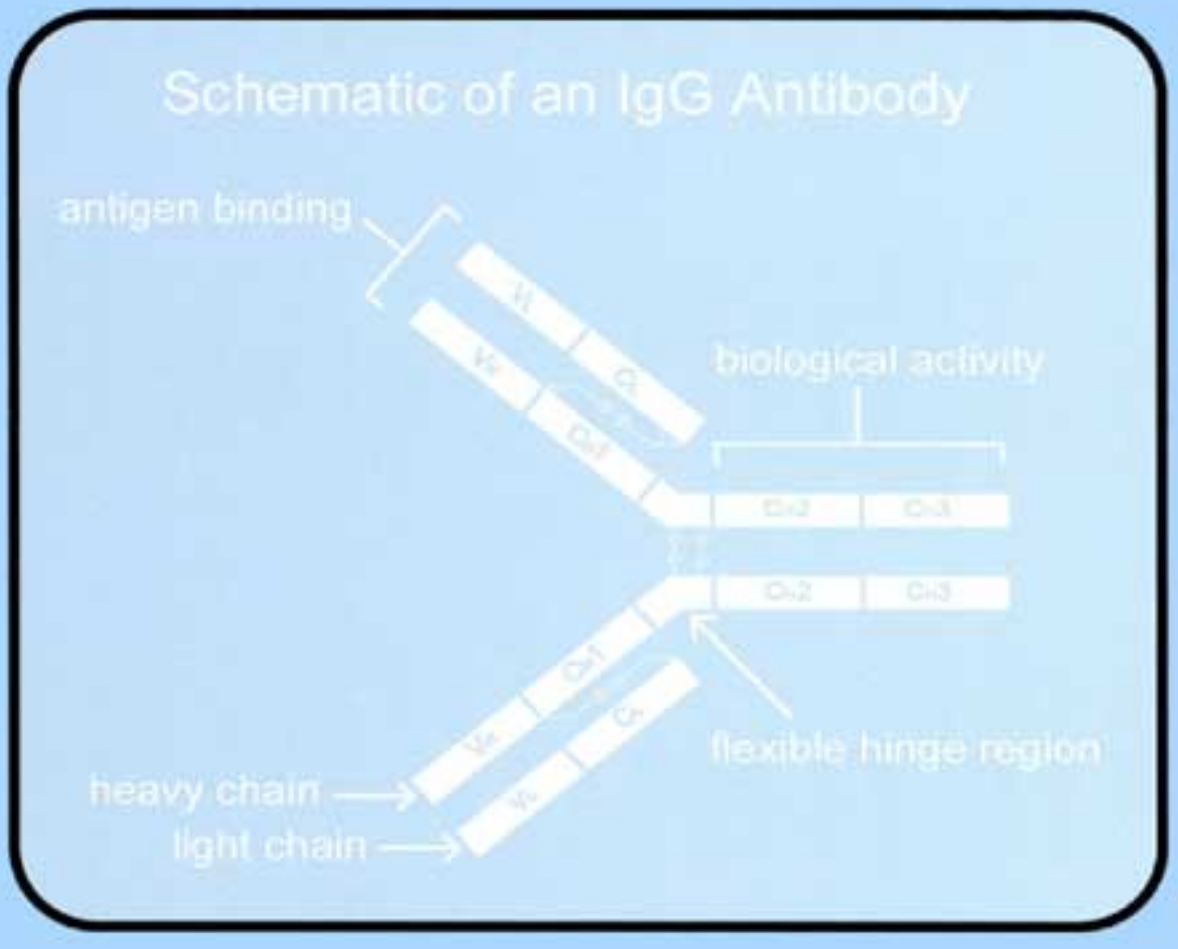
A Guide to Methods in the Biomedical Sciences

Ronald B. Corley



Springer

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Thanks to TLOML GOMD
and to all of our children, to whom I dedicate this.

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Acknowledgements

There are many people to thank for their help in making this book possible. A true debt of gratitude goes to the many students and technicians who have worked in my laboratory, and made bringing new methods to the lab so much fun. I have learned more from you than you did from me. You would never have known that some of your work would end up in a book like this! Thanks also to Alex for help with research, Kylle for all the proofreading, Kateri for help with the figures, and Kathy for bailing me out as needed.

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Introduction

We shall not cease from exploration
And the end of all our exploring
Will be to arrive where we started
And know the place for the first time.
T.S. Eliot (from "Little Gidding")

Students often remark to me how difficult it is to begin reading the primary, journal based literature or follow lectures in a biomedical discipline without some understanding of the methods being used. I can certainly understand their dilemma. While learning "the facts" in science does not necessarily require a working knowledge of HOW we arrive at a conclusion (though it certainly helps!), it is a very different process to begin to understand how we know what we know, and what the limitations of that knowledge might be. This requires first a basic understanding of the methods used to achieve that knowledge, which is followed later by a more detailed and sophisticated understanding of the nuances of the techniques used. In addition, because the tools of science are rapidly evolving, even sophisticated students (and faculty for that matter) can be challenged when beginning to read in a new discipline, where a whole new language emerges.

And, if it is difficult for students, it must be equally frustrating for the lay public to figure out how things are done. To be sure, it is not just students who should be interested in scientific methodology. The use of biomedical techniques is exploding in everyday life: from pregnancy and paternity testing to genetic testing for fetal development, tracing ancestry, and forensics, to new antibody based diagnostic procedures, it is hard to live in today's society and not be touched directly or indirectly by many of the same methods that are used in a biomedical laboratory.

So, how does a student new to science (or an interested lay person) begin to learn the language of techniques? Usually, s/he asks a faculty member, or a colleague, in hopes they might help. Or, the student might

scan the web in hopes of obtaining quick (and accurate) information. Or, if they have access to a laboratory, they might consult a classic techniques manual such as the "Red Book" (some excellent methods manuals are listed at the end of the book). However, methods manuals generally provide far more information than necessary at the beginning. Their sheer size can make simply leafing through one of these manuals a daunting task. My students have often said that they would like to have available a simple "pocket primer" describing some of the essential techniques, giving just enough information to get started, but not so much information to bury them in the details.

The purpose of this book is to attempt to meet that need, filling the void between no information and too much detail. It is intended to provide a basic description of common methodology, identify the type of information that can be obtained from a particular technique and, when appropriate, provide alternative approaches. I have always found the history of science interesting, so where possible I have included some historical background to the development of particular techniques. Significant scientific advances rarely occur without the development of new and exciting techniques that can be applied to solving problems. A number of technical advances were of sufficient value to earn for their discoverers a Nobel Prize and many major advances were accompanied by the development of new techniques. I have included some of these breakthroughs in this book as well, and provided where I thought appropriate the original references for some of these major discoveries.

But what techniques should be included? There are thousands of methods that have been developed in the various biomedical disciplines, and if the book was to remain compact the methods included would have to be limited in some way. In deciding on what these might be, I have had numerous discussions with faculty colleagues and students who have provided me excellent suggestions. In the end, I felt that to be the most useful that I would limit the methods not only to those that are frequently used, but to those used in several different disciplines as well. I have also divided the book into 6 basic sections to highlight the selected methods in protein chemistry, nucleic acids, recombinant DNA, antibody-based techniques, microscopy and imaging, and the use of animals in biomedical sciences. I also added a subsection on forensics, and tossed in a few other techniques that I thought would be of interest. In the end, I decided on which techniques "made the cut" (no, they were not selected solely on the basis of whether I've used them in my lab), and the organization of the book reflects what is probably my own idiosyncratic way of thinking. I think most of the basic methods are covered, but if you think a method that should have been included is not, let me know!

Chapter 1

DETECTION AND ANALYSIS OF PROTEINS

A. Introduction

Proteins are the body's building blocks. They are the second most abundant part of our bodies, comprising about 20% of our weight (the most abundant constituent, water, accounts for 70%). Proteins make up muscles, most of our enzymes are proteins, and the antibodies that protect us from pathogens are glycoproteins, or proteins that also contain carbohydrate sidechains. Proteins are biopolymers that consist of various mixtures of the 20 amino acids. The word "protein" is derived from a Greek root meaning "of first importance". Proteins were discovered in 1838 by Jöns Jacob Berzelius (1779–1848), a Swedish chemist. Berzelius also developed a system of chemical notation that is essentially the same basic system used today. Berzelius is one of three chemists who are considered the fathers of modern chemistry. The other two chemists are Antoine Levoisier (1743–1794) and John Dalton (1766–1844). Levoisier was the first to articulate and experimentally demonstrate the idea of the conservation of matter. He used quantitative methods to measure products of chemical reactions, allowing the composition of compounds to be determined with considerable accuracy. Levoisier also had the distinction of losing his head (literally!) during the French Revolution.

John Dalton made numerous contributions to the field of chemistry. He formulated the atomic theory, which proposed that elements were composed of atoms in fixed amounts and, therefore, each element had a fixed mass. His name is inextricably linked to measurements of weights of proteins. The basic unit is a "Dalton", or Da, which is defined as one-twelfth the mass of the most abundant isotope of carbon, ^{12}C, which is equal to $1.66053873 \times 10^{-24}$ grams. The association of Dalton's name with precise measurements of atomic mass seems ironic to me, in that Dalton was said to be a brilliant mind but not a particularly good

Table 1. Selected Nobel Laureates (Protein Chemistry)

Hermann Emil Fischer	structure of biomolecules; proof that protein molecules are chains of amino acids	1902*
Sir William Henry Bragg William Lawrence Bragg	use of x-ray diffraction for structural analysis	1915†
Jean Baptiste Perrin	sedimentation equilibrium	1926†
Theodor Svedberg	development and use of ultracentrifuge to determine molecular weight	1926*
George de Hevesy	use of isotopes as tracers	1943*
James Batcheller Sumner John Howard Northrop Wendell Meredith Stanley	chrystallography of proteins; protein purification and structure	1946*
Arne W.K. Tiselius	development of electrophoresis and adsorption to analyze proteins in complex mixtures	1948*
Archer John Porter Martin Richard L.M. Synge	development of partition (paper) chromatography	1952*
Linus Carl Pauling	discovery of chemical bonds	1954*
Frederick Sanger	structure and amino acid sequence of insulin	1958*
Max F. Perutz Sir John C. Kendrew	use of x-ray diffraction to solve structure of globular proteins	1962*
Herbert A. Hauptman Jerome Karle	development of methods to directly use x-ray diffraction information to solve structure	1985*
Richard R. Ernst	development of NMR	1991*
John B. Fenn Koichi Tanaka Kurt Wüthrich	structural analysis of macromolecules (mass spectrophoscopy; NMR)	2002*

*Nobel Prize in Chemistry
†Nobel Prize in Physics

experimentalist, even to the point of using inaccurate instrumentation for his measurements. But Dalton's curious mind also led him to be the first to identify color blindness, a condition that both he and his brother suffered from. In an attempt to permit the discovery of the molecular basis for his color blindness, he donated his eyes (not his head!) for study upon his death.

The discovery that proteins are composed of strings of amino acids was made by Hermann Fischer, who won the Nobel Prize in Chemistry in 1902 for his accomplishment (Table 1). The amino acids are joined together by peptide bonds, and consequently proteins are often referred to as polypeptides. Peptide bonds form between the carbon atom of the carboxyl group and nitrogen atom of the amino group of adjacent amino acids. The sequence of amino acids make up a protein identifies

its primary structure. The primary amino acid structure dictates local folding patterns within individual protein domains that are common to a number of different proteins. These folding patterns form the secondary structure of a protein. Linus Pauling, who won the Nobel Prize in Chemistry in 1954 for describing chemicala bonds, used both experimental approaches as well as models of small polypeptide chains to determine the orientation of the peptide bond. He found that because of the rigidity of the peptide bonds, amino acids would naturally assume common secondary structures stabilized by hydrogen bonds. The most common secondary structures are “beta-pleated sheets” and “alpha-helices”. The further folding of a protein that gives it its own unique overall structure is its tertiary structure. The tertiary structure of a protein is determine and also stabilized by chemical interactions such as hydrogen bonds, ionic bonds, Van der Waals forces and hydrophobic interactions. Further, many proteins form multisubunit complexes, and the way these interact identifies the quaternary structure of the protein.

The following sections outline common methods that are used to monitor proteins, analyze their composition and mass, and determine subunit structure in biomedicine. They also outline various methods used to determine secondary, tertiary and quaternary structures of proteins, and identify various post-translational modifications.

B. Basic methods for protein analysis

Determination of protein content

Three methods are commonly used to quantify proteins in biological samples. Two of these are colorimetric assays named after their inventors, Lowry, who developed the first simple method for protein quantitation in 1951 (1), and Bradford (2), who developed a more sensitive colorimetric assay in 1976. The fact that Lowry’s paper is one of the most frequently cited papers in history attests to the importance of methods to monitor protein concentration.

The Bradford assay is commonly used today due to its sensitivity and simplicity. It is based on the ability of Coomassie Brilliant blue dye, which was first used to dye wool, to bind to proteins at acid pH. Binding leads to a color change, which provides a measure of the amount of total protein present. The binding of Coomassie Blue is nonspecific and irreversible. Most Bradford assays today are quantitated using automated plate readers at an OD of 595 nm. The amount of protein can be quantitated by comparing the absorbance with a standard curve prepared using a purified protein such as albumin.