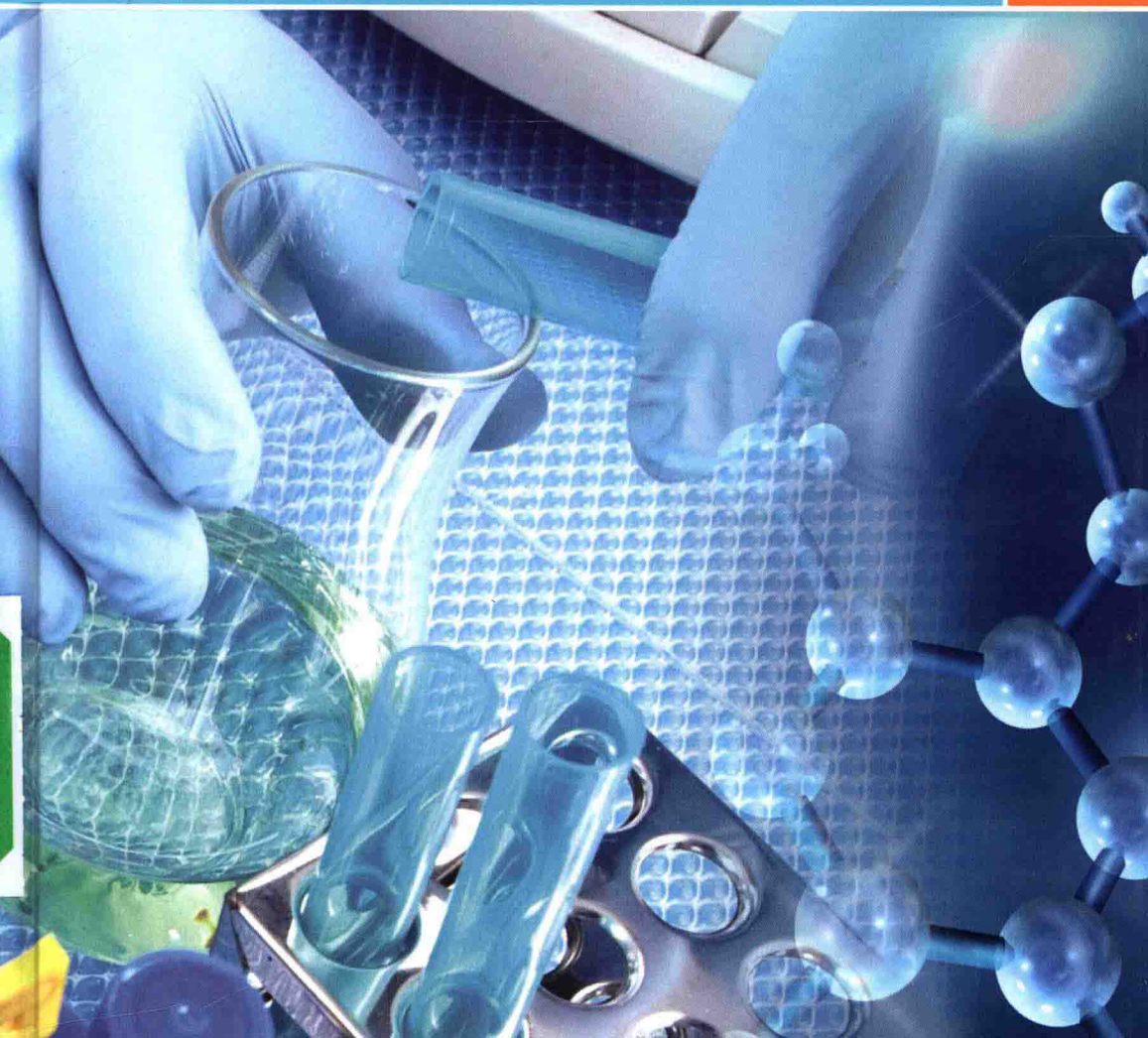
An Introduction to [redacted]ecular Biotechnology

Fundamentals, Methods and Applications

Tatyana Ivanovna Plekhanov
Editor



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Preface

The Hungarian agricultural engineer Karl Ereky foresaw in 1919 a time when biology could be used for turning raw materials into useful products. He coined the term biotechnology to describe that merging of biology and technology. Ereky's vision has now been realized by thousands of companies and research institutions. The growing list of biotechnology products includes medicines, medical devices, and diagnostics, as well as more-resilient crops, biofuels, biomaterials, and pollution controls. While the field of biotechnology is diverse, the focus of this guide is on molecular biotechnology.

Molecular Biotechnology is about the application of biological science at the molecular level. The 21st Century is called the Biotechnology Century based on the explosion of molecular technologies, and the impact these technologies are likely to have upon the world we live in. In the recent past, biotechnology has emerged as an important tool especially in the economic sectors of agriculture, livestock management, human health care and pharmaceutical industries, and for solving environmental and societal issues. As a result, many universities now offer various programmes in biotechnology and related fields. Already, the number of new products being developed for human and animal health is growing exponentially, and this trend is well and truly set to continue. Molecular Biotechnology is a fundamental component of modern biology. Molecular biotechnology is a vibrant competitive field of study that is formed by the union of recombinant DNA technology with biotechnology. Since the early 1970s, when recombinant DNA technology was first developed, there has been a veritable explosion of knowledge in the biological sciences. Since that time, with the advent of PCR, chemical DNA synthesis, DNA sequencing, monoclonal antibodies, directed mutagenesis, genomics, proteomics, and metabolomics, our understanding of and ability to manipulate the biological world have grown exponentially. The discovery that DNA was the key to life was the spark that lit the biotechnology revolution of the 20 century. DNA was the key, which allowed scientists to manipulate life on the molecular level. If

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the cells do not produce the correct proteins, don't worry, change its DNA. Can't wait long enough for nature to produce the correct strain? Change to DNA. From uncertain beginnings, molecular biotechnology has now established itself as the centrepiece of biotechnology. In fact, much of the population still equate genetic engineering and biotechnology as equivalent. We are now on the verge of a brave new future. Over the past two decades, biologists have acquired a very powerful tool in the form of molecular biotechnology. These techniques have led the analysis of biological systems in detail undreamed of only a generation ago.

Today, molecular biotechnology has given us several hundred new therapeutic agents, with many more in the pipeline, as well as dozens of transgenic plants. The use of DNA has become a cornerstone of modern forensics, paternity testing, and ancestry determination. Several new recombinant vaccines have been developed, with many more on the horizon. The list goes on and on. Molecular biotechnology really has lived up to its promise, to all of the original hype. It is already used widely in manufacturing insulin for diabetics, and is central to a range of other biomedical products. In the future, these technologies will continue to advance in areas such as developing increasingly sensitive forensic systems, new treatments for diseases and biological solutions for environmental problems. The methodology addresses many questions to arrive at an understanding of what is needed to pursue new types of treatments. The tools are designed to uncover the molecular roots of disease and pinpoint critical differences between healthy cells and diseased cells. Researchers often use multiple approaches to create a detailed picture of the disease process. Once the picture starts to emerge, it can still take years to learn which of the changes linked to a disease are most important. By determining which molecular defects are really behind a disease, scientists can identify the best targets for new medicines.

This book will help to gain a sound understanding of cellular processes, genetics and microbial systems - all of which are central to health, disease and genetic technologies - and learn about genomic technologies, bioinformatics, expression systems for the production of therapeutic proteins, plus microbial, plant, human and viral biotechnology. Scope of biotechnology, advanced techniques used in molecular biology and genetics, gene cloning technology and its applications, genetic engineering and how it revolutionized biotechnology, commercial potential of tissue culture technology and many other aspects are encompassed in this volume.

—Editor

Contents

Chapter 1

Techniques of Molecular Biology

Since the late 1950s and early 1960s, molecular biologists have learned to characterize, isolate, and manipulate the molecular components of cells and organisms. These components include DNA, the repository of genetic information; RNA, a close relative of DNA whose functions range from serving as a temporary working copy of DNA to actual structural and enzymatic functions as well as a functional and structural part of the translational apparatus; and proteins, the major structural and enzymatic type of molecule in cells.

Expression Cloning

One of the most basic techniques of molecular biology to study protein function is expression cloning. In this technique, DNA coding for a protein of interest is cloned (using PCR and/or restriction enzymes) into a plasmid (known as an expression vector). This plasmid may have special promoter elements to drive production of the protein of interest, and may also have antibiotic resistance markers to help follow the plasmid.

This plasmid can be inserted into either bacterial or animal cells. Introducing DNA into bacterial cells can be done by transformation (via uptake of naked DNA), conjugation (via cell-cell contact) or by transduction (via viral vector). Introducing DNA into eukaryotic cells, such as animal cells, by physical or chemical means is called transfection. Several different transfection techniques are available, such as calcium phosphate transfection, electroporation, microinjection and liposome transfection. DNA can also be introduced into eukaryotic cells using viruses or bacteria as carriers, the latter is sometimes called bactofection and in particular uses Agrobacterium tumefaciens. The plasmid may be integrated into the genome, resulting in a stable

transfection, or may remain independent of the genome, called transient transfection. In either case, DNA coding for a protein of interest is now inside a cell, and the protein can now be expressed. A variety of systems, such as inducible promoters and specific cell-signalling factors, are available to help express the protein of interest at high levels.

Large quantities of a protein can then be extracted from the bacterial or eukaryotic cell. The protein can be tested for enzymatic activity under a variety of situations, the protein may be crystallized so its tertiary structure can be studied, or, in the pharmaceutical industry, the activity of new drugs against the protein can be studied.

Polymerase Chain Reaction (PCR)

The polymerase chain reaction is an extremely versatile technique for copying DNA. In brief, PCR allows a single DNA sequence to be copied (millions of times), or altered in predetermined ways.

For example, PCR can be used to introduce restriction enzyme sites, or to mutate (change) particular bases of DNA, the latter is a method referred to as "Quick change". PCR can also be used to determine whether a particular DNA fragment is found in a cDNA library. PCR has many variations, like reverse transcription PCR (RT-PCR) for amplification of RNA, and, more recently, real-time PCR (QPCR) which allow for quantitative measurement of DNA or RNA molecules.

Gel Electrophoresis

Gel electrophoresis is one of the principal tools of molecular biology. The basic principle is that DNA, RNA, and proteins can all be separated by means of an electric field. In agarose gel electrophoresis, DNA and RNA can be separated on the basis of size by running the DNA through an agarose gel. Proteins can be separated on the basis of size by using an SDS-PAGE gel, or on the basis of size and their electric charge by using what is known as a 2D gel electrophoresis.

Macromolecule Blotting and Probing

The terms *northern*, *western* and *eastern* blotting are derived from what initially was a molecular biology joke that played on the term *Southern blotting*, after the technique described by Edwin Southern for the hybridisation of blotted DNA. Patricia.

Thomas, developer of the RNA blot which then became known as the *northern blot* actually didn't use the term. Further combinations of these techniques produced such terms as *southwesterns* (protein-

DNA hybridizations), *northwesterns* (to detect protein-RNA interactions) and *farwesterns* (protein-protein interactions), all of which are presently found in the literature.

Southern Blotting

Named after its inventor, biologist Edwin Southern, the Southern blot is a method for probing for the presence of a specific DNA sequence within a DNA sample. DNA samples before or after restriction enzyme digestion are separated by gel electrophoresis and then transferred to a membrane by blotting via capillary action. The membrane is then exposed to a labeled DNA probe that has a complement base sequence to the sequence on the DNA of interest. Most original protocols used radioactive labels, however non-radioactive alternatives are now available. Southern blotting is less commonly used in laboratory science due to the capacity of other techniques, such as PCR, to detect specific DNA sequences from DNA samples. These blots are still used for some applications, however, such as measuring transgene copy number in transgenic mice, or in the engineering of gene knockout embryonic stem cell lines.

Northern Blotting

The northern blot is used to study the expression patterns of a specific type of RNA molecule as relative comparison among a set of different samples of RNA. It is essentially a combination of denaturing RNA gel electrophoresis, and a blot. In this process RNA is separated based on size and is then transferred to a membrane that is then probed with a labeled complement of a sequence of interest. The results may be visualized through a variety of ways depending on the label used; however, most result in the revelation of bands representing the sizes of the RNA detected in sample. The intensity of these bands is related to the amount of the target RNA in the samples analyzed. The procedure is commonly used to study when and how much gene expression is occurring by measuring how much of that RNA is present in different samples. It is one of the most basic tools for determining at what time, and under what conditions, certain genes are expressed in living tissues.

Western Blotting

Antibodies to most proteins can be created by injecting small amounts of the protein into an animal such as a mouse, rabbit, sheep, or donkey (polyclonal antibodies)or produced in cell culture (monoclonal antibodies). These antibodies can be used for a variety of analytical and preparative techniques. In western blotting, proteins are first

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separated by size, in a thin gel sandwiched between two glass plates in a technique known as SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis).

The proteins in the gel are then transferred to a PVDF, nitrocellulose, nylon or other support membrane. This membrane can then be probed with solutions of antibodies. Antibodies that specifically bind to the protein of interest can then be visualized by a variety of techniques, including coloured products, chemiluminescence, or autoradiography. Often, the antibodies are labeled with enzymes. When a chemiluminescent substrate is exposed to the enzyme it allows detection. Using western blotting techniques allows not only detection but also quantitative analysis. Analogous methods to western blotting can be used to directly stain specific proteins in live cells or tissue sections. However, these *immunostaining* methods, such as FISH, are used more often in cell biology research.

Eastern Blotting

Eastern blotting technique is to detect post-translational modification of proteins. Proteins blotted on to the PVDF or nitrocellulose membrane are probed for modifications using specific substrates.

Arrays

A DNA array is a collection of spots attached to a solid support such as a microscope slide where each spot contains one or more single-stranded DNA oligonucleotide fragment. Arrays make it possible to put down a large quantity of very small (100 micrometre diameter) spots on a single slide. Each spot has a DNA fragment molecule that is complementary to a single DNA sequence (similar to Southern blotting). A variation of this technique allows the gene expression of an organism at a particular stage in development to be qualified (expression profiling). In this technique the RNA in a tissue is isolated and converted to labeled cDNA. This cDNA is then hybridized to the fragments on the array and visualization of the hybridization can be done. Since multiple arrays can be made with the exact same position of fragments they are particularly useful for comparing the gene expression of two different tissues, such as a healthy and cancerous tissue. Also, one can measure what genes are expressed and how that expression changes with time or with other factors. For instance, the common baker's yeast, *Sacch-aromyces cerevisiae*, contains about 7000 genes; with a microarray, one can measure qualitatively how each gene is expressed, and how that expression changes, for example, with a change in

temperature. There are many different ways to fabricate microarrays; the most common are silicon chips, microscope slides with spots of ~ 100 micrometre diameter, custom arrays, and arrays with larger spots on porous membranes (macroarrays). There can be anywhere from 100 spots to more than 10,000 on a given array. Arrays can also be made with molecules other than DNA. For example, an antibody array can be used to determine what proteins or bacteria are present in a blood sample.

Allele Specific Oligonucleotide

An allele-specific oligonucleotide (ASO) is a short piece of synthetic DNA complementary to the sequence of a variable target DNA. It acts as a probe for the presence of the target in a Southern blot assay or, more commonly, in the simpler Dot blot assay. It is a common tool used in genetic testing, forensics, and Molecular Biology research.

An ASO is typically an oligonucleotide of 15–21 nucleotide bases in length. It is designed (and used) in a way that makes it specific for only one version, or allele, of the DNA being tested. The length of the ASO, which strand it is chosen from, and the conditions by which it is bound to (and washed from) the target DNA all play a role in its specificity. These probes can usually be designed to detect a difference of as little as 1 base in the target's genetic sequence, a basic ability in the assay of single-nucleotide polymorphisms (SNPs), important in genotype analysis and the Human Genome Project. To be detected after it has bound to its target, the ASO must be labelled with a radioactive, enzymatic, or fluorescent tag. The Illumina Methylation Assay technology takes advantage of ASO to detect one base pair difference (cytosine versus thymine) to measure methylation at a specific CpG site.

Example

Figure: *Binding of the "S" ASO probe to "S" DNA (top) or "A" DNA (bottom).*

The human disease sickle cell anemia is caused by a genetic mutation in the codon for the sixth amino acid of the blood protein

beta-hemoglobin. The normal DNA sequence G-A-G codes for the amino acid glutamate, while the mutation changes the middle adenine to a thymine, leading to the sequence G-T-G (G-U-G in the mRNA). This altered sequence substitutes a valine into the final protein, distorting its structure.

To test for the presence of the mutation in a DNA sample, an ASO probe would be synthesized to be complementary to the altered sequence, here labelled as "S". As a control, another ASO would be synthesized for the normal sequence "A". Each ASO is fully complementary to its target sequence (and will bind strongly), but has a single mismatch against its non-target allele (leading to weaker interaction). The first diagram shows how the "S" probe is fully complementary to the "S" target (top), but is partially mismatched against the "A" target (bottom).

A segment of the beta-hemoglobin genes in the sample DNA(s) would be amplified by PCR, and the resulting products applied to duplicate support membranes as Dot blots. The sample's DNA strands are separated with alkali, and each ASO probe is applied to a different blot. After hybridization, a washing protocol is used which can discriminate between the fully complementary and the mismatched hybrids. The mismatched ASOs are washed off of the blots, while the matched ASOs (and their labels) remain.

In the second diagram, six samples of amplified DNA have been applied to each of the two blots. Detection of the ASO label that remains after washing allows a direct reading of the genotype of the samples, each with two copies of the beta-hemoglobin gene. Samples 1 and 4 only have the normal "A" allele, while samples 3 and 5 have both the "A" and "S" alleles (and are therefore heterozygous carriers of this recessive mutation). Samples 2 and 6 have only the "S" allele, and would be affected by the disease. The small amount of 'cross hybridization' shown is typical, and is considered in the process of interpreting the final results.

Alternatives

ASO analysis is only one of the methods used to detect genetic polymorphisms. Direct DNA sequencing is used to initially characterize the mutation, but is too laborious for routine screening. An earlier method, Restriction Fragment Length Polymorphism (RFLP) didn't need to know the sequence change beforehand, but required that the mutation affect the cleavage site of a Restriction Enzyme. The RFLP assay was briefly adapted to the use of oligonucleotide probes, but this

technique was quickly supplanted by ASO analysis of polymerase chain reaction (PCR) amplified DNA. The PCR technique itself has been adapted to detect polymorphisms, as allele-specific PCR.

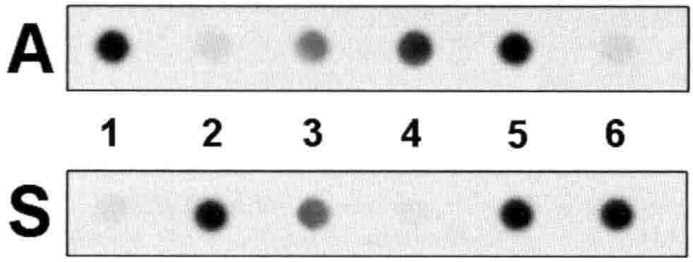

Figure: *Schematic of dot-blots using the "A" or "S" ASO probes.*

However, the simplicity and versatility of the combined PCR/ASO method has led to its continued use, including with non-radioactive labels, and in a "reverse dot blot" format where the ASO probes are bound to the membrane and the amplified sample DNA is used for hybridization.

History

The use of synthetic oligonucleotides as specific probes for genetic sequence variations was pioneered by R. Bruce Wallace, working at the City of Hope National Medical Center in Duarte, California. In 1979 Wallace and his coworkers reported the use of ASO probes to detect variations in a single-stranded bacterial virus, and later applied the technique to cloned human genes. In 1983 and 1985 Wallace's lab reported the detection of the mutation for sickle cell anemia in samples of whole genomic DNA, although this application was hampered by the small amount of label that could be carried by the ASO.

Fortunately PCR, a method to greatly amplify a specific segment of DNA, was also reported in 1985. In less than a year PCR had been paired with ASO analysis. This combination solved the problem of ASO labelling, since the amount of target DNA could be amplified over a million-fold. Also, the specificity of the PCR process itself could be added to that of the ASO probes, greatly reducing the problem of spurious binding of the ASO to non-target sequences. The combination was specific enough that it could be used in a simple Dot blot, avoiding the laborious and inefficient Southern blot method.

The Cell

The cell is one of the most basic units of life. There are millions of different types of cells. There are cells that are organisms onto

themselves, such as microscopic amoeba and bacteria cells. And there are cells that only function when part of a larger organism, such as the cells that make up your body. The cell is the smallest unit of life in our bodies. In the body, there are brain cells, skin cells, liver cells, stomach cells, and the list goes on. All of these cells have unique functions and features. And all have some recognizable similarities. All cells have a 'skin', called the plasma membrane, protecting it from the outside environment.

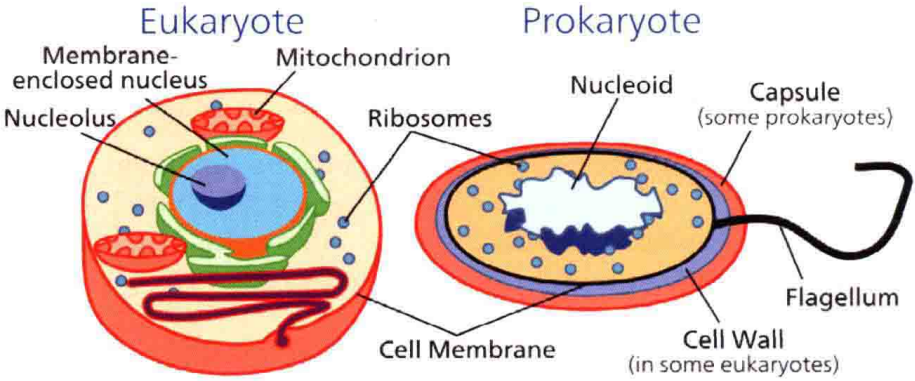

Figure: *The cells of eukaryotes (left) and prokaryotes (right)*

The cell membrane regulates the movement of water, nutrients and wastes into and out of the cell. Inside of the cell membrane are the working parts of the cell. At the centre of the cell is the cell nucleus. The cell nucleus contains the cell's DNA, the genetic code that coordinates protein synthesis. In addition to the nucleus, there are many organelles inside of the cell - small structures that help carry out the day-to-day operations of the cell. One important cellular organelle is the ribosome. Ribosomes participate in protein synthesis. The transcription phase of protein synthesis takes places in the cell nucleus. After this step is complete, the mRNA leaves the nucleus and travels to the cell's ribosomes, where translation occurs. Another important cellular organelle is the mitochondrion. Mitochondria (many mitochondrion) are often referred to as the power plants of the cell because many of the reactions that produce energy take place in mitochondria. Also important in the life of a cell are the lysosomes. Lysosomes are organelles that contain enzymes that aid in the digestion of nutrient molecules and other materials. Below is a labelled diagram of a cell to help you identify some of these structures.

There are many different types of cells. One major difference in cells occurs between plant cells and animal cells. While both plant and animal cells contain the structures discussed above, plant cells have

some additional specialized structures. Many animals have skeletons to give their body structure and support. Plants do not have a skeleton for support and yet plants don't just flop over in a big spongy mess.

This is because of a unique cellular structure called the cell wall. The cell wall is a rigid structure outside of the cell membrane composed mainly of the polysaccharide cellulose. As pictured at left, the cell wall gives the plant cell a defined shape which helps support individual parts of plants. In addition to the cell wall, plant cells contain an organelle called the chloroplast. The chloroplast allow plants to harvest energy from sunlight. Specialized pigments in the chloroplast (including the common green pigment chlorophyll) absorb sunlight and use this energy to complete the chemical reaction:

$$6\ CO_2 + 6\ H_2O + \text{energy (from sunlight)} \rightarrow C_6H_{12}O_6 + 6\ O_2$$

In this way, plant cells manufacture glucose and other carbohydrates that they can store for later use.

Organisms contain many different types of cells that perform many different functions. In the next lesson, we will examine how individual cells come together to form larger structures in the human body.

The cell is the functional basic unit of life. It was discovered by Robert Hooke and is the functional unit of all known living organisms. It is the smallest unit of life that is classified as a living thing, and is often called the building block of life. Some organisms, such as most bacteria, are unicellular (consist of a single cell). Other organisms, such as humans, are multicellular. Humans have about 100 trillion or 10 cells; a typical cell size is 10 μm and a typical cell mass is 1 nanogram. The largest cells are about 135 μm in the anterior horn in the spinal cord while granule cells in the cerebellum, the smallest, can be some 4 μm and the longest cell can reach from the toe to the lower brain stem (Pseudounipolar cells). The largest known cells are unfertilised ostrich egg cells which weigh 3.3 pounds.

In 1835, before the final cell theory was developed, Jan Evangelista Purkynì observed small "granules" while looking at the plant tissue through a microscope. The cell theory, first developed in 1839 by Matthias Jakob Schleiden and Theodor Schwann, states that all organisms are composed of one or more cells, that all cells come from preexisting cells, that vital functions of an organism occur within cells, and that all cells contain the hereditary information necessary for regulating cell functions and for transmitting information to the next generation of cells. The word *cell* comes from the Latin *cellula*, meaning, a small room. The

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