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Single neurons as experimental systems in molecular biology

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Abstract

The cellular and the inter-connective complexity of the central nervous system (CNS) necessitate's analysis of functioning at both the system and single cell levels. Systems neuroscience has developed procedures that facilitate the analysis of multicellular systems including multielectrode arrays, dye tracings and lesioning assays, and at the single cell level there have been significant strides in assessing the physiology and morphology of individual cells. Until recently little progress had been made in understanding the molecular biology of single neuronal cells. This review will highlight the development of PCR and aRNA procedures for analysis of mRNA abundances in single cells. Also, other procedures for the analysis of protein abundances as well as the association of RNA with proteins will also be summarized. These procedures promise to provide experimental insights that will help unravel the functional mechanisms regulating the cellular components of the CNS.

Contents

Abbreviations: PCR, polymererase chain reaction; RNA, ribonucleic acid; mRNA, messenger RNA; LTP, long-term potentiation; LTD, long-term depression; CNS, central nervous system; aRNA, amplified antisense RNA; RT-PCR, real-time polymerase chain reaction; cDNA, complementary deoxyribonucleic acid; GABA, gamma-amino butyric acid; NFT, neurofibrillary tangle; TUNEL, Tdt-mediated dUTP digoxigenin nick end labeling; RBP, RNA binding protein; RRM, RNA recognition motif; hnRNP, heteronuclear ribonuclear protein; rRNA, ribosomal RNA; snRNA, small nuclear RNA; NTP, nucleotide triphosphate; APRA, antibody positioned RNA amplification; FMRP, fragile-X mental retardation protein; FXS, fragile-S syndrome; CREB, cyclic adenosine monophosphate response element binding protein; DHPG, (RS)-3,5-dihydrosy-phenylglycine; GluR2, ionotropic glutamate receptor subunit 2; mGluR1, metabotropic glutamate receptor subtype 1; UTR, untranslated region; CamKII, calmodulin kinase II; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; RCA, rolling circle DNA amplification; IDAT, immuno-detection amplified by T7 RNA polymerase; ELISA, enzyme-linked immunosorbent assay; cdr, complementarity determining region; ScFv, single chain Fv fragment

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1. Introduction

In 1894 Cajal formalized the cellular analysis of the central nervous system (CNS) with the publication of his tome "Les nouvelles idees sur la fine anatomie des centers nerveux". His detailed studies provided the first systematic analysis of the two major CNS cell types, neurons and glia. The inter-connectivity of neurons was established and the detailed morphological polarity of neurons and glia was discovered using light microscopy and various dyes that would contrast the cellular membranes or organelles with the general tissue background. These studies provided the intellectual framework from which it was possible to ask how CNS cells communicate with one another, how morphologically similar cells functionally differ from one another, and how morphological polarity is established and maintained.

These early anatomical investigations showed that neurons contacted one another receiving information on their cell bodies and dendrites and passing the information to other cells through their axons. Cajal called this idea "the law of dynamic polarization". The sites through which neurons communicate with one another have since been labeled as synapses. Synapses are oriented such that the axon of a presynaptic neuron abuts the dendrite of a post-synaptic neuron creating an area at which the neurons communicate with one another. This structural polarity is one of the key hallmarks of how neurons function. Given the morphological and functional polarity of neurons it is reasonable to ask whether this polarity modulates the functioning of neurons.

To investigate this question it is necessary to analyze neurons as individual units as well as individual neurons in ensemble connection with other neurons and glia. This level of analysis requires utilization of methodologies that provide single cell resolution. This was accomplished for functional analysis of neuronal channels through the development of whole-cell and patch-clamp recording procedures. These electrophysiological analyses led to the discovery of neuronal physiologies including long-term potentiation (LTP) and long-term depression (LTD).

Ideally, the molecular analysis of neuronal functioning would highlight mechanistic aspects of RNA transcription, RNA-processing and transport, mRNA translation, protein modification, as well as mRNA and protein degradation. Each of these biological processes involves the functioning of many proteins and additionally, in some cases, RNAs. For example, mRNA abundance is a dynamic property of a cell that represents a balance between RNA transcription and RNA degradation. Transcription of DNA into RNA requires the concerted activity of hundred's of proteins while RNA degradation is more poorly understood, but is thought to involve between 3 and 10 proteins. In general, the quantitation of these processes is approximated by measuring the cellular mRNA abundance using techniques such as Northern Blotting, in situ hybridization, PCR, and microarray analysis.

An important aspect of these types of analyses is the sample that is being analyzed. While model systems, such as drosophila or mouse, are often used in neuroscience, the analysis of human samples is more limited because tissue sources are scarce. Indeed, often the only tissue available for analysis is from pathological tissue specimens. While some fresh pathological tissue specimens are available for research as by-products of surgery, most are fixed tissue samples. Consequently, for the foreseeable future, the analysis of the cell biology of human samples must rely upon the analysis of fixed tissues.

This chapter will highlight the need to examine multiple biological processes simultaneously in a selected cellular environment. The need for this follows from the observations that all diseases have multigenic consequences and all of cellular physiology results from the coordinate regulation of multiple genes and gene products.

2. Single cell mRNA abundance analysis from fixed tissues and live cells

Single-cell gene expression analysis becomes necessary when cell populations of interest are limited, phenotypically heterogeneous, or anatomically isolated. Clinically relevant examples would include the evaluation of soft tissue biopsies or cerebrospinal fluid specimens for suspected cancer. Fundamental research examples would include the analysis of mechanisms of oocyte maturation (in which only one cell develops at a time), neuronal phenotypic variation within isolated nuclei or cortical layers of the central nervous system (CNS), or disease states (such as Alzheimer's disease, AD) in which sub-populations of cells are specifically targeted by degenerative processes (Callahan et al., 1998; Steuerwald et al., 2000; Bonaventure et al., 2002). The more recent realization that cells within seemingly homogeneous populations are actually heterogeneous at the level of gene expression further supports the need for highly sensitive and quantitative single-cell approaches to their study (Eberwine et al., 1992, 2001; Pape et al., 2001; Theilgaard-Monch et al., 2001; Elowitz et al., 2002).

Multiple methods are now being used to identify targets for single-cell gene expression analysis. As is discussed elsewhere in this review, single living cells can be identified based upon phenotypic criteria such as morphology, fluorescent protein expression, and membrane current physiology. The use of immunocytochemical techniques in fixed cells can greatly increase the investigator's ability to isolate cells of interest through the detection of specific antigen expression in addition to morphology and anatomic localization. Taking advantage of these cellular identification techniques, various molecular biological assays have been developed that allow for the isolation and amplification of mRNA from single-cells or from sub-cellular regions.

RNA amplification can be achieved using either the reverse transcription polymerase chain reaction (RT-PCR) or the amplified antisense RNA (aRNA) procedure. RT-PCR is

a relatively straightforward method of amplification where mRNA is reverse transcribed into cDNA using either random or poly-T primers and reverse transcriptase. The 5′ ends of the newly synthesized cDNA can be modified to include a sequence that allows the binding of a 5′. After this manipulation specific primers for both the 5′ and 3′ ends can be used for the PCR. aRNA amplification involves reverse transcription of mRNA with a poly-T primer linked to a bacteriophage promoter sequence (usually the T7 promoter) or a random dodecamer with a bacteriophage RNA promoter site (Fig. 1). After second-strand synthesis is completed, RNA polymerase is used to make RNA transcripts from the bacteriophage promoter-linked construct. This aRNA product can then be used as template for cDNA synthesis and conversion into double stranded cDNA so that subsequent rounds of RNA polymerase-based RNA amplification can be performed. This procedure is quite robust, as ∼2000 transcripts can be generated from each DNA template (Phillips and Eberwine, 1996) when using high concentration T7 RNA polymerase.

Both RT-PCR and aRNA amplification have been used to amplify transcripts from single living cells (Mackler and Eberwine, 1993; Telfeian et al., 2003). RT-PCR appears to be suitable for amplification of a relatively small number of genes (<20), as a portion of the reverse-transcription reaction can be added to separate PCR reactions, each containing gene-specific primers. Alternatively, multiplex PCR can be performed but quantitation of such complex PCR data is quite difficult. While RT-PCR is widely used to amplify cellular RNA, this technique suffers from skewing of the abundances of the original RNA population due to the exponential nature of the amplification process. Therefore, PCR is generally more difficult to make quantitative than other procedures (Lambolez et al., 1992; Dixon et al., 2000; Liss, 2002). However, the advent of real-time PCR technology has made such quantitation more reliable (Steuerwald

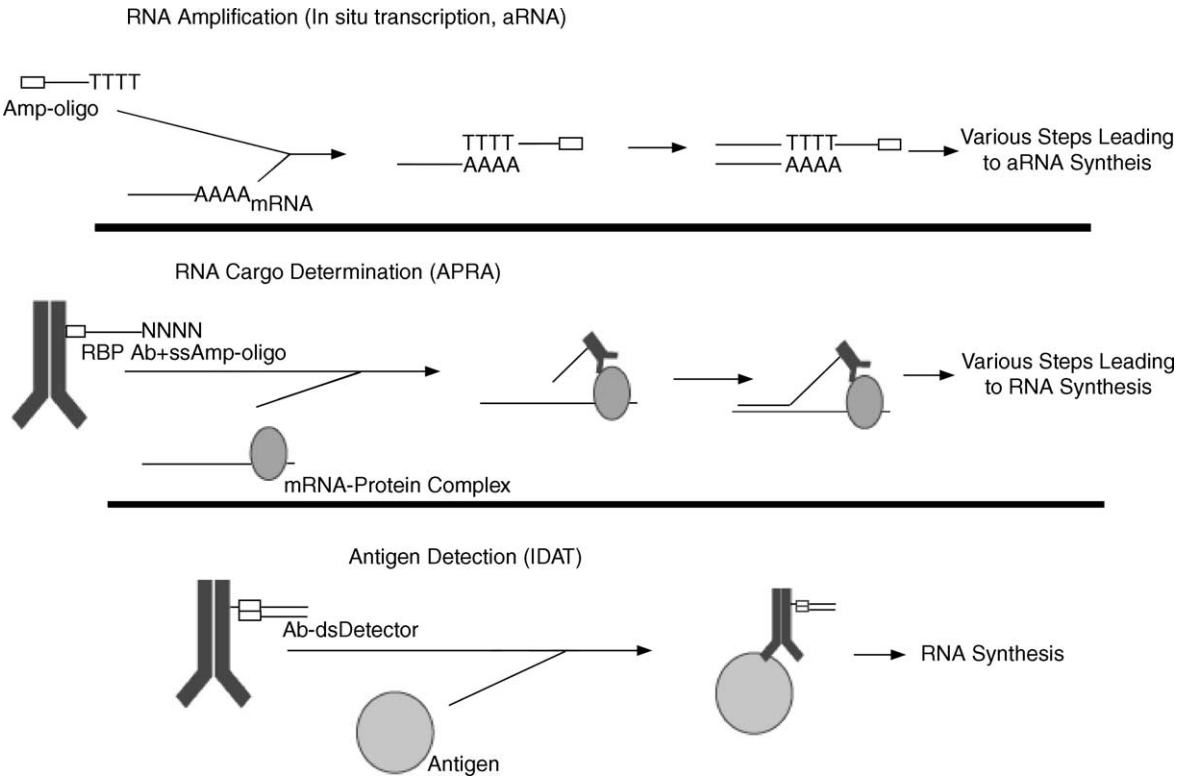

Fig. 1. Schematic of the aRNA, APRA and IDAT Procedures. These procedures permit the detection and quantification of mRNAs (aRNA), RNAs associated with a RNA binding protein (APRA) and proteins (IDAT). Each procedure uses a short DNA sequence containing a T7 RNA polymerase promoter site. Detection of the molecules to be quantified occurs through the linear amplification of the DNA sequence 3′ of the T7 RNA polymerase promoter site. The aRNA amplification procedure (top panel) utilizes a short oligonucleotide sequence containing a poly-T at its 3′ end and a T7 RNA polymerase promoter on the 5′ end. This oligonucleotide is annealed to the poly-A tail of mRNA. The mRNA is copied into cDNA using the oligonucleotide as a primer and after various procedural steps a double-stranded cDNA (dscDNA) containing the T7 RNA polymerase promoter is synthesized. This dscDNA is amplified into RNA using T7 RNA polymerase. In the middle panel depicting the APRA procedure, an antibody directed against a RNA binding protein (RBP) is attached (covalently or noncovalently) to a single stranded DNA containing containing a T7 RNA polymerase promoter site (5′ end) and a degenerate sequence (3′ end). This antibody-DNA complex is used in immunohistochemistry and the remaining steps of the APRA procedure are detailed in the body of this manuscript. The key feature is the in vitro synthesis of cDNA copies of mRNAs that are in close proximity to the RBP. The IDAT procedure depicted in the bottom panel shows an antibody that will specifically bind to a particular antigen and is in complex with a double stranded DNA containing the T7 RNA polymerase promoter site. The antibody–antigen complex is detected by the T7 RNA polymerase mediated amplification of the DNA that is attached to the antibody.

et al., 2000). These techniques have most-often been applied to the amplification of RNAs isolated from living cells, and will not be discussed in detail.

For global expression profiling, aRNA amplification facilitates a million fold amplification of mRNA from as few as two rounds of aRNA amplification, thereby creating enough material for the subsequent molecular analyses. As T7 RNA polymerase amplifies the DNA template linearly, any skewing that might result in amplification rate would be linear as opposed to the exponential skewing that occurs with PCR. In other words, since T7 RNA polymerase binds to the T7 RNA polymerase promoter in a consecutive manner and proceeds at a standard rate of ≈100 base/s to create RNA molecules from the substrates; several T7 RNA polymerases may be bound to a single template depending upon the length of the cDNA. Amplification is therefore exclusively dependent on the number of templates and the length of the templates. A cDNA template, X, that is 10 times the length of another template, Y, will have 10 times more T7 RNA polymerase on it as compared to the other template Y, but Y will initiate 10 times more often then X so if there are equivalent amounts of these mRNAs the amount of amplified aRNA will be equal. At least 20 cycles of PCR are required to amplify mRNA 1,000,000-fold, whereas only two rounds of aRNA amplification are required to produce equivalent amounts. Moreover, mistakes or inefficient amplification occurring in the early rounds of RT-PCR amplification would propagate these inaccuracies in the final PCR product. Those errors which result from multiple rounds of amplification are inherent to the RT-PCR but not to the aRNA amplification procedure. Because of these differences, aRNA amplification has become popular in the generation of probes for expression profiling from large amounts of tissue as well as from single cells (Brooks-Kayal et al., 1998; Nair et al., 1998; Telfeian et al., 2003).

T7 RNA polymerase-mediated antisense RNA (aRNA) amplification approaches take advantage of the linear nature of the transcriptional process to amplify mRNA in a highly reproducible and quantitative manner that maintains the complexity of the original mRNA population (Van Gelder et al., 1990; Cheetham et al., 1997; Morrison et al., 2000), and have been further empowered by the development of in situ transcription techniques that allow for first strand cDNA synthesis to be achieved in cells prior to their isolation (Tecott et al., 1988; Van Gelder et al., 1990; Crino et al., 1996). The products of mRNA amplification can be used to identify and quantitate the expression of known mRNAs (by slot blots or custom arrays) and/or of novel transcripts (by EST-based micro- or macroarrays, cDNA library generation and sequencing, Taqman PCR-based RNA quantitation, or PCR-based techniques such as SAGE or differential display from native or experimentally manipulated single cells. Dependent upon the type of data being generated, one or more of these approaches may also be utilized to confirm subsets of the microarray data.

Single cells of interest can be identified for analysis by multiple methods. Chow et al. (1998) amplified RNA from single, morphologically identified pyramidal neurons from multiple brain regions of Alzheimer's disease and control brains to evaluated differences in gene expression profiles by slot blot analysis. Neurons of interest can also be identified within specific nuclei/subnuclei of the brain or peripheral nervous system using Nissl staining and neuroanatomic localization (Luo et al., 1999; Bonaventure et al., 2002). Using these techniques, combined with laser capture microdissection of single neurons, the cells analyzed were shown to exhibit highly individualized gene expression profiles using T7 RNA polymerase-based amplification and cDNA microarray analysis. Single cells can also be identified through the detection of specific antigen expression by immunohistochemistry. Several groups have exploited this detection mechanism to isolate and evaluate single abnormal cells independently from their normal, neighboring counterparts in various models of human disease. For example, Hemby et al. (2002) identified human entorhinal cortex layer II neurons by neurofilament staining and demonstrated differences in gene expression between individual cells in schizophrenic and neurologically normal brains. Similarly, Crino et al. (1996) used the expression of neuronal precursor protein antigens to identify, isolate, and then generate molecular profiles of individual abnormal cells in CNS tubers. In further studies, these techniques were used to demonstrate that GABA and glutamate receptor subunit mRNAs were differentially expressed in single dysplastic, versus normal, neighboring neurons in cortical dysplasia (Crino et al., 1997, 2001). Ginsberg et al. (1999, 2000), in a series of reports, successfully demonstrated differential gene expression patterns not only in individual neurons affected by the degenerative process of Alzheimer's disease (neurofibrillary tangle, or NFT, positive by immunohistochemistry) when compared to neighboring, unaffected (NFT negative) hippocampal neurons, but also revealed the presence of specific patterns of mRNA sequestration within single pathological inclusion bodies such as NFTs, neuropil threads, and senile plaques. In a further application, apoptotic neurons produced by lateral fluid percussion injury in rats were identified and isolated away from TUNEL-negative (non-apoptotic) cells and analyzed for differences in gene expression in a model of human head trauma (O'Dell et al., 2000).

The analysis of mRNA populations from fixed cells provides important insight into the molecular underpinning of many human diseases. There are several advantages for fixing single-cells prior to expression analysis. RNases are inactivated by fixation (Tongiorgi et al., 1998). Cell membranes are made less elastic, which can aid the dissection of cells from the extracellular matrix as well as dissection of sub-cellular regions, such as dendrites, from whole neurons. However, strong fixation can cause base modification and protein cross-linking that may inhibit the initial reverse transcription step, leading to shorter cDNAs and subsequent amplified nucleic acids (Eberwine et al., 1992, 1993; Crino

et al., 1996; Masuda et al., 1999). Perhaps the greatest limitation of expression analysis of fixed cells is the inability to measure many of the physiological characteristics of cell prior to cell harvest.

Single live-cell analysis has been particularly useful in the isolation of neurons that have been selected for expression analysis on the basis of their physiological profile. Single neurons can be patch-clamped for the measurement of membrane capacitance, conductance, or other parameters (Mackler and Eberwine, 1993; Brooks-Kayal et al., 1998; Nair et al., 1998). Once a cell has been identified and the physiological parameters determined, the cellular contents are aspirated into the recording electrode and expelled into a sample tube for the reverse transcription reaction (Brooks-Kayal et al., 1998; Telfeian et al., 2003).

Analysis of live cells is also advantageous when working with very small amounts of mRNA (<1 pg), where the negative effects of fixative-induced RNA modifications and cross-linking may be problematic. Expression profiling of dendrites and the growth cones of axons have been successfully performed on live neurons (Miyashiro et al., 1994; Crino and Eberwine, 1996). Patch-clamp pipettes may be utilized as scalpels to dissect the neuronal process from the cell soma. After dissection, the neuronal process can be aspirated into the pipette and transferred to a sample tube for analysis. Expression profile analysis of neuronal processes has resulted in the identification of many mRNAs that are selectively localized in the dendrites, and potentially, the perisynaptic space. These data have been crucial in the development of the synaptic plasticity model, that selective protein synthesis near the synapse is crucial for the development of the synapse and regulation of synaptic signaling (Mackler et al., 1992; Miyashiro et al., 1994; Crino and Eberwine, 1996).

These basic methods have also been modified to amplify RNA populations within sub-cellular regions to assess not only gene expression, but also mRNA trafficking patterns, in single cells (Fig. 2). Miyashiro et al. (1994) were early innovators of this application, demonstrating not only that individual neurites from rat hippocampal neurons (identified by morphology) expressed different assortments of glutamate receptor subunit mRNAs, but that these populations varied within different segments of the neurites, and between neurites and cell bodies of the same neurons. Crino et al. further identified dendrite- and dendrite growth cone-specific transcripts using similar methods, suggesting that mRNAs are transported to peripheral cellular processes in a highly-regulated manner to support crucial cell functions and synapse formation/maintenance (Crino and Eberwine, 1996; Crino et al., 1998). These latter publications went on to show that translation and post-translational modifications occur within the dendrite itself, as will be detailed elsewhere.

Another application of live neuron expression profiling was illustrated in the work of Mackler and Eberwine in which neurons that had undergone tetanic stimulation were

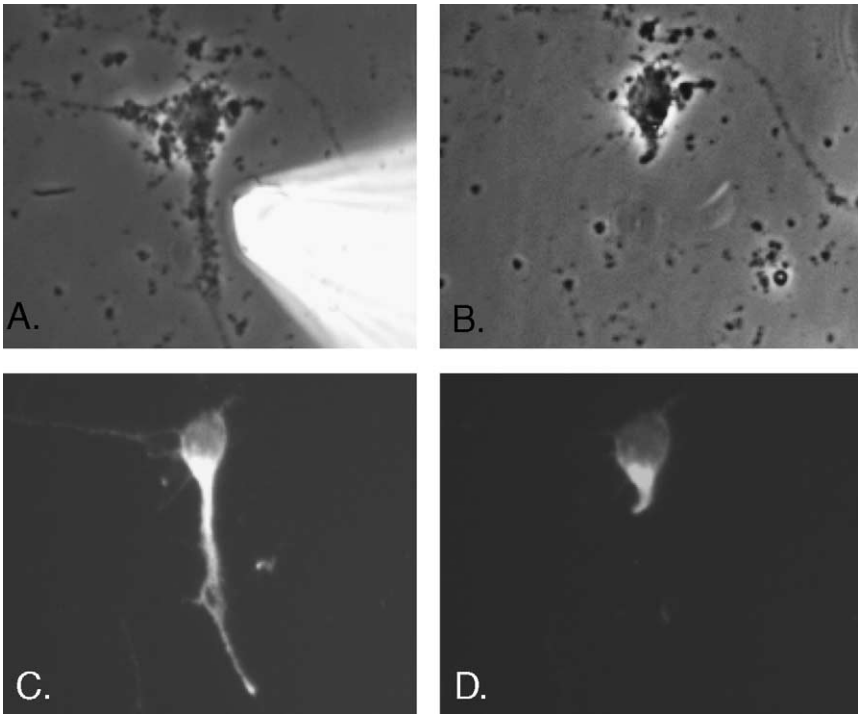

Fig. 2. The mechanical dissection of a neuronal dendrite. An individual neuron is shown in phase (panel A) and upon fluorescence from transfected GFP (panel C). The patch pipette used to sever the process is shown in panel A. Panels B and D show the remaining cell soma after the dendrite has been severed and harvested into the patch pipette. This mechanical severing is quite easy and can yield approximately 5 dendrites/min.

expression profiled (Mackler et al., 1992). The goal of these experiments was to determine which genes responded to such neuronal stimulation by increasing or decreasing mRNA abundances. Among the changes were increases in calmodulin dependent protein kinase II and GABA-A receptor β-1 subunit mRNAs while a decrease was observed in protein kinase C mRNA. These studies were done before the advent of microarrays and consequently were limited in their analysis. But even with these limitations, this study described the first coordinate changes in single cell gene expression associated with physiological manipulation.

3. RNA binding protein mRNA cargo analysis

While not generally appreciated, mRNAs within cells are associated with RNA binding proteins (RBPs). Trafficking of mRNAs to different cellular regions and all other aspects of RNA metabolism require the association of RNAs with RBPs. For example, RBPs facilitate RNA synthesis and degradation as well as mRNA translation into protein. The association of RBPs with RNAs is therefore an important aspect of cellular biology that has, unfortunately, been difficult to examine. Recently, various procedures have been developed to examine these interactions in a quantitative manner. Before discussing these procedures, a summary of the complexities of these interactions will place the technical issues into the proper scientific context.

Genetic and biochemical reports have characterized the specific properties of RBPs and the RNAs with which they interact. In addition, functional studies demonstrate the crucial role RBPs play as cellular integrators exerting control on RNA metabolism, pre-mRNA splicing and editing events, shuttling of mature transcripts between the nucleus and cytoplasm, and the stability and translational control of trafficked mRNAs (Maquat and Carmichael, 2001). The disruption of RBP function may underlie the pathogenesis of particular forms of epilepsy (Musunuru and Darnell, 2001), rheumatism (Fritsch et al., 2002), cancer (Barbouti et al., 2003), motor neuron disease (Pellizzoni et al., 1998), and mental retardation (Turner et al., 1996).

Many RBPs can be classified by the presence of phylogenetically-conserved *trans*-acting protein domains or motifs, which are known, or hypothesized, to directly recognize *cis*-acting RNA sequence elements. The RNA recognition motif (RRM), DEAD/DExH-box, hnRNP K homology (KH) domain, and double-stranded RNA-binding domain (dsRBD) are four of the most common motifs used to classify RBPs. Often, RBPs contain some mixture of RNA-binding motifs which gives rise to a set of RNA-binding modules which are known, or thought, to lead to cooperative binding (Cobianchi et al., 1988; Nadler et al., 1991).

The RRM is typically 80–100 amino acids which possess two short consensus sequences, the RNP2 octamer and the RNP1 hexamer, and several highly-conserved hydrophobic residues located outside these consensus regions (Kenan et al., 1991). The RRM is highly conserved throughout evolution and present in pre-mRNA-, mRNA-, pre-rRNA-, and snRNA binding proteins. Mutational analysis of various amino acid residues within RNP1 and RNP2, have pinpointed several conserved amino acids as essential for RNA-binding activity (Scherly et al., 1989; Lutz-Freyermuth et al., 1990). These studies suggest that the β-sheets, their inter-connecting loops, and the contiguous amino and carboxy-termini of the RRM are juxtaposed to direct the specificity of RNA binding (Gorlach et al., 1992; Oubridge et al., 1994).

The DEAD box and related DexD/H proteins have seven to eight conserved motifs. These domains serve to unwind areas of double-stranded RNA via the use of NTP hydrolysis. This RNA helicases or “unwindase” activity is rarely processive, as is the case of DNA helicases, since RNA does not often have long segments of continuous double-stranded regions. The enzymatic core of the eIF4A-like DEAD box proteins are characterized by two discrete domains connected by Walker A and B nucleotide triphosphate binding motifs (Fry et al., 1986). A cleft formed by domains 1–2 and domain 3 acts as the RNA recognition platform where a number of conserved amino acids serve to intercalate the bases of the substrate RNA (Tanner and Linder, 2001).

The dsRBD is a ∼65–70 amino acid sequence which confers highly specific double-, but not single-stranded RNA binding (St. Johnston et al., 1992). Three regions of the dsRBD structure interact with nascent mRNAs: the amino- and carboxy-terminal α-helices and the loop between β-strands 1 and 2 (Ryter and Schultz, 1998). The minor groove of the RNA interacts with three-turn amino-terminal α-helix and the loop connecting β-strands 1 and 2. The major groove of the interacting RNA contacts with residues in the carboxy-terminal α-helix. It is unclear if unidentified sequence elements may confer RNA binding specificity (Manche et al., 1992; St. Johnston et al., 1992; Krovat and Jantsch, 1996).

The KH domain is ∼70 amino acids characterized by a set of conserved hydrophobic residues, an invariant Gly-X-X-Gly segment, and a variable loop. The Gly-X-X-Gly motif and the variable loop connecting the second and third β-strands form an α-helix/β-sheet vise-like pocket, composed almost exclusively of aliphatic side chains, that serves as the RNA recognition surface (Lewis et al., 2000). Mutagenesis of the KH domain in a number of proteins support the necessity of the Gly-X-X-Gly motif (Jones and Schedl, 1995) and then variable loop (Baehrecke, 1997) as lynchpins for RNA recognition.

One RBD of interest containing a KH domain is the fragile-X mental retardation protein (FMRP). In addition to two KH domains, FMRP contains another motif known to bind RNA, the RGG box. Mutations in the KH domain or gene silencing of FMRP are sufficient to cause fragile-X syndrome (FXS), the most common form inherited mental retardation (Turner et al., 1996). Dendritic spine development is abnormal in FXS patients (Rudelli et al., 1985) as

well a mouse model of FXS (Comery et al., 1997). Despite the accumulated knowledge about the pathogenesis of FXS, the physiological role of FMRP is not well understood. Recently, several laboratories have devised strategies to identify the endogenous substrates of the FMRP RNA-binding activity (Brown et al., 2001; Darnell et al., 2001; Miyashiro et al., 2003). We have used the specificity of a well-known anti-FMRP antibody to position a priming oligonucleotide in close proximity to RNAs bound within the FMRP mRNP complex. We have referred to this technique as antibody-positioned RNA amplification (APRA) (Fig. 1). The priming oligonucleotide contains a T7 RNA polymerase promoter that is 5′ to a long linker sequence and a 15 nucleotide degenerate sequence that primes the in situ transcription of mRNAs with accessible sequence bound within the FMRP messenger ribonucleoprotein (mRNP) complex. The resulting single-strand cDNA is then converted into double-stranded cDNA and processed through the aRNA amplification procedure. When APRA-derived aRNA probes are then used to screen macroarrays for putative targets of the intrinsic RNA-binding activity of FMRP, several hundred candidates were observed from our initial macroarrays which contain ∼1100 cDNA elements. A subset of these candidates (∼80) was tested in more conventional in vitro protein–RNA interactions to determine if there were able to bind to recombinant FMRP directly. Approximately 60% of this subset were shown to bind to FMRP specifically in vitro. These FMRP cargoes can be grouped into many functional classes with some being involved in cell signalling while others in regulating gene expression.

These data emphasize the efficiency and specificity of the APRA procedure in identifying substrates of FMRP. We have begun to investigate other classes of RBPs to determine the RNA cargoes they interact with. The presence of very specific antibodies to these RBPs has greatly facilitated some of these studies. The identification of native targets of different RBPs, especially those involved in disease pathogenesis, may help facilitate further understanding of the developmental sequelae underlying the physiological importance of RBPs.

4. Translational control of mRNA expression in single cells

The presence of mRNA in neurites is now well documented, however, understanding the regulation of its translation into protein has been far more challenging. While not yet fully understood, it is becoming increasingly apparent that localized protein synthesis in dendrites is required for some aspects of synaptic plasticity. When the regulation of this translation is disrupted, it can ultimately contribute to various kinds of pathogenic disorders, for example, FXS.

Many procedures have been developed in order to study localized protein synthesis in neurons (Rao and Steward, 1991; Feig and Lipton, 1993; Weiler and Greenough, 1993; Mayford et al., 1996; Weiler et al., 1997; Wu et al., 1998; Ouyang et al., 1999). Although these methodologies, including hippocampal slices, synaptosome and synaptoneurosome preparations, have strongly suggested the presence of regulated protein synthesis in neurites, they are contaminated with misconstruing factors from neurons or glia and are often made from pooled tissue samples. Given these impurities they will not be discussed any further.

As previously described, isolated dendrites are pure preparations of dendrites. Since they are devoid of contaminating cellular factors unlike other procedures, they offer many advantages when studying translational phenomena. Using these preparations, the translational dynamics of transfected mRNAs can be examined in separated sub-cellular compartments (e.g. soma versus dendrites) (Crino and Eberwine, 1996) (Fig. 3). Crino et al. (1998) first demonstrated that transfected mRNA for CREB could be translated into protein in isolated dendrites. It was later shown that translation of transfected mRNA for GluR2 tagged with the epitope for c-myc is up-regulated following DHPG stimulation, an mGluR1 and mGluR5 agonist, and inserted into the membrane (Kacharmina et al., 2000). These results highlighted the fact that naked, nonendogenous mRNAs could be translated into protein by the existing machinery in dendrites and their synthesis regulated by certain kinds of stimulation. More importantly, these data also demonstrated that newly translated protein could undergo appropriate post-translational modifications. However, functionality of the freshly translated, nonendogenous protein has yet to be demonstrated.

Improvements on isolated dendrite procedures have recently been made in combination with live fluorescent microscopy techniques that have provided more details about the temporal and spatial regulation of protein synthesis in dendrites. Recent data has demonstrated that translation of the mRNA for green fluorescent protein occurs in fixed areas along transfected dendrites following DHPG stimulation (Aakalu et al., 2001; Eberwine et al., 2001; Job and Eberwine, 2001a). These “hotspots” are not static. In some, the rate of translation of GFP is exponential, whereas in others the rate is linear, resembling protein translation in the cell soma (Job and Eberwine, 2001a,b).

Additional elements residing in the 5′- and 3′-untranslated regions (UTRs) of dendritically localized mRNAs also likely play a role in regulating their translation. While most of these elements are not well characterized, certain other *cis* acting factors in the 3′-UTRs of CamKII and Tau, for example, have been defined that are sufficient for their dendritic and axonal localization, respectively (Aronov et al., 2001; Blichenberg et al., 2001). There are most likely other *cis* elements, not unlike those just described, that affect the translational efficiencies and stabilities of several dendritically localized mRNAs.

In the future it will be necessary to dissect more precisely the properties described above and their role in regulating protein synthesis in isolated dendrites. Defining elements

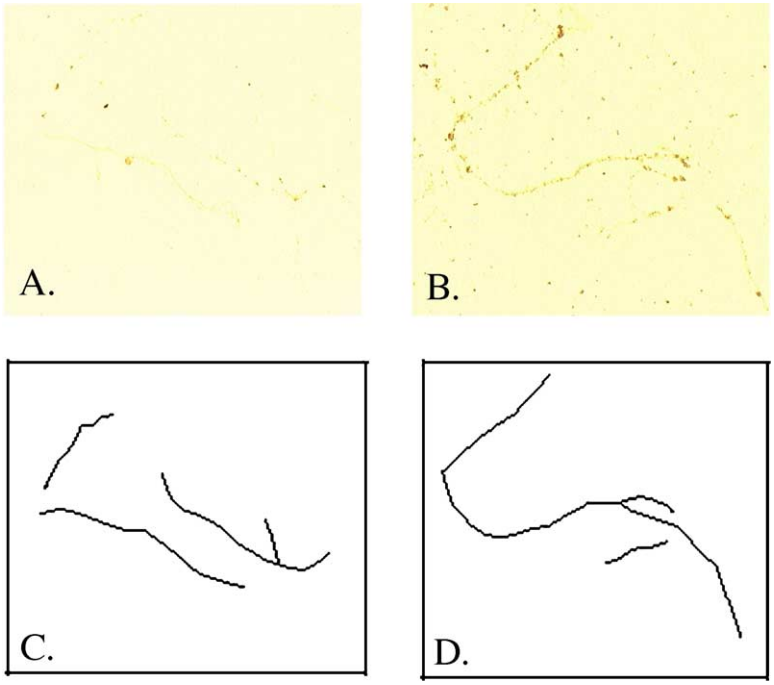

Fig. 3. mRNA translation occurs in neuronal dendrites. Panel A shows an isolated dendrite that has been transfected with a reporter mRNA that upon translation gives rise to a c-myc epitope that can be detected with antibodies. The basal level of translation is quite low. Upon stimulation of translation by the metabotropic glutamate receptor agonist, DHPG, a large increase in translation occurs as evidenced by the brown immunoprecipitate resulting from translation of the transfected mRNA (panel B). Panels C and D are line drawings of the dendrites shown in phase in panels A and B, respectively.

in mRNA and characterizing their effect on the spatial and temporal regulation of protein synthesis in respect to the activity state of dendrites is essential to our understanding of disease, learning, and memory.

5. Small sample and single cell proteomics procedures

Expression profiling of mRNA(s) highlights various mRNAs whose abundances may be altered under different experimental circumstances. Often it is erroneously assumed that protein abundances corresponding to these mRNAs are altered in a similar manner. It is clear from numerous studies that protein levels do not necessarily parallel mRNA abundances. mRNA expression profiling is not a direct readout of protein abundance differences. Expression profile data should therefore be viewed as providing candidate mRNAs whose protein abundances may be altered. Given this concern, a distinct step in quantitating cell biological function is the direct analysis of protein abundances.

The term “proteome” was proposed in 1995 as a description of the protein complement expressed from the genome in a cell or a tissue. Proteomics refers to the global identification, quantitation and understanding of proteins as well as the unraveling of their functions in cells under normal conditions and stress, such as diseases and drugs or environmental challenges. The classical proteomics methodologies are mass spectrometry and two-dimensional (2D) gel electrophoresis. Two-dimensional gel electrophoresis is a combination of isoelectric focusing and denaturing polyacrylamide gel electrophoresis (SDS-PAGE) in which proteins are separated based on their Pi and molecular mass (Peng and Gygi, 2001). Mass spectrophotometric analysis of these isolated proteins, after parsing into smaller peptides by proteases, often permits the identification of the protein based upon the mass of the peptide fragments (Morris, 1980). Protein profiles generated in this manner have allowed scientists to characterize thousands of proteins that are expressed in a given tissue with great resolution (Aharoni et al., 2002; Bergquist et al., 2002; Domon et al., 2002). Although 2D gel electrophoresis is a powerful analytical tool, it has limitations: it does not have enough resolution and dynamic range to separate all cellular proteins, it lacks sensitivity, it requires large amounts of tissue as starting material, and it cannot detect proteins with a molecular mass greater than 100,000 Da.

Using proteins isolated from tissue homogenates loses the cellular specificity that will be necessary to understand the potential roles of any peptide or protein in cellular functioning. Also an understanding of regulation of protein levels in a particular cell type requires finer resolution that protein homogenates provide. In an effort to overcome this issue, Caprioli and colleagues have reported using mass spectrometry to analyze the peptide complement of tissue sections (Caprioli et al., 1997; Chaurand and Caprioli, 2002; Chaurand et al., 2002). While an interesting and potentially exciting proteomics approach, more research effort and better methodological descriptions are required to

permit the ready utilization of this methodology in multiple laboratories.

More recent methods have been developed to improve the sensitivity of protein detection including antibody-based detection using immuno-PCR and RCA (rolling circle DNA amplification) techniques. In the immuno-PCR approach, a linker molecule is used to attach a marker DNA to the antibody in the antibody–antigen complex. The marker DNA is then amplified in a PCR reaction. Although this technique increased the sensitivity of detection 10^5-fold over alkaline phosphatase-conjugated ELISA, the amplification is nonlinear therefore making it difficult to make quantitative measurements of the amount of signal relative to the amount of protein in the sample (Ruzicka et al., 1993). In immuno-RCA an oligonucleotide primer is covalently attached to the antibody, then in the presence of a circular DNA template, amplification by DNA polymerase is carried out resulting in a long, single-stranded DNA molecule containing hundreds of copies of the circular DNA sequence. RCA overcame some of the sensitivity problems associated with immuno-PCR (Schweitzer et al., 2000) but it is difficult to quantitate and has not been widely used.

IDAT, immuno-detection amplified by T7 RNA polymerase, is a new technique developed in this laboratory (Zhang et al., 2001), which overcomes many of the obstacles faced by the traditional proteomics methods (Fig. 1). It combines the specificity of antibody–antigen interaction with the sensitivity of detection based on RNA amplification by T7 RNA polymerase. IDAT is based on attachment of a reporter DNA, which is a double-stranded oligonucleotide containing the T7 promoter, to an antibody. After binding of the antibody to the antigen, T7 RNA polymerase is used to amplify RNA from the double-stranded DNA that is coupled to the antibody in the antibody–antigen complex. Amplified RNA is labeled with radioisotope and detected by gel electrophoresis. As described earlier in this chapter the creation of RNA by T7 RNA amplification is linear (Van Gelder et al., 1990; Chee et al., 1996) therefore permitting the quantitation of antibody bound to antigen. The density of the band in the gel reflects the abundance of the antibody bound to the antigen in the sample. IDAT can be used with a protocol configuration similar to that used in the sandwich ELISA, where one antibody, immobilized to a solid support, is used for capturing the target antigen, and another, coupled to an oligonucleotide containing the T7 promoter, is used for detection. Using this method, Zhang et al. (2001) were able to detect $p185^{her2/neu}$ receptor from the crude cellular lysate at 10^{-13} dilution, which is 10^9-fold more sensitive than the conventional ELISA method. They also showed the presence of $p185^{her2/neu}$ in the cytoplasmic extract of a single, cultured rat hippocampal neuron harvested using a microcapillary tube approach, thus demonstrating the robustness of IDAT technology for single-cell resolution of protein antigens. Based on these data, IDAT appears to be the most sensitive assay system developed to date. Further with regard to the subject of this chapter, the IDAT procedure has been shown to have single cell sensitivity (Zhang et al., 2001).

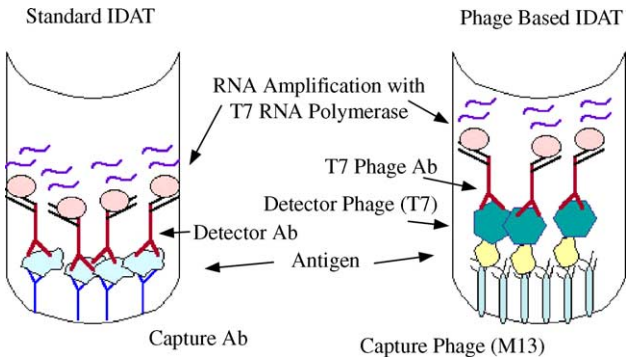

Fig. 4. Schematic of the IDAT procedure for detection of proteins. Depicted in this drawing is the standard IDAT procedure utilizing selective antibodies (left panel) and the phage-based IDAT procedure that can be used as a general proteomics platform (right panel). The procedures are essentially the same with only a few differences reflecting the different goals of each procedure. In the general proteomics platform two phage display libraries in two distinct phage vectors are used to identify antigens. There are many variations on this type of general proteomics platform including the use of a single phage display library.

In our efforts to develop a general proteomics platform, single-chain Fv fragments (ScFv) or complementarity determining region (CDR) peptides can be substituted for the antibody in IDAT. A large-scale protein screening can be envisioned using two phage display libraries, combined with the sensitive detection methodology provided by IDAT (Fig. 4). Both phage display libraries are enriched for clones of interest by biopanning against a target protein lysate. Individual clones picked at random out of one of the panned libraries (library 1) are immobilized on a microtiter plate. Clones are incubated with the target protein lysate, and then detected with the second phage display library (library 2). Due to the biopanning performed for both phage libraries, a large number of the selected library 1 clones will capture a protein from the target lysate, and clones from the second phage library are likely to bind to the captured proteins. Positive wells containing a sandwich consisting of phage from library 1, target protein, and phage from library 2, will be detected using an antibody that is conjugated to an oligonucleotide containing the T7 promoter and that recognizes the phage from library 2. This antibody is a universal detector since it will detect any phage from the second library. Phage from the second library will be present in the well only if the first phage library bound to an antigen that also binds to phage from the second library. Specificity of detection is provided by the initial immobilizing phage, as well as by the second detector phage population. This allows the detection of multiple antigens without having a specific antibody to each antigen and without coupling each specific antibody with the amplification oligonucleotide. RNA amplified by T7 RNA polymerase will be either radiolabeled and separated in gel electrophoresis or detected using a fluorometer and a fluorescent RNA binding dye. The experimental configuration is very similar to that

of the conventional sandwich ELISA. Two different phage particles are used instead of the capture and detector antibodies, and instead of the enzyme-conjugated secondary antibody the detection is based on IDAT assay using an antibody recognizing the second phage.

Proteins captured by the immobilized library 1 clones can be identified through performing a second biopanning step. Phage library 2 is screened for peptides binding to the library 1 clones, which were positive in the IDAT assay. The peptide displayed on the surface of a library 1 clone, which originally bound to a protein from the protein lysate, is likely to capture the same protein sequence from phage library 2. After three to four rounds of biopanning the positive wells are again detected based on IDAT assay using an antibody against the second phage coupled with the oligonucleotide. Positive wells at this point contain an immobilized library 1 clone interacting with a library 2 clone through their peptide tags. Library 2 clones from the positive wells are then isolated and the DNA sequences encoding for the peptide tags are obtained. The sequence of the peptide reveals the binding epitope of the corresponding target protein, based on which the target protein can be identified.

The protein screening should be both qualitative and quantitative. Sequence information will lead to identification of proteins in the sample. Signal intensity in the phage-IDAT assay, in both radiometric and fluorescence-based modification, reflects the abundance of the protein in the sample, based on the linear RNA amplification by T7 RNA polymerase. Different conditions can be compared by comparing IDAT data of different protein lysates run in parallel on identical plates, against identical sets of clones.

The use of the phage display libraries provides a large collection of protein tags, which can be screened with ease, and the sequence information is readily available. The tags are displayed on the surface of well-characterized phage particles; therefore, all clones can be detected with a single, commercially available antibody. Fluorescence-based detection provides an improvement over the radiometric method: it eliminates the need for gel electrophoresis and allows for the screening of large numbers of clones in a microplate format. With robotic assistance, this technology provides a powerful tool for multiple proteomics applications in research and medicine. With these modifications the IDAT may be useful in the detection and quantification of antigens from a wide variety of samples, such as tissue samples or single cells, as well as body fluid, food or water, and is likely to be an important tool in both research and diagnostic applications. Antigens that can be detected include proteins, lipids and metabolites and their modifications, essentially anything for which detector molecules are available.

6. Proteomics on fixed tissue sections

Highly sensitive antigen detection is critical to the efficiency of immunohistochemical analysis, a methodology commonly employed in the identification of pathological markers in both the diagnosis of and research pertaining to various human diseases (Cummings et al., 2002; Elzagheid et al., 2002). Traditionally, immunohistochemistry involves detection of an antigen with an antibody (Ab) that will specifically bind to the antigen followed by observation of the antibody–antigen complex under a microscope. This visualization is achieved either by direct labeling of the primary Ab (prior to Ab incubation) or by indirect labeling via a tagged secondary Ab that will specifically bind the primary antibody (Elzagheid et al., 2002). Common tags include fluorescent labels, peroxidase and alkaline phosphatase. However, immunohistochemistry is often plagued by a lack of sensitivity and the inability to simultaneously detect more than a few antigens at a time due to paucity of distinguishable labels. Numerous attempts have been made to optimize antigen detection by altering various aspects of the traditional technique (McQuaid et al., 1995; Shi et al., 1996; Frost et al., 2000; Shi et al., 2001). It has been demonstrated, for example, that changes in pretreatment conditions, such as boiling, heating, and exposing tissue to formic acid, can alter an antibody's ability to detect antigen in Alzheimer's tissue (Cummings et al., 2002). These findings, although significant, focus on specific disease and antibodies, lacking broad-based applicability to immunohistochemistry.

As described previously, the recently developed and highly sensitive IDAT technique (Zhang et al., 2001) could potentially fulfill the need for increased sensitivity in immunohistochemistry. Slight modifications to the IDAT procedure would be necessary to perform IDAT on tissue sections. This can be envisioned through the use of double-stranded oligonucleotide-coupled antibody as an immunohistochemical probe on fixed tissue sections. After appropriate incubation times, the antibody–antigen complex would be detected, in situ, by using a labeled nucleotide in the generation of RNA with T7 RNA polymerase. Given the linearity of the RNA amplification procedure the amount of product is a direct measure of the amount of antibody bound to immobilized antigen in the tissue section. For multiple antigen detection on a single tissue section, a different length oligo could be attached to each distinct Ab to be used, so that the presence of a particular antigen in the tissue section is reflected by the generation of a RNA product of a particular length. Once this procedure is fully developed it should be possible to perform a complex proteomics analysis of archival human tissue.

7. Discussion

This manuscript highlights some of the technical advances that permit analysis of the biochemical properties of single neuronal cells. There is a significant amount of work ahead of us, and others, in generating these data, including RNA expression profiles, protein profiles, translation rates, and RNA cargo analysis described in this chapter. This information is

a necessary component of the identification of a cell's physiological state. However, as in many scientific disciplines, the ability to examine the system has advanced at a faster pace than our understanding of what the biology is telling us. With massive amounts of data being generated, one of the most important next steps to take is to put this information into a useful biological context. For example, it is impossible at this time to look at an expression profile or protein profile and know which of the myriad of changes are integral to disease onset or progression. In particular, the analysis of expression profiling data has emphasized determination of mRNA abundance differences that, in turn, has permitted hypothesis to be generated concerning the biological significance of these changes based upon the presumed function of the encoded protein. Universally, the expression profiling analysis software has emphasized statistical significance of the data but has not properly dealt with the biological importance. This is particularly true of mRNA abundances which can be dramatically distinct from the corresponding protein levels and even more distinct from the functional protein abundances. Some have attempted to overcome this problem by thinking of the mRNA in terms of the protein that it encodes and the functional pathway in which the protein functions. Such approaches are timely but still limited.

One way of analyzing this wealth of information is to resort to the traditional way of doing science, examination of one or a few mRNAs or proteins in detail. This is very time-consuming and likely will provide too narrow of a perspective on the biological role of that mRNA or protein in the physiology being studied. This is not an indictment of the traditional way of doing science but rather highlights the type of information that is needed to assess the biological importance of any particular gene under the physiological conditions being studied. It may be appropriate to reassess the types of information that are being sought on a genomics and proteomics scale. All of biology, as highlighted by examples in this chapter, results from two or more molecules interacting with one another in a particular biological context. This can be formalized in both a chemical and biological context. Perhaps, most simply, it is possible to envision each biological process in a cell as being dictated by the following characteristics: affinity, on and off rates, rate of reaction, abundance, and position within the cell. Affinity can be defined as the avidity with which two or more molecules interact with one another. While affinity is a static number, its relation to biology is quite dynamic. Biology comes into play in this discussion in the "on and off" rates which is the time it takes for two or more molecules to associate and dissociate. These rates are influenced by the abundance of the interacting molecules and the conformation of the molecules that may help or hinder the interactions. This is distinct from the rate of reaction, which is the rate at which the biological consequence of the molecules interacting occurs. Finally, the position in the cell where the interactions occur is critical given different salt concentrations, pH, and abundances of biomolecules in different cellular regions. Knowing this information for cellular small molecules, RNAs, and proteins should permit the generation of a predictive biology. Such information may permit better intervention or regulatory strategies to be developed.

If this hypothesis is correct, then what is needed is not a retrenching into traditional biology, but rather a new set of genomics and proteomics methodologies that permit the rapid, high-throughput characterization of these parameters at the cellular level. The marrying of these data with that currently being generated promises to provide insight into biological processes that has been coveted but heretofore unavailable. As more information is generated, these concepts will be reduced to algorithmic form.

Over the last century through careful detailed analysis of neuroanatomy, individual genes and proteins we have taken the first steps towards understanding how cells function. The ability to globally detect and quantitate cell biological processes in a spatially defined manner now makes it possible to more rapidly define those coordinated activities that result in cellular responses. There are many challenges ahead in defining these biological processes that result from these coordinated responses but the path is clear and treadable.

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