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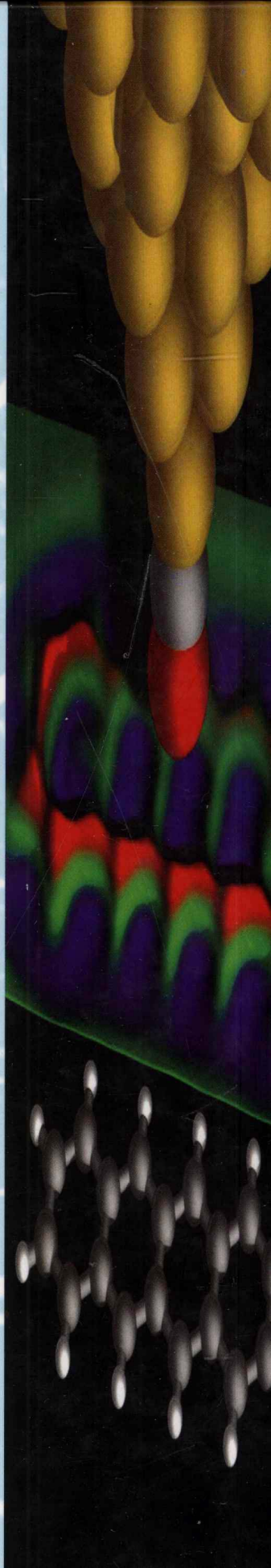
Single Molecule Biology

单分子生物学



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原版引进

科学出版社

Single Molecule Biology

单分子生物学

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科学出版社
北京

图字:01-2009-7223 号

This is an annotated version of
Single Molecule Biology
By Alex E. Knight.

ISBN:978-0-12-374227-8

Authorized English language reprint edition published by the Proprietor.
ISBN 13: 978-9-81-272557-8

Elsevier (Singapore) Pte Ltd.
3 Killiney Road
#08-01 Winsland House 1
Singapore 239519
Tel: (65) 6349-0200
Fax: (65) 6733-1817

First Published 2011
<2011>年初版

图书在版编目(CIP)数据

单分子生物学=Single Molecule Biology:英文/(英)奈特(Knight, A. E.)著. —北京:科学出版社,2011

ISBN 978-7-03-031094-1

Ⅰ.①单… Ⅱ.①奈… Ⅲ.①分子生物学-英文 Ⅳ.①Q7

中国版本图书馆 CIP 数据核字(2011)第 090421 号

责任编辑:孙红梅 李小汀
责任印制:钱玉芬/封面设计:耕者设计工作室

科学出版社 出版
北京东黄城根北街 16 号
邮政编码:100717
http://www.sciencep.com

北京住信达欣艺术印刷有限公司 印刷

科学出版社发行 各地新华书店经销

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2011 年 6 月第 一 版 开本:787×1092 1/16
2011 年 6 月第一次印刷 印张:23 1/2
印数:1—1 500 字数:550 000

定价:118.00 元

(如有印装质量问题,我社负责调换)

导　读

我们一般从高中开始接触分子生物学，获得基本的分子生物学知识。例如，DNA是遗传信息的载体，可自我复制，并通过转录将遗传信息传递到信使RNA（mRNA）上；DNA的复制主要由一个被称为DNA聚合酶的大分子承担，RNA的合成则主要在RNA聚合酶的催化下进行；信使RNA分子上相邻的三个碱基能决定一个氨基酸，称为三联体密码子；转运RNA（tRNA）一端携带氨基酸，另一端的反密码子与信使RNA上的密码子配对；核糖体结合信使RNA模板，通过转运RNA识别模板上的密码子并转运相应的氨基酸，按照信使RNA上的信息依次连续合成蛋白质。从这段描述中我们已经看出，尽管生命系统十分复杂，人们大都习惯于以单个生物分子为描述对象，依据单个分子的行为来思考问题和构建模型。与此相对应，人们一直期待着能够在单分子水平直接研究基本生命过程。

二十世纪九十年代以来，物理学家发明了许多新型的观测和操控技术（如光镊、磁镊、单分子荧光等），将分子生物学与物理学的交叉领域推进到单分子水平。一方面，人们能够在体外直接操纵并检测单个分子的运动和变化，或通过施加外力改变生化反应途径，研究化学能与机械能的相互转化；另一方面，人们正逐步实现对活细胞内单个分子的实时追踪，研究细胞内部的各种物理化学过程。这些新技术揭示了关于生物大分子和活细胞的全新知识，引起了广泛关注，其中一部分已被及时补充到新版的分子生物学教科书上。由Alex Knight主编的《单分子生物学》（*Single Molecule Biology*）是一本可用作教科书的专著，从生物学的角度对这个迅速成长起来的新领域做了完整和权威的介绍。无论背景是物理学还是生物学，对单分子研究感兴趣的人都可以将该书用作该领域更深层次探索的启蒙和起点。通过本书前言和目录的中译文，读者可以了解本书的内容和写作目的，故不再赘述。下面我们只从学术的角度讨论一下单分子生物学的重要性。

由于实验手段的限制，分子生物学中几乎所有重要的结论都是基于生化实验中一些分子的浓度变化做出来的，是许多分子活动的一个平均结果，即集群（系综）平均。集群平均的研究结果无疑是正确的，但它往往掩盖了各个分子的个性，所以需要根据实际的单个分子的活动来验证。生物学家很早就知道，许多生物分子在细胞中不仅数量极少（纳摩尔量级，某些生物大分子的数目甚至低达每细胞1～10个），在细胞内部的分布也很不均匀。这和试管内的均匀反应体系完全不同。体外集群平均得到的结果不能直接推广到细胞中的生物分子。特别需要注意：生命过程是由一个个生物分子来实现的。要理解生命过程，除了必须了解生物分子的集群效应，还必须了解单个分子的运动或特性，二者互相补充。

生物单分子和细胞是高度异质的复杂小系统。从物理学上讲，这类系统的异质性是其生物学功能的基础。它们的行为不能简单地用平均场理论来描述。这类系统将受到各种内源或外源噪声的显著影响，表现为行为上的大涨落。例如近年来人们发现，许多功

能蛋白（如肌红蛋白）并非具有唯一天然构象，而是同时存在多种构象。蛋白质分子在这些构象之间涨落形成的特殊分布（而不是某个特定的构象）才是该种蛋白质行使生物功能的基础。又如，在细胞体内各种基本分子过程（如基因表达）中，源于分子低拷贝性质的分子数的小涨落可能被正反馈机制放大，导致整个过程发生非平衡相变，从而使细胞表现出完全不同的行为。上述两种涨落在生物系统中比较典型，但其特征至今没有得到明确的理解。对于这类问题，传统的热力学和统计物理学显然并不适用，人们需要从理论和实验上同时拓展、甚至发展新的热力学和统计物理学的概念和方法。

以上我们从生物学和物理学两方面阐述了单分子生物学的重要性和必要性。单分子生物（物理）学本质上是一个交叉学科，是由对生命现象感兴趣并掌握了相关技术的物理学家与对物理学有一定程度的认识并乐于应用新技术的生物学家共同开创的。这种相互融合的合作氛围在相当大范围内还不是十分浓厚。物理学者往往怵于一些复杂的生化研究过程，他们惊异于生物学家挖掘复杂对象中有用信息的能力。例如，他们惊异于生物学家居然能从如此复杂的细胞中发现并提取未知蛋白质；生物学家需要技术支持，但往往对仪器的期待过高，希望一打开仪器的开关就能开展有效的测量。这显然不利于走在学科发展的最前沿，因为新仪器特别是还没有商业化的仪器总是需要复杂的操作技巧。不仅如此，物理学家与生物学家的交流还存在语言上的障碍。由于所受的训练不同，很自然思维方式也不一样，在交流过程中往往各说各话。我个人理解，单分子生物物理是少有的双方可以有共同语言和思维方式的研究领域。不仅如此，单分子生物物理也是少有的学科交叉成功的典范。对生命现象感兴趣的物理研究人员或学生，不妨以单分子生物物理研究为切入点，从而了解分子生物学的前沿；而不满足于对生命现象仅仅做定性描述的生物学研究者或学生，也可以从单分子生物研究中攫取技术力量和思维灵感，引领分子生物学的潮流。

单分子生物物理尚处于发展的早期，还有太多的事情要做。我们希望本书，加上其他一些已经出版的单分子生物物理方面的优秀书籍，能够激发更多的研究人员参与到这一有可能改变分子生物学研究范式的研究中来。

李明
中国科学院物理研究所
二零一一年五月

前　言

本书旨在对“单分子”研究方法如何帮助我们理解生物系统及过程作一个总结。本书的各章节都是专门为本书而编写的，主要针对高年级本科生或一年级研究生。我们希望各种专业背景的读者都能读懂本书，这对一个交叉学科的发展来说是必需的。当然，具备一些生物学知识会更有帮助。本书并没有打算包罗万象，也不可能面面俱到。我只希望它能成为该领域更深层次探索的启蒙和起点。

在第一章里，我尽可能地为刚接触本领域的读者提供了一些背景知识。后面的章节都由该领域的学术带头人所撰写。每章都涵盖一个用单分子方法阐释的生物学系统。最后，附录提供了一些本领域常用的缩写、符号和单位的参考；作为英国国家测量局的工作人员，我还特别提供了一些国际计量单位的注释及其在生物学上的使用。

Alex Knight
特丁顿国家物理实验室
2008 年 8 月

（李明　译）

Preface

This book is intended to act as an overview of the ways in which "single molecule" methods have contributed to our understanding of biological systems and processes. The chapters have been written specially for the book and are aimed at the level of a final year undergraduate or a first-year PhD student. The hope, therefore, is that the book should be accessible to readers from a wide variety of backgrounds, as I feel is essential for this field of research, which is intrinsically interdisciplinary. Some biological knowledge, however, will be a benefit. The book is by no means comprehensive – nor could it hope to be – but I hope that it will provide a primer, and a starting point for further exploration.

In the first chapter, I have striven to give some background to the reader new to the field. The subsequent chapters are all written by leaders in their fields, and each covers a biological system that has been illuminated by the single molecule approach. Finally, the Appendix is intended to provide a useful reference on abbreviations, symbols and units that are commonly encountered in the field; in particular, as a scientist working at the UK's national measurement institute, I wanted to include some notes on the SI and its use in biology.

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August 2008

Acknowledgments

Thanks are due to many people for helping me to put this book together. First of all, at the National Physical Laboratory, I must thank Marc Bailey for support and encouragement and Anna Hills for reviewing drafts. My work on this book has been supported by NPL's Strategic research programme, and also by the National Measurement System of the Department for Innovation, Universities and Skills (DIUS).

Elsewhere, thanks are also due to Edward Bittar for suggesting the book in the first place; to Justin Molloy for his encouragement, and for providing such a striking cover image; and to the team at Academic Press/Elsevier including Luna Han, Gayle Luque and April Graham.

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Introduction: The "Single Molecule" Paradigm

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Summary

A new experimental paradigm, based on the detection of individual molecules, has been making great strides in the dissection of biomolecular function in vitro in the past two decades. A technological convergence – of improved detectors, probes, microfluidics and other tools – is leading both to an explosion of this area of research and its development into a tool for investigating processes in living cells.

Key Word

single molecule detection

The "Single Molecule" Paradigm

Imagine a busy motorway, packed with all kinds of vehicles. Now imagine that you are trying to describe the traffic on that motorway (see Figure I.1). You could try to summarize it by a single number; the average speed of the traffic would be a good example. This gives a good indication as to whether the traffic is flowing or obeying the speed limit, but it does not tell you much more. Sports cars may be tearing along in the outside lane, more cautious drivers cruising in the center lane, while trucks rumble along in the slow lane. Indeed, some vehicles may be pulled over on the hard shoulder. What's more, vehicles will occasionally change lanes, slow down, or accelerate. We don't get a full picture of this diversity from a single number, but this is the kind of measurement of molecular properties, quantities, or behavior that we usually make in the life sciences.

For example, if we measure the properties of a molecule by a spectroscopic technique, such as fluorescence spectroscopy, we are likely to be measuring the average characteristics of

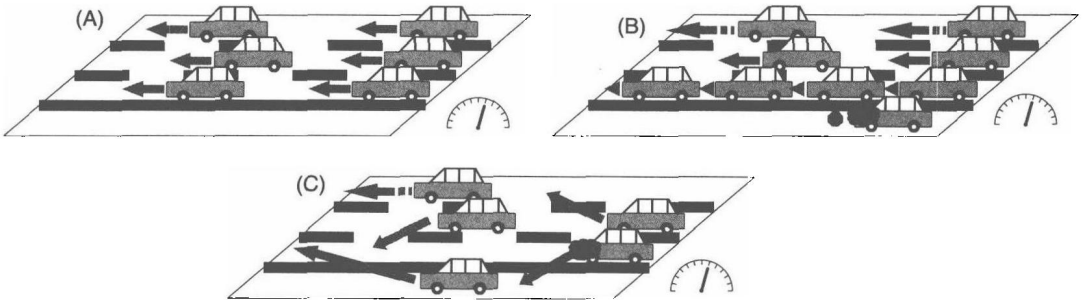

Figure I.1: Single molecule detection can unravel differences between molecules in a population. This figure illustrates the "motorway" analogy of single molecule experiments used in the text. When a single number is used to describe the properties of a population of molecules – represented by cars on a motorway – it gives no information about how the properties vary within that population. For example, if we know only the average speed of the cars on the motorway, we do not know if all the cars are moving at the same speed (A) or whether their speeds differ (B). This is known as *static heterogeneity*. Furthermore, it may be that the cars are changing speed – or stopping and starting – and again this is not apparent from the average speed (C). This is known as *dynamic heterogeneity*

a very large ensemble of molecules. If our cuvette holds 3 ml, and our sample is of a protein at 1 mg/ml, then for a typical protein of a molecular weight 50 000 Da we have 60 nmol of protein in the cuvette. This may sound like a relatively small amount, but it corresponds to 36 000 000 000 000 000 individual molecules. This huge number arises because Avogadro's constant (N_A), the number of entities in a mole, is such a huge number – approximately 6×10^{23}. Viewed from this perspective, we are looking at a very large sample indeed!

So in most techniques, even if the quantities are, in molar terms, tiny, any measurement we make is an average across many millions or billions of molecules. The usual approach is to assume that all the molecules are the same. But this is often not the case, particularly for the complex molecules that are found in biology; the molecules may have different properties (sports cars, trucks – or breakdowns) and indeed, these properties can change over time (switching lanes) – and moreover in a random (or stochastic) fashion. Sometimes the ensemble, "averaged" measurement is good enough. But at other times, we need much more understanding of the molecules – in fact, we need a whole new approach.

This new approach is one that has been developing steadily over the past two decades, and now appears to be undergoing something of an explosion. This is the "single molecule"

approach. A rather inelegant name, perhaps, but this describes a philosophy where molecules are thought of – and measured – as individual entities.[1] It is important not to get too fixated on the word "single" – even if you measure a single molecule, it does not tell you much; after all, how can you be sure that it is representative? So even single molecule experiments may characterize hundreds or thousands of molecules, for as in other fields of biology, good statistics are vital. Indeed, often what we are interested in is the shape of the *distribution* of our property of interest.

This is the key point, then: not that we analyze a sample at the absolute limit of detection (although we do), but that we treat all the molecules in that sample as individual entities.

When we consider such tiny samples, the conventional units of quantity become somewhat ungainly. A single molecule is approximately 1.66 yoctomoles[2]; a zeptomole corresponds to approximately 600 molecules. Therefore in this type of work, experimenters tend to report on numbers of molecules rather than molar quantities[3] – see Figure I.2.

Why Single Molecules?

So what are the advantages of observing or measuring single molecules? The reaction of many, on hearing about single molecule detection, is to assume that the benefit is in the ability to detect and even quantitate very small amounts of material. While this is true up to a point, it misses the main advantages of the single molecule approach, as will be shown later. Another common (but somewhat more acute) reaction is that one cannot infer much from looking at a single molecule: how does one know this molecule is typical? This is an excellent point, but in reality, "single molecule" experiments are never really done with *single* molecules. In fact, it is the name that is misleading – really we are interested in performing *discrete* molecule experiments, that is, experiments where we observe a group of molecules as a population of discrete individuals rather than as an undifferentiated *ensemble*. This implicitly requires that we can, in some sense, detect a single molecule but this alone would never make sense as an experimental design.

The continuous improvements in analytical science have pushed detection limits to extraordinarily low levels – picomoles or femtomoles, for example – so it is natural that single molecule detection techniques, where we are reaching the ultimate detection

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[1] Bustamante has suggested the term *in singulo* to denote "single molecule" experiments, contrasted with *in multiplo* to denote "bulk" or "ensemble" measurements (Bustamante, 2008).

[2] The less familiar SI prefix yocto- indicates a factor of 10^{-24}, whereas zepto- indicates 10^{-21}. See appendix.

[3] Moerner (1996) has wittily suggested the adoption of a new unit, the guacamole, corresponding to a single molecule, where the prefix guaca- corresponds to 1/Avocado's number.

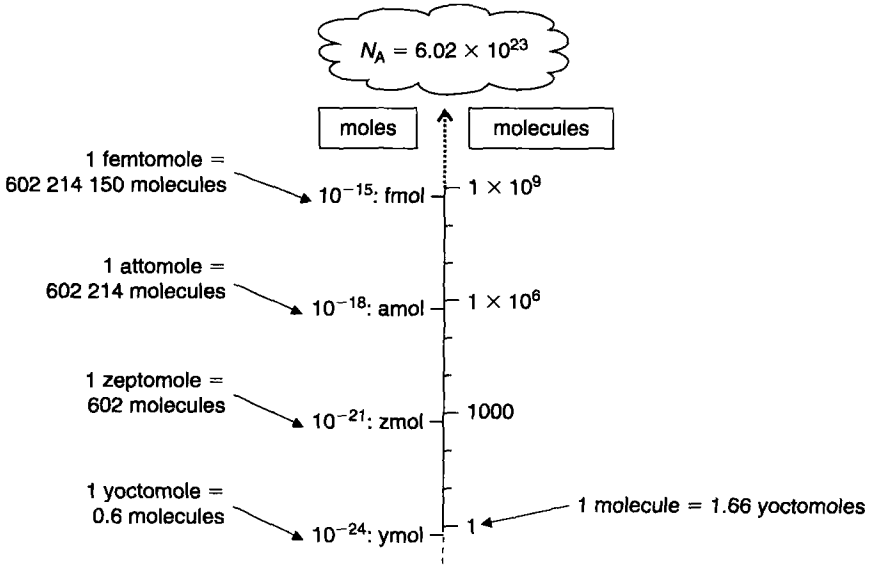

Figure I.2: The scale of single molecule detection. Historically, we tend to quantify molecules in terms of the mole, where a mole contains N_A molecules (where $N_A = 6.02 \times 10^{23}$) – a very large number (see appendix). For large biological molecules, we tend to deal with much smaller quantities – submultiples of the mole – and as measurement techniques increase in sensitivity we are dealing in smaller and smaller quantities. Once we get into the subattomole range, it becomes more convenient to think in terms of numbers of molecules. This logarithmic scale provides a quick comparison between moles and molecules. The less familiar SI prefixes of zepto- and yocto- are brought into play to express these tiny quantities; a zeptomole is approximately 600 molecules, whereas a yoctomole is less than 1 molecule. Since molecules are discrete entities, at this scale they are best quantified by a counting approach and expressed as numbers of molecules

limit of yoctomole sensitivity (Figure I.2), should be seen in this light. However, as the sample sizes become smaller, the number of molecules likewise becomes smaller, until the random statistical variations in the numbers of molecules counted – known as "shot" or "Poisson" noise – become a significant factor. A more significant problem with the real-world application of these techniques simply for detection and quantitation is the sampling issues. The detection volumes for most single molecule techniques are typically very small; how can we be sure that our detection volume accurately reflects the concentration of the molecule in the larger sample? Also, as with many microscale techniques, there are questions about purification and handling of the sample and losses due to the

molecule of interest being retained in matrices or on surfaces. This is not to say that quantitative results on numbers of molecules cannot be obtained, within stated uncertainty limits, but rather that this is not the true strength of this approach.

Let us now try to summarize the reasons why single molecule approaches are useful:

1. Static heterogeneity: By identifying *subpopulations* of molecules within a sample, we may be able to understand more about the characteristics of the molecule and its mechanism. For example, different molecules may experience different local microenvironments and thus exhibit different activities; or there may be a variety of different conformational states. Obtaining detailed statistics is a benefit of the single molecule approach.

2. Dynamic heterogeneity: Often the behavior of interest concerns the *transitions* of the molecule between different states; for example, where an enzyme is binding to a substrate molecule, we may wish to know the rate of release of the product. These transitions are often lost in ensemble measurements because of the intrinsic averaging, or special tricks have to be used to "synchronize" the molecules. We may also be interested in rare or transient states of the molecule, which are obscured in bulk measurements.

3. Microscopic properties: The molecules may have properties which are key to their function, but which can only be measured at a microscopic, single molecule scale. For example, the activity of myosin motor proteins in muscles can be measured on a larger scale, such as a whole-muscle fiber or a myofibril, because they are organized into arrays that integrate their forces and displacements. In contrast, many other sorts of myosins, such as myosin V or myosin I (see Chapter 1, this volume), operate as individuals and their activity can thus only be measured at the single molecule level. Another example is when we are looking for a change in the *orientation* of molecules (Figure I.3). Where the molecules are randomly orientated, changes in the orientation of individual molecules make no difference to the orientation of the population. This is important in the study of rotary motors such as the F_1 ATPase, where direct single molecule observations proved the hypothesis that these were rotary motors, and have since enabled a detailed dissection of the mechanism (Noji et al., 1997).

4. Trace detection: Notwithstanding the comments above, an advantage of the single molecule approach is that very small amounts of material are typically needed. This is obviously a benefit where samples are difficult to obtain (e.g., a low-abundance protein) but could also be a benefit where large numbers of experiments are required,

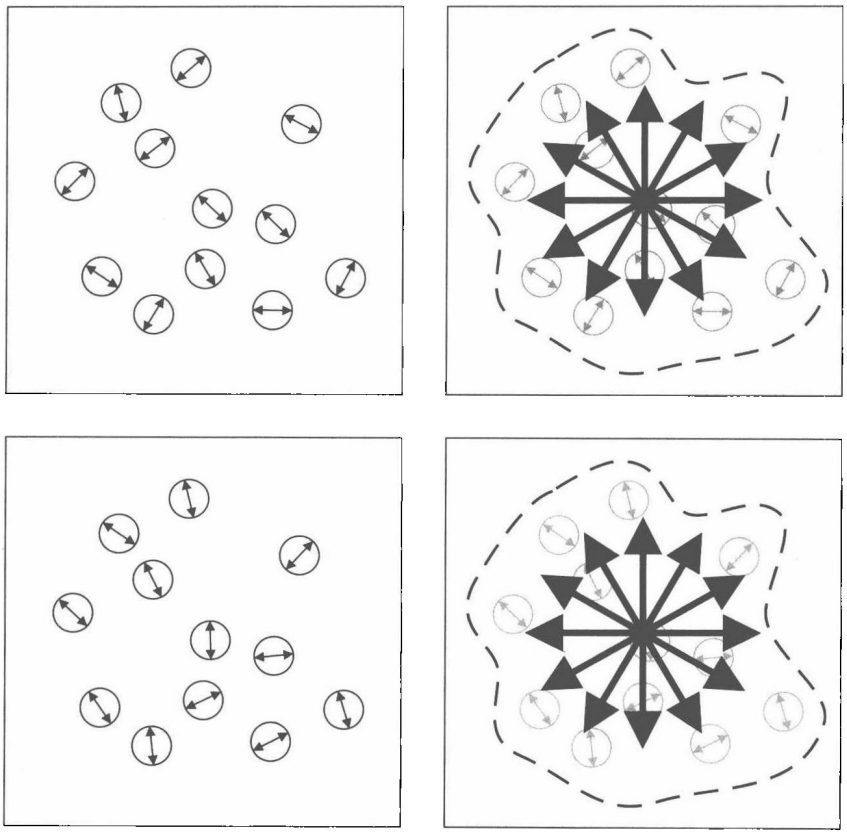

Figure I.3: Single molecule detection can reveal characteristics obscured in the bulk. This figure illustrates how some properties can only be meaningfully measured at the single molecule level. At top left, a population of molecules (circles) is randomly oriented (arrows). By single molecule approaches, the orientation of each molecule may be determined. At right, a bulk method will only conclude that there is no net orientation in the sample. In the lower panels, the sample is remeasured at a different point in time. The single molecule approach detects changes in the orientations of the individual molecules, but the bulk approach reports no change

as in some high-throughput screening methods, for example, in genomics, drug discovery, or systems biology. For example, microarray or microfluidic devices can be used to screen large numbers of samples (see, e.g., Chapter 10, this volume).

5. Spatial information: In many (but not all) approaches, images of molecules or precise localizations or distances are obtained. This spatial information can be of great

benefit. For example, single molecule FRET can be used to observe conformational changes in molecules or complexes; single molecules can be localized in cells or the colocalization of molecules can be observed.

6. "Digital" detection: The discrete (or "digital") nature of single molecule detection is very different from the conventional "analogue" approach. It can lead to more accurate measurements because "noise" or "background" signals are more readily distinguished from the molecules of interest or their behaviors (depending on the type of experiment). For example, molecules can be counted or steplike "digital" state changes can be observed.

7. Direct approach: Bulk methods often require that the behavior of molecules is *inferred* rather than measured directly. Often this inference relies upon a model or assumptions about the system. In contrast, single molecule approaches are more direct and typically less model dependent. However, single molecule approaches may also introduce artifacts (e.g., due to the introduction of labels), which must be carefully allowed for in the experimental design.

In summary, the single molecule approach has many advantages. We should remember, though, that most problems in biology are solved through the application of a variety of complementary tools, and indeed this is demonstrated in the subsequent chapters.

Life as a Molecule

To perform and interpret single molecule experiments, we must have some understanding of the microscopic world in which the molecules exist. In some ways it is surprisingly similar to our familiar macroscopic world and in others, startlingly different.

Let us start out by thinking about the *scale* of the phenomena we are measuring. Biological molecules are the natural world's equivalent of nanotechnology (and usually far superior in their capabilities); their characteristic dimensions are typically of the order of nanometers.[4] Similarly, the forces that molecules can exert are of the order of

[4] Some biological molecules can, of course, be much larger in one dimension. The obvious example is DNA, with a diameter of 2 nm and a variable, but often very great, length. For example, the genome of λ phage, often used in laboratory experiments, is approximately 16 μm long: an aspect ratio of approximately 8000. Many DNA molecules are far, far longer than this; for example, human chromosome 1 if fully extended would have a contour length of approximately 84 mm. Some proteins can also reach dimensions of the order of micrometers, such as titin, an important component of muscle. Still greater lengths can be achieved by filaments assembled from many smaller protein subunits, such as the cytoskeletal filaments actin and tubulin.