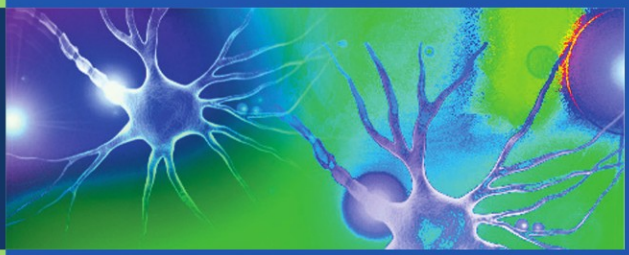
H.R.H. Patel • M. Arya
I.S. Shergill *Editors*



Basic Science Techniques in Clinical Practice

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H.R.H. Patel, M. Arya, and I.S. Shergill (Eds)

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Foreword

As medical research evolves, the scientific methodology and practice becomes ever more complex. The editors of this book have brought together a timely piece of work which will truly help future health professionals, whether they embark on research or just wish to understand what laboratory methods are available.

An important area of the book looks at statistics, study design, and analysis. This is particularly helpful when students, researchers, and clinically active people are trying to understand evidence-based medicine.

As the editors have put it, simple but informative text should inspire and give confidence to all people considering undertaking high-quality research. I envisage this to be a key book for the future of medical research and strongly recommend it.

Professor Sir Ara Darzi

Preface

Medical researchers including doctors, nurses, medical students, and allied health professionals have traditionally undertaken a period of research as part of their career pathway. Research is poorly conceptualized by many and often never formally discussed during the training process. Interestingly, recruiting to scientific and academic research is currently a major problem, with the main perceived disadvantage being a financial loss of earnings. However, the aims of clinical research are primarily to allow an understanding of scientific methodology and practice, such that the same principles can be applied to clinical medicine with the significant advantage of enhanced patient care. Through personal experience and feedback from others, one of the striking features of basic scientific research is that the underlying philosophy is invariably different to clinical practice and many clinicians can become easily disillusioned. Many health professionals experience a "culture shock" as they move into a research environment. This is due to several factors; some practical and some more fundamental. Simply speaking, medical professionals are usually thrown in at the deep end and expected to "swim". It is evident that the value of basic knowledge and support at the beginning of the research period is therefore vital, to build a suitable platform to carry out good quality research. In this book, we have tried to cover the main areas in research, allowing anyone to set up and complete research projects in clinical research as well as in basic science research.

We have endeavored to advise on the common basic science techniques that are currently popular in research, providing new information, as well as updating areas that may be familiar to most health professional. A host of international authors have provided their unique perspective in their field of specialist interest, representing institutions from around the world.

The book has three sections. Section A considers study design and research governance which are core subjects to understand

before embarking on any project. Section B concentrates on basic science techniques. Modern laboratory techniques have been covered for laboratory-based research. These should provide enough detail to allow readers to take up their technique with confidence, as well as allowing a reference point to more experienced researchers. The final section tackles data analysis and the presentation of the results, both in the oral and written format.

We hope this simple but informative text inspires and gives confidence to all people considering undertaking high-quality research.

Enjoy!

Hiten Patel
Manit Arya
Iqbal Shergill

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Chapter 1
Research Governance

S.J. Vyas, M. Arya, I.S. Shergill, and H.R.H. Patel

INTRODUCTION

The Department of Health (DOH), United Kingdom, regulates the conduct of medical practice in the country. Its scope of action extends beyond the same, as it also defines and formulates criteria pertaining to performing research activity. Indeed, research governance (RG) is more like research regulation, and the DOH would largely assume the role of the regulation.

Research governance oversees a broad range of regulations, principles and standards of good practice that exist to achieve, and continuously improve, research quality across all aspects of health care in the United Kingdom and worldwide.[1]

The DOH is responsible for setting health and social care policy in England. The department's work sets standards and drives modernization across all areas of the National Health Service (NHS), social care, and public health. As well as policy documents, the DOH also issues guidance on implementation of policies. The research governance framework (RGF) for health and social care defines the broad principles of good RG and is key to ensuring that health and social care research is conducted to high scientific and ethical standards. The first issue of the RGF was issued in March 2001 and a later updated in April 2005.[2,3]

These guidelines and principles of RG apply to everyone connected to health-care research, whether a chief investigator, care professional, researcher, their employer(s), or support staff. This list includes any health-related research which involves humans, their tissues, and/or data.[1]

Examples of such research include the following:

- Analysis of data from a patient's medical notes
- Observations
- Conducting surveys
- Using noninvasive imaging
- Using blood or other tissue samples

- Inclusion in trials of drugs, devices, surgical procedures, or other treatments

It is now important ethically and medicolegally that any individual/group/department or institution involved in any kind of the above mentioned research activity be aware of these guidelines and follow any kind of healthcare research process. It therefore becomes their responsibility and obligation to be well informed of the protocols before embarking on any such exercise. Therefore, institutions would implement their RG procedures which would govern and monitor the research process and identify any violations of the set system of procedure by audit cycles.

The Internet/World Wide Web has hundreds of examples of RG and policy documents. Each university/academic institution and government body (e.g., General Medical Council, Medical Research Council, National Health Service) has its own rule sets regarding the type of research activity that takes place within its institution. While there will be subtle differences in each of these, the basic principles of RG embedded in the constitution of each of these institutions/organizations would be similar.

WHY WE NEED RESEARCH GOVERNANCE

Research governance is needed for the following purposes:[1,2]

- Safeguard participants in research
- Protect researchers/investigators (by providing a clear framework to work within)
- Enhance ethical and scientific quality
- Minimize risk
- Monitor practice and performance
- Promote good practice and ensure lessons are learned

In broad terms RG ensures that health and social care research is conducted to the highest scientific and ethical standards. The legal implications of the RGF would apply to everyone involved in research, health, or social care setting using NHS resources or involving NHS patients.

HISTORY OF DEVELOPMENT OF RESEARCH GOVERNANCE[1,2]

It was as early as the 1930s that the seeds of RG were sown. There was a mistake in the formulation of a children's syrup in the United States, which caused a number of deaths. Waking up to this call the US Food and Drug Administration (FDA) thought it necessary to issue guidelines and bring about tight regulation of the healthcare industry.

The Nuremburg Code (1947) was one of the first attempts to regulate the ethics of medical research. It was written shortly after World War II, following revelations at the Nuremberg Trials that unethical research was carried out by certain members of the medical profession during the Nazi period in Germany. The code has 10 requirements and begins with the now widely recognized principle that voluntary consent of human participants in research is paramount.

The code has since been superseded by documents such as the Declaration of Helsinki (1964), Good Clinical Practice (1996–1997), and the European Union Directive on Good Clinical Practice issued in 2005 (2005/28/EU).[4,5]

There have since been a number of directives issued to regulate healthcare research such as the following:

2001: European Union Directive on Clinical Trials (2001/20/EC)[6]
2001: Research Governance Framework for Health and Social Care (UK)
2004: Medicines for Human Use (Clinical Trails) Regulations (UK)
2004: Human Tissue Act 2004 (UK)
2005: Mental Capacity Act 2005 (UK)

All these acts and guidelines, as stated above, are laws by themselves and need to be complied with as part of any research being undertaken. Failure to do so may have medico-legal implications, and hence it is imperative that all participating bodies are aware of the legal obligations within the existing framework.

The European Union Directive on Clinical Trials Act 2001 (2001/20/EC) is a good example.[4,5,6] This is a legal document which sets out how the system of procedure in the conduct of clinical trials evaluating the safety and efficacy of a medicinal product in humans should be performed. The directive was introduced to simplify and harmonize the administration of clinical trials across the European Union by establishing clear, transparent procedures. More importantly the directive ensures uniformity in conduct of clinical trials across member states and hence ensures uniformity in the conduct of these multicentred trials across different nations. From this, RG requirements, in relation to investigational medicinal products, have evolved in response to a number of different factors:

1. Mistakes or problems with medicinal products historically have increased the need for product regulation

2. Abuses of humans rights and incidents of fraud have increased the need for tighter ethical controls (e.g., Tuskegee Syphilis Study)[2]
3. A divergence in regulations and guidance in different countries has caused duplication of research, raising concerns about the:
   - Cost of clinical trials
   - Ethical implications of repeating studies
   - Need to rationalize and harmonize RG requirements

Obviously to safeguard research and take care of the ethical issues involved in research a RGF has been set up. The RGF outlines the principles of good governance for all research within the remit of the Secretary of State for Health. This includes clinical and nonclinical research (Table 1.1).[3]

TABLE 1.1. RGF basic principles and guidelines (not exhaustive)[3]

Basic criteria	Personnel who are directly or indirectly affected by the RGF	Standard for health care covered under following five domains
Promotion and protection of public health	All personnel involved in social care and public health at primary, secondary, and tertiary levels of health and allied care	Ethics
Undertaken by NHS, nongovernmental health agencies and Department of Health	Managers and staff of all professional groups irrespective of the level of seniority	Science
Undertaken by, within, or on behalf of social care agencies	For all those involved in clinical research, directly or indirectly	Information
Sets out principles, requirements, and standards, and defines mechanisms to deliver them		Health, safety, and employment
Improves research and safeguards the public		Finance and intellectual property

SALIENT FEATURES OF THE RESEARCH GOVERNANCE FRAMEWORK

The following summary depicts the RGF guidelines more specifically: [3]

Research involving patients in general and NHS patients in particular should ensure that the ethical guidelines are met with and the basic ethical issues are complied with. The research has to be approved by the Hospital Ethical Review Committee.

1. Complete informed consent of the participants should be obtained and this is the obligation of the Ethical Review Committee to ensure that it is done in a just and transparent manner.
2. Provisions should be available to the participants to withdraw themselves from the research process, whenever they want to.
3. Respect the multi-cultural nature and diversity of human society and conditions.
4. Ensure patient confidentiality and the confidentiality of data.
5. Take into account the risk to participants and provision for adequate compensation wherever necessary.
6. Base current research in the light of the existing evidence. Hence, peer reviews and the strength of the current evidence should be taken into consideration when planning research.
7. Research should be peer reviewed by experts in the field from time to time.
8. Findings and data should be made available to independent bodies and individuals for review from time to time.
9. Freedom of information: findings should be made available to people who may benefit from such research, general public at large and the research should be communicated in professional channels through appropriate channels and should be open to criticism.
10. Health and safety of participants should be assured at all times.
11. Financial and intellectual property rights must be respected at all times.
12. Strict guidelines for the recording and reporting of adverse outcomes exist and these must be maintained as per the guidelines laid down in the standard operating procedures (SOP).
13. Clinical trials involved in animal and human research must be registered with a central registering authority and their findings should be accessible to the public.

The RGF is incorporated into the constitution of the DOH. They are a leader in the field of implementing standards of RG in the field of health and social care. As of April 2004, the responsibility of ensuring that adequate standards are maintained and kept up, were passed on to the Strategic Health Authority, who ensure tight quality control and implementations of the research regulations in the NHS.[7] Thus RG has become a part of the NHS quality agenda. The framework of the RG is closely allied with the framework for clinical governance. Individual NHS bodies now report directly to the DOH on research and clinical governance issues. As a part of the national implementation plan for the United Kingdom, a network of research management and governance offices was established in April 2003.These offices ensure maintaining and compliance of the research procedures in primary, community, and social care. The responsibility for the national RG standards and policy will remain with the United Kingdom DOH. There is also a control assurance standard for RG that has been commissioned to assure quality control in RG.[8]

CONCLUSION

Regulation of research has evolved considerably since medieval ages, when most research and discovery was less controlled, but the product of relentless persuasion and thinking of the human mind. This applies mostly to medical discoveries such as administration of the first anesthetic (Lister), penicillin (Fleming), and small pox vaccine. One wonders if such strict regulations existed, whether it would have been possible to discover these landmark medical innovations. We can believe that had mankind not taken these risks then, we would not have seen so many advancements in modern medicine as we see today. Alternatively, we have become defensive as we protect ourselves from the speculated and feared side effects of new discovery. It is more like a fear and the insecurity of the unknown. However, regulation of research is equally important so as to direct and organize it while providing maximum protection to the participants and not compromise their safety in today's day and age.

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Chapter 2
Designing Health Studies

Rumana Z. Omar, Julie A. Barber, and Gareth Ambler

Study design is a fundamental aspect of research. If the design of a study is poor, no amount of clever analysis will provide reliable results. The results from poorly designed studies could be meaningless, and many resources will have been wasted, not to mention the possible risk to the subjects, which would be unethical. Therefore, it is essential that researchers invest adequate time and effort in designing their studies appropriately. It would be advisable to involve a medical statistician or an epidemiologist at the design stage of a study.

This chapter is divided into two parts. In the first part, the important aspects of study design are discussed. The second part focuses on the types of studies that are commonly encountered in health research.

ESSENTIALS OF A STUDY DESIGN

Specifying the Research Question

The first step should be to provide a clear research question, for example, the hypothesis to be tested. The research question should be justified on the basis of a thorough literature review of relevant previous research.

Selection of Subjects

Study subjects should be selected to ensure that they are representative of the population to which the results of the study will be applied *(target population)*. For example, studies including only patients referred to one particular hospital or volunteers may not always provide a representative sample of a more general population. The *source* of subjects and the *inclusion and exclusion criteria* need to be clearly defined.

Specifying the Primary Outcome

It should be decided in advance which outcome measure is of major interest. The analysis of this outcome should be used to

provide the study's main conclusions. Information on other outcomes could be collected, but these should be considered to be of secondary importance. Any interesting findings among the secondary outcomes should be interpreted cautiously, possibly as ideas for further research than as definitive results.

Inclusion of a Control Group

For studies investigating disease associations, it is not enough simply to consider what happens to subjects who are exposed (for example, to a treatment or risk factor); we need also to know what happens to subjects who are not exposed. This comparative role is undertaken by a *control group* of subjects. A control group is a set of subjects who either have not been exposed to the risk factor or treatment under investigation, or do not have the outcome of interest, depending on the type of design used. Subjects could also act as their own controls—for example, drawing comparisons between what happens before and after an exposure.

Confounding

In a study population, there could be differences in the characteristics of the subjects, such as age and sex, which may affect outcome, and which may also be related to the exposure of interest. For example, in comparing the operative mortality between two surgical techniques, the differences between the outcomes of the two operations could be due to the procedures. It could also be due to differences in the preoperative patient characteristics, which could also affect the choice of the technique. In statistical terms, the effects of operation and patient characteristics are said to be confounded (see Figure 2.1). It is important

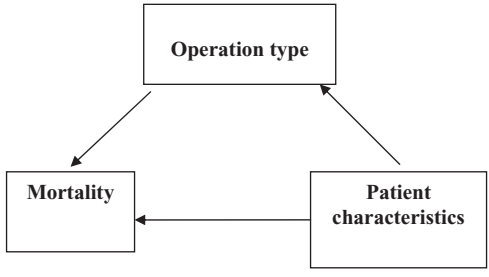

FIGURE 2.1. Confounding parameters when comparing two operative groups.

to identify potential confounders and make plans for dealing with these at the design stage of a study.

Confounding may be controlled through study design or in analysis. Two common methods for controlling confounding at the design stage are *matching* and *randomization*. In *matching* subjects in the groups under comparison are matched on factors that are considered as potential confounders. Randomization uses *random allocation* to assign patients to comparison groups to ensure that patients in the different groups only differ in their characteristics by chance. In analysis, one could adjust for confounding variables using statistical techniques such as stratification or multiple regressions. In order to do this, it is important to plan at the design stage what information should be collected on potential confounders.

Sample Size

When planning a study it is important to estimate the number of subjects required. Otherwise it could be impossible to tell if a study has a good chance of producing worthwhile results. In general, the larger a study is the greater its power and precision. Power is concerned with testing for effects (for example a difference between two treatments) and is defined as the probability that a study will be able to detect a clinically important effect if it really exists. Precision of a sample estimate is determined by the width of a confidence interval for the estimate. Statistical formulae are available to calculate sample size for a study.[1]

Bias

The presence of bias in a study may affect the validity of its findings. Steps should be taken at the design stage to avoid bias. The most commonly occurring biases are described below:

Selection bias: Stems from an absence of comparability between groups being studied or nonresponse. This could be avoided by selecting comparable groups, which are representative of the target population and taking effective strategies to minimize or handle missing data.

Assessor/response bias: If the assessor or respondent is aware of the exposure and/or disease status of the subjects this may influence their assessment/response. The exposure and disease conditions should be concealed from the assessors and responders if possible to avoid this type of bias. This is known as *blinding*.

Recall bias: For retrospective studies, subjects may find it difficult to provide accurate information on past exposures. The ability to recall may vary between subjects who have the disease and those who do not. This is particularly the case for exposures for which information is not usually recorded—for example, dietary habits. It is important to phrase questions carefully to avoid recall bias.

Writing a Protocol

A study design should start with the writing of a protocol. It should lay out systematically the various stages of the study described above and include strategies for data collection and achieving completeness, data entry, storage and validation of data, a broad statistical analysis plan, and the responsibilities of the study personnel. An analysis plan ensures that analysis for the study is performed in an objective way, thus avoiding data dredging, which may show spurious relationships.

TWO TYPES OF STUDIES

Health research studies may be broadly divided into two types: randomized controlled trials and observational studies.

Randomized Controlled Trials

In randomized controlled trials, a health intervention is planned for a specific outcome, and its effectiveness for that particular outcome is evaluated. Randomization is used to allocate patients into the intervention and control groups. Further design considerations for trials include the following factors.

Concealment of Random Allocation

When using randomization in trials, it is essential that the treatment allocation system prevents the person entering patients from knowing the next treatment allocation in advance. A common way of doing this is to use a series of sealed opaque envelopes, each containing a treatment specification. For drug trials, the allocation may be carried out by the pharmacy, which produces numbered bottles that do not indicate the treatment contained. In many large multicenter studies, patients are enrolled by telephoning a central office.

Blinding

Blinding is a technique used to minimize *response bias*. By blinding treatment allocation from both the patient and assessors, it is possible to eliminate response bias—this is known as *double*

blinding. Where it is only possible to blind either the assessor or patient, the study is *single blinded*. It is important to achieve the maximum degree of blindness when designing a trial.

Placebos

If there is no existing *standard* beneficial treatment, then it is reasonable to give the control group *placebos* instead of any active treatment. Placebos are identical in appearance to the active treatment, but are pharmacologically inactive. Placebos are used because the act of taking a treatment may itself have some benefit to the patient, so that part of any benefit observed in the treatment group could be due to the knowledge/belief that they had taken a treatment. Moreover, for a study to be double blind, it is necessary for the two treatments to be indistinguishable. When the control treatment is an alternative active treatment rather than a placebo, the different treatments should still be indistinguishable, if possible.

Protocol Violations

A common problem relates to patients who have not followed the protocol—for example patients who receive the wrong treatment or do not take their treatment, known as *noncompliers*. If this does occur, it is advisable to keep all randomized patients in the trial and analyze groups as randomized. This is known as an *intention-to-treat* analysis.

Types of Trials

Parallel Group Trials

In the simplest type of trial design, one group of subjects receives the planned intervention and is known as the intervention group. A group of subjects does not receive the planned intervention and is known as the control group. The outcome is compared between the two groups. Subjects are allocated to the intervention and control groups using randomization. This ensures that each subject has an equal chance of being allocated to either group, and groups differ only with respect to their intervention. An example is a trial where patients with type 2 diabetes were randomized to the angiotensing converting enzyme (ACE) inhibitor Ramipril or placebo (on top of standard treatment). The outcome of interest is the occurrence of cardiovascular events in patients.[2] This is the most frequently used design for trials.

Crossover Trials

The most common alternative is the *crossover trial,* in which all patients are given both the intervention and control treatments

in a sequence. Here randomization is used to determine the sequence of the treatments. For example, a crossover trial was carried out to evaluate the effectiveness of oral morphine for the management of refractory dyspnoea.[3] Patients were randomized to receive four days of oral morphine, followed by four days of identically formulated placebo, while the other half received placebo followed by morphine.

Strengths and Limitations of Parallel and Crossover Trials

In a crossover trial, the variability is less as the comparison is within subjects and hence a smaller sample size is needed. However, this design is only suitable when:

- Treatment periods are fairly short to minimize the risk of drop out for other reasons
- Conditions are chronic and cannot be cured
- There is no carry over of effect of intervention from one period to the next. It may be possible to have a *wash-out* period between the intervention periods, to reduce the risk of carry over

Observational Studies

In observational studies, associations between health outcomes and exposure to risk factors or preventative measures are observed in subjects without any planned intervention. Observational studies may be classified as 1) descriptive (includes case report/series and cross-sectional) or 2) analytic (includes cohort and case-control studies). Descriptive observational studies are used to describe disease patterns and trends. Often these studies are used to generate hypotheses and plan health programs. Analytic studies may be used to estimate or test relationships between an exposure to a risk factor or a preventative measure, and a health outcome.

Case Report/Series

A case report is a detailed profile of a single patient, reported by one or more clinicians. For example, a report was published on a 40-year-old woman who developed pulmonary embolism after use of oral contraceptive.[4]

A case series is an expanded version of a case report that includes a series of patients with a given health condition. For example, a study was conducted on 12 children who had received the measles-mumps-rubella (MMR) vaccine and were referred to a gastroenterology unit and had a history of normal development followed by a pervasive developmental disorder (PDD).[5]

An important point to note here is that case report/series focuses on a single group of patients and crucially does not include a control group.

Cross-Sectional Study

In this type of study design, all information on a group of subjects is collected at a single time point. There is no typical format, and each study may be designed to meet the need of the researcher. This study design includes subjects who are exposed to the risk factor or preventative measure under investigation, as well as those who are not. The outcome of interest is compared between the exposed and unexposed groups. This design is particularly suitable to estimate the prevalence of a disease and to examine health trends. It may also be used to examine the association between a risk factor or a preventative measure and a health outcome. An example is the Health Survey of England conducted to monitor health trends, and estimate prevalence of certain diseases and certain risk factors associated with this outcomes.[6]

Strengths and Limitations of the Descriptive Observational Studies

A case report/series does not include control groups and, hence, cannot be used to investigate associations or causation between exposure and outcome. These studies may only be used for descriptive purposes. There are also issues about whether the subjects included in a case report/series are representative of the study target population. These types of studies could be used for reporting or sharing experiences on rare health conditions.

A cross-sectional study has no rigid format and is therefore more prone to bias. It is a simple design, which could be used when resources are limited to give some idea about disease associations. It is not suitable for studying disease causation, as it provides no information about the order of events, i.e., whether disease preceded or followed exposure.

Cohort Study

In a cohort study, a group of subjects is identified according to the research objectives and followed over the study period, until the subjects drop out, have the event of interest, or reach the end of the study period. The event rate is compared between the group of subjects exposed to the risk factor, or any preventative measure under investigation, and the unexposed group. A cohort study may be both prospective and retrospective. A retrospective

cohort study could be about patients with a past history of exposure to certain factors, experiencing health conditions that were also observed in the past.

An example of a cohort study is a study investigating the association between stable partnership and development of AIDS or death in HIV patients receiving highly active antiretroviral therapy (HAART) treatment.[7] In this study, patients were followed from the first follow-up visit after receiving HAART to their last follow-up visit within the study period or death or development of AIDS. The conclusion from this study was that stable partnership was associated with a slower rate of progression to AIDS or death in HIV patients receiving HAART.

Case-Control Study

Case-control studies are always retrospective. They examine how exposure to retrospective factors contributes to current health conditions. A group of subjects with the outcome of interest is recruited according to some prespecified inclusion criteria *(cases)*. Another group of subjects who have not experienced the outcome of interest is recruited as *controls*. Exposure to the risk factor of interest is then compared between the cases and controls. Cases and controls may be matched on important confounding characteristics. For example, a matched case-control study was used to investigate the association between the MMR vaccine and PDD.[8] The exposure of interest was the MMR vaccine. Subjects with a diagnosis of PDD, while registered with a general practitioner (GP), were recruited as cases. Subjects with no diagnosis of PDD, matched on age, sex, and GP to the cases, were recruited as controls. The conclusion was that there was no association between MMR vaccine and PDD.

Strengths and Limitations of Analytic Observational Studies

Cohort studies are particularly suitable for studying rare exposure. For example, to investigate the association between long-term chronic exposure to radiation and risk of cancer, a cohort of workers from a nuclear plant may be recruited. It may also be used to examine risk factors that change over time (temporal relationships), estimate incidence rates of a disease, and study multiple outcomes. However, this design may not be suitable for rare outcomes, as it could be time consuming to observe all the events required to achieve the desired sample size. Alternatively, a large number of subjects may have to be recruited. Both of these could prove to be expensive.

TABLE 2.1. Appropriateness of study design types

Study Objective	Type of Study Design
Evaluating health intervention	Randomized controlled trials
To study natural history of a disease	Cohort and case-controls studies
To examine disease associations	
To estimate disease prevalence	Cross-sectional studies
To examine health trends	
To generate hypothesis	
To report observation of rare conditions for descriptive purposes	Case-report/series
To generate hypotheses	

Case-control studies are particularly suitable for rare outcomes. It is cheap and fast. However, they can be more prone to bias than cohort studies, depending on the way the controls are selected. For example, if volunteers are selected as controls, they may not be representative of the study target population. Using hospital controls could distort the effect of exposure if controls are recruited from patients who are more likely to be exposed to the risk factor of interest compared to the members of the target population. For example, in a case-control study examining the association between non-steroidal anti-inflammatories (NSAIDs) and the development of gastric ulcer, patients in the gastroenterology unit of a hospital are recruited as cases. Selecting controls from the rheumatology unit of the same hospital would not be appropriate, as they are likely to use more NSAIDs than the general population.

Reliability of Different Study Designs

A properly conducted randomized trial is the most reliable study design. Causal effect may only be directly inferred from such studies. Drawing causal inference from analytic observational studies is more difficult. Bradford Hills suggests several conditions that need to be met before causal inferences could be drawn from an analytical observational study.[9] Cohort studies are usually less prone to bias than case-control studies. The use of case-report/series is very limited. The appropriateness of each type of design for health studies is summarized in Table 2.1.

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Chapter 3
Immunohistochemistry

Philippa Munson

INTRODUCTION

Immunohistochemistry describes the localization of antigens in histological and cytological preparations using antibodies. It is now recognized as an essential element and a major tool, both in diagnostic and research-orientated cellular pathology. The technique involves the detection of specific or highly selective cellular epitopes with an antibody and appropriate labelling system. Immunohistochemistry can be performed on cytological preparations, frozen sections and paraffin-embedded histological sections.

The first point of call for any researcher wishing to perform immunohistochemistry should be the local immunohistochemistry laboratory. Immunohistochemistry is a speciality that is constantly changing and improving, and the local immunohistochemistry laboratory will have more experience and more up-to-date methods than most research laboratories. This should therefore be done, even when in possession of an existing protocol from other researchers. It is also wise to refrain from purchasing any reagents without first consulting the laboratory and to ask the histology laboratory to perform the section cutting. Although microtomy can be learnt in a few weeks, it is a skill that takes years to perfect and poor section quality can severely affect the interpretation of the immunohistochemistry.

BASIC IMMUNOHISTOCHEMISTRY

The basics of immunohistochemistry involve the use of an antibody to detect a specific epitope, which is then visualized using a detection system and chromogen.

Fixation, be it with alcohol or formalin, will mask some antigens to a certain extent. When this occurs, some form of antigen retrieval will be needed to re-expose the antigen. This has to take place before applying the primary antibody (Table 3.1).

TABLE 3.1. Glossary of immunohistochemical terms

Antigen	A molecule that induces the formation of an antibody
Epitope	A single antigenic determinant (functionally it is the portion of a antigen that combines with antibody paratope)
Antibody	A molecule produced in response to an antigen. It has the property of combining specifically with the antigen that induced its formation
Fixation	The process of preservation that is necessary for both cytological and histological specimens
Taking sections to water	The wax in the sections must be removed and the sections brought through a gradient of alcohols before immersing them in water and performing immunohistochemistry.
Antigen retrieval	Aka antigen unmasking epitope retrieval. This is the use of enzymatic or heat-mediated methods that "reverse" the effects of fixation, enabling the antibody to combine with the antigen
Peroxidase block	If peroxidase is the enzyme used in the detection system, any peroxidase in the section must first be saturated with its substrate (hydrogen peroxide) to prevent endogenous staining
Primary antibody	The first antibody to be applied to the section, i.e., the antibody that identifies the antigen under investigation
Secondary antibody	Aka bridge or link antibody. It attaches to the primary antibody. This may be labelled with biotin, fluoroscein or another molecule or be used in its unlabeled form
Tertiary layer	The third (and usually final) layer that attaches either directly to the secondary antibody (if the secondary is unlabeled) or to the molecule with which the secondary antibody is labelled (e.g., biotin). The tertiary layer will be labelled with an enzyme that works with the chromogen to produce a colored product
Chromogen	The solution that enables a colored product to be formed over the site of the antigen. The chromogen is usually used in solution with the substrate of the enzyme that has been used in the tertiary layer NB. The secondary, tertiary, and chromogen are collectively known as the detection or labelling system.

(*Continued*)

TABLE 3.1. (*Continued*)

Counterstain	The histochemical dye, usually hematoxylin, that is applied to the section last, enabling morphological identification of the tissue components
Dehydrate, clear, and mount	The process by which sections are dehydrated through graded alcohols, cleared in xylene, and mounted using a synthetic mountant

The basic steps of an immunohistochemistry protocol are:

fixation/processing/embedding
↓
section cutting/microtomy
↓
dewaxing sections and taking to water
↓
antigen retrieval
↓
peroxidase block
↓
primary antibody
↓
secondary antibody
↓
tertiary layer
↓
chromogen
↓
counterstain
↓
dehydrate, clear and mount sections

METHODOLOGY

Immunohistochemistry methodology starts with the process of fixation. The length of time a sample spends in fixative is very important as under- or over-fixation can lead to problems with proteolytic antigen retrieval.

Samples should not be kept in fixative indefinitely (24 hours is optimal for formalin). If a delay is experienced between sample collections, they should be taken to the histology laboratory for processing once they have spent 24 hours in fixative.

If the antibody of interest is going to be used on patient samples, it is imperative to determine first that there is an available antibody that works on paraffin-embedded sections; otherwise retrospective studies will be exceedingly difficult.

Fixation

Fixation is essential for tissue and antigen preservation. The most important reactions that take place are those that stabilize the proteins. The general principle is that the fixatives form cross-links between proteins, thereby stabilizing the cytoskeletal structure.

Formaldehyde (formalin) is the fixative of choice for routine histology; therefore, any retrospective studies using patient samples will involve the use of immunohistochemistry on formalin-fixed, paraffin-embedded blocks. The aldehydes form cross-links between protein molecules, the reaction being with the basic amino acid lysine.

These cross-linking "methylene bridges" ensure that the structures of intracytoplasmic proteins are not significantly altered. However, they can also have the effect of "masking" antigens, therefore, tissue that has been fixed in formalin will generally require some form of antigen "unmasking," i.e., antigen retrieval.

Alcoholic fixatives are generally used for frozen sections or cytological preparations, as they are poor penetrators of tissue. They preserve most proteins in a relatively undenatured state, although some antigen retrieval may be necessary. It is wise to refrain from fixing frozen sections in glutaraldehyde or paraformaldehyde, as these will usually mask the antigen, and effective antigen retrieval is very difficult to perform on frozen sections.

Antigen Retrieval

Antigen retrieval is the method by which antigens that have been masked through fixation and processing are unmasked prior to immunostaining. There are two main methods for antigen retrieval: proteolytic and heat-mediated. Of these two, heat-mediated antigen retrieval (HMAR) is the most effective on the majority of antigens; however, the correct antigen retrieval method must be determined for each antibody.

Proteolytic digestion generally utilizes trypsin, chymotrypsin, protease, or proteinase K enzymes. The majority of these need to be used at 37°C, with correctly prepared pH solutions. The main pitfall of proteolytic digestion is that the digestion time has to be tailored to the fixation time, i.e., the longer a tissue has

been in fixative, the more methylene bridges will have formed, requiring a longer time in enzyme to break these down. If the antigen under investigation is localized in the membrane, then HMAR is generally preferable to enzyme digestion.

Heat-mediated antigen retrieval was first described by Shi et al.[1] (1991) and has proved to be a revolutionary technique as many antigens previously thought to have been lost or destroyed by fixation and processing can now be recovered and demonstrated;[1] however, the theory behind HMAR methods remains unclear.

There are two major factors in HMAR: the antigen retrieval solution and the cooking method used. A number of different retrieval solutions with different pHs are available. The most commonly used are citrate (pH 6.0) and some form of Tris-EDTA (pH 9.0). These retrieval solutions can be used in microwave ovens, pressure cookers, decloakers, or on some automated immunostainers. Any of these cooking methods are acceptable, provided they are performed and used in a consistent manner. HMAR methods do require strong adhesives on the slide to prevent section detachment. The slides must then be thoroughly drained before being heated to at least 60°C for at least one hour.

The main advantage of HMAR over proteolytic digestion is that heating times to retrieve antigens tend to be uniform, regardless of the amount of time spent in fixative.[2] This is in contrast to the variability in digestion times required when using enzymes.

The main pitfall with HMAR is that extreme care must be taken not to allow the sections to dry, as this destroys antigenicity. Sections are particularly susceptible to drying when being removed from a hot solution, therefore, the solutions should be flushed from the container with cold running tap water. The slides can then be removed when the fluid is cool.

Antibodies

The vast majority of primary antibodies available for use on human tissue are made in either rabbits or mice. It is essential to check (before purchasing) that the required primary antibody is available that works on formalin-fixed, paraffin-embedded tissue. Again, liaison with the local immunohistochemistry laboratory will be helpful. Generally speaking, monoclonal murine antibodies are preferable to polyclonal antibodies, as they tend to be more specific. See *Animal Tissue* section for more specific guidelines on selecting antibodies for tissue other than human.

Antigens and epitopes: An antigen can be defined as a molecule (protein, carbohydrate, or lipid) that binds with an antibody. One antigen is composed of a number of epitopes or antigenic determinant groups. An epitope consists of a small amino acid sequence and which is what binds to the variable region of the antibody. Due to differences in their manufacture, a monoclonal antibody will only recognize one epitope on an antigen whereas a polyclonal antibody will recognize many epitopes on an antigen. One analogy is that of a Christmas tree and lights. If the antigen is the tree and the epitopes are the lights, then a monoclonal antibody will only recognize the red lights on a particular tree, whereas a polyclonal antibody will recognize all the colors of lights on that particular tree.

Staining Methods/Detection Systems

There is a wide variety of systems available today, but the best and most reliable of these are the avidin-biotin and polymer-based systems. Both of these methods are available in prediluted forms, which are generally preferable where immunohistochemistry is not performed on a regular basis.

Avidin-Biotin Methods

These methods were first described by Heggeness and Ash[3] (1977) and utilize the high affinity of the glycoprotein avidin for biotin, a low molecular weight vitamin.[3] Avidin is present in egg white and is composed of four subunits that form a tertiary structure possessing four specific binding sites for biotin. Egg-white avidin contains some oligosaccharide residues that possess an affinity for some tissue components. As a result, a similar molecule, streptavidin (extracted from the culture broth of the bacterium *Streptomyces avidinii*), is generally used as this molecule does not contain the oligosaccharide residues.

Biotin (vitamin H) can be conjugated to both antibody and enzyme molecules. Up to 200 molecules of biotin can be conjugated to one antibody molecule, often with the aid of spacer arms. By spacing the biotin molecules, streptavidin is given room to bind and is able to maximize its strong affinity for biotin.

In a *streptavidin-biotin complex*, the streptavidin and biotinylated enzyme are supplied as two separate reagents, which need to be added together 30 minutes before use. The streptavidin can be added in slight excess so that the biotinylated enzyme does not saturate all of the biotin-binding sites. Either peroxidase or alkaline phosphatase can be used as the enzyme label.

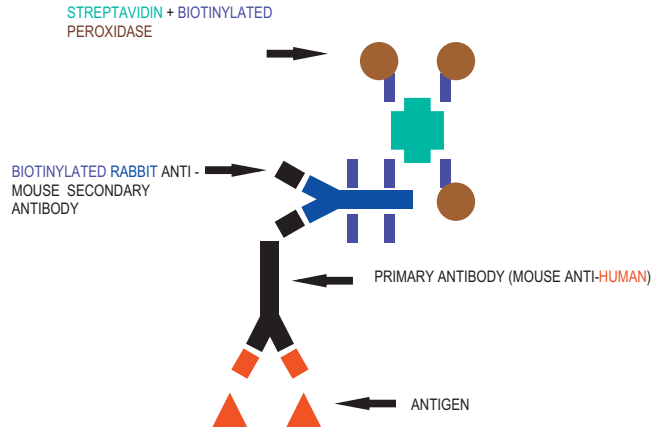

FIGURE 3.1. Primary streptavidin-biotin complexing.

In a *labelled streptavidin* technique, the streptavidin molecule is directly labelled with the enzyme of choice. This is the technique favored by companies producing prediluted reagents, as the streptavidin-biotin complex is not stable for long periods.

Avidin-biotin techniques provide high sensitivity as the high affinity of streptavidin for biotin enables, firstly, a very stable complex to be formed and, secondly, many enzyme molecules to be deposited at the antigenic site (Figures 3.1 and 3.2).

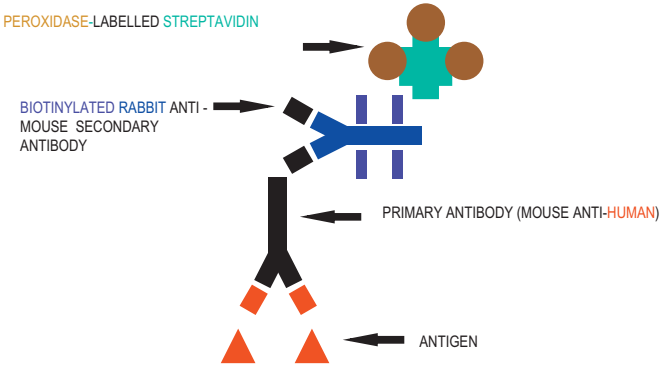

FIGURE 3.2. Secondary streptavidin-biotin complex labeling.

Polymer-Based Methods

Polymer-based methods can be either a two- or three-layer system. In the two-layer system, the secondary antibody is part of the polymer molecule. The secondary antibody is conjugated to the polymer as are a large number of enzyme molecules. The EnVision kit, available from Dako, U.K., is an example of a two-layer polymer system. Vyberg and Neilsen[4] (1998) reported comparable sensitivity between the Dako kit and a three-stage avidin-biotin system (Figure 3.3).[4]

In the three-layer system, the secondary antibody is applied unconjugated, then the tertiary antibody which is conjugated to the polymer along with enzyme molecules. The Novolink Polymer kit, available from Novocastra Laboratories, U.K., is an example of a three-layer polymer system (Figure 3.4).

The main advantage of polymer-based systems is that they can be used on tissues containing a lot of endogenous biotin without producing background staining. (See *Background* section.)

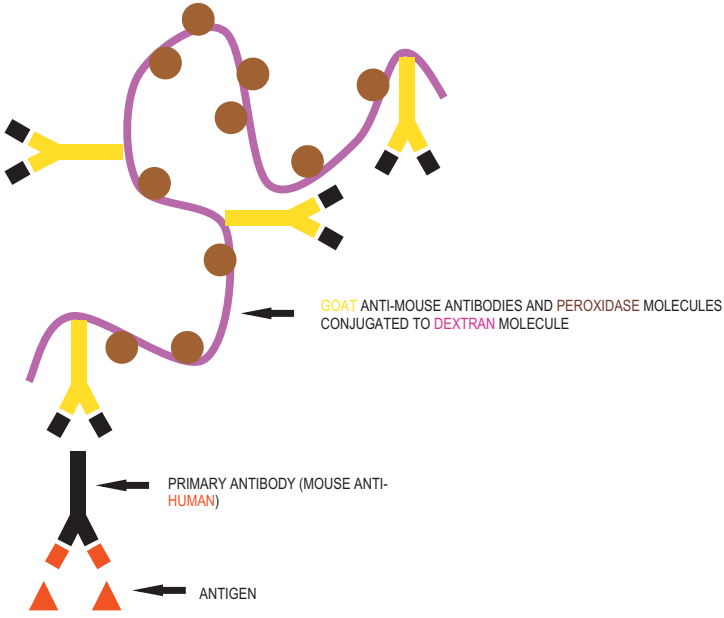

FIGURE 3.3. Two layer polymer-based labeling.

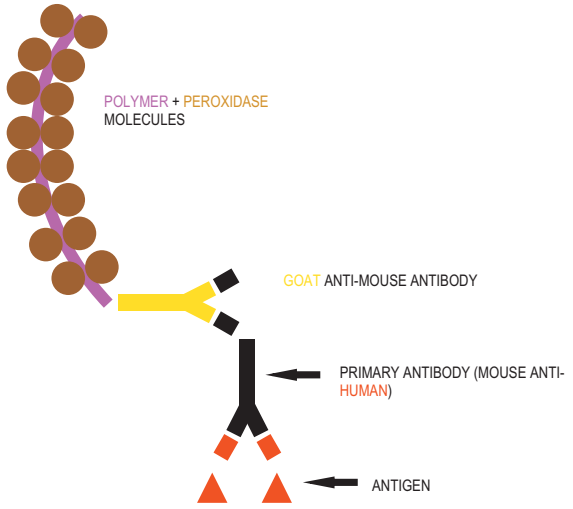

FIGURE 3.4. Three layer polymer based labeling.

Enzyme Labels and Chromogens

Enzymes are the most widely used labels in immunohistochemistry, and incubation with a chromogen using a standard histochemical method produces a stable, colored reaction end product suitable for the light microscope. In addition, the variety of enzymes and chromogens available allow the user a choice of color for the reaction end product.

Horseradish peroxidase labelling + DAB (3,3'diaminobenzidine tetrahydrochloride) were first described by Nakane and Pierce in 1966,[5] and this is still the most commonly used combination of enzyme and chromogen in immunocytochemistry. DAB precipitates to a brown reaction end product when in the presence of peroxidase and hydrogen peroxide (hydrogen peroxide is in solution with the DAB). A by-product of the reaction of hydrogen peroxide (the substrate) with peroxidase (the enzyme) is an oxygen radical, which acts on DAB and precipitates it at the antigenic site.

Some endogenous pigments (e.g., melanin, lipofuchsin, haemosiderin, and formalin pigment) can mimic the appearance of DAB, and in these situations, it may be preferable to use another chromogen, e.g., 3-amino-9-ethylcarbazole (AEC), which produces a red end product.[6,7]

Alternatively, a different enzyme can be used in the detection system. Alkaline phosphatase is the most widely used alternative to peroxidase and can be developed using a number of chromogens, most usually Fast Red, Fast Blue, or NBT/BCIP (nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate). Alkaline phosphatase can also be useful when the target tissue contains a lot of endogenous peroxidase, e.g. tissue containing red blood cells, etc. (See *Background* section.)

Animal Tissue

When using animal tissue, the detection system components need to be chosen with care. First, the primary antibody must be able to recognize that particular species; this information will be on the specification sheet. The secondary antibody then needs to recognize the primary antibody without cross-reacting with the host tissue. For example, if the host tissue is mouse, the primary antibody should be raised in another animal, e.g., rat. The secondary antibody will then be raised in another animal, e.g., rabbit, and be anti-rat.

Occasionally, the only available primary antibody will be raised in the same species as the host tissue. In these circumstances, there are commercial kits available to facilitate these staining procedures.

TROUBLESHOOTING/OPTIMIZATION

Optimizing Primary Antibodies

The two main factors involved in optimizing a primary antibody are antibody dilution and antigen retrieval. Guidelines may be given on the antibody specification sheet, however, if none is available, one approach is to pick a starting dilution (e.g., 1/50) and test known positive tissue using a number of different retrieval methods. A negative control must always be included when optimizing a new antibody to ensure any staining seen is appropriate.

Background

The major causes of background staining in immunocytochemistry are hydrophobic and ionic interactions and endogenous enzyme activity. *Hydrophobicity* is a property shared by most proteins and confers stability on the tertiary structure of peptides. It may also take place between different protein molecules and impart stability to immune complexes.

Proteins are rendered more hydrophobic by aldehyde fixation, and the extent of hydrophobic cross-linking of tissue proteins is primarily a function of fixation. Therefore, factors such as time, temperature, and pH of fixation can be optimized to avoid excessive cross-linking.

Connective tissue (e.g., collagen, elastin, laminin), epithelium, and adipocytes are especially prone to hydrophobic cross-linking, as are immunoglobulins. Methods to decrease this type of background include the addition of detergent/surfactant, e.g., Tween 20, to the buffer, or the use of a blocking protein applied prior to the primary antibody. The blocking protein must be of the type that can compete effectively with IgG for hydrophobic binding sites in the tissue. Therefore, the blocking protein must contain proteins identical to those in the link antibody in order to prevent nonspecific binding of the secondary antibody, e.g., normal swine serum for polyclonal antibodies when the secondary antibody would be swine anti-rabbit.

Generally, most existing immunocytochemical protocols will be optimized to reduce hydrophobic binding, most usually through the addition of detergent to the wash buffer.

Ionic interactions result when proteins of opposite charges meet. Most IgG class antibodies have a net negative surface charge at a buffer pH of 7.0–7.8. Ionic interactions can be expected if tissue proteins have a net positive surface charge. These interactions can be reduced by using diluent buffers with higher ionic strength. As a rule of thumb, buffers used during immunocytochemical procedures should be between pH 7.0 and pH 8.0, even during the DAB stage.

Endogenous Enzyme Activity

If enzymes similar to those used as the label are present in the tissue, they may react with the substrate used to localize the label and give rise to interpretation problems. False-positive reactions produced in this way can be eliminated by inhibiting the endogenous enzyme activity prior to staining.

Peroxidase results in the decomposition of hydrogen peroxide and is a common property of all hemoproteins, myoglobin, cytochrome, and catalases. The most frequently used method is saturation of the endogenous peroxidase with its substrate (hydrogen peroxide), usually in the form of 0.5% hydrogen peroxide in a diluent (water, buffer, or methanol).[8]

When staining specimens that contain large amounts of endogenous peroxidase, it is often easier to use a different enzyme, i.e., alkaline phosphatase, in the detection system.

As for endogenous peroxidase, endogenous *alkaline phosphatase* activity is quenched by the addition of its substrate (levamisole) in the chromogen solution. Levamisole will inhibit many types of alkaline phosphatase activity. However, the alkaline phosphatase used in the detection system is usually intestinal in nature and remains unaffected by levamisole.

Endogenous Biotin

Biotin is a vitamin and coenzyme that is found in liver, kidney, and a variety of other tissues. Biotin binds specifically and with a very high affinity to avidin and streptavidin. Its endogenous activity is most pronounced in frozen sections. It is possible to block endogenous biotin with successive incubations of 0.1% avidin and 0.01% biotin. The avidin blocks the endogenous biotin, and the dilute biotin blocks any free sites left on the avidin. Alternatively, if the tissue under investigation contains large amounts of endogenous biotin, a different detection system can be used, e.g., a polymer-based method, which does not use biotin in its labelling system.

Washing

Thorough washing of slides in between the various steps of an immunohistochemical technique is essential. Most protocols use a tris buffered saline (TBS), although phosphate buffered saline (PBS) can be used. As mentioned above, the addition of a detergent to the buffer wash will help reduce some background staining; however, this should not be used when staining frozen sections, as there is a higher risk of section detachment when dealing with these specimens. It is worth remembering that it is difficult to over wash sections whereas under washing them will result in dirty, patchy staining.

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Chapter 4

Cell Culturing: A Beginner's Guide to Understanding the Basics of Cell Culturing

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INTRODUCTION

The fundamental aspect of cell culturing is to understand the type of cells that the investigator wishes to grow. There are many differences between the cell types; however, the easiest method of categorizing them is into primary cells and cell lines.

Two types of cultured cell types:

- Primary cells
- Cell lines

Primary Cells

These are cells derived directly from tissue samples/biopsies which have heterogeneous nature of cells with variable growth friction. The cells are extracted directly from the tissue and grown directly in specified optimal cell culture Medias.

Cell Lines

These are cells subcultured from primary cells but now have been manipulated in the laboratory so that they last longer and can go through more cell passages with growth friction of 80% or more, than the original primary cells before they change their morphology. On the whole, they tend to be more resilient than primary cells. However, due to their manipulation, they may not mimic in-vivo cells as closely as the unmanipulated primary cells. Subcultured cells separated from primary cells create homogeneous cell lineages. They ascertain specific properties by the process of cloning or physical cell separation, thus leading to so-called cell strains.

FUNDAMENTALS OF CELL CULTURING

The reason for undertaking cell culturing is that the growth of the cells can be mimicked as closely as possible to its growth in its normal host environment. By growing the cells in cell culture flasks or cell culture dishes, the investigator may build up the cell numbers and then use these cells to conduct experiments. These in-vitro cell experiments may be performed, and later the final effects on the cells may be investigated.

The cells are delicate and prone to bacterial and fungal infections from the environment, so all precautions are used to minimize contamination. Bacterial contamination is a difficult problem. In this respect, mycoplasma-species contamination is a more difficult problem to deal with than the growth of other bacteria and fungi. To combat contamination, the single-use disposable equipment and aseptic techniques are applied (Figure 4.1).

Methods to prevent cell contamination:

- Gloves
- Sterilized equipment
- Laminar flow cabinet
- Singe-se pipettes
- Resterilization of equipment

What Happens When an Infection Occurs?

Usually a bacterial or fungal infection would mean that the cells would need to be safely discarded to prevent cross contamination. However, when the cells are "precious," an attempt to treat the contamination may be made. Free-floating contaminants in the media are physically removed, and the cells are washed numerous times with phosphate buffered saline (PBS). Loose lids or covers are replaced with new sterile ones. An antibiotic mix is added to the media, and the antibiotic medium is added to the cells. The cells are then cultured for 2 to 3 days. If the cells remain uncontaminated, then they are returned back to a normal media for another 2 to 3 days to confirm that no contamination remains.

First-choice antibiotics/antifungal agents for cell culture:

- Gentamicin
- Streptomycin
- Penicillin G
- Amphotericin B (antifungal)

FIGURE 4.1. Cell culturing in a laminar flow cabinet. Note the scientist is using a battery-operated "pipette boy" to draw the media up into the pipette. (Courtesy of P.N. Vaiude, Honorary Clinical Fellow, Barts and the London NHS Trust.)

Media: Their Function in Cell Culturing

Media provide the food and supplements that are needed for the growth of cells. Over the years, scientists have searched for the ideal media needed for the growth of various cell types. There is now an exhaustive list of media which are suited for the different cell types. So, when the investigator is growing a cell type, the investigator must be careful to select the correct medium. Also, as part of an investigator's experiment, the investigator may add or remove certain substances from the media and monitor the cells' response to the changes in their environment. A medium may be a solid, broth, or solution. Figure 4.2 shows the Caplan-1 and Paca-3 cell strains growing in solution medium.

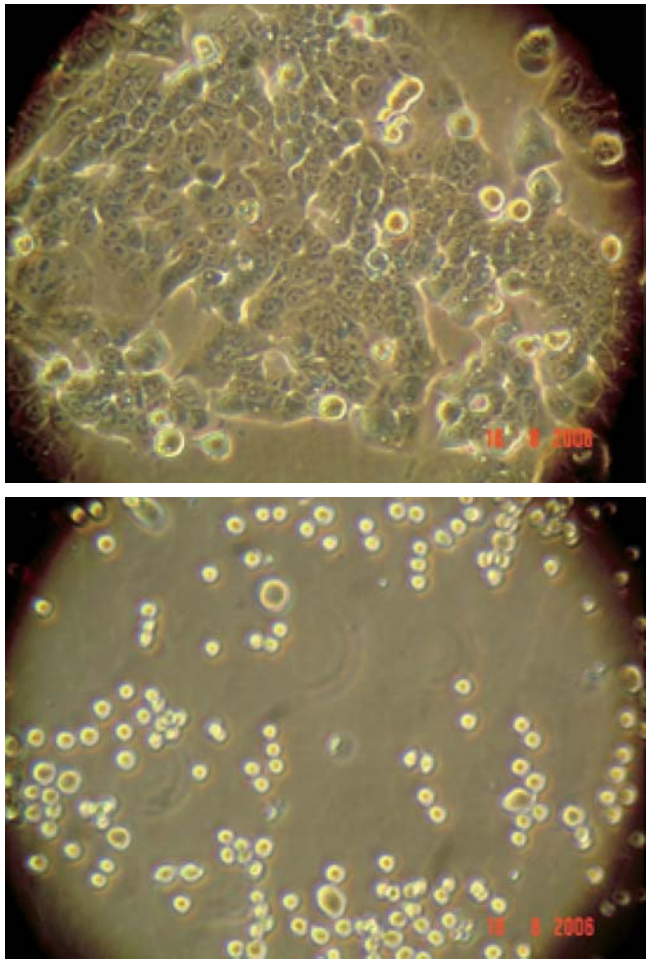

FIGURE 4.2. Caplan-1 (A) and Paca-3 (B) cell strains growing in solution medium. (Courtesy of P.N. Vaiude, Honorary Clinical Fellow, Barts and the London NHS Trust.)

Support Cells

Certain cells need "feeder" cells to support their growth, especially during the initial stages when their cell numbers are low. Once the cells of interest have reached an adequate number and are able to support themselves, the "feeder" cells are removed, and the cells are allowed to grow on their own momentum. An

example of this is the use of "3T3" cells from mice for keratinocytes (skin epithelial cells). These cells are gamma irradiated before use so that they can provide support for the keratinocytes when they are initially extracted from the biopsies and are low in number. The 3T3 cells are irradiated so that they can provide support but then die off within a few days; otherwise they will overtake the cells being cultured and kill them.

Incubation

Ideal conditions for the growth of cells are normally maintained for maximal growth. This usually includes incubating the cells at a suitable temperature, environmental moisture, and an adequate environmental CO_2 concentration. Humidified CO_2 incubators with shelves are usually available with these preset conditions for cell culture. They are regularly cleaned out with a detergent or 70% alcohol, and fungicide/bactericide is placed in the humidified water to prevent cell infections during incubation.

CELL EXTRACTION

There are many means of extracting cells, and the one that we will describe is for fibroblasts from tissue biopsies.

Handling Tissue Biopsies

Tissue biopsies should be taken immediately from the time of the operation/procedure and kept in a suitable transport media. (An example is E4 [DMEM Eagle's medium] medium with 4 mls of glutamine and an antibiotic mix.) Ideally, the cell extraction should start immediately, but it may be done up to a maximum of 48 hours later if the samples are kept in the transport media at a constant fridge temperature of 4°C–6°C.

Fibroblast Extraction

Primary fibroblasts are fairly resilient cells that proliferate quickly and do not need support cells for their growth. However, like all cells, they will need their ideal medium and environmental conditions (Figures 4.3 and 4.4).[1]

Fibroblast extraction steps:

- Take tissue biopsy
- Place on a Petri dish
- Using a sharp scalpel cut the tissue into tiny pieces
- Moisten cut biopsy tissue
- Leave for 30 minutes
- Add medium and close Petri dish with its cover

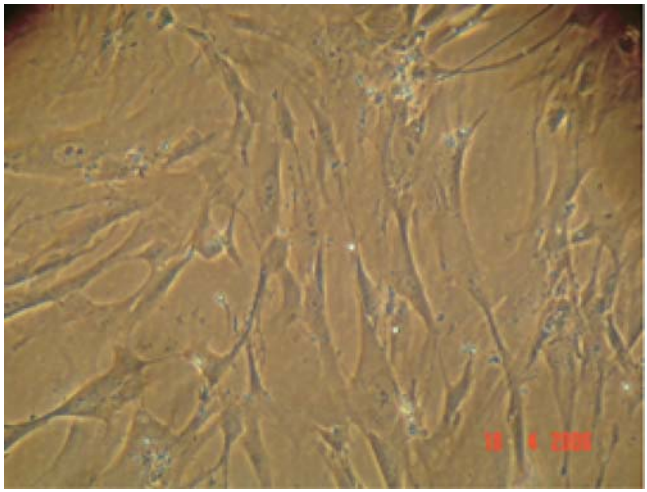

FIGURE 4.3. Fibroblasts growing out from the explanted tissue sample on a cell culture flask. The picture was taken with a camera mounted on a light microscope. Magnified ×20. (Courtesy of P.N. Vaiude, Honorary Clinical Fellow, Barts and the London NHS Trust.)

FIGURE 4.4. The investigator is using a light microscope to monitor the progress of his cells, which are bathed in media and growing in cell culture flasks. (Courtesy of P.N. Vaiude, Honorary Clinical Fellow, Barts and the London NHS Trust.)

- Incubate and inspect at least twice-weekly
- When the fibroblast outgrowths from the biopsy are adequate, remove the tissue
- Add Trypsin to remove the fibroblast from the plate
- Transfer the fibroblasts to a 25 cm^2 tissue culture flask
- Add medium to the flask
- When the flask is confluent (full of cells), trypsinize again
- Transfer the fibroblasts to a larger 75 cm^2 tissue culture flask
- Change the medium at least twice-weekly
- Cells can be grown for many passages before their morphology changes
- The cells can also be stored in liquid nitrogen for use later. (Use standard cell-freezing protocols

CONCLUSION

Cell culturing techniques have developed and significantly improved over the decades and have enabled major scientific leaps to important fields in cancer study, wound healing, and, not least, stem cell technologies.[2]

Glossary:

- Cell lines—manipulated primary cells (engineered cells)
- In vitro—out of the body/natural environment; in a laboratory vessel
- In vivo—in the body/natural environment
- Passage—to remove/detach the cells from the culture disk/flask
- Primary cells—cells directly from the tissue (unmanipulated cells)
- Trypsinize—to add trypsin

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Chapter 5
Flow Cytometry

P. Erotocritou, M. Arya, S.N. Shukla, and H.R.H. Patel

WHAT IS FLOW CYTOMETRY?

The measurement of the physical and chemical characteristics of cells is called *cytometry*. *Flow cytometry* is the technique where these measurements are made individually of single particles (cells, nuclei, chromosomes), suspended within a stream of liquid, as they pass through a laser light source.

All parameters measured can be divided into two main groups: 1) those related to light scattering, which mainly reflects the size of the cell and its internal complexity and 2) those related to fluorescence. These are associated with the presence of one or more fluorochromes inside the cell or attached to the cell surface membrane, either naturally (autofluorescence) or artificially (e.g., using fluorochrome conjugated monoclonal antibodies).

The light scatter and emitted fluorescence of *each* particle is collected, filtered and converted to digital values which are stored on a computer. The essential feature of flow cytometry is that individual measurements are made for each particle within the suspension, rather than measuring an average property for the entire population, thus allowing subpopulations to be detected within the overall population. An extremely large number of particles can be evaluated in a very short time; some systems can run particles at rates approaching 100,000 particles per second whilst simultaneously collecting 10–20 parameters from each particle. Additionally, flow cytometry can assess particles of most sizes—these range from particles below the resolution limits of visible light, as they can be detected by their fluorescent signatures; to those particles as large as several thousand microns.

There are many applications of flow cytometry in both basic and clinical research. The technology permits rapid and accurate measurements of multiple cell and intracellular characteristics that include DNA/RNA cell content, mitochondria and chromosomes, enzyme activity, membrane potential, the measurement of intracellular pH or ions such as calcium, the detection

and quantification of cell antigens, the analysis of multidrug resistance.[1,2,3,4]

HISTORICAL ASPECTS OF FLOW CYTOMETRY AND THE FLOW CYTOMETER

The foundations of flow cytometry began with the seminal work by Caspersson and Schultz in 1938.[5] They showed that the DNA content of unstained cells doubled during the cell cycle, when measured by ultraviolet and visible light absorption. Following this, in the 1940s it was shown by Papanicolaou[6] that cancerous cells from cervical cancer could be identified by observing the staining patterns obtained by staining tissues with specifically designed dyes.[6]

Coons and Kaplan[7] in 1950 demonstrated fluorescence antibody methods improved detection of antigens,[7] which suggested that it was more beneficial to measure fluorescence than absorption. Fluorescin has since remained the most common label for quantitative immunofluorescence analysis, and now for routine flow cytometric applications in immunology and hematology.

Moldovan initially established the principle of the automated flow analysis of single cells by reporting a photoelectric method for counting individual cells flowing through a capillary tube mounted on a microscope stage.[8] Coulter[9] modified this system and built the forerunner of modern day flow cytometers. In this machine blood cells in saline suspensions passed one by one through a small orifice being detected by changes of electrical impedance at the orifice.[9]

In the 1960s, Kamentsky[10] designed a cytometer that measured individual cell absorption and scatter. The apparatus was used to measure the nucleic acid content and light scattering of unstained mammalian cancer cells in a flow stream.[10] Very soon afterwards, Fulwyler[11] described the first flow sorter.[11] This instrument allowed biological cells to be separated according to their volume, which was determined as they passed through a Coulter aperture.

Thus, flow cytometry was based mainly on fluorescence and light scattering, and was originally used in the detection of nuclear DNA and cell surface antigens. The applications of this technique have since expanded rapidly.

FLUORESCENCE

This is a key concept in flow cytometry and is the property of molecules to absorb light energy at one wavelength, called the excitation wavelength, and then rapidly re-radiates some of that

energy as light of a longer or emission wavelength. Fluorescence is always of a lower energy, and therefore longer wavelength, than the exciting light.

When a compound absorbs light, electrons are initially raised from the ground state to an excited state and then subsequently return to the ground state via numerous routes; some routes do not result in fluorescence, such as the loss of the energy by heat, but certain molecules lose energy by a process of radiative transfer, termed fluorescence. When stimulation of the fluorescent compound is stopped by removing the exciting light source, fluorescence emission immediately stops.

Immunofluorescence allows the visualization of cellular features or structures by linking them to a fluorescent molecule. These fluorescent molecules can either be dyes which bind directly to structures within or on the cell, or fluorochromes conjugated to a ligand, such as monoclonal antibodies. The fluorochromes and dyes used for flow cytometry must be compatible with the laser utilized by the cytometer.

The characteristic distribution of radiated energy for a fluorochrome is called its emission spectrum. Each fluorochrome has a clear, distinct peak in its emission spectrum represented visually by a characteristic color for that fluorochrome (Table 5.1). This property allows flow cytometric assays to use multiple fluorochromes in a single experiment thus allowing the simultaneous evaluation of different cell features, without having to be concerned about significant overlap or interference from individual emission spectra.

TABLE 5.1. Photomultiplier detection of light emission produced by Common Fluorochromes/Dyes in the Becton Dickinson FACScan

Photomultiplier	Light emission wavelength detected (color)	Fluorochrome
FL1	515–545 nm (green)	**FITC**
FL2	564–606 nm (yellow-orange)	**PE/PI**
FL3	650–675 nm (red)	**PE-CY5**

Key: FITC, fluorescein isothiocyanate; PE, phycoerythrin; PI, propidium iodide;
PE–CY5, a tandem conjugate of two fluorochromes (PE and Cyanine-5) in which the energy is transferred from the first to the second.

LIGHT SCATTER

The flow cytometer is able to detect and quantify light scatter signals as well as immunofluorescence. The light scatter signals are due to the laser light of the flow cytometer reflecting and refracting off the cells. Two types of light scatter are quantified:

1) Orthogonal or side light scatter, which is light scatter measured at a right angle (90 degrees) to the direction of the laser beam. This is detected in the side channel, the intensity of the reading correlating with the granularity of the cell.

2) Forward or low angle scatter, which is light scatter measured along the same axis that the laser light is traveling or near 180 degrees from the point at which the laser beam intersects the cell. This measurement correlates with cell size and cell surface properties, such as cell ruffling. Therefore not only can it be used to distinguish cell size, but also live from dead cells.

COMPONENTS OF THE MODERN FLOW CYTOMETER

Fluidic System

The fluidic system focuses the cells/particles into a fine stream, which are moved individually to intersect the laser light source. Furthermore, the fluidic system is vital in cell sorting by flow cytometry. Initially, the sample of fluorescently labelled suspension of single cells flows rapidly through a narrow channel in the instrument, where a small amount of cell suspension joins a larger amount of cell free buffer (sheath fluid). These two streams do not mix, and consist of an inner sample stream surrounded by an outer sheath stream (coaxial flow). The coaxial stream ensures the cells are centered in the flowing stream, passing the laser beam optimally centered, in addition to being spaced out sequentially and passing the laser beam individually.

To ensure the sample fluid is flowing continuously, positive air pressure is applied to the sample reservoir and sheath fluid. A purge line is connected to the sheath inlet to allow a vacuum to be applied for clearing blockages and air bubbles.

Optical System and Analysis

The optical system has two components:

1. A light source, most frequently a laser, which is necessary to excite sufficiently the cells, in addition to the lens and mirrors required to focus and direct the laser beam to the flow chamber through which the cells are passing. As mentioned earlier, the

lasers, which emit light at specific peak emission wavelengths, need to be matched with appropriate fluorochromes used for immunofluorescent staining analyzed on the cytometer. The flow channels through which the coaxial stream passes is positioned vertically to the laser beam.

2. A variety of mirrors and filters that absorb certain wavelengths while transmitting others are needed for the collection of light emitted by the cells or scattered from them. These include dichroic mirrors, which reflect light above a specific wavelength, while permitting light below that wavelength to pass through; long pass filters (permit only light above a specified wavelength to pass through); short pass filters (permit only light below a specified wavelength to pass through); band pass filters (permit light within a specified wavelength range to pass through).

These mirrors and filters also act to separate and direct emissions of varying wavelengths to the corresponding detectors or photomultiplier tubes (PMT). Within the PMTs the incoming light is converted into electronic signals. Subsequent electronic and computational processing of these signals results in graphic display and statistical analysis of the measurements being made.

There are cytometers now available that are capable of analyzing up to 13 parameters for each cell (forward scatter, side scatter and up to 11 colors of immunofluorescence).[12] This allows for cell size, lipid content, protein content, DNA content, enzyme activity to name a few characteristics for each cell to measured. Thus, allowing for a multidimensional representation of a population to be obtained. With most cytometers, it is almost always possible for at least 1,000 cells to be analyzed per second, whilst with appropriate specimens some cytometers are able to analyze up to 100,000 cells per second, with no marked reduction in data quality.[13]

Color Assignment

The signal emitted by a fluorochrome is detected by its corresponding PMT and then converted to a parameter that can be acquired. The series of optical filters and mirrors used ensures that only specific regions of the spectrum reach each PMT. The PMT detectors in the flow cytometer are labeled FL1, FL2, FL3 and onwards depending on how many are present, with light of specific emission wavelength only been detected by each.

The Becton Dickinson FACScan contains an argon laser with an emission at 488 nm, with three PMTs detecting fluorescence specific wavelengths produced through excitation of several commonly used fluorochromes/dyes (Table 5.1) and two for detecting side and forward scatter.

A wide variety of fluorochrome conjugated ligands and dyes are available for directly estimating cellular parameters such as DNA content. The simplest method utilizes a fluorescent dye that binds preferentially to DNA, allowing for cellular DNA levels to be detected. These DNA dyes, such as propidium iodide are termed stoichiometric, as the number of molecules of the dye bound is equal to the number of DNA molecules. Fluorochrome conjugated ligands are used in measuring membrane potential, enzyme activity, calcium flux, pH, the density and distribution of cell-surface and cytoplasmic determinants and receptors (Table 5.1).

DATA ANALYSIS

Histograms and Dot Plots

When light from a specific region of the spectrum is detected by a PMT, it is converted via an amplifier to a voltage, this voltage being proportional to the intensity of fluorescence. These voltages are then processed through a series of linear and log amplifiers. Using a logarithmic scale to measure fluorescence is indicated in most biological situations, and allows for a distribution to be normalized. Also, a logarithmic scale is important when there is a broad range of fluorescence, as is often encountered with biological distributions, because this type of amplification allows for an expansion of the scale for weak signals with a compression of the scale for strong or specific fluorescence signals.

These voltages are a continuous distribution and are converted to a discrete distribution by an analog to digital converter (ADC) which positions each signal according to the level of fluorescence into a specific channel. These channels correspond to the original voltage generated by a specific light event detected by the PMT detector. Consequently the ADC assigns a channel number based on the pulse height for individual events; brighter specific fluorescence events yield a higher pulse height and thus a higher channel number when displayed as a histogram. The greater the resolution of the ADC, the closer this reflects the continuous distribution. The ADCs in the FACScan are 10-bit, meaning they divide data into four decades across 1024 channels

with 256 channels per decade. The number of decades is fixed for the FACScan.

The data gained through flow cytometry is most often represented as histograms or dot plots (Figure 5.1). The histogram graph has fluorescent intensity on the x-axis with the number of cells in each channel on the y-axis. With the FACScan, histograms have 1024 channels displayed on the x-axis using a 4-log decade logarithmic scale (256 channels per decade), with higher channel number corresponding to a greater signal intensity of a fluorescence/scatter after amplification (Figure 5.1). A histogram can only represent the intensity of a single parameter (scatter or fluorescence).

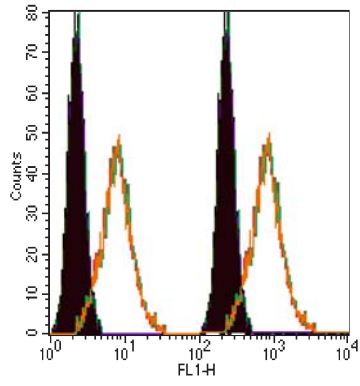
Dot plots (bivariate display, two parameter histograms, scattergram, bitmap) unlike histograms show the correlated distribution for two parameters, such as forward scatter versus side scatter, with each cell being represented as a dot, positioned on the x and y axes according to the intensities detected for that cell. Dot plots can be divided into quadrants allowing for the number of particles in each of the defined areas to be counted (Figure 5.1).

Histograms and dot plots both allow for discrete subpopulations of cells with different intensities to be identified.

Gating and Negative Controls

Normally data only from single, living cells is wanted and data from cell debris, dead cells, and clumps of two or more cells needs to be eliminated. Single cells are distinguished from subcellular debris and clumps of cells according to size. Living cells are distinguished from dead cells by forward scatter (dead cells have lower values) and side scatter (dead cells have higher values). These differences remain even after formaldehyde fixation, despite the fact that after fixation, all the cells are dead. Due to the above, the computer used can be configured to display the fluorescence signals only from single living cells (Figure 5.1). This is termed scatter-gated fluorescence analysis, where a specified set of scatter properties is set to identify fluorescence signals from a desired group of particles only. It is possible to "gate" on any set of signals in order to analyze the specific data of either the main cell population or a subpopulation.

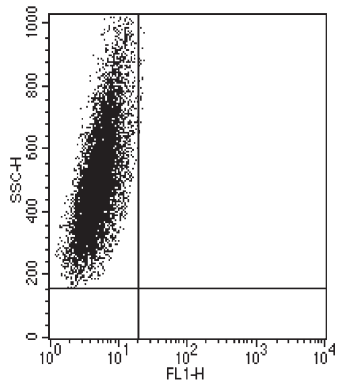
On dot plot graphs quadrant gates can be set using background levels of fluorescence of either the respective unstained negative control (fluorochrome-conjugated secondary antibody only) or fluorochrome isotype matched control population of cells (when using a fluorochrome-conjugated primary antibody)



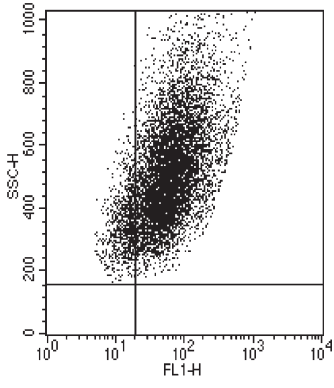
Histograms

Negative Control – contains secondary antibody only

Contains primary and secondary antibody



Dot plot - negative control – contains secondary antibody only



Dot plot - contains primary and secondary antibody

This figure shows representative flow cytometry histograms and dot plots. On both the histograms and dot plots the x-axis shows fluorescence intensity (channel number) on 1024 channels encompassing 4-log decades (i.e. logarithmic scale).
On the histograms the y-axis shows cell counts in each channel, whereas on the dot plots the y-axis shows side scatter intensity - SSC (channel number) on a linear scale.
Quadrant gates were set on the dot plots using the background levels of fluorescence of the respective unstained negative control (contains only the fluorochrome-conjugated secondary antibody without the primary antibody).
Data due to dead cells/cell debris and non-specific binding of the secondary antibody being have been gated out of the final results

FIGURE 5.1. Histograms and dot plots.

(Figure 5.1). The fluorochrome isotype matched control primary antibody has no specificity to any epitope on the cells being analyzed, is the same isotype and species used at the same concentration and bound to the identical fluorochrome at the same fluorochrome/protein ratio as the antigen specific primary antibody. Both of these negative control samples are needed to highlight the amount of nonspecific binding that is present with the antigen specific antibody.

When quadrant gates are used on the graph, each quadrant then demonstrates the following:

1. Lower-left quadrant represents cells negative for the descriptors on both the x- and y-axis.
2. Upper-right quadrant represents cells dual-positive for the descriptors on both the x- and y-axis.
3. Upper-left quadrant represents cells positive for the y-axis descriptor, but negative for the x-axis descriptor.
4. Lower-right quadrant represents cells positive for the x-axis descriptor, but negative for the y-axis descriptor.

PRINCIPLES OF FLOW CYTOMETRY SAMPLE PREPARATION

The aim of sample preparation is to produce a monodisperse suspension; this is a suspension of single cells with minimal aggregation. Furthermore, the sample should contain minimal cell debris, dead cells and clumps as the clumps can cause disruption of fluid flow or block the tubes within the flow cytometer. In addition, it is important to ensure that preparative methods used will not bias results. For instance, enzymatic preparative techniques can alter cell-surface antigens and affect cell viability.

Once a monodisperse suspension is gained the cells are labelled by incubation with a fluorescent tag under appropriate conditions. This may be a fluorescent dye, fluorescent conjugated antibody or ligand, which is specific for the "antigen" to be measured. The fluorescent probes and monoclonal antibodies may bind nonspecifically and it is essential that care is taken to minimize the chances of cross-reactions. Data acquisition can occur with the cells either alive or fixed with substances such as formaldehyde. The advantage of formaldehyde fixed cells is that the light scatter and fluorescent properties are maintained and data can be acquired several weeks later.

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Chapter 6

Western, Northern, and Southern Blotting

Stephan Schlickeiser and Uwe Pleyer

INTRODUCTION: THE HISTORY OF SOUTHERN, NORTHERN, AND WESTERN BLOTTING

In the early 1970s, the possibility of mapping whole genomes arose, due to the prior discovery of bacterial enzymes that cut DNA at specific "restriction sites," and the development of recombinant DNA technologies and gene cloning. Nevertheless, one could not identify one single gene among thousands of fragments of DNA—until Edward Southern[1] introduced his eponymous powerful DNA transfer and probing technique in 1975.[1] He realized that restriction fragments can first be separated electrophoretically on an agarose gel and then be transferred to a nylon membrane by capillary action—the same way that blotting paper absorbs ink. Afterward, the blotted membrane can be incubated with a radioactive probe specific for the gene fragments of interest, which in turn become visible by placing an x-ray film on top of the membrane.

In 1977, gene expression analysis ascended dramatically when George Stark and colleagues replicated the configuration of Southern's transfer apparatus in an effort to transfer cellular RNA to chemically activated cellulose paper.[2] This technique was named "Northern blotting"—as a pun on Southern's name.

Two years later, Stark developed an early protein blotting technique, relying on overnight capillary transfer from gel to activated cellulose paper.[3] Harry Towbin's[4] faster and simpler approach for electroblotting proteins to nitrocellulose membranes is clearly preferred today.[4] The name "**Western blot"** was first given to the technique by W. Neal Burnette[5] in 1981, though.[5]

Definitions:

Southern blot: DNA is detected with a hybridization DNA or RNA probe.

Northern blot: RNA is detected with a hybridization DNA or RNA probe.

Western blot: Protein is detected with a complementary antibody.

BASIC PRINCIPLES AND METHODS: ELECTROPHORESIS, GEL BLOTTING, AND DETECTION

All three blotting techniques use a very similar methodology. Molecules separated by electrophoretic procedures are transferred to a membrane that is especially suited to support the detection of fragments with a particular DNA sequence, single species of RNA, or proteins.

Gel Electrophoresis: Size Separation

A simple way to identify one particular molecule amongst others, that are related but not identical, is to distinguish them by their physical characteristics such as size or mass. The gel electrophoresis is a technique that permits a size based separation of macromolecules by their movement in an electric field. The buffer conditions, such as pH and ion composition, provide a negative net charge of the nucleic acid fragments or proteins, which will be moved from the cathode to the anode. The gel usually consists of a cross-linked polymer matrix. Its sub-microscopic pores will impede large molecules and let smaller fragments migrate faster through the gel.

Sample Preparation: Southern and Northern

DNA is first isolated by common extraction protocols. Afterward, the purified genomic or plasmid DNA is partially digested by restriction endonucleases. These enzymes cut double-stranded nucleic acids at certain sequences. The resulting mixtures of different-sized linear fragments are then loaded side-by-side into "wells" formed in an agarose gel.

RNA is single-stranded. Due to their natural instability, work with RNA molecules requires much more care. All procedures need to be done with sterile, RNAse-free supplies. Furthermore, RNA has to be pretreated with denaturing formaldehyde to prevent formation of base-paired secondary structures.

Sample Preparation: Western

Samples from cell culture are lysed in an extraction buffer containing proteinase inhibitors. Tissue samples are cooled or frozen rapidly and homogenized using mechanical force. The cell debris is removed by sharp centrifugation. Further centrifugation steps

can be used to isolate cytosolic or nuclear fractions. The resulting lysates are then assayed for whole protein content, so that from each sample equal amounts of protein can be taken for the most commonly used sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Before applying the samples to the gel, they have to be boiled one to five minutes in a denaturing buffer solution (e.g., Laemmli's buffer). This buffer contains a tracking dye optionally; reducing agents, such as dithiothreitol (DTT) or 2-mercaptoethanol; and, most importantly, SDS. Heat and reducing agents destroy tertiary protein folding and quaternary protein structures by breaking up disulfide bonds and preventing their reformation simultaneously. SDS is an anionic detergent that denatures secondary and nondisulfide-linked tertiary structures, thus unfolding and linearizing the proteins completely. Furthermore, the anionic SDS binds to most of the proteins in a uniform ratio of approximately 1.4 g SDS per 1.0 g protein. Hence, it applies a negative charge to each protein in proportion to its mass (number of amino acids) so that they may be separated in an electric field strictly by their length or size, respectively.

Agarose Electrophoresis: Southern and Northern

As mentioned above, DNA and RNA strands, which have a negatively charged phosphate backbone, will move towards the anode if an electric current is applied to the gel matrix.

Shorter fragments will move faster because they are able to slip through the agarose mesh networks more easily. Agarose concentrations of 0.5% to 2% are normally used for the separation of fragments of 100 base pairs (bp) up to several kilobases (kb). For an ideal resolution of larger molecules (up to 750 kb), the agarose concentration has to be lowered. Inconveniently, these gels are very fragile and have long running times.

The voltage normally applied to a gel is 100 mV (about 5 to 8 V/cm). Higher voltages will shorten the running time to less than one hour, but as well lead to a decrease in resolution. Buffers used for agarose electrophoresis are in general tris acetate EDTA (TAE) and sodium boride (SB). TAE has a relatively low buffering capacity but provides the best resolution, whereas SB has the highest buffering capacity, allowing shorter running times for fragments smaller than 5 kb (voltages up to 350 mV).

After completion of the separation, bands corresponding to nucleic acids fragments of different length can be visualized using a fluorescent dye (e.g., ethidium bromide). Fragment-size determination is typically done by comparison to strands of

known length. Size standards are commercially available (DNA ladders).

For high resolution of short DNA fragments or RNA molecules (<100 bp), polyacrylamide gels are commonly used.

Sodium Sodecylsulfate–Polyacrylamide Gel Electrophoresis: Western

Samples with the denatured proteins are placed into the lanes of the stacking site of a polyacrylamide gel submerged in a suitable buffer. One lane is reserved for a molecular weight standard in order to determine the size of unknown proteins, usually in Dalton. An electric current of about 5 to 50 mA is commonly applied across the gel. Movement through the stacking gel forces all proteins to focus in one sharp front at the boundary to the resolving gel. Thus, different sized proteins may resolve in sharp and distinct bands at the end of the separation. To achieve longer retention of proteins and higher resolutions respectively, one can enhance the grade of polymerization of the resolving gel by increasing the percentage of acrylamide. Typically, resolving gels are made in 8% to 15%. The stacking gel (5%) is poured on top of the readily polymerized resolving gel to form a sharp transition. A gel comb defines the sample lanes. The recipe for a 10 mL 10% polyacrylamide gel could be:

Resolving polyacrylamide mixture dH_20 mL		Stacking polyacrylamide mixture (5%) dH_20 mL	
30% acrylamide mix	4.0	30% acrylamide mix	4.0
1.5M Tris pH 8.8	3.3	1.0M Tris pH 6.8	3.3
10% SDS	2.5	10% SDS	2.5
10% ammonium persulfate	0.1	10% ammonium persulfate	0.1
TEMED	0.1	TEMED	0.1
(not to add before everything is ready)	0.004	(not to add before everything is ready)	0.004

Note: Monomeric acrylamide is toxic!

Following electrophoresis, the gel is stained (e.g., with Coomassie Brilliant Blue or silver stain), to visualize the separated proteins. The crucial step is to take an image of the gel before transferring to the membrane, because the molecular weight marker cannot be shown after blotting.

To better characterize one particular protein, it is possible to use a two-dimensional gel electrophoresis, which separates the

proteins of a single sample according to their isoelectric point (1st dimension) and their molecular weight (2nd dimension).

Blotting: Transfer to Solid Support

After the DNA fragments, RNA molecules, or proteins have been separated according to their size, they have to be transferred to a solid membrane in order to make these molecules accessible to specific detection. The membranes or filters are made of nitrocellulose, nylon, diazo-modified cellulose, or polyvinylidene fluoride (PVDF). These materials allow binding with high capacities by either crosslinking with single-stranded nucleic acids or strong hydrophobic and charged interactions with proteins. Therefore, the relative positions of the molecules are not altered.

Since DNA fragments in the agarose gel are double stranded, they have to be denatured by pretreatment with an alkaline solution (typically containing sodium hydroxide).

There are two methods to transfer the molecules. The first method is to place the membrane on top of the gel that lies on a stack of wet filter paper. Then pressure is applied by either suction *(vacuum blot),* or by placing another stack of dry paper towels and a weight on top of the membrane and gel *(capillary blot)*. Thus, molecules will move from the gel onto the membrane with a flow of buffer by simple capillary action.

The second method takes advantage of the molecules' negative charge *(electro blot)*. In an electrophoretic chamber, the membrane is placed face-to-face with the gel between two large electrode plates (Figure 6.1). These electrodes can either be positioned in a tank filled with suitable buffer *(tank blot),* or membrane, gel, two buffer soaked stacks of filter paper and electrodes can be assembled akin to a sandwich *(semidry blot)*. Pay attention to the right polarization, because the negatively charged molecules will move towards the anode!

Afterward, nitrocellulose membranes are baked, and nylon membranes are exposed to ultraviolet light to covalently link DNA or RNA to the solid support. As proteins usually stick very tightly to the membrane, they don't need to be crosslinked.

Efficiency and integrity of the blotting process may be controlled by reversible staining of the membrane, e.g., with Ponceau S.

Detection and Localization: Complementarity and Hybridization

The basic principle behind the specific detection of any target macromolecule is the sequence-specific or shape-specific molecular recognition that occurs when a complementary probe mol-

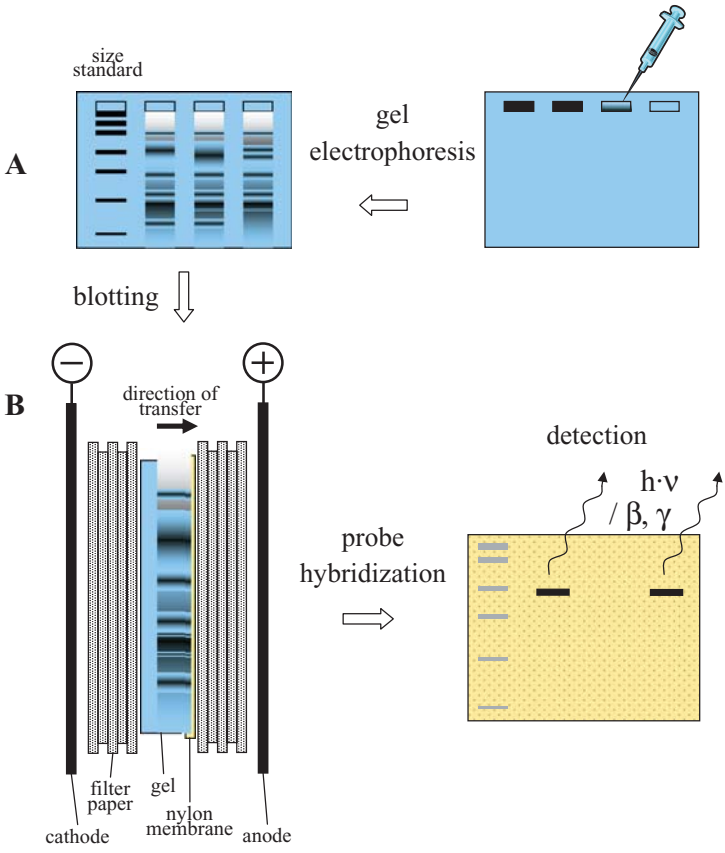

FIGURE 6.1. Gel loading and blot electro/blotting.

ecule binds to the target. A high complementarity between both molecules results in a highly stringent formation of a probe-target complex. Hence, hybridization reactions will specifically occur in the presence of a large number of similar but noncomplementary molecules. This hybrid complex can then be located if the probe molecule bears a detectable function. The most commonly used probes in Southern and Northern blotting are short radioactive (^{32}P or ^{125}I), fluorescent- or digoxigenin (DIG)–labeled DNA or RNA single strands (100 to 500 bp) that have to be reverse

complement to the target sequence. In Western blotting, a two-step hybridization with a primary antibody against the target epitope and a secondary antibody directed at a species-specific portion of the primary antibody is preferred. The secondary antibody is typically tagged with radioactivity or linked to a reporter enzyme, which drives a colorimetric or chemiluminescent signal.

Blocking

Because the used membranes are most suitable to bind protein and nucleic acids, nonspecific binding between probes and the material has to be blocked. These nonspecific interactions are prevented by soaking the membranes in a solution containing a high concentration of DNA (e.g., herring or salmon sperm DNA) in case of Southern and Northern hybridization. For antibody-based Western hybridization, protein is used for blocking, typically 5% nonfat dry milk or 3% bovine serum albumin (BSA). The presence of a detergent in the blocking solution, like Tween 20 at 0.05% is also important.

Hybridization

The labeled probe is added to the blocked Southern, Northern, or Western blot membrane in a buffered saline solution, containing a small percentage of detergent and nonfat milk or BSA. Under gentle agitation, the probe molecules are allowed to bind for a period of hours. The recommended final DNA or RNA probe concentration is 2 to 10 ng/ml. A primary antibody is generally incubated with the filter in dilution between 0.5 and 5 µg/ml. The secondary antibody can be added after the unbound primary antibody is removed by washing the membrane.

Washing

After hybridization, the membrane is rinsed repeatedly in several changes of buffer to wash off any unbound, excess antibody, or nucleic acid probe, in order to avoid unspecific background signals.

Detection

If the probe is radioactive, the pattern of hybridization is visualized on x-ray film by autoradiography. The membrane is pressed against the film, which in turn is exposed, for a few minutes up to weeks, wherever the probe bound. Because nonradioactive detecting methods are safer, quicker, and cheaper, nowadays autoradiography is scarcely used.

The secondary antibody, which can be directed against protein-bound primary antibodies as well as DIG-labeled DNA or RNA probes, is usually linked to biotin, a fluorescence dye, or to reporter enzymes. Alkaline phosphatase *(colorimetric detection)* converts a chromogenic dye to a colored reaction product visible on the membrane. Most frequently used is horseradish peroxidase *(chemiluminescence detection)* which, if provided with hydrogen peroxide and a chemiluminescent agent (e.g., luminol), produces fluorescence in proportion to the amount of bound protein. The light is detected by a photographic film, and more recently by CCD cameras, which create a digital image of the blot's localization pattern.

The use of a polyclonal antibody, that binds severalfold to one primary antibody, as well as the enzyme catalyzed chemiluminescent emission of light, provides strong signal amplification (Figure 6.2). Therefore, secondary "enhanced chemiluminescence" (ECL) detection is considered to be one of the most sensitive detection methods for blotting analysis. By this way, down

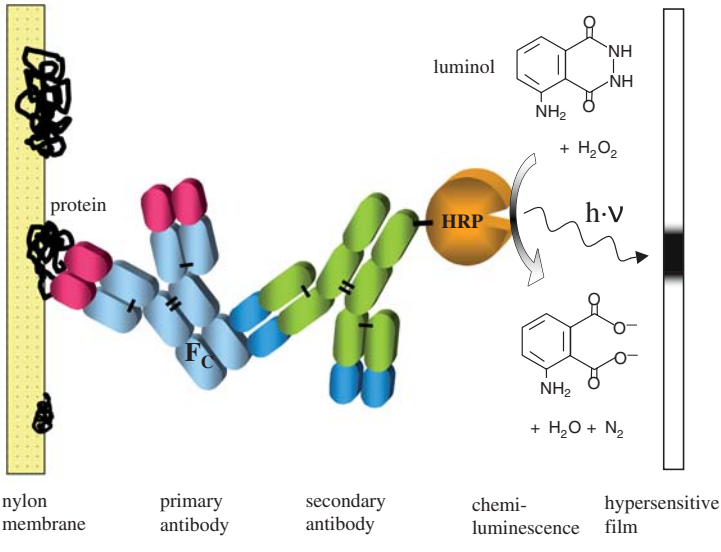

FIGURE 6.2. Enhanced chemiluminescence (ECL).

to 10 ng of protein, as well as less than 10 nucleic acid molecules are detectable.

Stripping

After detection with one particular probe, this can almost completely be removed by stripping the membrane. The membrane has to be submerged with a special stripping buffer and, in case of Southern and Northern blots, heated to 94°C. Then the membrane is ready for the next hybridization with a different probe. This process can be repeated up to 20 times.

KEY POINTS

- Southern blots are used to identify DNA, Northern blots for RNA, and Western blots for protein, respectively
- Molecules of DNA cut with restriction enzymes, RNA denatured with formaldehyde, and protein denatured with SDS are separated according to their size by agarose gel electrophoresis (Southern, Northern) or SDS-PAGE (Western)
- The separated molecules are transferred to a solid nylon or nitrocellulose membrane by capillary action (Southern, Northern) or electrophoresis (Western)
- The blotted membranes are blocked with extensive DNA (Southern, Northern) or protein (Western) to prevent unspecific binding of probes
- The blocked membranes are hybridized with a target-specific ssDNA or RNA probe (Southern, Northern), or antibody probe (Western)
- The probes are labeled with radioactivity, fluorescence dyes, DIG or reporter enzymes
- A labeled secondary antibody directed against DIG or primary antibody is applied in a second hybridization step
- After hybridization, unbound probes are extensively washed off
- Bound probes are detected by autoradiography, fluorescence, or enzymatic chemiluminescent emission of light
- Southern blotting can be used for DNA fingerprinting and genome mapping, northern blotting for gene expression profiling, and Western blotting for protein characterization, identification as well as expression analysis

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Chapter 7
Fluorescent In Situ Hybridization

Fiona Campbell and John M.S. Bartlett

INTRODUCTION

In situ hybridization (ISH) technique was introduced by Gall and Pardue[1] in 1969. At that time the technique was limited by the use of radioactively labelled probes that were subsequently visualized by autoradiography. The development of interphase cytogenetics in the 1980s and fluorescent labels in 1986[2] has seen the technology applied in a number of fields. Although fluorescent in situ hybridization (FISH) is a valuable research tool, it is now also a technique employed in the diagnostic laboratory. It is currently a standard tool in cytogenetics laboratories, where it is used for the diagnosis of hereditary disorders, chromosomal aberrations, and hematologic cancer markers. More recently, the technique has been applied to formalin-fixed, paraffin-embedded cells and tissues. The application of FISH to detect gene amplifications (HER2 in breast cancer),[3] gene rearrangements (BCR-ABL in leukaemias),[4] microdeletions,[5] chromosomal duplication, and viral infections (HPV) highlights the importance of this methodology, not only in the clinical setting but in wider research applications.

This chapter serves to highlight the key points in the applications of FISH technology.

BASIC PRINCIPLES

Fluorescent in situ hybridization is based on the ability of complimentary single-stranded nucleic acid molecules to hybridize to each other, and therefore allow the demonstration of specific genes or chromosomes in their cellular environment. The techniques are simple and involve the pretreatment of the tissue or cellular preparation to unmask the target DNA and the hybridization of a specific DNA probe, of complimentary base sequence, to this target.

Fluorescent in situ hybridization can be performed on either fresh or archival tissues and unstained cytologic preparations. Standard FISH protocols can be divided into 5 basic steps:

1. Probe and sample preparation
2. DNA unmasking (pretreatment and digestion)
3. Probe and target DNA denaturation
4. Probe and target DNA hybridization
5. Posthybridization wash and visualization

METHODOLOGICAL ASPECTS OF FISH

Probe and Sample Preparation

Probes

The main requirement for FISH is the development of a DNA probe with homology to the target region. In most instances, these probes are commercially available, and these are highly recommended, as the "in-house" development of such probes needs to be subject to rigorous quality control measures. The probe DNA can be labelled by nick translation, polymerase chain reaction (PCR), random priming, or chemical conjugation. All probes must have Cot-1 DNA added, as this represents repetitive sequences of the human genome and addition of it to the probe will suppress nonspecific hybridization.[6] Cot-1 DNA is usually supplied with commercial probes.

All FISH procedures using commercial probes should be carried out according to manufacturer's instructions for optimal results, as they may differ slightly from the method detailed below.

Sample Preparation

Fluorescent in situ hybridization methodologies are most commonly applied to isolated cells from whole blood or other bodily fluids, frozen-tissue sections, or formalin-fixed paraffin-embedded (FFPE) tissues.[1,2] Cytologic preparations and frozen-tissue sections can be prepared on to silane-coated slides or charged slides and fixed in an acid: alcohol mix, for example, 1 part acetic acid with 3 parts methanol.

Formalin-fixed paraffin-embedded tissue sections can also be prepared by sectioning at 3–4 μm thickness and mounting onto silane-coated or charged slides. Formalin fixation is used to prevent tissue degradation and to preserve morphology but can also affect the retention of nucleic acids. Most pathology laboratories use neutral buffered formalin to fix tissues, and this provides an optimal platform for tissue morphology assessment. Neutral buffered formalin acts as a strong oxidizing agent, promoting divalent protein-protein and protein-nucleic acid cross-links in fixed tissues. This fixation is followed by dehydration and

heating (in paraffin wax) to "embed" tissue for sectioning. Each of these effects can alter nucleic acids and intracellular proteins, and result in a reduction in the ability for macromolecules (such as FISH probes) to enter cells. The early steps of ISH/FISH protocols are designed to reverse this by breaking down cross-links between macromolecules and rehydrating the tissue section.

DNA UNMASKING: PRETREATMENT AND DIGESTION

De-waxing and Rehydration

These steps are common to most histological staining methods, including routine hematoxylin and eosin (H&E) staining, various special stains, and many immuno-histochemical staining techniques. After sectioning and incubation in a 56°C oven, the tissue sections must have the residual paraffin wax removed and need to be rehydrated through graded alcohols to distilled water.

Acid Permeabilization

Following de-waxing and rehydration a number of different steps may be used to increase tissue permeabilization and to allow probe access. Each of these steps has the potential to cause tissue damage and result in the loss of tissue morphology, causing the technique to fail. Thus, tissue permeabilization is a balance between allowing probe access and retaining tissue architecture. The most frequently used pretreatment protocols involve one or more steps, using acid, detergents, and/or reducing agents with the aim to permeabilize tissue prior to protease digestion steps, which break down the cellular proteins to enhance probe penetration, and to reduce autofluorescence.

Most commonly, incubation in 0.2 normal hydrochloric acid is used to remove protein from the tissue and improve probe access. This incubation step is thought to reverse some of the effects of formalin fixation.

Reducing Agents

Sodium metabisulphite, sodium thiocyanate,[3] and MES are used to break disulphide bonds formed by formalin fixation and aid subsequent protease digestion, and therefore, increase probe access.

Using both acids and reducing agents prior to protease digestion ultimately reduces the amount of exposure to proteolytic enzymes and therefore minimizes the amount of tissue damage and any loss of morphology.

Protease Digestion

As mentioned above, this step needs to be performed with minimal tissue damage. This is achieved by varying the times the

tissue is exposed to the proteolytic enzymes, and by assessing the tissue section microscopically before proceeding to the hybridization steps. The digestion can be assessed by mounting the slides using a nuclear stain known as DAPI (4,6-diamidino-2-phenylindole-2-hydrochloride). Protease digestion can be performed using proteinase K or pepsin. The protease digestion will remove any proteinaceous material from the tissue section, and the extent of this digestion will depend on the nature of the tissue being treated. Hemorrhagic tissue and those tissues with large amounts of adipose will show areas of sparse, widespread nuclei, whereas those tissues with dense collagen will retain much of the stroma even after substantial digestion times. The main importance with protease digestion is the nuclear appearance. The nuclei should be clear and evenly spread, not touching, overlying, or clumping together. If a tissue section is under-digested, the nuclei will be hard to see underneath the protein. If a tissue section is over-digested, the nuclear boundaries may be lost, or the nuclei will clump tightly together so that they do not appear as individual nuclei.

This step is the most critical step in the entire FISH procedure. One of the most common reasons for a failed result is due to inadequate or inappropriate digestion times.[3] It is recommended that various digestion times be evaluated for individual tissue sections even when protocols from manufacturers do not include this option; frequently, commercially derived protocols underestimated the duration of protease digestion required. It is also extremely important that tissue digestions are routinely checked microscopically to ensure that optimal tissue digestion is achieved before proceeding to the hybridization step.[4–6]

Probe and Target DNA Denaturation

Tissue DNA denaturation can be performed using heat, chemicals, or a combination of both. Commonly tissue DNA is denatured using a solution containing formamide at 72°C for 5 minutes on a flat-bed-heated stage.

Probe DNA are generally heated to 95°C for approximately 5 minutes, although there are some commercial probes that do not require denaturation.

Probe and Target DNA Hybridization

Hybridization between the probe DNA and the target tissue DNA is performed, usually on a flat-bed-heated stage at 37°C–45°C, for at least 14 hours in most instances. Humidity control is critical in some procedures. Hybridization temperatures are chosen to ensure maximum binding of the probe to the target DNA.

POSTHYBRIDIZATION WASH AND VISUALIZATION

Posthybridization Wash

Posthybridization washes are essential to remove any excess unbound probe and any nonspecifically bound probe. The stringency of the posthybridization wash can be determined by salt and formamide concentrations and by temperature.[7] Increasing temperature and formamide concentrations, and decreasing salt concentrations, will decrease hybridization. Each of these methods results in removing the hydrogen bonds required for probe-target DNA binding, and, therefore, the removal of any nonspecifically bound probe.

Visualization and Scoring/Interpretation of Results

The results of the FISH procedure are visualized using epifluorescence microscopy.

QUALITY ASSURANCE

Examples of a FISH protocol for formalin-fixed, paraffin-embedded tissue sections are listed below.

This pretreatment method has been adapted from the PathVysion pretreatment protocol (Abbott Diagnostic Labs., UK). If many slides are to be treated, steps 2–19 can be performed on a VP2000 automated tissue processing station, substituting the 2xSSC washes with distilled water. The VP2000 can accommodate up to 50 slides per run. All glassware should be rinsed with distilled water before use.

Slide Pretreatment

1. Cut 4-μm tissue sections, pick up on silane-coated slides, and bake overnight in a 56°C oven. Store at room temperature until required
2. Set up two water baths, one at 37°C and one at 80°C. Place one Coplin jar per 5 slides to be treated in each water bath. Fill those at 80°C with 8% sodium thiocyanate (pre-treatment solution) pH 6.5–7.0 and those at 37°C with 0.2 *N* HCl pH 2.0 ± 0.2 for protease digestion, but *do not* add the protease at this time
3. Immerse slides into xylene for 5 minutes at room temperature to remove the paraffin wax
4. Repeat step 3 with fresh xylene
5. Transfer slides to 99% ethanol for 5 minutes at room temperature to remove xylene from the tissue sections
6. Repeat step 5 with fresh 99% ethanol

Acid Permeabilization

7. Remove slides and allow them to air-dry before immersing in 0.2 *N* HCl for 20 minutes at room temperature
8. Remove slides and wash in distilled water for 3 minutes
9. Wash slides in 2xSSC buffer for 3 minutes at room temperature

Pretreatment (Reducing Agent)

10. Carefully place slides into the sodium thiocyanate solution at 80°C and incubate for 30 minutes
11. Remove slides and rinse in distilled water. Transfer to 2xSSC buffer for 5 minutes at room temperature
12. Wash in fresh 2xSSC buffer for a further 5 minutes and, during this step, add the protease to the 0.2 N HCl at 37°C and mix well

Protease Digestion

13. Incubate the slides in the protease at 37°C for the recommended digestion time. This incubation time will vary depending on the tissue type, tissue fixation method used, and the concentration and activity of pepsin used
14. Remove slide and immerse in 2xSSC buffer for 5 minutes at room temperature
15. Repeat step 14 with fresh 2xSSC buffer
16. Immerse slides in 10% neutral-buffered formalin for 10 minutes at room temperature
17. Place slides in 70% alcohol for 1 minute at room temperature
18. Place slides in 85% alcohol for 1 minute at room temperature
19. Place slides in 99% alcohol for 1 minute at room temperature
21. Air-dry slides. Mount using a mountant containing DAPI (4,6-diamidino-2-phenylindole-2-hydrochloride) and apply glass cover slips
21. Assess the tissue digestion with a 100-W fluorescence microscope that incorporates a filter specific for the excitation and emission wavelengths of DAPI. If digestion is optimal, proceed to step 22. If sections are under-digested, proceed to step
22. Repeat steps 13 to 21 and reassess the digestion. If sections are over-digested, discard the slides and repeat the procedure using new sections, reducing the incubation time in protease

22. Place slides into 2xSSC buffer until the cover slips wash off. Place slides into fresh 2xSSC buffer for 5 minutes
23. Completely dry slides in 56°C oven

Denaturation

24. In a fume hood, check the pH of the denaturing solution (49 mls formamide, 7 mls 20xSSC, 14 mls distilled water) and apply 100 µl to each slide. Cover with temporary cover slips made from strips of Parafilm cut to size. Place slides on a flat-bed-heated stage (e.g., Omnislide) and incubate at 72°C for 5 minutes
25. Remove the slides from the Omnislide and transfer a fume hood. Remove the temporary cover slips
26. Within the fume hood, place the slides in 70% alcohol for 1 minute at room temperature
27. Place slides in 85% alcohol for 1 minute at room temperature
28. Place slides in 99% alcohol for 1 minute at room temperature
29. Remove the slides and leave to air dry at room temperature.

Hybridization

30. Work in reduced light; apply 10 µl of the appropriate probe to a 22 × 26 mm cover slip. Invert the slide gently on to the cover slip, taking care to avoid air bubbles
31. Seal the edges of the cover slip with rubber cement
32. Incubate the slides on the Omnislide with a light-shielding lid, overnight at 37°C

Posthybridization Wash

33. Place one Coplin jar containing 50 mls of posthybridization wash buffer (2xSSC, 0.3% NP40) per 5 slides to be washed into a water bath set at 72°C. Fill a Coplin jar or staining dish with posthybridization wash buffer and keep at room temperature
34. Work in reduced light; remove the slides from the Omnislide
35. Use forceps, remove the rubber cement from around the edges of the cover slips and place the slides into the dish of posthybridization wash buffer at room temperature until the cover slips fall off
36. Check the temperature of the posthybridization wash buffer is at 72 ± 1°C before proceeding

37. Remove slide from the room temperature buffer and drain off excess fluid. Place into the 72°C buffer for 2 minutes. Do not put more than 5 slides into each Coplin jar

38. Remove slides from the posthybridization wash buffer and allow to air dry in the dark

39. Mount slides in mounting media containing DAPI. Seal the edges of the cover slip with nail varnish

Visualization and Scoring Slides

40. Use a fluorescent microscope, scan the slide and identify areas for scoring

41. Score the slides using the preferred method. In most cases, this will involve counting the appropriate signals within a predefined number of nonoverlapping cell nuclei. This will produce an overall ratio for the whole slide, a result for the average signals per number of cells counted

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Chapter 8

Quantitative Reverse Transcriptase Polymerase Chain Reaction

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INTRODUCTION

Since the first documentation of real-time polymerase chain reaction (PCR),[1] it has been used for an increasing and diverse number of applications, including mRNA expression studies, DNA copy number measurements in genomic or viral DNAs,[2–7] allelic discrimination assays,[8,9] expression analysis of specific splice variants of genes[10–13] and gene expression in paraffin-embedded tissues,[14,15] and laser captured microdissected cells.[13,16–19] Therefore, quantitative reverse transcriptase polymerase chain reaction (Q-RT-PCR) is now essential in molecular diagnostics to quantitatively assess the level of RNA or DNA in a given specimen. Q-RT-PCR enables the detection and quantification of very small amounts of DNA, cDNA, or RNA, even down to a single copy. It is based on the detection of fluorescence produced by reporter probes, which varies with reaction cycle number. Only during the exponential phase of the conventional PCR reaction is it possible to extrapolate back in order to determine the quantity of initial template sequence. The "real-time" nature of this technology pertains to the constant monitoring of fluorescence from specially designed reporter probes during each cycle. Due to inhibitors of the polymerase reaction found with the template, reagent limitation or accumulation of pyrophosphate molecules, the PCR reaction eventually ceases to generate template at an exponential rate (i.e., the plateau phase), making the end point quantitation of PCR products unreliable in all but the exponential phase. Examples of fluorescent reporter molecules include dyes that bind to the double-stranded DNA (i.e., SYBR® Green) or sequence-specific probes (i.e., TaqMan® products or Molecular Beacons Probes). The automation of the reaction as a whole has enabled Q-RT-PCR assays to be easy to perform with higher sensitivity and more specificity.

This chapter introduces important aspects of Q-RT-PCR, including comparison between conventional PCR and Q-RT-PCR methodology, fluorogenic and sequence-specific probes, methods for quantitation of Q-RT-PCR products, common terminology, and a review of instrumentation.

UNDERSTANDING THE FUNDAMENTALS

Quantitative reverse transcriptase polymerase chain reaction is the reliable detection and measurement of products generated during each cycle of the PCR process, which are directly proportional to the amount of template prior to the start of the PCR process. Holland and co-workers[20] demonstrated that the thermostable enzyme Thermus aquaticus (i.e., Taq) DNA polymerase had 5′ to 3′ exonuclease activity. This group also showed that cleavage of a specifically designed target probe during PCR by the 5′ nuclease activity of Taq polymerase can be used to detect amplification of the amplified product.[20] An oligonucleotide probe, which was designed to hybridize within the target sequence, was introduced into the PCR assay. This probe was labeled with ^{32}P at its 5′ end and was nonextendable at its 3′ end to ensure it could not act as a primer. Annealing of probe to one of the PCR product strands during the course of amplification generated a substrate suitable for exonuclease activity. Also, during amplification, the 5′ to 3′ exonuclease activity of Taq DNA polymerase (when the enzyme extended from an upstream primer into the region of the probe) degraded the probe into smaller fragments that could be differentiated from undegraded probe. This dependence on polymerization ensured that cleavage of the probe occurred only if the target sequence was being amplified. After PCR, cleavage of the probe was measured by using thin-layer chromatography to separate cleavage fragments from intact probe. The introduction of dual-labeled oligonucleotide fluorogenic probes allowed the elimination of post-PCR processing for the analysis of probe degradation.[21] The probe has a reporter fluorescent dye at the 5′ end and a quencher dye attached to the 3′ end. While the probe is intact, the close proximity of the quencher significantly decreases the fluorescence emitted by the reporter dye. A fluorescence signal is only emitted on cleavage of the probe, based on the fluorescence resonance energy transfer (FRET) principle.[22]

In the real-time quantitative TaqMan® assay, a fluorogenic nonextendable probe, termed the “TaqMan” probe, is used (Figure 8.1).[23] The probe has a fluorescent reporter dye attached to its 5′ end and a quencher dye at its 3′ terminus. If the target

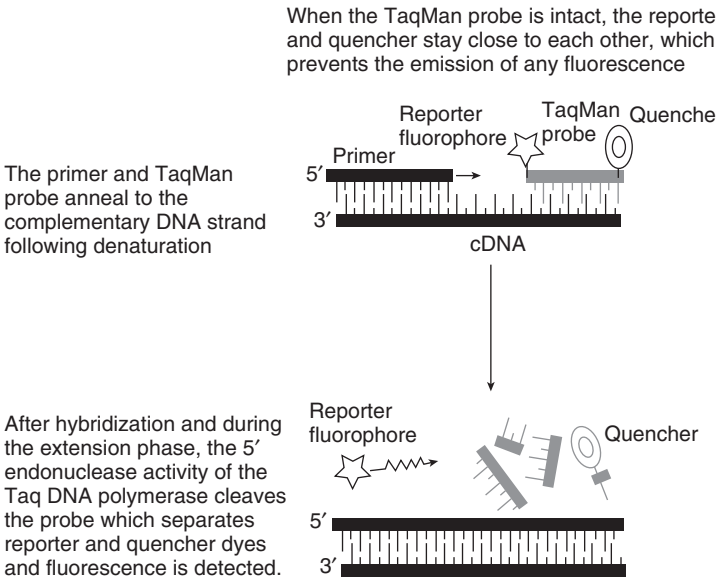

FIGURE 8.1. Hydrolysis probes (e.g., TaqMan assay). (From Ref. 73)

sequence is present, the fluorogenic probe anneals downstream from one of the primer sites and is cleaved by the 5′ nuclease activity of the Taq polymerase enzyme during the extension phase of the PCR. While the probe is intact, FRET occurs, and the fluorescence emission of the reporter dye is absorbed by the quenching dye. Cleavage of the probe by Taq polymerase during PCR separates the reporter and quencher dyes, thereby increasing the fluorescence from the former. Additionally, cleavage removes the probe from the target strand, allowing primer extension to continue to the end of template strand, thereby not interfering with the exponential accumulation of PCR product. Additional reporter dye molecules are cleaved from their respective probes with each cycle, leading to an increase in fluorescence intensity proportional to the amount of amplicon produced. The various available chemistries for real-time PCR are described later in this review.

Using any of the developed chemistries, the increase in fluorescence emission during the PCR reaction can be detected in real time by a modified conventional PCR thermocycler. The computer software constructs amplification plots using the fluo-

rescence emission data that are collected during the PCR amplification (Figure 8.2). Figure 8.2 demonstrates a representative amplification plot and defines the important terms associated with it.

- Baseline: The baseline is defined as the PCR cycles in which a reporter fluorescent signal is accumulating but is beneath the limits of detection of the instrument. By default, the computer software sets the baseline from cycles three to 15; however, this often needs to be changed manually utilizing software supplied with each particular thermocycler.
- ΔRn: A computer software program calculates a ΔRn using the equation Rn = Rnf – Rnb, where Rnf is the fluorescence emission of the product at each time point and Rnb is the fluorescence emission of the baseline.[23,24] The ΔRn values are plotted

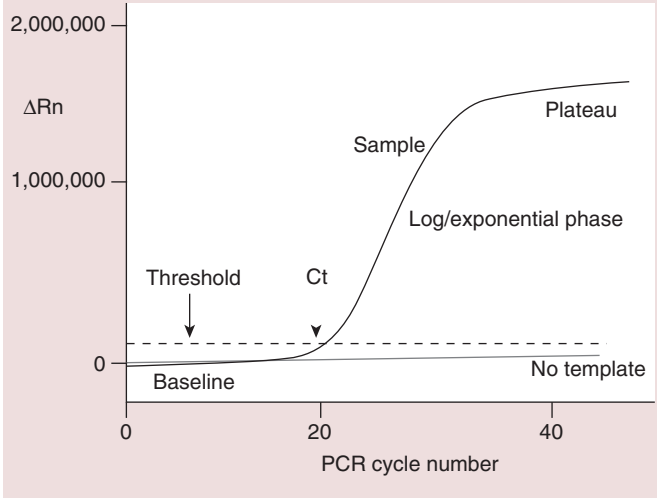

FIGURE 8.2. Model of a single amplification plot illustrating the nomenclature commonly used in real-time quantitative PCR. (From Ref. 73)

versus the cycle number. During the early cycles of PCR amplification, ΔRn values do not exceed the baseline.

- Threshold: An arbitrary threshold is chosen by the computers, based on the variability of the baseline. It is calculated as ten times the standard deviation of the average signal of the baseline fluorescent signal between cycles three to 15. A fluorescent signal that is detected above the threshold is considered a real signal that can be used to define the threshold cycle (Ct) for a sample. If required, the threshold can be manually changed for each experiment so that it is in the region of exponential amplification across all amplification plots.
- Ct: This is defined as the fractional PCR cycle number at which the reporter fluorescence is greater than the minimal detection level (i.e., the threshold). The Ct is a basic principle of real-time PCR and is an essential component in producing accurate and reproducible data.[1]

The presence of more template at the start of the reaction leads to a fewer number of cycles passing before reaching the point at which the fluorescent signal is recorded as statistically significant above background.[24] This Ct value will always occur during the exponential phase of target amplification, which occurs during the early cycles of PCR. As reaction components become limiting, the rate of target amplification decreases until the PCR reaction is no longer generating template at an exponential rate (plateau phase), and there is little or no increase in PCR product. This is the main reason why Ct is a more reliable measure of starting copy number than an endpoint measurement of the amount of accumulated PCR product. During the exponential phase, none of the reaction components is limiting, and therefore, Ct values are very reproducible for replicate reactions with the same starting copy number.

DISCUSSION OF METHODS TO QUANTIFY REAL-TIME POLYMERASE CHAIN REACTION RESULTS

Standard-curve or absolute quantitation: As shown by Higuchi and co-workers, the plot of the log of initial target copy number for a set of known standards (five- or tenfold serial dilution) versus Ct is a straight line (the standard curve).[1] Quantitation of the amount of target in the "unknown" samples of interest is accomplished by measuring Ct and using the standard curve to determine starting copy number. The most common source of a known sample is a plasmid for the gene of interest, and the standard curve is generated based on a serial dilution of a starting

amount. Another option, and easier to generate if a plasmid is unavailable, is the use of a synthetic single-stranded sense oligonucleotide for the entire amplicon. The advantage of this approach is that it significantly simplifies the process of obtaining a standard curve for amplicons up to 100 bp, which encompasses most real-time PCR amplicons. Furthermore, it is also less susceptible to bias when quantified by a spectrophotometer due to the relative purity of the oligonucleotide. Together with the greater precision of measurement of the standard and the possibility of calculating the moles of oligonucleotide (hence, number of copies); it is possible to approximate the number of copies of a template in an unknown sample, although not in terms of absolute copy number. One final option for a standard curve is to use a cell line with a known copy number or expression level of the gene of interest. The standard curve method is used in circumstances when absolute quantitation is critical for the investigator (e.g., when measuring a small number of genes in either a few or many samples)[25,26] or in quantitation of viral load.[27–29]

Relative quantitation: Relative quantitation is also known as the comparative threshold method (2-Ct method). This method eliminates the need for standard curves, and mathematical equations are used to calculate the relative expression levels of a target relative to a reference control or calibrator, such as a nontreated sample or RNA from normal tissue. The amount of target, normalized to an endogenous housekeeping gene and relative to the calibrator, is then given by 2-Ct, where Ct = Ct (sample) – Ct (calibrator), and Ct is the Ct of the target gene subtracted from the Ct of the housekeeping gene. The equation thus represents the normalized expression of the target gene in the unknown sample, relative to the normalized expression of the calibrator sample. For this calculation to be valid and in order to obtain reliable results, it is imperative that the amplification efficiencies of the housekeeping and target gene are approximately equal and at or above 90%. This can be established by looking at how Ct (of both sample and calibrator) varies with template dilution. If the plot of complementary DNA (cDNA) dilution versus Ct is close to zero, it implies that the efficiencies of the target and housekeeping genes are very similar. If a housekeeping gene cannot be found whose amplification efficiency is similar to the target, the standard curve method is then preferable. Alternatively, new primers can be designed and/or optimized to achieve a similar efficiency for the target and housekeeping gene amplicons.

CONTROLS IN QUANTITATIVE REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION: USE OF THE "HOUSEKEEPING" GENE

In real-time quantitative PCR experiments specific errors will be introduced due to minor differences in the starting amount of RNA, quality of RNA, or differences in efficiency of cDNA synthesis and PCR amplification. In order to minimize these errors and correct for sample-to-sample variation, a cellular RNA is simultaneously amplified with the target, which serves as an internal reference against which other RNA values can be normalized. The most common genes used for normalization, termed "housekeeping" genes, are β-actin, a cytoskeletal protein, and glceraldehyde-3-phosphate dehydrogenase (GAPDH), a glycolytic enzyme,[30] and ribosomal RNA (rRNA). These genes should theoretically be expressed at a constant level among different tissues of an organism, at all stages of development, and their expression levels should also remain relatively constant in different experimental conditions.

However, none of these housekeeping genes are ideal. It has been shown that *GAPDH* expression levels are altered by glucose, insulin, heat shock, and cellular proliferation; and β-actin levels may also be modified by experimental treatments.[31–35] rRNA production is less likely to vary under conditions affecting mRNA transcription.[36,37] However, it is not always a good representative of total mRNA population in a cell as rRNA is expressed at a much higher level than mRNA.

Other alternative housekeeping genes have been proposed, but none have been entirely satisfactory, and no single unequivocal reference gene has yet been identified. Some authors have suggested the use of several housekeeping genes in a single experiment and that the mean expression of these multiple housekeeping genes can be used for normalization.[38] Importantly, selection of the housekeeping gene for each specific experiment should be made very carefully as the reliability of the results depends on the choice of the most relevant housekeeping gene according to the cells of interest and specific experimental treatments.

AMPLICON DETECTION STRATEGIES

Two general chemistries are available. These include double-stranded (ds) DNA-intercalating agents (DNA-binding dyes) and fluorescent probes. The former includes SYBR Green I or ethidium bromide and is the simplest and most cost-effective method, as amplicon-specific labeled hybridization probes are not required. SYBR Green I only fluoresces when intercalated into

dsDNA. The intensity of the fluorescence signal is therefore dependent on the quantity of dsDNA present in the reaction. The main disadvantage of this method is that it is not specific, because the dye binds to all dsDNAs formed during the PCR reaction (i.e., nonspecific PCR products and primer-dimers).

With fluorogenic probes, nonspecific amplification due to mispriming or primer-dimer artifact does not generate signal, as specific hybridization between probe and template is necessary for fluorescence emission. Also, fluorogenic probes can be labeled with different and distinguishable reporter dyes, thus allowing the detection of amplicons that may have been produced by one or several primer pairs in a single PCR reaction—termed multiplex real-time PCR. However, different probes must be developed to detect different sequences. The various chemistries are now described in more detail.

Double-stranded DNA-intercalating agents (DNA-binding dyes): SYBR Green I is a nonsequence-specific fluorogenic minor groove DNA-binding dye that intercalates into dsDNA (it does not bind to single-stranded DNA). SYBR Green 1 exhibits little fluorescence when unbound in solution but emits a strong fluorescent signal upon binding to dsDNA.[39] An increase in the fluorescence signal occurs during polymerization, and this decreases when DNA is denatured. Fluorescent measurements are performed at the end of the elongation step of each PCR cycle to monitor the increasing amount of amplified DNA. The advantage of this technique is that it is relatively cheap, as it can be used with any pair of primers for any target. However, as the presence of any dsDNA generates fluorescence, specificity of this assay is greatly decreased due to amplification of nonspecific PCR products and primer-dimers.[40] Generating and comparing melting curves (plotting fluorescence as a function of temperature) using the LightCycler™ (Roche Molecular Diagnostics) (or RotorGene, Smart Cycler, iCycler, Mx4000) is one method of increasing the specificity of the reaction.[40] A characteristic melting peak at the melting temperature™ of the amplicon will distinguish it from amplification artifacts that melt at lower temperatures at broader peaks. It is possible to set the software to acquire fluorescence above the primer-dimers' melting temperature but below that of the target. Another controllable problem is that longer amplicons create a stronger signal. Usually, SYBR Green is used in singleplex reactions; however, when coupled with melting-point analysis, it can be used for multiplex reactions. The SYBR Green I reaction has been used for many applications (e.g., viral load detection[41] and cytokine quantifaction.[42–44]

Hydrolysis probes (e.g., TaqMan probes): This chemistry has already been outlined earlier in this review (Figure 8.1). A forward and reverse primer and a probe are used. The efficiency of the assay is mainly dependent on 5′ to 3′ nuclease activity; the most commonly used enzyme is Taq polymerase,[20] but any enzyme with 5′ nuclease activity can be used.[45] The oligonucleotide probe has a covalently bonded fluorescent reporter dye and quencher dye at the 5′ and 3′ ends, respectively. Various fluorescent reporter dyes are in use including 6-carboxyfluorescein (FAM), tetrachloro-6-carboxyfluorescein (TET), hexacholoro-6-carboxyfluorescein (HEX), or VIC. Quenchers include either 6-carboxytetramethylrhodamine (TAMRA) or 4-(dimethylamino-azo) benzene-4-carboxylic acid (DABCYL). When the probe is intact, the proximity of the reporter and quencher dyes permits FRET, and fluorescence emission does not occur. During PCR amplification, the probe anneals to the target, and Taq polymerase cleaves the probe, allowing an increase in fluorescence emission. The increase in fluorescence intensity is directly proportional to the amount of amplicon produced. The TaqMan chemistry is the most widely used real-time PCR assay and has been used for multiple purposes.[32,46,47]

TaqMan minor groove-binding probes have more recently been developed. In this chemistry, the standard TAMRA quencher at the 3′ end is replaced by a nonfluorescent quencher, and a minor groove-binder molecule is also incorporated at the 3′ terminus. The latter stabilizes the probe-target complex by folding into the minor groove of the dsDNA. Additionally, the Tm of the probes is increased, allowing the use of very short oligoprobes (14 nucleotides in length) and providing more accurate allelic discrimination. Thus, TaqMan minor groove-binding probes are ideal for detecting single nucleotide polymorphisms[48,49] and for the quantitative analysis of methylated alleles.[50]

Dual hybridization probes: This method has been convincingly validated in studies using the LightCycler instrument. Two hybridization probes are used—one carries a donor fluorophore at its 3′ end, and the other is labeled with an acceptor fluorophore at its 5′ end. After the denaturation step, both probes hybridize to their target sequence in a head-to-tail arrangement during the annealing step. This brings the two dyes in close proximity, allowing FRET. The donor dye in one of the probes transfers energy, allowing the other one to dissipate fluorescence at a different wavelength. The measured fluorescence is directly proportional to the amount of DNA synthesized during the PCR reaction. The specificity of this reaction is therefore increased as a fluo-

rescent signal is only detected when two independent probes hybridize to their correct target sequence. This method has been widely used for detection of minimal residual disease after therapy[51,52] and viral load quantification.[53,54]

Molecular beacons: Molecular beacons also contain covalently bound fluorescent and quenching dyes at either end of a single-stranded DNA molecule. However, they are also designed to adopt a hairpin or stem-and-loop structure while free in solution to bring the fluorescent dye and the quencher in close proximity for FRET to occur.[55] The loop portion of the molecule is complementary to the target nucleic acid molecule, and the stem is formed by the annealing of complementary arm sequences on the ends of the probe sequence. The close proximity of the fluorophore and the quencher in this hairpin configuration suppresses reporter fluorescence. When the probe sequence in the loop hybridizes to a complementary nucleic acid target sequence during the annealing step, a conformational change occurs that forces the stem apart. This results in a linear structure and thus separation of the fluorophore from the quencher dye (FRET does not occur) and an increase in fluorescence emission. A new hybridization takes place in the annealing step of each cycle, and the intensity of the resultant fluorescence indicates the amount of accumulated amplicon at the end of the previous cycle. Molecular beacons remain intact during PCR, and they must rehybridize to the target sequence each cycle for fluorescence emission. Molecular beacons are especially suitable for identifying point mutations.[56–58]

Scorpions: Similar to molecular beacons, scorpions adopt a stem-and-loop configuration with a 5′ fluorophore and 3′ quencher. The specific probe sequence is held within the hairpin loop, which is attached to the 5′ terminus of a PCR primer sequence by a nonamplifiable monomer (termed the PCR stopper). This chemical modification prevents PCR from copying the stem-loop sequence of the scorpion primer. During PCR, scorpion primers are extended to form amplicon. In the annealing phase, the specific probe sequence in the scorpion tail curls back to hybridize to the complementary target sequence in the amplicon, thus opening up the hairpin loop. This prevents the fluorescence from being quenched, and a signal is observed.[59] As the tail of the scorpion and the amplicon are now part of the same strand of DNA, the interaction is intramolecular.

The benefits of scorpions derive from the fact that the probe element is physically coupled to the primer element, which means that the reaction leading to signal generation is a

unimolecular event. This contrasts to the bimolecular collisions required by other technologies such as TaqMan or molecular beacons. The benefits of a unimolecular rearrangement are significant in that the reaction is effectively instantaneous and the fluorescence signal much stronger. Also better discrimination and specificity are achieved using scorpions. Scorpion probes have been used for viral load and mutation detection.[60,61]

Duplex scorpions are a modification of scorpions. However, in contrast to scorpions (or molecular beacons), the fluorophore and quencher dye is separated onto different and complementary oligonucleotides. The advantage of duplex scorpions is the significantly greater separation between the quencher and reporter fluorophore, which decreases fluorophore quenching when the probe is bound to the target, resulting in better signal intensity compared with conventional scorpions.[62]

DESIGNING A PRIMER, PROBE, AND AMPLICON

Great care should go into the design of an assay. Primers, probes, and amplicons are designed to very exacting specifications, and the TaqMan system provides its own primer/probe design software from Applied Biosystems known as Primer Express, which is one of the most widely used oligonucleotide design programs for developing real-time quantitative PCR assays. Primer3, a free program from Massachusetts Institute of Technology (MA, USA), can also be used to generate good real-time PCR assays, including designs incorporating an internal hybridization probe. The amplicon for the PCR product should be as small as reasonably possible, usually 50–150 bp in length for designs using hybridization probes (and less than 300 bp for SYBR Green assays). Shorter amplicons amplify more efficiently and are more tolerant of reaction conditions. The optimal length for single-stranded primers is 15–20 bases with a G/C content of 20%–80%. Their Tm should be in the range of 68°C–70°C for TaqMan primers. Molecular beacon and hybridization probe-associated primers can have a wider range of Tm, but the Tm of any one pair should be similar (i.e., not differ by more than 1°C–2°C). Nonspecific priming is minimized by selecting primers that have only one or two G/Cs within the last five nucleotides at the 3′ end. If using a SYBR Green I approach, the PCR primers must not form an appreciable amount of primer-dimer bands. A melting curve analysis of each product is needed to ensure that the fluorescent signal observed is from the desired PCR product. In mRNA expression assays using a hybridization probe, the probe sequence should span an exon/exon boundary if possible. Having the probe Tm 8°C–10°C

higher than that of the primers ensures that the probe is fully hybridized during primer extension. TaqMan probes should not contain a G at their 5′ ends due to the quenching effect of a G in this position on reporter fluorescence, even after probe cleavage.

PUSHING THE TECHNOLOGY FURTHER: MULTIPLEX QUANTITATIVE REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION

The term multiplex Q-RT-PCR is used to describe the use of multiple fluorogenic probes for the discrimination of multiple amplicons in a single tube. The main advantages of multiplexing over single-target analysis are the ability to provide internal controls, lower reagent costs and preservation of precious low quantity samples. The main restrictions of this technique have been the limited number of available fluorophores, fluorescence emission from quenching dyes, and the common use in real-time instruments of a monochromatic light source. The introduction of nonfluorescent quenchers, which have no inherent fluorescence, has been a breakthrough that has allowed an increase in the number of spectrally discernable fluorogenic probes used per reaction. Initial Q-RT-PCR instrumentation contained optimized filters to minimize overlap of the emission spectra from the fluorophores. Newer systems have used either multiple light-emitting diodes, which span the whole visible spectrum, or a tungsten lamp, which emits light over a broad range of wavelengths. However, despite these advancements, only four-color multiplex reactions are usually possible,[63,64] of which one color may be used for an internal control. One recent development is the introduction of combinatorial fluorescence energy transfer tags,[65,66] which will help to boost the development of multiplex real-time PCR.

EQUIPMENT REVIEW

There are a variety of instruments available on the market, each of which has its own individual characteristics. Great care should be taken when choosing which instrument to buy, and it is important to match the instruments capabilities with laboratory needs. Cost should not be the only factor when making a choice; the cheaper models cannot compensate for the variance in the optics and therefore are not capable of detecting smaller differences. The higher-throughput instrument may be more than is needed. The ABI Prism® 7700 Sequence Detection System (SDS) from Applied Biosystems was the first commercially available thermocycler for real-time PCR, but has now been discontinued.

Continuous fluorescence wavelength laser light detection from 500–660 nm allowed multiplex PCR on this machine.

The ABI Prism 7700 has more recently been replaced by the ABI Prism 7900HT, which has similar specifications to the 7700 SDS but is completely automated and designed especially for very high-throughput applications (384 samples per run). Another recent introduction is the less expensive ABI Prism 7000 SDS. It retains the Peltier-based 96-well block thermal cycling format of the ABI 7700, but replaces the laser with a tungsten–halogen lamp that simultaneously illuminates all sample wells. The software supplied with the instrument is much more user friendly and is Microsoft Windows–based, which allows easy export of data and amplification plots. One of the major advantages of the ABI instruments is the collection of data from a passive reference signal to normalize each reaction for variances in the optics of the system. In addition, Applied Biosystems have launched the Applied Biosystems 7300 and 7500 Real Time PCR systems, which represent less expensive alternatives.

The low-priced LightCycler from Roche Molecular Biochemicals induces fluorescence excitation by a blue light-emitting diode that is read by three silicon photodiodes with different-wavelength filters, allowing detection of spectrally distinct fluorophores. Therefore, multiplex PCR can be performed. A complete PCR run of 30–40 cycles is performed in 20–30 min, but only a limited number of samples (maximum 32) can be analyzed simultaneously. As the LightCycler analyzes the specificity of the results by performing melting curves, it makes the use of dsDNA-binding dyes such as SYBR Green I more reliable. However, as samples need to be in capillaries rather than tubes, it is less practical for the investigator.

The iCycler iQ from BioRad Instruments has a tungsten-halogen lamp allowing excitation of a wide range of fluorophores (400–700 nm). It is able to multiplex four different fluorophores per sample tube. Also, it has an optical module, allowing fluorescence emission to be viewed during the course of PCR amplification. Furthermore, the 96 samples are tracked simultaneously, thereby providing a fast assay. A recently launched module allows it to amplify 384 samples at any one time.

A new option is the Mx4000® Multiplex from Stratagene. This sequence detector instrument is able to detect multiple fluorescence PCR chemistries, including TaqMan and hybridization probes, and molecular beacons. The light source for the Mx4000 system is a quartz tungsten–halogen lamp that generates a broad excitation range of 350–750 nm, and there are four photomulti-

plier tubes with a detection range of 350–830 nm. The instrument is ideal for performing multiplex PCR. Importantly, the system contains an integrated personal computer that operates independently from the instrument's embedded microprocessor, which gives some protection against data loss.

The Smart Cycler System has recently become available from Cepheid. The system can be operated with molecular beacons, scorpions, hybridization probes, TaqMan probes, or SYBR Green I. An advantage of this system is its high flexibility, as it contains 16 different modules. Each module can be individually programmed and has its own optical subsystem, and can detect four different fluorophores in one reaction. Different operators can define the parameters for each reaction and different runs can be carried out at the same time for individual experiments. A disadvantage of the basic system is the small sample number (maximum 16); however, this can now be increased to 96 wells per run.

The Rotor Gene™ 3000, designed by Corbett Research, is a centrifugal thermal cycler comparable with the LightCycler. It uses four separate light-emitting diode light sources that excite at 470, 530, 585 and 625 nm. Excitation is detected using six filters and photomultipliers at 510, 555, 610, 660, 580, and 610 nm. The design of this instrument is radically different from all other instruments: the real-time reactions are carried out in standard microfuge tubes inside a 36- or 72-well rotor that spins at 500 rpm. This is meant to remove any temperature equilibration time and nonuniformity, and sample-to-sample variation of less than 0.01°C is claimed.

CONCLUSION

The introduction of real-time PCR technology has revolutionized the field of molecular diagnostics and has enabled the shift of molecular diagnostics toward a high-throughput, automated with lower turnaround times. It allows the sensitive, specific, and reproducible quantification of mRNA. Real-time PCR assays are characterized by a wide dynamic range of quantification of 7–8 logarithmic decades, a high technical sensitivity (<5 copies) and a high precision (<2% standard deviation).[32] Also, no post-PCR steps are required, thus avoiding the possibility of cross contamination due to PCR products. The disadvantages of real-time quantitative PCR when compared with conventional PCR include the fact that:

- Amplicon size cannot be monitored without opening the system
- The limited multiplex capabilities of existing instruments

- The incompatibility of several systems with some fluorogenic chemistries

Real-time PCR technology is only as reliable as the accompanying controls and associated quality assurance programs. This includes the quality of standards and choice of housekeeping gene (the search for the ideal housekeeping gene or protocol is ongoing), the use of suitably controlled standard curves and the need to fully optimize, validate, and evaluate each and every new assay against previously standardized assays. Without such care, real-time PCR will provide an enormous amount of fast but inaccurate data.

The contemporary competition for Q-RT-PCR technology is microarray. However, due to current microarray technologies requiring a large amount of starting material and displaying a limited dynamic range for quantification expression levels of selected genes, true quantification experiments will continue to be conducted using real-time PCR methods.[67,68] Therefore, a combination of both technologies, in which the screening of the involved genes is performed by microarrays and the precise quantification and high throughput screening is performed by real-time PCR, is the ideal method. Similarly, real-time PCR technology will continue to be combined with advanced microdissection techniques[13,16–19] or nucleic acids obtained from paraffin-fixed archival samples.[14,15] The detection and analysis of minimal residual disease[51,69] and viral loads will remain an important application. Also, it will be possible to measure gene expression or DNA copy number in specific cells that are isolated with difficulty and are present in only very small numbers. Combining techniques for sorting fetal cells or DNA from the maternal circulation with real-time PCR will enable early prenatal diagnostics of numerous congenital disorders using minimally invasive procedures.[70–72] Real-time techniques will inevitably be used in the analysis of clinical samples to aid clinicians in prognosis and management of patients with a variety of diverse disease states. Limiting this application at present is the lack of universal agreement on basic issues such as quality and quantity control of RNA, storage standards, guidelines for analysis and reporting of results, and standardization of protocols. These assays are likely to be increasingly utilized as an important area of molecular diagnostics in the future.

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Chapter 9

Proteonomics: High-Throughput Structural Biology—Methods for Cloning, Protein Expression, and Purification

William K. Gillette and James L. Hartley

INTRODUCTION

The problems associated with expressing and purifying human proteins, especially in *Escherichia coli*, the primary host organism for high-throughput (HTP) applications, are well-documented and have plagued researchers for decades. Low yields due to toxicity, recombinant protein insolubility, and poor purification are just some of the problems that result[1] in typical success rates from 2%–20% when expressing eukaryotic proteins in *E. coli* (Service). HTP structural genomic (SG) projects, such as NIH's protein structure initiative (PSI) begun in 2000. Initially, the PSI focused on technology development to provide highly automated procedures for cloning, expression testing, protein purification, and protein crystallization, thus addressing production problems by increasing throughput. The development of these techniques has allowed PSI centers and other similar initiatives around the world to deposit over 2,000 novel protein structures in the Protein Dada Bank (PBD) as of January 2006 (PSI, http://www.nigms.nih.gov/Initiatives/PSI/). Nevertheless, despite the expenditure of significant resources,[2] the rate of discovery is much less than hoped for at the beginning of the initiative due to bottlenecks at every stage of the pipeline[1] as the problems mentioned above persist. Also, the citation rate of structures from SG centers is significantly lower than that for the top structural biology laboratories,[3] suggesting that the current HTP approaches are not as successful in determining the structures of more difficult, and perhaps, more significant proteins. The phrase "picking the low hanging fruit" is often used as an analogy to describe this situation. Accordingly, in the second

phase of the PSI that began in mid-2005, four PSI centers are focusing on high throughput production, while the remaining six centers are specializing in specific areas, such as higher eukaryotic proteins (especially human), membrane proteins, and proteins relevant to disease.

CURRENT APPROACHES

HTP Cloning

The combination of open reading frame (ORF) identification by genome sequencing projects and the availability of HTP cloning methods such as the recombination-based Gateway cloning system[4] of Invitrogen and ligation-independent cloning or LIC,[5] has enabled the creation of large numbers of "ORF clones," bypassing technical problems inherent in using pooled cDNA libraries.[6] A schematic of the HTP techniques used by SG centers to proceed from cloning to protein purification is pictured in Figure 9.1.

Once sequence-verified, ORF clones can be used to generate a wide variety of expression clones by simple *in vitro* recombination techniques without the need for subsequent sequence validation. A variety of recombinational cloning systems (reviewed in Marsischky and LaBaer)[6] are used by HTP production facilities as well as ligase-independent cloning (LIC) and the standard cloning of PCR fragments by endonuclease/ligase cloning.[6] A major liability of the latter approach is the limitation on the number of expression constructs that can be reasonably created.[7] This is due to the effort required for their construction and the introduction of sequence errors from faulty primers and the PCR used in the creation of each clone. A downside to recombination cloning is the addition of more non-native amino acids from the translated recombination sites to the desired protein molecule. Since more than 1,400 papers using Gateway cloning have been published, any detrimental effects of these amino acids are probably minor.

Expression clones can be constructed to test many variables that affect protein expression and purification. Chief among these are the use of affinity tags, solubility tags, and vector sequences required for the expression in a given expression system (bacteria, mammalian, yeast, etc.). Although there are trends,[8–10] there is no way to predict *a priori* the best expression construct and system for a given protein. The possible combinations (e.g., a limited set might include 3 affinity tags, 3 solubility fusions, N- and C-terminal locations, 3 expression hosts) to test

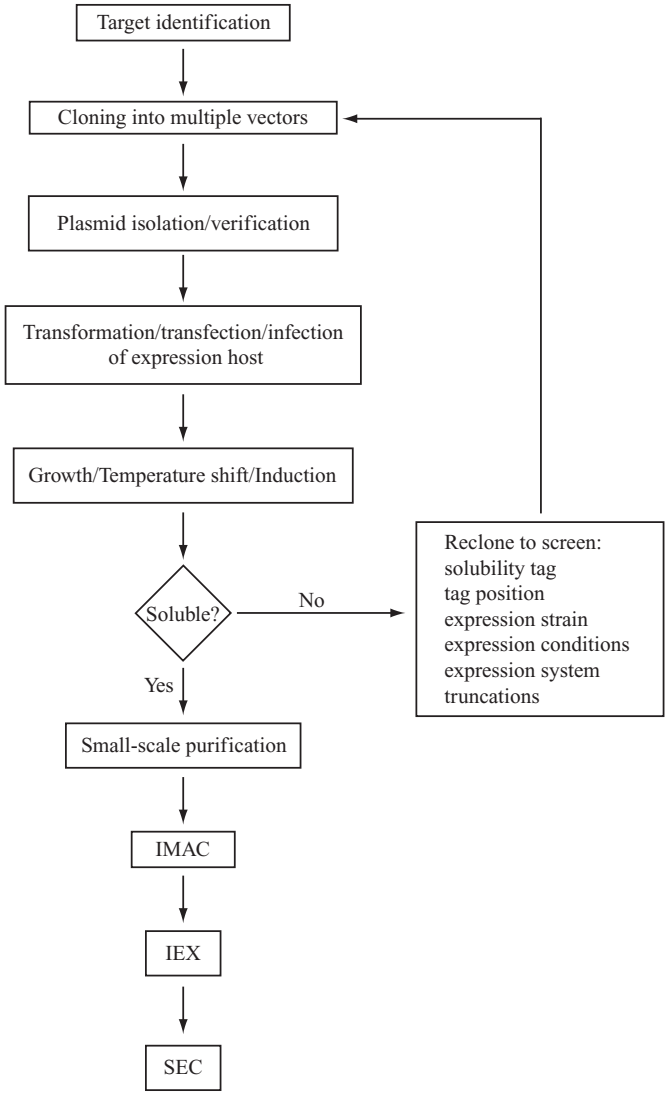

FIGURE 9.1. Schematic of the typical workflow for HTP protein production. IMAC (immobilized metal-ion affinity chromatography), IEX (ion exchange chromatography), SEC (size exclusion chromatography).

can become quite numerous and hence the need for automation-friendly methods at all steps of HTP SG.

Hexa-histidine tags are by far the most commonly used affinity tag in HTP applications due to the low cost, ease of subsequent downstream purification, and the relatively modest addition of non-native amino acids. However, this tag does not enhance the solubility of the fused target protein, thus solubility enhancing proteins are often cloned in-frame with the target protein to improve solubility.[11] Commonly used solubility partner proteins are maltose binding protein (MBP), NusA, and thioredoxin.[8] Reports of differential success with these and other fusion proteins are widespread in the literature, however, MBP, NusA, and thioredoxin are widely reported as the most useful.[8,10,11]

Expression Systems

Several expressions systems have been adopted by the majority of HTP SG centers: *E. coli*, baculovirus, and mammalian cell culture (HEK293 cells). Additional approaches are and will be coming available to address particular problems of recombinant protein expression.[12] For example, a Rhodobacter expression system shows promise for the expression of membrane proteins,[13] and both *Saccharomyces cerevisiae* and *Pichia pastoris* have been reported as amenable to HTP production of human proteins for structural genomics.[14]

Of the available expression systems, *E. coli* is preferred for HTP production, despite the limitations mentioned above, for its ease of use and reduced cost. Advantages include a robust growth rate, inexpensive media, a well-defined genetic system, a variety of induction systems, and the availability of strains and techniques for different applications. For example, isotopically labeled amino acids important for NMR and x-ray crystallography can be incorporated into the protein with the use of labeled precursors in the media. Although *E. coli* has limited capability for post-translational modification of proteins that can be important for folding and activity,[15] this can be beneficial for structural studies due to the reduction in sample heterogeneity. Strains with low protease levels (e.g., BL21) and modifications to express tRNAs present at higher levels in eukaryotes than bacteria can be the difference between no expression and high levels of soluble protein.[16] The major drawback to recombinant expression in *E. coli* is the lack of the proper protein folding machinery, which often leads to mis-folding and/or aggregation. However, new solubility and affinity tags are frequently generated for protein expression in *E. coli*,[17–20] and the flexibility of the system allows

the co-expression of chaperones and potential binding partners that might enhance proper folding and protein stability. The robustness and flexibility of the system will likely prove important when screening difficult proteins. The use of *E. coli* extracts is also becoming a viable option and is particularly attractive for the expression of toxic proteins and in the screening of additives and co-factors to enhance solubility.[21]

The baculovirus/insect cell expression system is a robust expression system capable of producing levels of protein similar to that from *E. coli*, with the added benefit that the proteins are more likely to be soluble.[21,22] Additionally, insect cells are capable of post-translational modifications that are sometime necessary for activity.[15] Although the pipeline for production is significantly longer for the baculovirus system compared to *E. coli*, the techniques needed for cloning, viral production, insect cell culturing, infection, and harvest have all been readily adapted to HTP methods and can lead to protein structure determination.[21]

Expression in mammalian systems is attractive due to the possibility of obtaining correctly folded proteins. However, mammalian cell culture is costly; yields are considerably lower, and generally not amenable to HTP techniques. These factors have limited the use of mammalian expression system in HTP applications to the HEK293 cell line.[23] As the difficult proteins are processed through the SG pipeline, this system and others as yet to be developed will likely play an important role.

Expression Conditions

The conditions under which a protein is expressed have some of the most dramatic effects on the success of expression. Variables included temperature, levels of inducer, media formulations, aeration, time of induction, and time of harvest. The most dramatic of these variables is the temperature during induction. It has long been known that lowering the temperature at the point of induction can realize dramatic improvements in protein solubility in *E. coli* (Schein and Noteborn).[24] For this reason, HTP protein expression in *E. coli* is routinely carried out at temperatures ranging from 4°C–30°C.[7,8,25] Temperature also can affect solubility in the baculovirus/insect cell expression system (manuscript in preparation).

The auto-induction system for *E. coli* recently described by Studier[26] obviates the need for monitoring culture ODs and the addition of the expensive inducer IPTG when using the traditional T7 RNA polymerase transcription system widely favored

in HTP applications. With this procedure, batch cultures of high ODs, normally only obtained in controlled fermentation experiments, can be achieved. The use of this system is being adopted by several HTP applications (Protein Expression Meeting, CHI PepTalk, San Diego, 2006). A limitation on autoinduction is the difficulty in timing reduction in culture temperature to maximize both yield and solubility (Protein Expression Meeting, CHI PepTalk, San Diego, 2006).

Expression and Solubility Screening

Standard methods of detergent-based cell-lysis or sonication are easily amenable to HTP techniques to produce lysates from the harvested expression material. After the insoluble material is removed, either by filtration or centrifugation, a variety of screening methods are used to determine the amount of soluble protein in the lysate. One approach is to assay for the His-tagged protein, using an antibody specific for the His tag. Antibodies against many of the other commonly used affinity and solubility tags are also available. By comparing the signal from whole cell lysates to signal from clarified lysates, the amount of expressed and soluble material can be determined. If the tag assayed is N-terminally located, this approach detects all tagged species with no differentiation between full-length proteins and truncated species. Another approach is to assay the samples directly by SDS-PAGE. However, this is a time-consuming technique, and recently HTP separation of proteins by size using microfluidic technology has been achieved.[27,28] Alternatively, some groups avoid the analysis of soluble protein altogether and proceed directly to the first step in HTP purification.[7]

Purification

Immobilized metal-ion affinity chromatography (IMAC) is most often the first step in the purification of His-tagged target proteins. If expression optimization has been performed and the target is expressed as a fusion to a solubility tag such as MBP, a His tag is included in the construct for purification. HTP formats for IMAC enable purification without the need for expensive chromatography workstations in this first stage of screening. Indeed, the entire protein production process from gene cloning through expression and to purification can be performed in 96-well format using IMAC.

Well-expressed, soluble proteins are often >90% pure after a single IMAC step. For many uses, proteins expressed as fusions

must be cleaved with an appropriate protease to separate the target from the solubility protein. An example of a commonly used approach is to express the protein in the format, His_6-MBP-tev-POI, where an N-terminal His tag is fused to the solubility protein MBP upstream of a TEV protease recognition site, TEV,[29,30] and POI is the protein of interest. After cleavage with TEV (in this example the TEV protease is His-tagged to enable removal after cleavage), the POI is released. TEV protease cleaves the site ENLYFQ/X, where X can be any amino acid except proline.[31] Thus, in theory, a protein can be cloned in such a way as to produce the wild type sequence after TEV cleavage. In practice, this procedure can fail at several points in a protein dependent, and as yet, unpredictable manner. Some proteins are resistant to TEV protease cleavage, while others precipitate after being released from the solubility partner and some proteins do not separate well from the components of the TEV protease digestion in the second IMAC step designed to remove all His-tagged proteins from the sample. Still, the benefits afforded by solubility tags (allowing the purification of intractable proteins) warrants their continued use in HTP SG.[29]

At this point, some proteins are sufficiently pure, while other proteins may require an additional chromatographic step to remove contaminants (typically ion-exchange chromatography). A final gel filtration step (size exclusion chromatography) to remove soluble aggregates is also often employed. Successfully purified proteins can be used for buffer optimization studies to refine the chromatographic conditions and are scaled up to provide the quantities necessary for structural studies, usually 10–100 mg.

ADDITIONAL APPROACHES

While HTP approaches have greatly improved the ability to screen large numbers of samples and advances in cloning and the introduction of new affinity and solubility tags have led to the successful purification of proteins that were previously intractable, the inherent problems of protein production in heterologous expression systems still remain. It is expected that as the structure of the relatively tractable proteins are determined, what remains are especially difficult proteins. Notable in this group are membrane proteins, which are the target of 60%–70% of pharmaceutical drugs.[32]

One lesson that has been learned repeatedly in the study of proteins is that we do not know the rules that govern protein

behavior. Thus, additional techniques and approaches will be needed to address the problems associated with protein production. We have developed such an approach named POET, for Pooled ORF Expression Technology,[33] that can improve the efficiency of a majority of the processes discussed here (Figure 9.2). The basic approach of POET is that working with pools of known ORFs, instead of the individual clones, finesses the complexities of protein expression by improving the efficiency of any step in the protein production pipeline by a factor of n, where n is the number of ORFs in a given pool. We have applied POET to a pool of 688 *C. elegans* ORFs. A high percentage of ORFs identified in this experiment yielded expressed, soluble, purified proteins in agreement with POET predictions. Standard techniques of recombinational Gateway cloning, protein expression, and purification are applied to the pool. The resulting purified proteins are analyzed by standard proteomic techniques (two-dimensional gel electrophoresis (2DGE) and mass spectrometry) to identify and quantify the successfully purified proteins. Recent results in our lab suggest that application of a new mass spectrometry approach[34] to a purified POET protein pool can produce quantification without the need for 2DGE. If validated, this would allow the automation of all steps in the POET process, further increasing the efficiency of the approach. The ability to quantify protein abundance by mass spectrometry would be an exceptional improvement in the many proteomic fields.

The POET method is useful in identifying proteins that are likely to be successfully expressed and purified in isolation under a given set of experimental conditions. As proteins that are relatively easily expressed and purified are characterized and the remaining uncharacterized proteins are tested under more conditions, the success rate will decrease, and techniques that improve the efficiency of the process, such as POET, will be needed.

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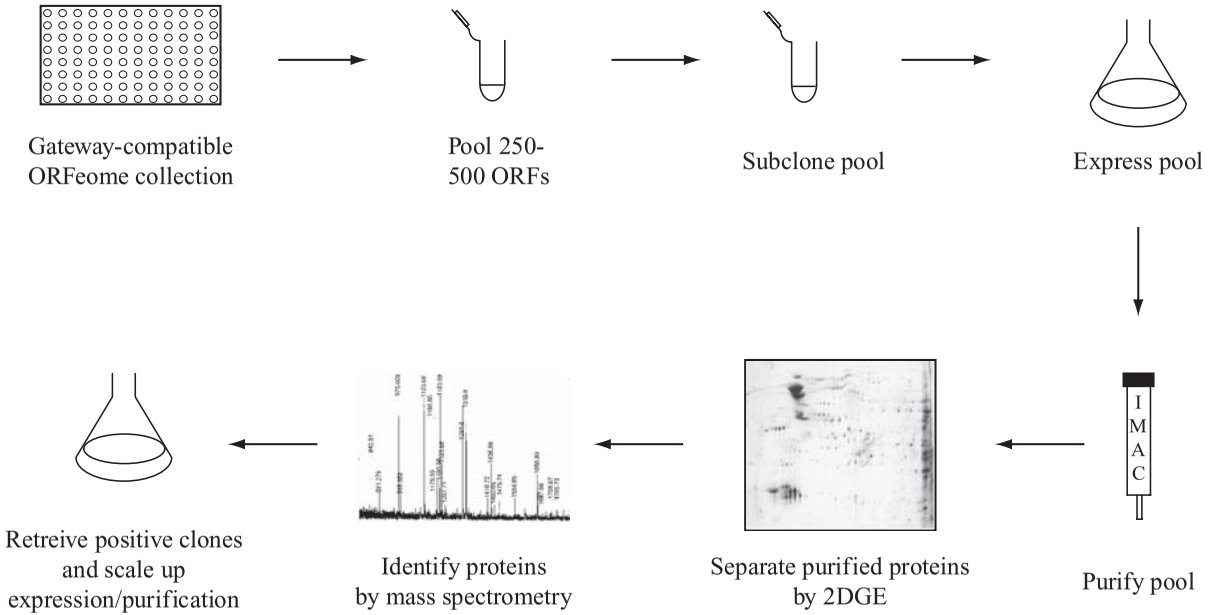

FIGURE 9.2. Summary of POET method for screening an ORF clone collection for expressed, soluble proteins that can be purified. IMAC (immobilized metal-ion affinity chromatography).

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Chapter 10
DNA and Tissue Microarrays

Maysa M. Abu-Khalaf, Lyndsay N. Harris, and Gina G. Chung

DNA MICROARRAY

Methodology

Although techniques such as RT-PCR and in situ hybridization (ISH) can give information about gene expression, they are limited in scope as typically one gene product is evaluated with each assay. The advent of transcriptional profiling using DNA microarray has revolutionized the field of molecular medicine as measurement of thousands of genes simultaneously in a given sample provide a vast amount of data for new disease classifications and biomarker discoveries. DNA microarray-based gene expression profiling relies on nucleic acid polymers, immobilized on a solid surface, which act as probes for complementary gene sequences.[1] Microarrays typically contain several thousand single-stranded DNA sequences, which are "arrayed" at specific locations on a synthetic "chip" through covalent linkage. These DNA fragments provide a matrix of probes for fluorescently labeled complementary RNA (cRNA) derived from the sample of interest. The expression of each gene in the sample is quantified by the intensity of fluorescence emitted from a specific location on the array matrix which is proportional to the amount of that gene product (Figure 10.1).[2]

Several different microarray systems have been developed, using either 25-mer or 60-mer oligonucleotides or cDNA as probes. It is important to note that technical differences between various types of arrays can influence the subsets of genes detected, especially when analyzing 12,500+ transcripts per slide. Two main types of microarrays are used for evaluation of clinical samples: cDNA or spotted arrays, and oligonucleotide microarrays[3]

1. cDNA array: Spotted arrays are constructed using annotated cDNA probes from commercial vendors, or by reverse transcription

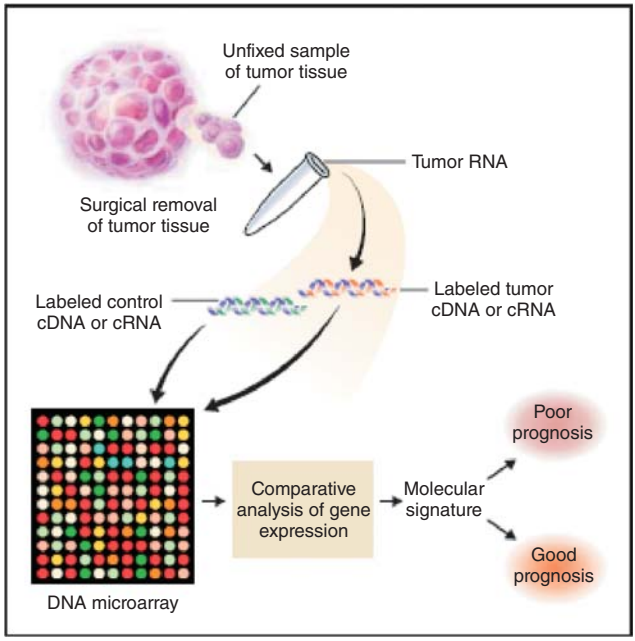

FIGURE 10.1. Reprinted from Sauter G, Simon R. Predictive molecular pathology. *N Engl J Med* 2002;347:1995–1996. (Copyright 2002 Massachusetts Medical Society)

of mRNA from a known source used to generate cDNA probes for specific gene products which are then systematically spotted on glass slides.[3] For sample testing, human Universal RNA standards (serial 10-fold dilutions) are extracted and reverse transcribed in parallel along with test samples and labeled with a fluorescent dye (e.g. Cy3). cRNA from test samples is also labeled with a fluorescent dye (e.g., Cy5). Each Cy5-labeled test sample cRNA together with the Cy3-labeled reference probe is hybridized to the spotted microarray simultaneously. Fluorescence intensities of the scanned images from the hybridizations are quantified, normalized, and corrected to yield the transcript abundance of a gene as an intensity ratio with respect to that of the reference sample.[4] Arrays are generally analyzed with a confocal laser scanner, which allows quantification of gene expression as a relative value at each coordinate on the slide.

2. Oligonucleotide microarrays: Oligonucleotide arrays use 25 base pair DNA oligomers to be printed onto the array matrix.[5]

These arrays, designed and patented by Affymetrix, use a combination of photolithography and combinatorial chemistry, which allows the simultaneous generation of thousands of probes. Oligonucleotide probes for different genes can be deposited or synthesized directly on the surface of a silicon wafer in a predetermined order. Sequences are designed to minimize cross-reactivity, however, some nonspecific hybridization will usually occur. Therefore, the Perfect Match/Mismatch (PM/MM) probe strategy is utilized, in which a second probe that is identical to the first except for a mismatched base (the MM probe) is laid adjacent to the PM probe. After sample hybridization, signal from the MM probe is subtracted from the PM probe signal to account for the nonspecific binding.

Data Analysis

Relative levels of expression on the microarrays are analyzed using sophisticated statistical techniques. There are two main types of multisample analyses: *class discovery* (creating new classes based on differences in expression among samples) and *class prediction* (using samples from known biologic classes to identify a list of genes whose expression pattern can be used to predict the class of a new sample).[6]

The first step of analysis is normalization of the raw data which maximizes the likelihood that the measurements of differential expression are not artifacts of the hybridization reaction.[6] Data are then filtered to select those genes with the largest magnitude of differences in expression for further analysis.

To discover new subgroups based on the gene expression patterns of biologically similar samples (class discovery), *unsupervised analysis* is used. This technique uses clustering algorithms to group specimens according to similarities in their transcriptional profile. For class prediction, *supervised analysis* is used, whereby the gene expression profile of one defined group is compared to another and a list of genes is generated which distinguishes the two groups. Once a set of genes is identified in the "training set," they are ranked by their power to predict the group to which each sample belongs by cross-validation. Typically, one sample at a time is left out, the classifier is trained on the remaining samples, and the sample is then classified based on its correlation to a predictor set generated from the remaining samples. The independent predictive ability of the gene list ("classifier") is ideally performed on a separate set of samples. Often this is achieved by dividing the original set into a training set and a validation set.

The predictive success with either technique is used to calculate the error rate of the predictive genes. It should be noted that leave-one-out cross-validation generally overestimates classification accuracy, and so ultimately, an independent validation set is required.[6]

Sample Preparation Issues

For clinical applications of gene profiling, an important consideration is the isolation of sufficient mRNA from the tumor samples of interest. Adequate numbers of cells of interest must be isolated from heterogeneous tissue extracts, and these specimens must be preserved in a way that does not degrade the quality of the RNA. With respect to preservation, most clinical samples are preserved using aldehyde-based chemicals such as formalin, which has been reported to degrade the quality of RNA.[6] Alternatively, flash-freezing samples by immersion in liquid nitrogen can maintain the quality of mRNA. Samples, however, must be frozen as soon as possible after excision to avoid RNA degradation or changes due to ischemia.

Clinical Applications

Molecular phenotyping of human tumors using micorarray technology has provided insights into tumor subtypes and their attendant clinical outcomes. For example, using microarray technology, Sorlie et al. demonstrated that invasive breast cancers could be divided into four main subtypes by unsupervised analysis.[7] These groups were distinguished by differences in their expression profiles: a "luminal cell-like" group expressing ER; a "basal cell-like" group expressing keratins 5 and 17, integrin β4, and laminin, but lacking ER; an Erb-B2 positive group; and a "normal" epithelial group, typified by genes characteristic of basal epithelial cells and adipose cells. A subsequent study by this group showed that these subtypes have specific clinical outcomes, which are reproducible in other data sets.

Other investigators have used supervised analysis to identify a signature profile in breast cancer patients at very low risk for distant relapse.[8] This 70-gene prognostic signature was able to delineate a group of patients with very low risk of distant recurrence in an independent dataset which appeared to perform better than traditional prognostic tumor characteristics as defined by St. Gallen or NIH criteria.[9]

While microarray profiling has provided important insights into the biology of disease, applying this technology to the study of response to therapy is likely to benefit patients in a more

immediate way. Recent studies suggest that subsets of genes identified by microarray profiling can be used to predict response to a chemotherapy used in breast cancer, the taxanes. Application of this method has lead to the identification of tau[10] and apoptosis genes[11] as potential markers associated for resistance to taxane-based therapy. Furthermore, not only was high expression of tau correlated with a lack of response to paclitaxel in vivo, down-regulation of tau via small interfering RNA caused an increase in sensitivity to paclitaxel in vitro.[10]

In a preoperative trial of trastuzumab and vinorelbine, pretreatment tissue core biopsies were used to perform transcriptional profiling using Affymetrix U133+2 Gene Chips (Harris et al. *Clin Can Res* in press). Unsupervised analysis for class distinction revealed three top-level clusters; all tumors which achieved a pathologic complete response to this combination therapy were in one cluster whereas resistant tumors (defined as lack of response or progression within 1 year) fell in another distinct cluster. Larger T4 tumors were more frequent in the "nonresponse cluster" ($p = 0.02$), and supervised analysis comparing differential expression patterns between T4 vs. lower stage tumors showed that both under and overexpressed genes of the basal lineage were more frequent in T4 tumors ($p < 0.00001$). Supervised analysis of nonresponding tumors showed higher expression of several growth factors (HGF, IGF-1, PDGF, pleotropin), growth factor receptors (c-met, leptin receptor), and the PI3Kinase regulatory subunit p85 and MAP2.

TISSUE MICROARRAY

Construction

Tissue microarrays complement the large scale genomic/proteomic discovery approach of DNA arrays by allowing the simultaneous analysis of DNA, RNA, or protein in large numbers of samples per single experiment (as opposed to DNA arrays which look at large numbers of gene products simultaneously in a test sample). By linking these data to relevant outcome information, e.g., survival, these analyses can give insight into the clinical significance of a given biomarker.

Although the concept of standardizing and streamlining immunohistochemistry (IHC) techniques have been previously reported,[12] Kononen et al.[13] first described a device for the construction of TMAs that could be feasibly accessible to many labs (Figure 10.1). The bulk of the time spent in TMA construction is the collection of the appropriate paraffin-embedded "donor"

tissue blocks and identification of the area of tissue of interest (e.g., invasive tumor). The "recipient" or "master" arrays are then assembled by taking a core tissue specimen from hundreds of separate donor blocks (e.g., different patient tumor blocks) and re-embedding them into the recipient block. Typically, cores are 0.6 mm in diameter spaced at 0.7–0.8 mm, which allows up to 1000 samples to be placed on a recipient block. Larger diameter cores can also be taken in certain instances (for example, when tissue heterogeneity is expected to be greater), although this reduces the number of cores that can be taken from the donor block and that can be placed into the recipient block. Depending on the thickness of the samples, 100–200 5-μm sections can be cut from the recipient block for transfer onto a standard glass slide using an adhesive tape transfer method. The resultant slide can then be analyzed for a variety of molecular targets at the DNA, RNA, or protein level. Redundant arrays can also be constructed by obtaining multiple cores from the donor blocks and placing them at identical coordinates in recipient blocks.

Because both cut slides as well as blocks may be subject to antigen oxidation and degeneration, some facilities store recipient blocks in sealed nitrogen chambers and coat cut slides in paraffin to minimize these effects.[14] In addition, because tissue blocks are three-dimensional structures that can change as more sections are cut; most facilities employ a quality control monitoring system (e.g., every 10th section stained with H&E to assess tissue representatively).

Advantages and Criticisms of Tissue Microarrays

There are several advantages to TMAs. First and most significant is the amplification of tissue resources. A conventional block would be exhausted by 50–100 cuts, and analysis of 50 antibodies on 250 specimens would require 12,500 slides. This approach to tissue analysis is a prohibitive task that also very quickly exhausts tissue resources. As an example using TMAs, up to 400 master blocks can be made from a 1-cm tissue section. These can each be cut as many as 200 times, allowing the evaluation of 80,000 unique reagents. As the TMA technology becomes more commonly utilized and incorporated into clinical trials, this may be of particular importance. First, this allows the efficient organization and storage of archived tissue blocks in many pathology departments. At the Yale TMA Facility, radiofrequency identification tags are used in the tissue blocks for this purpose. Second, tissue collection for biologic correlative studies is now standard in clinical trials, however, the samples are often small and obtained

by invasive procedure specifically for research purposes. TMA technology significantly increases the number of experiments that can be performed from these precious resources as well as assist in the distribution of samples to other investigators.

Other advantages of TMAs relate to the high-throughput format. Large numbers of different types of tissues (benign and malignant), xenograft tissues, cell lines, or recombinant proteins can be readily integrated into the arrays to serve as intra- and interslide references. Only a limited amount of antibody and other reagents, similar to what is used for a whole section, are required. In addition, because hundreds of samples can be studied in one experiment, common variables that can affect reproducibility, such as antigen retrieval, reagent concentrations, and washing times, can be standardized.

A common criticism of TMAs relates to the issue of tissue and tumor heterogeneity—whether a small core is representative of the entire tumor. Indeed, this argument can be expanded to whole tissue sections and blocks of tumor, as large surgical resections are only semirandomly sampled by the pathologist. Nevertheless, many investigators have shown concordance rates of approximately 95% between 2–4 0.6 mm TMA spots and whole sections for common biomarkers such as estrogen receptor and progesterone receptor in breast cancer.[15,16] Furthermore, they were able to reproduce known clinicopathologic correlations with the TMA-based studies.[16] Similar validation studies have been performed in numerous other tumors, including those felt to be more inherently heterogenous such as Hodgkin's lymphoma,[17] pancreatic carcinoma,[18] and soft-tissue sarcomas.[19] Although TMAs are best used as epidemiology-based research tools to examine relative expression of molecular markers in large cohorts of patients, these studies suggest that diagnostic application to individual clinical patients may also be appropriate if used judiciously.

Applications

Over 600 studies have been published using TMA technology since Kononen's initial description in 1998. Most of these studies were performed on various malignancies to study different molecular markers using IHC. When linked to a clinical endpoint, e.g., survival or response to a specific therapy, they have the ability to assess rapidly the prognostic or predictive value respectively of the marker of interest. TMAs have also been used to validate genes discovered by genomic surveys such as DNA microarrays. A variety of TMAs spanning tumor development

and progression has been described such as normal breast tissue, atypical hyperplasia, in situ carcinoma, invasive carcinoma, nodal metastases, and distant metastases. Similar arrays have been described for prostate and for pancreatic carcinomas. Other techniques such as fluorescence in situ hybridization (FISH) or mRNA in situ hybridization (mRNA-ISH) can be readily adapted to TMAs. Finally, TMAs may prove to be a useful technology beyond cancer research. TMA spots can be arrayed with parental and modified cell lines, genetically engineered animal tissues, noncancerous diseased tissues, and multiorgan/multitumor tissues. In the last model, a miniature animal system can be represented for rapid and global assessment of a specific marker, although optimization of antibody titers across such disparate tissues can be problematic.

Quantitative Analysis

Whereas analysis or "scoring" of chromagen-linked IHC stains of TMAs is relatively straightforward with a bright-field microscope, it is time-consuming and laborious, often making this the rate-limiting step. More importantly, because judgments of intensity are subjective and limited, intra- and interobserver reproducibility is difficult. Many efforts to produce a more automated and quantitative analysis of IHC stains have been developed, and more recently, several commercially available programs have become available.[19–21] We have developed an automated quantitative technology that uses modified IHC with immunofluorescence-based detection rather than optical density, which allows increased sensitivity and dynamic range.[22] Using molecular tags to define tumors (i.e., cytokeratin for epithelium) and localize subcellular compartments (i.e., DAPI for nucleus), protein expression is assessed on a continuous scale by co-localization algorithms. This technology has been applied to the study of a variety of biomarkers in numerous different cancers.[23,24] In addition, because a molecular tag is simply defined by a molecule with specificity for a defined/localized antigen, one can envision studying in situ quantitative co-localization to subcellular compartments such as Golgi or mitochondria and stromal compartments such as endothelial cells. As the TMA technology becomes increasingly utilized, the need for more sophisticated biostatistical strategies to rigorously organize and analyze these data becomes even critical. Simple spreadsheet and standard statistical software packages will most likely be inadequate. Clustering algorithms analogous to those employed for gene expression arrays may be further adapted and utilized in the future.

DNA and tissue microarray: key points

- Transcriptional profiling has substantially advanced molecular medicine by producing new disease classifications and by serving as a paradigmatic methodology for biomarker discoveries. DNA arrays allow the simultaneous measurement of thousands of gene products by hybridizing cRNA from a sample of interest to immobilized nucleic acid polymers on a solid surface.
- Expression levels are analyzed mainly by class discovery (which creates new biologic categories based on sample differences of expression) or class prediction (which identifies new profiles of expression that can predict known biologic classes).
- Molecular profiles of human tumors using micorarray technology can provide insights into newly defined tumor subtypes and aid in prediction of clinical outcomes and response to specific treatments.
- TMA technology is a powerful tool for the study of biomarker development and clinicopathologic correlations in large numbers of tissue samples.
- The TMA platform allows the analysis of DNA, RNA, and protein levels in morphologically intact tissue.
- The high-density placement of small 0.6-mm cores of tissues amplifies tissue resources and allows biomarker analysis on large numbers of samples rapidly and with standardized experimental conditions.
  - Several-fold TMA cores have been shown to be representative of whole sections in a variety of cancers, but new tumor cohorts should be carefully assessed for heterogeneity.
  - A variety of automated image analysis technologies have been described to produce readings of TMAs more rapidly and reproducibly.
  - TMAs can readily validate potential biomarkers identified in DNA microarray experiments, assess prevalence of biomarkers in commonly prevalent cancers, and can be incorporated into clinical trials for retrospective investigations into the molecular mechanisms of therapies.

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Chapter 11

Basic Scientific Techniques in Recording Cellular Data

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Basic electrophysiological techniques have been used for years to address fundamental biological questions in the peripheral and central nervous system. Recently these have evolved to include optical as well as electrical recording methods. This chapter, describes some of these methods and their application to the study of neuronal organization in the neonatal mouse spinal cord.[1,2]

The major topics that are covered here include electrical recording setup, extracellular recordings, intracellular recordings, morphology of single cells by intracellular staining, optical recording, calcium-sensitive optical imaging, voltage sensitive recordings, and intrinsic signal imaging.

ELECTRICAL RECORDING SETUP

To perform physiological experiments, investigators have to equip the lab with many specialized pieces of equipment. These are used to amplify, record, and analyze the data acquired from the animal nervous system. To illustrate such techniques, we describe experiments that are performed in our laboratory on the development and operation of locomotor circuits in the spinal cord of the neonatal mouse. However, the basic principles we describe here also apply to studies in other parts of the nervous system.

Setting up an electrophysiological laboratory, one has to keep in mind that experiments should be reproducible and manageable. The tissue under investigation must be kept viable and as close as possible to the *in vivo* physiological condition. Application of modern recording methods usually necessitates removing the tissue from the animal where it can be studied more conveniently than *in situ*. Once removed, the tissue must be maintained alive in a bathing medium containing all the important ions and buffers that are required for physiological function. In addition, an energy supply must be provided—typically glucose—and the bathing solution must be oxygenated. The tissue is then placed in a chamber where physiological studies can be performed.

One great advantage of studying the spinal cord in isolation is that reflexes and even complex behaviors are preserved. These can then be examined without anesthesia or the constraints imposed by whole animal *in vivo* studies. Indeed, it is possible to activate locomotor-like behavior in which the motoneurons supplying the limbs discharge in a manner closely resembling overground locomotion in the intact animal. To understand how these circuits function, it is necessary to define the properties of individual neurons, their connections with each other and their operation in the locomotor circuit.

Studies of connectivity between neurons are usually accomplished by electrically stimulating one class of neuron and recording the responses in its presumed neuronal targets. For instance, a muscle cell will contract, or a nerve fiber will initiate an impulse, if an electric current of suitable size and duration is passed through the tissue. The conductors which are used to deliver the current to the tissue are called "electrodes." The apparatus which produces the electric current is called the "stimulator." Stimuli can be delivered singly or in trains of pulses depending on the requirements of the experiment.

Specialized instruments are used to record the result of these stimuli. The instruments vary both in their sensitivity and in their ability to follow slowly or rapidly occurring events. The highest demands are found in experiments where a combination of steady and rapidly-changing potentials of small size must be recorded simultaneously. This requires an "amplifier" and recording system with a flat frequency response from zero to several tens of KHz and a maximum sensitivity sufficient to detect changes of a few microvolts. Traditionally, the instrument of choice to visualize these amplified voltages has been the oscilloscope but personal computers containing circuitry to convert analog signals into digital form are now more commonly used to display, process, store, and analyze the signals.

EXTRACELLULAR RECORDINGS

Extracellular recordings involve the acquisition of electrical signals from a population of neuronal processes (e.g., axons) or the electrical "field" generated in the space surrounding the cell bodies or dendrites of a group of neurons. These electrical signals are typically very small and require substantial amplification. One method for acquiring and recording such small signals is by the use of "suction" electrodes. Suction electrodes, comprise a glass or plastic tipped capillary filled with a conductor (usually saline) into which a nerve or a piece of central nervous system is drawn. With such electrodes, it is possible to record two types

of signal from a nerve or fiber tract. The first corresponds to the electrical activity accompanying action potentials as they flow along the nerve. In the spinal cord such signals are generated in motoneurons by spinal circuits and flow out of the cord along motor nerves where they can be recorded (Figure 11.1D, spikes). A second type of record corresponds to slow synaptic activity at the motoneuron cell body and dendrites that can be recorded as an electrotonic signal at the tip of the suction electrode (Figure 11.1D, slow potential). Both types of signals are measured as the voltage across the nerve/electrode junction resistance. A more detailed explanation of the electrical model used to explain this type of recording has been published.[3]

Among the important design considerations of these electrodes are 1) the inner diameter of the capillary electrode must be chosen carefully to accommodate the nerve or fiber tract under study, 2) a flexible piece of plastic tubing must be attached to the other end of the electrode and connected to a small syringe to produce the negative pressure required to draw the nerve into the capillary, and 3) two chlorided silver wires are used (one inside the capillary and the other outside) to record the difference in voltage across the two wires or electrodes. In this way, signals that are common to both wires can be cancelled out, leaving only the response from the wire detecting the neural signal. Suction electrodes can also be used to deliver a stimulus to the tissue by applying a brief current or voltage pulse across the electrodes.

Multiple electrodes are used to study complex behaviors such as locomotor activity where at least four extracellular electrodes are required to detect the alternating rhythmic activity between the two sides of the spinal cord and between the rostral and caudal segments.

INTRACELLULAR RECORDINGS

One of the most important signals that can be recorded from the nervous system is the electrical potential across the membrane of individual neurons. This measurement is accomplished either by inserting a very fine glass electrode (sharp electrode) inside a neuron or by sealing a blunter electrode (patch electrode) to the membrane and then rupturing the membrane under the tip to gain access the cell interior (Figure 11.1D). The electrodes are connected to specialized amplifiers that can compensate for their high capacitance and resistance which would otherwise filter the electrical signals. These electrical signals provide information about the electrical properties of the individual neurons, their connections with other neurons and their activity during complex behaviors such as locomotion.

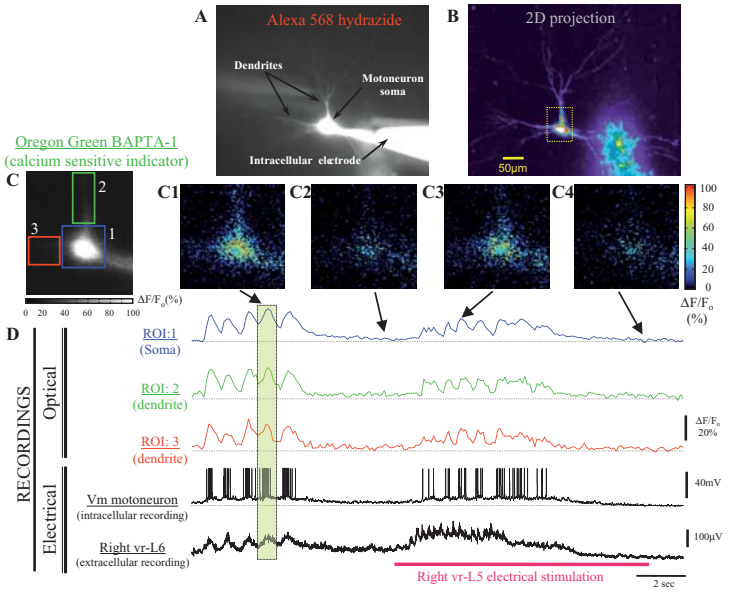

FIGURE 11.1. Combined electrical and optical recordings from neonatal mouse spinal motoneurons. **(A)** Image of a neonatal motoneuron during intracellular recording and stained with a florescent marker (Alexa 568 hydrazide). **(B)** Confocal image of the same motoneuron after withdrawal of the intracellular micropipette. The dotted box indicates the area shown in the panels below (C-C4). **(C)** The motoneuron was also injected with a calcium-sensitive dye (Oregon Green BAPTA-1). An averaged image of the intracellular florescence is illustrated together with 3 regions of interest (ROI) (blue: soma, green, and red: primary dendrites). **C1–4**, confocal images showing the changes in fluorescence of the calcium dye during spontaneous activity (C1), quiescence (C2 and C4) and evoked bursting (C3). **(D)** Optical signals measured from the 3 ROIs shown in C (blue, red, and green), are shown together with the intracellular membrane potential (Vm motoneuron) and spike discharge and slow potential recorded extracellularly from the ventral root of the same spinal segment (Right vr-L6). The box in green highlights the increased somatic and dendritic optical signal corresponding to the burst of action potentials recorded intracellularly. Electrical stimulation of an adjacent ventral root (denoted by the horizontal violet bar) evoked a similar episode of bursting and optical activity.

MORPHOLOGY OF SINGLE CELLS BY INTRACELLULAR STAINING

The morphological identification of intracellularly recorded neurons provides a powerful means of studying structure-function relationships in the CNS. Intracellular electrodes can be

used to inject a dye inside the cell in order to reveal its cytoarchitecture. Ideally, the intracellular label should have the following characteristics: Firstly, it should be easily ejected from the intracellular micropipette without clogging; secondly, it should be water soluble and diffuse quickly so that the cell can be filled in a reasonable time; thirdly, it should not interfere with the function of the neuron and finally, it must be easily visualized in the light or fluorescence microscope.

Horseradish peroxidase (HRP) was one of the first successful intracellular labels. Since then, low molecular weight markers such as the biotin-lysine complex (biocytin; MW = 372) and neurobiotin (MW = 323) have been widely employed. In addition, several fluorescent markers (Lucifer Yellow, Alexa hydrazides) have been used to visualize the injected cells using a fluorescent microscope (Figure 11.1A, B). The use of fluorescent markers is particularly useful when it is necessary to visualize the neuron while obtaining intracellular recordings. Most of the intracellular markers carry a positive or negative charge which is determines how the marker is injected to the cell. For example, biocytin is negatively charged and requires negative current pulses for injection whereas neurobiotin is positively charged and therefore requires positive current.

OPTICAL RECORDINGS

Optical recording methods offer another set of powerful tools for investigating neuronal and network function. They have the advantage of being noninvasive and are capable of resolving the activity of many cells simultaneously. Currently, optical techniques fall into three categories. First, fluorescent probes of intracellular ion concentration (e.g., calcium, sodium and chloride); second, direct measurement of membrane potential using voltage-sensitive dyes; third, intrinsic signal imaging which monitors the changes in tissue properties (light scattering, hemoglobin oxygenation) that accompany neuronal activity. In the spinal cord, we have used all three methods to monitor the activity and spatio-temporal dynamics of individual neurons and neuronal populations during several reflex and complex behaviors. [4]

CALCIUM-SENSITIVE OPTICAL IMAGING

Calcium-sensitive dyes exhibit the largest changes in fluorescence on binding to their target ion.[5] This is, in part, because calcium ions undergo much larger changes in intracellular concentration than other ions (often 100-fold), and so provide a very easily detected indirect signal of neuronal activity. A critical component of experiments involving ion-sensitive dyes is the loading of the neurons under investigation. Several successful approaches have

been reported, ranging from direct injection into the tissue using a membrane-permeable type of dye (AM),[6] retrograde loading,[7] and electroporation.[8] The particular loading method employed will be dictated by the requirements of the study.

Ion-indicator dyes change their florescence when the dye binds the free ion in question. Of course, care must be taken when using such dyes not to "buffer" the ions which can change their dynamics and possibly the neuronal function being studied. For this reason, the lowest useable concentration of the dye should be employed. When using conventional epifluorescence microscopy, these changes in florescence are monitored with sensitive charged coupled device (CCD) or intensified video cameras. Such devices usually operate at 30 frames/sec but specialized cameras can employ much higher frame rates.

Alternatively, the florescent signals can be detected using confocal or multiphoton microscopy (Figure 11.1). Multiphoton microscopy exploits the fact that florophores exposed to very brief laser pulses can absorb two photons at a time instead of the usual single photon. Each of the absorbed photons is approximately double the wavelength of the single photon that is normally absorbed. This has several major advantages. First, long wavelengths penetrate biological tissue with less scattering than shorter wavelengths and can visualize labeled cells up to several hundred micrometers below the surface. Second, because the probability of the florophore absorbing two photons is highest at the focal plane, only a thin slice of tissue is fluorescent, thereby resulting in reduced phototoxicity. Finally, 2-photon microscopy allows the use of nondescanned detectors which do not use a pin-hole to achieve confocality and, as a result, collect both the direct and scattered light emitted from the fluorophore.[9]

VOLTAGE SENSITIVE RECORDINGS

Several classes of dye have been introduced that monitor neuronal membrane potential. In general, these are lipophilic dyes that diffuse into the neuronal membrane where their fluorescence and orientation in the membrane is influenced by the transmembrane potential. They are usually bath-applied to the tissue in question although they may also be introduced into defined neuronal populations by retrograde loading.[10] Highly specialized equipment must be used to detect the changes in fluorescence accompanying changes of membrane potential because these changes are often extremely small (1/1000 of the resting light level). As a result, the detectors must have great sensitivity and a very wide dynamic range (up to 17 bits). Typically, this involves the use of an array of photodiodes (128–464) that are coupled to

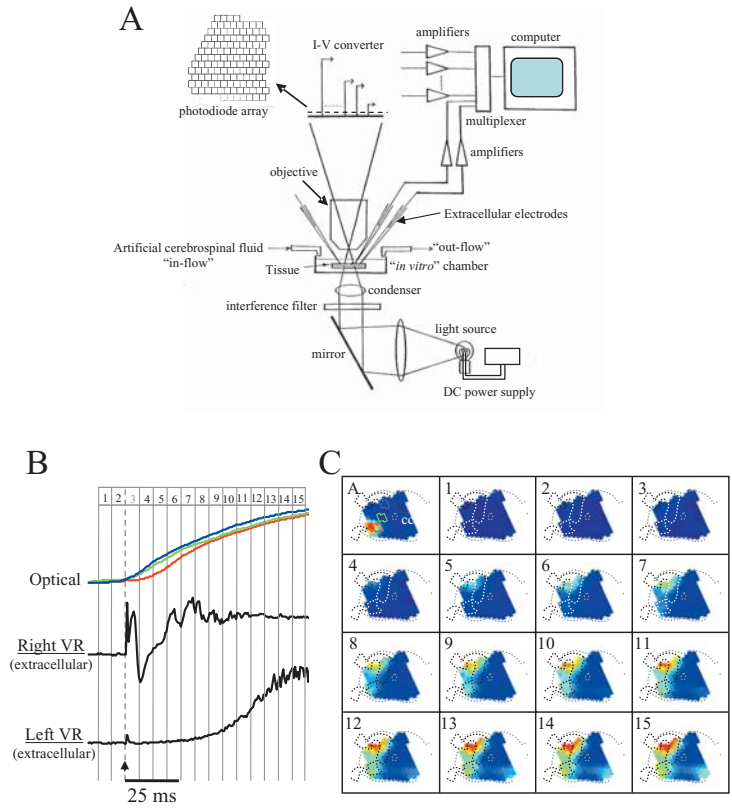

FIGURE 11.2. Combined electrical and voltage-sensitive optical signals. This figure shows the spread of activity at the beginning of an episode initiated by a single stimulus to the ipsilateral (right) dorsal root in the neonatal chick spinal cord slice *in vitro*. (**A**) diagram of the apparatus used for optical and electrical recordings. The slice preparation was continuously perfused with artificial cerebrospinal solution in an *in vitro* chamber on the stage of a modified Nikon microscope (OPTIPHOT, Japan) mounted on an air table. (**B**) Right and left ventral root (VR) electrical responses together with the optical signals from three different cord regions (ventral/lateral motor column: red; intermediate: green; dorsal: blue). Each trace was averaged from 4–6 adjacent diodes (indicated on panel A of part **C**) and was normalized to its peak amplitude. Data were obtained at a sampling interval of 0.64 ms. The gray lines indicate the timing of 10 frame averages (each 6.4 ms) corresponding to the pseudocolored images shown in C. The dotted gray line corresponds to the onset of the dorsal root stimulus (arrowhead). (**C**) Montage of the pseudocolored optical signals from the array, superimposed on the outline of the cord (dotted gray lines). The outlines of the motor nucleus and the primary afferents are shown by dotted black lines. The first image (A) shows a pseudocolored image of the cord following antidromic stimulation of the ipsilateral ventral root to identify the location of motoneurons (indicated by the red-outlined diodes). cc—central canal. The arrow in panel 12 shows the activity propagating to the contralateral lateral motor column. Data from an E11 chick spinal cord embryo.

individual amplifiers under computer control. An example of this type of recording is shown in Figure 11.2.

INTRINSIC SIGNAL IMAGING

One may notice that when neural tissue becomes active, its light absorption changes. Although these changes are not fully understood, they are believed to originate from activity-dependent alterations in neuronal birefringence and tissue volume. Whatever the source of these changes, they can be exploited to provide a noninvasive measure of neural activity in the absence of dye-labeling and its attendant phototoxicity. One disadvantage of these signals over those originating from voltage-sensitive dyes is that they are significantly slower and cannot be used to detect rapid events such as individual action potentials. Nevertheless, we have successfully employed this technique to visualize the patterns of activity accompanying locomotor-like behavior in the isolated mouse cord.

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Chapter 12

Presenting and Publishing Research Data

Howard A. Bird

INTRODUCTION

Publication of research is an important though often neglected aspect of the research pathway. Although often perceived as the final link in the chain, an ever increasing emphasis on audit dictates that the research ultimately with be judged on the quality of publications emanating from it. In turn, this will influence the availability of future research grants. Therefore, it is sensible to plan likely outlets for any research findings at the very earliest stage of an application for funding. If, on consideration, there are unlikely to be any obvious outlets for the results, the would-be researcher should give very careful thought to the value of embarking on the research project in the first place.

In recent years, academia has been driven by the "Holy Grail" of the Research Assessment Exercise with its dependence on placing research publications in journals where they not only attract attention but from which they will be regularly quoted by other researchers. Essential to this is the concept of impact factor, an index that is not without its critics.

Mercifully, an increasing number of funding bodies are starting to attach equal importance to other important benefits from research, including the development of a critical mind that leads to writing and awarding of higher research degrees such as MD and PhD, and the possible immediate benefits the research might have to clinical practice. Quite clearly, the longer the period devoted to research the greater the chances of higher quality publication. Ultimately, this is still likely to be audited by the grant awarding body and the quality of the research and the way in which it is presented is likely to have impact on future funding opportunities.

Sources of funding include major government or independent bodies such as the Medical Research Council or the Wellcome Trust; at present arguably the most respected sources of

funding in United Kingdom universities. For National Health Service (NHS) employees, funding may have been derived from central or local NHS funds. Alternatively, funding may be derived from the larger charities such as the British Heart Foundation, Diabetes United Kingdom or the Arthritis Research Campaign, or even the smaller "disease-based" charities of modest size (such as the Scleroderma Association) or of very small size (such as the Ehlers-Danlos Support Group), to give two examples from my own speciality. Sometimes local university funds may be tapped. A department may be the beneficiary of donations or bequests that are intended to be used for research purposes. Increasingly, applicants are looking towards Europe for largesse amongst members of the European Community.

Industry still contributes to a substantial proportion of healthcare research worldwide. This type of research is less likely to be directed to fundamental research and more likely to be linked to industrial products that ultimately will be sold at a profit to satisfy shareholders. This represents the easiest source of research funding but arguably the one with the greatest number of strings attached and one less likely to command the respect of university or NHS research directly, however important this source might be for the regular provision of "oft funds."

Each funding body is likely to offer their own guidelines on the publication and dissemination of results and the would be researcher is strongly advised to check what is expected of them and which outlet for the results will be judged most acceptable by the funding body.

OUTLETS FOR RESULTS

A variety of outlets are available. Most respected and therefore most desirable is to publish in a peer-reviewed journal, preferably one with high-impact factor. Although the choice of journal is wide, in reality this may be quite restricted according to the area in which the researcher is working. An editor, in selecting material for publication, has obligations not only to the author but also to the readership, the editorial board and, to a lesser extent, the publisher. Journals with the highest impact factors are likely to have achieved this because they are publishing high-quality fundamental research in basic science, of appeal to a large discriminating audience, or because they are "mainstream" medical journals, taking articles only of the widest interest and therefore those likely to be most quoted.

The majority of publications emanating from research will be placed in a more specialized journal, inevitably with a lower

impact factor. If the speciality is a relatively small one, the impact factor will always be significantly lower than in a more popular speciality, even for the "best" journal. In general, the impact factors for rheumatology journals are always higher than those for journals of rehabilitation, though not as high as for journals in gastroenterology or cardiology, both of which are larger specialities worldwide.

Less satisfactory alternatives are to publish in a chapter (though this normally requires an invitation), which is likely to be less critically peer reviewed, or even to write a book, which will be subject to very little peer review at all though the publisher, in commissioning it, will obviously take soundings.

A better ploy is to start by presenting work as a talk or poster presentation to a learned society. Most speciality societies referee submissions competitively. Poster presentations are particularly valuable for young researchers since they enable the researcher to meet other experts in the field and allow for discussion, which may well lead to modification of the work. In turn, this acts as a "dress rehearsal" before submitting a formal paper to a journal. Indeed, the assessors who may later review it may even have visited the poster!

Less satisfactory outlets are talks to industry-sponsored symposia or even press releases. Sometimes the sponsors will require a talk, perhaps as part of a review after one year, and then as a prerequisite to further funding.

PUBLICATION IN PEER-REVIEWED JOURNALS

Ideally, research will be published as an original paper. If the editor feels, on submission, that the work is a little thin for a full-length paper, alternative outlets may be suggested. Several journals publish "brief communications" or might suggest revamping the work in the style of a "letter to the editor." Although this may come as a disappointment to the author, a letter to the editor published in a high-quality journal may well enhance a curriculum vitae more than a full-length paper in a journal of low repute.

Sometimes, particularly for industry-sponsored work, publication may have to be in an industry-sponsored symposium published as a supplement to the journal. These make a lot of money for journals and although normally subjected that the peers review them, this may be less searching than the conventional review for an article in the main journal.

Editorials perhaps command most attention but these are normally only likely to be written on the invitation of the editor,

though co-authorship of an editorial with a senior colleague is always a good ploy.

THE SPECTRUM OF JOURNALS

Even when the research is being planned, it makes sense to consider which journal will be targeted for publication in due course.

For the best quality work of general interest, an international mainstream independent journal such as the *Lancet*, *British Medical Journal*, *Journal of the American Medical Association*, or *New England Journal of Medicine* is likely to be selected.

For more specialized work, the researcher will focus on journals publishing research in that speciality. To take my own speciality, which is rheumatology, at the time of writing, the choice is quite varied, with some 20 respected peer-reviewed journals worldwide.

Inevitably, the major American journals have the widest readership and, therefore, carry the highest impact factors. *Arthritis and Rheumatism* publishes basic science and clinical research applied to basic science, or the sister journal *Arthritis Care and Research* with its more clinical flavor is likely to represent a first choice. The second choice would be divided between the other major North American journals, *The Journal of Rheumatology* (published in Canada), or one of the two major general European journals, *Annals of the Rheumatic Diseases* or *Rheumatology*. That both of the last two are published from the United Kingdom reflects the desirability of publishing in an English-language journal, as well as the foresight shown in the establishment of what is now the Arthritis Research Campaign years ago with the encouragement that has been given to research in arthritis in the United Kingdom over many decades.

If a paper bounces from this group, the author can choose either to move to the house journal of one of the smaller national societies (e.g., *Clinical and Experimental Rheumatology* from Italy; *Scandinavian Journal of Rheumatology* or *Clinical Rheumatology*, originally from Belgium) or to one of the disease-based journals such as *Lupus* or an osteoarthritis journal.

IMPACT FACTOR

This is one of several indexes by which the quality of journal is compared. Inevitably, it has been subject to some criticism and there are alternative ways of measuring this. Table 12.1 provides the definitions for impact factor, immediacy index, and cited half-life. Table 12.2 gives impact factors for a variety of

TABLE 12.1. Impact factor and variance

Impact Factor ("Impact")
A measure of the frequency with which the "average article" in a journal has been cited in a particular year. Thus, the impact factor of journal X would be calculated by dividing the number of all current citations to articles published in journal X during the previous two years by the number of articles ("source items") journal X published in those two years.

Immediacy Index ("Immediacy")
A measure of how quickly the "average article" in a specific journal is cited. Thus, the immediacy index of journal X would be calculated by dividing the number of all current citations of current source items published in journal X by the total number of articles journal X published in that year.

Cited half-life ("Cited Half")
The number of publication years going back from 1990, which account for 50% of the total citations received by the cited journal in the current year. The "cited half-life" thus indicates for how long articles in journal X stayed "alive," i.e., were considered worthwhile as citations.

general journals, rheumatological journals, and rehabilitation journals.

The impact factor is a measure of the frequency with which the "average article" in a journal has been cited in a particular year. It is based on an analysis of the journal's content in the previous two years and that has sparked some criticism. Some

TABLE 12.2. Selected impact factors (2005)

General	
New England Journal of Medicine	34.8
Nature	30.9
Science	29.7
Cell	26.6
Lancet	18.3
British Medical Journal	7.2
Rheumatology	
Arthritis and Rheumatism (USA)	7.2
Annals of the Rheumatic Diseases	3.8
Rheumatology	3.7
The Journal of Rheumatology	2.9
Rehabilitation	
Clinical Rehabilitation	1.0

have suggested that the immediacy index, based on a shorter period of the most recent year, is a fairer index of the "cutting edge" quality of research. Others have favoured the cited half-life, which surveys a much longer period, as a better measure of "durability."

Although an index based on two years analysis probably represents the best compromise, other sorts of errors can creep in. The change in name of a journal will cause it to drop temporarily in the tables. A new journal will take some time to appear in the tables. It is also theoretically possible for an editor to "fudge" the analysis by the exclusive publication of articles that automatically boost impact factors (such as those describing a new disease for the first time or defining diagnostic criteria), however boring to the readership such a journal might become.

WRITING THE PAPER

It is prudent to look through several papers previously published in the journal to which you will be submitting, if only to capture the style. A wider survey of recent issues of the journal will also give an impression of whether the research paper you will finally produce would be of interest to that particular journal or whether, as a matter of policy, it would be directed elsewhere by the editor.

Research is nowadays rarely done in isolation and the novice is likely to have senior colleagues, probably as co-authors, to whom he or she can turn when the paper is being written. In general, the introduction is likely to have been already written and referenced as part of a preceding grant application. If this can be slanted slightly towards the style of the journal, perhaps even quoting papers previously published in the same journal that have prompted the study, that is obviously helpful. The section "patients and methods" is also likely to be lifted almost directly from the protocol. The "results" section should write itself if the research has been well designed and executed. Again, it is prudent to check previous issues of the journal to judge the extent to which results should be presented as tables and diagrams and the depth of the statistical analysis that particular journal will require. The "discussion" section is perhaps the hardest part to write and should be well balanced and objective, perhaps suggesting future research projects that the most recent one has prompted. The "summary," typically 150 words, is normally written last of all.

For the references, the majority of journals use Vancouver style, but this also should be checked. Computer programs are

available to adapt references to a different style if the first journal rejects it.

ASSESSMENT

Editors and journals vary in the style of assessment. A small number use a single assessor and, typically, major international general journals have a professional committee that considers papers as well. The editors of speciality journals tend to send papers to three assessors, one of whom may well be a member of the editorial board. Since the composition of the board is published in the journal, it is therefore normally possible to predict whom one of the assessors might be! If a paper spans several disciplines the group of referees is likely to be representative of each of the various disciplines. Sometimes papers are sent routinely to a statistician; in other cases, this is only done if one of the first groups of assessors specifically requests it.

Once a decision is made, this is relayed back to the author, normally with copies of the different assessors comments, the assessor usually also receiving copies of each others comments, which not only educates assessors as well as authors but also provides an element of internal audit.

Authors are sometimes disappointed if the editor's final decision does not at first seem to be in line with the feedback provided for authors. This is because, in addition to providing authors feedback, each assessor has the opportunity to make comments to the editor in confidence that the authors will not see. An editor may also decide to weight the opinion of a senior and particularly trusted assessor more highly than that of a trainee assessor to whom the paper has also been sent. Ultimately, an author may also fall foul of the pages available to be filled (which correlates with the cost of the subscription to readers) and the particular balance of contents in recent issues of the journal, the editor needing to keep the readership happy as well as the authors. If a junior researcher has had work rejected for one of these reasons, this should normally be explained in a letter from the editor.

It is unusual for a paper to be accepted first time without any change. If reconsideration of the work is offered subject to the changes suggested by assessors, the author is well advised to make these changes since it then becomes extremely hard for the editor and the original assessors to reject it.

If the rejection seems unfair and the assessors' comments particularly critical, the author can always appeal to the editor with a set of reasonable arguments supplied to him/her. In this

situation, the editor may sometimes arrange for reassessment or may take the additional view of a further assessor, typically a senior member of the board. Invariably, demand outstrips the space available, particularly in a popular journal, and it is not unusual for papers to do the rounds of several journals one after another, the paper getting a little better each time as a result of the multiple assessors' comments! In general, papers are offered to journals with a lower impact factor if they have been turned down by one with a high-impact factor. Where a choice has to be made between several journals of equal impact factor, priority would normally be given to one that either specializes in publishing in that particular area or one for which the author feels a particular rapport with the editor, perhaps as a result of previous publications in that journal.

FUTURE TRENDS

Publication of results is not just to enhance the curriculum vitae. It is also seemed important by ethics committees since a failure to disseminate the results of research might be considered unethical if patients had been exposed to risk by participation in a study from which nothing was then learnt. A discussion of future dissemination therefore forms an integral part of ethics committee submission and would also be of interest to the research directorate of a trust since publicity through dissemination of research can only enhance its reputation.

Funding charities will also be keen to ensure that their money has been spent in optimal fashion. Here, a broader view may be taken. Given that the use of impact factors can be justifiably criticized, though may still represent the best compromise, are stakeholders best satisfied with a large output of papers that are only occasionally cited or is payback better with a smaller number of papers that are cited more frequently? Many are now deeming successful MD and PhD dissertations as almost important since these emphasize the educational importance of research to the researcher and seed research for future generations. Put another way, the teaching component of research may be as important as the research itself. By contrast, the NHS, as an organization responsible for patient care, may attach most importance of all to changes in clinical practice that might result from the research, especially if this demonstrates options for saving the managers budget by recommending less expensive clinical practice that is equally as effective as more expensive older methods.

It should also be noted that within two to three years major journals are going to insist on an international standard of ran-

domized controlled trial number allocation at the start of the research project as a prerequisite for consideration for later publication when this is appropriate. This is perceived as an audit of randomized controlled clinical trials, both ensuring their quality and also encouraging the publication of results, if they are good or bad to the financial interests of the funding body.

Ten key points

1. Plan publication from outset.
2. Aim for publication in a peer reviewed journal.
3. Select on the basis of impact factor amongst other things.
4. Benefits in care may be as important as advances in fundamental knowledge.
5. A higher research degree is also a prestigious outcome.
6. Aim to present as a poster before submitting to journal.
7. Read the instructions to authors and follow them.
8. Concede the majority of points made by assessors when revising.
9. Try several journals in turn if necessary.
10. Publication as a letter in good a journal may be better than as a paper in a poor journal.

Chapter 13
Analyzing Health Studies

Julie A. Barber, Gareth Ambler, and Rumana Z. Omar

OVERVIEW

This chapter will consider commonly used methods for describing and analyzing data. We begin with an introduction to some important basic statistical concepts and then focus on some of the most well used methods of analysis for observational and intervention studies. The two types of data we will discuss in detail are *continuous* and *binary* data. For further reading, we recommend consulting a medical statistics textbook.[1,2,3,4]

TYPES OF DATA

Numerical Data

Numerical data are either *continuous* or *discrete*. Continuous variables can take any value within a given range; examples are height, weight, and blood pressure. Discrete variables take only certain values, usually integers. Number of GP visits per year and other count variables are examples of discrete variables. Discrete variables may be treated as continuous if they have a wide range of values.

Categorical Data

A variable is categorical if individuals are assigned to one of a number of categories. The simplest form is a *binary* variable with two categories, for example, alive/dead, pregnant/not-pregnant, and cancer/cancer-free. With more than two categories, the variable is either *nominal*, where there is no ordering of the categories, such as with blood group (A/B/AB/O), or *ordinal*, where there is ordering such as with pain score (none/mild/moderate/severe).

Data Structure

Understanding the structure of data is essential, as it determines the appropriate method of analysis. Most statistical methods assume that subjects or observations are *independent* of one

another. In some cases, however, we may have data where the observations are not independent, such as with paired data where subjects are measured twice (e.g., crossover trials) or are matched (e.g., matched case-control studies). In general, *clustered* data occur where sets of subjects or observations are clustered within larger units (e.g., patients within a general practice, or measurements taken repeatedly over time for each patient).

DESCRIBING DATA

It is important to perform a descriptive analysis, as it allows us to gain a basic understanding of the data. This helps to select the appropriate method of analysis as well as identify problems such as *outliers* (observations inconsistent with the rest of the data) and *missing values*. We can examine and summarize the data using *graphs*, *tabulations*, and *descriptive statistics*.

Continuous Data

Displaying and Assessing the Shape of the Data

Histograms are commonly used to display continuous data. The range of the variable is typically divided into intervals of equal width and the data displayed as a set of vertical bars with heights proportional to the number of observations in each interval. Often the histogram shows a symmetrical, "bell-shaped" *normal* distribution for the data. This *distribution* is fundamental to statistics with many statistical methods requiring assumptions of *normality*. A more reliable graph to assess normality is the *normal plot*. This plots the data against the values you would expect to observe if the data were actually Normally distributed; hence a straight line of points indicates Normality.[1]

Descriptive Statistics

We summarize continuous data with measures of central tendency (the location of the middle of the distribution) and spread (the *variability* of the distribution). The appropriate measures depend on the research question and the shape of the distribution of the data.

Mean and Standard Deviation

The *mean* (or *average*) and *standard deviation (SD)* are the most commonly used measures for summarizing continuous data. The standard deviation (and its square, the *variance*) quantifies the average distance of the observations from the mean. These measures are sensitive to extreme observations in the data. When the

data have a Normal distribution, approximately 95% of the data lie within 2 standard deviations of the mean.

Median and Interquartile Range

For non-normal distributions, it may be preferable to use *quartiles* to summarize the data, because these are less affected by extreme observations. The upper, median, and lower quartiles are the values that divide the data into quarters after arranging the data in ascending order. The *median* measures the central tendency and splits the data in half. The difference between the lower and upper quartiles is known as the *interquartile range*, and measures variability.

Categorical Data

To summarize categorical data, we calculate the number and proportion (or percentage) of subjects that fall into each category. For a graphical illustration of categorical data, one could use a *bar chart*, which is similar to a histogram but displays bars for each category.

ANALYZING DATA

As mentioned in the chapter on study design, we usually collect data from a *representative sample* of individuals in order to make *inferences* about the target *population* of such individuals. For example, to evaluate the effectiveness of a new treatment for breast cancer, we might evaluate the treatment on a sample of breast cancer patients from two UK centres, and then draw conclusions about the likely usefulness of this treatment for all breast cancer patients.

However, *statistical uncertainty* arises because we have information for only one of many potential samples that could have been taken from the population. The two basic methods of quantifying this uncertainty are *estimation* and *hypothesis testing*.

Estimation

We use our study sample to estimate population characteristics. For example, the mean blood pressure amongst a sample of British men might be used to estimate the true mean blood pressure for all British men. We could be interested in descriptive measures such as proportions or means, or comparative measures, such as relative risks, or differences in means between two groups.

Sampling Distributions

A sample estimate is unlikely to be exactly equal to the true population value and different samples will provide different

estimates. The distribution of estimates from different samples is called the *sampling distribution* and is used to quantify statistical uncertainty. Because we only have one sample, we often have to make *assumptions* about this distribution (e.g., Normality). When estimating population characteristics using a sample, it is essential that we report on the *statistical uncertainty*. This is achieved using either *standard errors* or *confidence intervals*.

Standard Errors

The *standard error (SE)* is used to quantify how precisely the population characteristic is estimated by the sample. It is the estimated standard deviation of the sampling distribution with smaller values of the standard error indicating higher precision and less uncertainty. Larger sample sizes give smaller standard errors and hence more precise estimates.

Confidence Intervals

It is often more useful to construct a range for the likely values of the population characteristic. The sample estimate and its standard error can be used to provide such a range in the form of a *confidence interval*. Conventionally we calculate 95% confidence intervals, which we can think of as the interval likely to contain the true population value with a probability of 95%. Because these intervals are obtained from the sampling distribution of the parameter, the formal interpretation of confidence intervals is in terms of taking many samples from the population. If we took 100 samples from the population and calculated a 95% confidence interval for each, we would expect 95 of these intervals to include the population value.

The upper and lower confidence limits are often calculated as

$$\text{estimate} \pm \text{multiplier} \times \text{SE}$$

where the multiplier is derived from the sampling distribution. For example, an estimate of the average age of patients undergoing elective cardiac bypass graft surgery is 60.5 years (SE = 1.7 years) based on a sample of 31 such patients from a London hospital. A 95% confidence interval showing the likely range for the true average age is 57.0 to 64.0 ($60.5 \pm 2.0 \times 1.7$).

Hypothesis Testing

The sample data can be used to test a predefined statistical hypothesis about a population characteristic. Typically this *null hypothesis* describes situations of no effect or no difference. For example, when investigating the possible link between respira-

tory infection and sudden infant death, we would test the null hypothesis that there was no link. There are many statistical tests suitable for particular hypotheses and types of data, and all produce probability statements called *P-values*.

P-values

To test our hypothesis, we consider how much evidence there is in the data *against* the null hypothesis. The amount of evidence is quantified in the form of a P-value, which lies between 0 and 1. Large P-values suggest we have little evidence that the null hypothesis is untrue, and so we *do not reject the null hypothesis*. Large P-values do *not* tell us that the null hypothesis is true. Small P-values tell us we have evidence against the null hypothesis, and we would *reject the null hypothesis*.

Formally, P-values are the probability of observing similar or more unlikely data than our own sample when the null hypothesis is true.

Statistical Significance

Often we refer to small P-values as statistically *significant* and large P-values as *nonsignificant*. A cut-off of 0.05 conventionally defines statistical significance, but this is arbitrary and hence should be used cautiously.

Over Interpretation of P-values

It is important to note that small P-values do not necessarily imply clinically important effects, and large P-values do not tell us there is no effect. By examining the estimate and confidence interval, we can make a better interpretation. The size of the estimate allows us to judge clinical significance. Wide confidence intervals may indicate that a large P-value has occurred because of low *power* (limited sample size) (see design chapter on study design for definition of power).

EXAMPLES

Using examples, we illustrate the basic methods of analysis for continuous and binary outcomes and look at the interpretation of statistical results. We focus on the comparison between two groups of data, both independent and paired. The results shown can be obtained using any good statistical software.

Two Independent Groups of Continuous Data

To investigate the relationship between behavior and risk of heart disease, we use the cholesterol levels from 60 men

categorized into two behavior groups: type A: urgent, aggressive and ambitious; type B: relaxed, noncompetitive and less hurried.[1]

Describing the Data

The data are shown in the format required for most statistical software (Table 13.1). Cholesterol level is a continuous outcome and its distribution in each behaviour group can be examined using histograms and Normal plots (Figure 13.1). Cholesterol

TABLE 13.1. Cholesterol levels (mmol/l) by behaviour type. Format required for analysis: *id* = patient identifier, *cholesterol* (mmol/l), behavior *group* (0 = type A, 1 = type B)

Id	Cholesterol	Group	id	cholesterol	Group
1	236	1	31	178	1
2	209	1	32	242	1
3	253	0	33	273	0
4	250	1	34	164	1
5	156	1	35	185	1
6	281	1	36	153	0
7	251	0	37	218	1
8	201	1	38	187	1
9	257	1	39	212	0
10	203	1	40	248	0
11	230	0	41	255	0
12	210	0	42	158	1
13	291	1	43	234	0
14	278	0	44	268	0
15	263	0	45	194	1
16	241	0	46	188	1
17	270	0	47	212	0
18	227	1	48	272	1
19	186	1	49	165	1
20	236	1	50	212	1
21	228	1	51	260	0
22	246	1	52	218	1
23	185	0	53	261	1
24	212	1	54	244	1
25	280	0	55	207	0
26	244	1	56	248	0
27	294	1	57	304	0
28	276	1	58	204	0
29	173	0	59	230	0
30	202	1	60	181	1

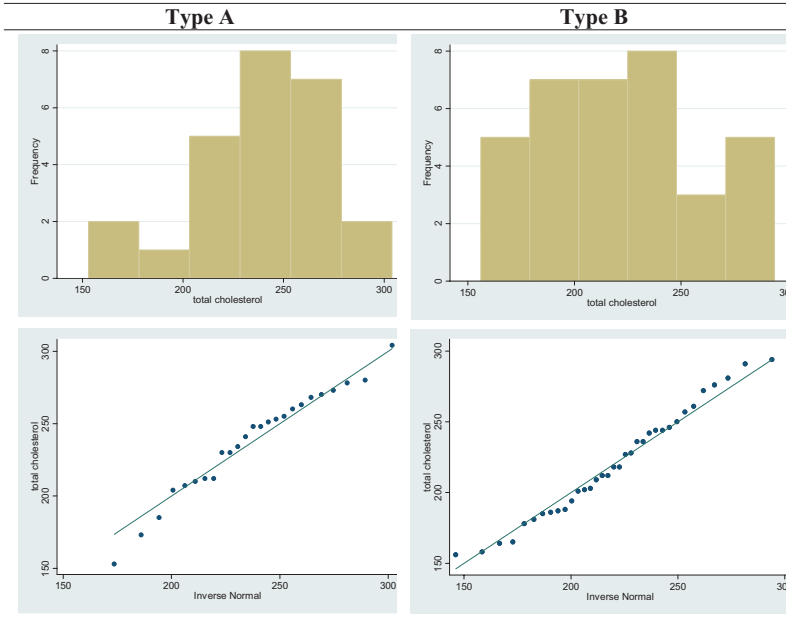

FIGURE 13.1. Histograms and Normal plots of cholesterol levels by behaviour type.

levels in each group appear to be approximately Normally distributed.

Summary statistics suggest that mean cholesterol for type A men is higher than for type B (Table 13.2). The standard deviations in the groups are reasonably similar.

Analysis: Two-Sample T-test

To formally compare sample means between two independent groups, we use *parametric* methods based on the *t-distribution*

TABLE 13.2. Mean (SD) cholesterol level by behaviour type

	Type A	Type B
Mean (mmol/l)	237.7	220.1
SD (mmol/l)	36.2	38.6
N	25	35

(which is related to the Normal distribution). These assume that the data in both groups come from Normal distributions and the groups have equal population standard deviations. The first assumption is important for small samples; we should check Normality if group sizes are less than 30. Our sample size is moderate, but the distributions appear approximately Normal (Figure 13.1), and the standard deviations are similar (Table 13.2), so we can go ahead and use these methods.

We are interested in whether cholesterol differs by behavioral type, so the parameter of interest is the difference in mean cholesterol between the groups. This is estimated as 17.6 mmol/l with a standard error of 9.9 mmol/l. The latter is relatively small, indicating some precision. A 95% confidence interval for the difference is –2.1 to 37.4 mmol/l (approximately $17.6 \pm 2 \times 9.9$). We can think of this as the interval within which we are 95% confident that the true difference in mean cholesterol in the population lies. The confidence interval suggests that type A personalities may, on average, have higher cholesterol values (i.e., differences greater than zero). However, the interval also includes the possibility of no difference in means (i.e., a difference of zero).

We use the *two-sample t-test* to test the null hypothesis that mean cholesterol levels are the same in each group. The t-test gives a P-value of 0.08. This indicates an 8 in 100 chance of obtaining a sample like ours if there is really no relationship between behavior and cholesterol. That is, there is some evidence that cholesterol levels differ by behavior type.

Violation of Assumptions

If the assumptions of the t-test are violated, we may use a different approach. *Welch's test* is suitable if the data are approximately Normal but the variances are unequal.[4] When there is non-normality, transforming data to a scale where assumptions are met may be useful; for example, analyzing log-transformed data and reporting results in terms of *geometric means*. Alternatively, we may use a *nonparametric* method, which does not require Normality assumptions. The *Mann-Whitney U* (or *Wilcoxon Rank Sum test*) is the nonparametric counterpart of the two-sample t-test. The latter compares the medians of the two groups, assuming that their distributions have identical shapes.

Two Paired Groups of Continuous Data

An example of paired data is a matched case-control study to investigate risk factors for cardiovascular disease (CVD) in

TABLE 13.3. Mean (SD) triglyceride level for each CVD group, and the group of differences

	CVD	no CVD	CVD—no CVD
Mean (mmol/l)	1.77	1.29	0.49
SD (mmol/l)	0.19	0.14	0.23
N	22	22	22

patients with Systemic Lupus Erythematosus (SLE).[2] Twenty-two patients with SLE and CVD were individually matched by age, sex, and ethnic group to patients with SLE but no CVD. We consider the continuous outcome triglyceride concentration.

Describing the Data

Summary statistics suggest that mean triglyceride concentration is higher in the CVD group (Table 13.3). However, because data are paired, we need to summarize the differences between the paired measurements. We create a new variable containing the differences between the concentrations for CVD and matched non-CVD patients. The mean (SD) of these differences is 0.49 (0.23) mmol/l (Table 13.3). A Normal plot of these differences suggests approximate Normality (Figure 13.2).

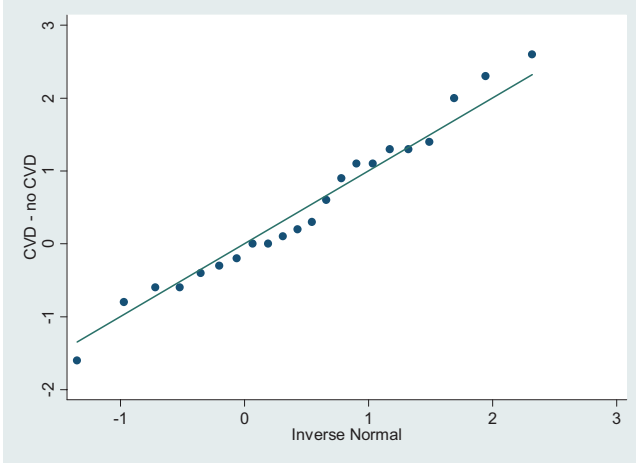

FIGURE 13.2. Normal plot of the differences in triglyceride levels between matched CVD groups.

Analysis: The Paired T-test

The usual parametric approach for paired data is the *paired t-test* method. This assumes that the differences between paired measurements have a Normal distribution. For small samples, this should be checked using a Normal plot. The parameter of interest is the mean of these differences. We can obtain an estimate of this mean and its standard error and confidence interval.

For the triglyceride data, the paired t-test methods seem appropriate (Figure 13.2). The mean (SE) of these differences is 0.49 (0.23) mmol/l, indicating a reasonable level of precision. A 95% confidence interval for the mean of the differences is 0.01 to 0.96 mmol/l. This suggests the true mean difference is likely to be positive, indicating that SLE patients with CVD have a higher mean triglyceride concentration.

A *Paired T-test* formally tests the null hypothesis that the mean difference is zero. For the triglyceride data, this gives a P-value of 0.045. Thus, we have evidence to reject the null hypothesis that there is no relationship between CVD and triglyceride concentration (i.e., that the mean difference is zero). This result is consistent with the interpretation of the 95% confidence interval.

Violation of Assumptions

If the differences between paired measurements are severely non-normal, it may be preferable to transform the original data or use a nonparametric method such as the *Wilcoxon signed rank test*.

5.3 Two Independent Groups of Binary Data

A trial was performed to compare neurocognitive impairment after cardiac bypass graft surgery. Sixty patients were randomized to either off-pump or on-pump surgery, and the binary outcome was neurocognitive impairment (yes/no) one week following surgery.[7]

Describing the Data

The complete dataset can be summarized as a 2×2 table (Table 13.4). The overall risk of impairment in the study was 27/60 (45%).

Analysis

When comparing groups of binary data, the parameter of interest is the estimated risk difference. For this trial, the difference is

TABLE 13.4. 2 × 2 table summarising neurocognitive impairment in the two groups

	Impaired	Not impaired	Total
Off-pump	8 (26.7%)	22	30
On-pump	19 (63.3%)	11	30
Total	27	33	60

36.7% (63.3% minus 27.7%) in favor of the off-pump group with a standard error of 3.7%. Assuming Normality, a 95% confidence interval for the risk difference is 13.3% to 60.1%. The interval is not close to zero, suggesting that off-pump surgery reduces the risk of neurocognitive impairment. The Normality assumption is considered acceptable if the observed values in the 2 × 2 table all exceed 10; this is approximately the case here.

To test the null hypothesis that the risk difference is zero, we use a *chi-squared test*. If the null hypothesis is true, we would *expect* the risk of impairment to be 45% in both groups; thus we would expect about 13.5 subjects in both groups to have neurocognitive impairment (for details on expected values, see the textbooks referenced). These expected numbers are very different from the *observed* numbers, and the P-value for the chi-squared test is 0.004, suggesting a clear difference between the two types of surgery.

The chi-square test is only valid when the sample size is sufficient. For a 2 × 2 table, all the expected values in the table should ideally exceed 10. This is the case here (Table 13.5).

Violation of Assumptions

If the sample size is too small, we may use *Fisher's Exact Test* and exact methods to obtain confidence limits.

TABLE 13.5. 2 × 2 table summarising neurocognitive impairment in the two groups: observed (expected) values

	Impaired	Not impaired	Total
Off-pump	8 (45% × 30 = 13.5)	22 (16.5)	30
On-pump	19 (13.5)	11 (16.5)	30
Total	27	33	60

TABLE 13.6. Outcome pairs breast cancer stud

Recurrence? (hormone)	Recurrence? (no hormone) Yes	No	Total
Yes	9	6	15
No	18	14	32
Total	27	20	47

Paired Binary Data

A study to assess the effect of hormone treatment on patients with node-positive breast cancer recruited 47 women. These were all given hormone treatment and were matched (on age, tumor grade, and number of nodes affected) to 47 women who did not receive hormone treatment. The binary outcome was tumor recurrence or death within 3 years.

Describing the Data

Since the data are paired, we arrange the data by pairs of outcomes (Table 13.6). The risk of a recurrence after hormone treatment is 31.9% (15/47), compared with 57.4% (27/47) for the no hormone group.

Analysis

The risk difference is 25.5% in favor of the hormone treatment, and the standard error is 9.7%. Assuming Normality, the 95% confidence interval is 6.5% to 44.6%. To formally compare the groups, we use *McNemar's test*. This gives a P-value of 0.014, showing evidence of a beneficial effect of hormone treatment. This test is valid provided that both discordant pairs in the table exceed 5. We have 6 and 18 discordant pairs.

Violation of Assumptions

If the sample size is too small, we can use exact methods for testing and calculating confidence intervals.

CONCLUSION

We have described the most basic methods for summarizing and analyzing two groups of continuous or binary outcomes. More advanced methods exist for different types of data, more than two groups, and other types of associations. *Regression* is used

to explore relationships between variables, or to adjust for confounders. Data clustered within larger units require more sophisticated methods. When conducting a research study, we strongly recommend that you consult a statistician regarding all aspects of the study, including design, data collection, analysis, and interpretation

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Chapter 14
Future

H.R.H. Patel, I.S. Shergill, and M. Arya

Research is the future, and the future is research! We believe that this mantra is the fundamental basis for successful basic science and clinical research applicable to medical practice. As is very much apparent, many characteristics of current medical practice require medical staff to have at least some exposure to basic science research. In the future, we anticipate many institutions worldwide will provide specific courses on clinical research for all health professionals, as is occurring in UK professional regulatory bodies. For the near future, well-established techniques will continue to be used, refined and, combined, such as the use of tissue microarrays, immunohistochemistry, and fluorescent in situ hybridization. Moreover, newer techniques will be introduced at the protein, RNA, and DNA levels, allowing the researcher a wide array of tools to address important scientific and clinical hypotheses. Notably, on the horizon is the widespread introduction of nanotechnology. We believe the use of nanotechnology in research has no bounds, if properly regulated, and will allow rapid advancements in research as well as potentially providing the scientific basis for cures of currently terminal diseases.

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