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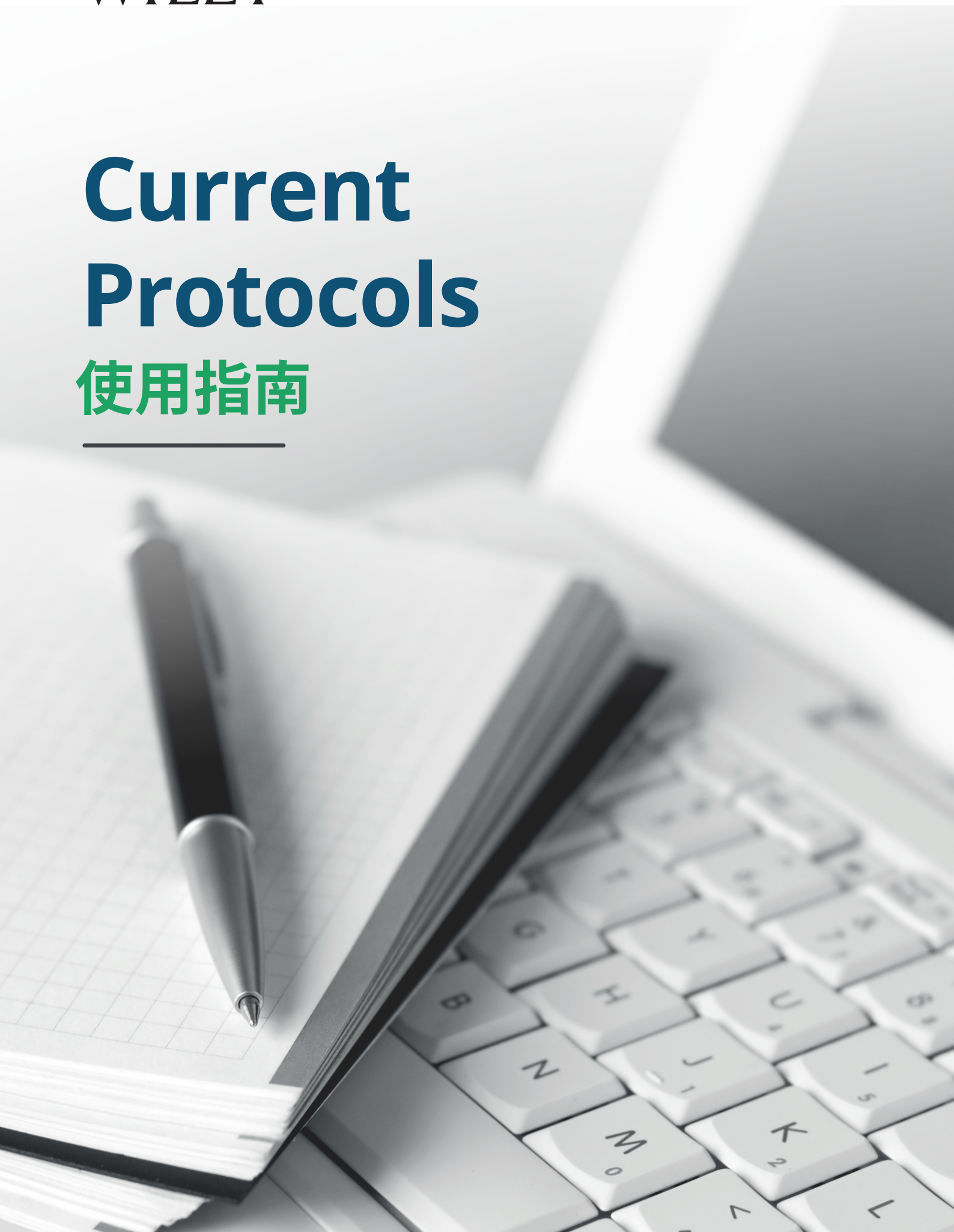

目录

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Current Protocols包含近25,000篇详细的分布式实验流程，为研究人员提供可靠、有效的解决方案，以确保结果的重复性，为科研成果铺平道路。有17本合集，既有适合初级研究者的Essential Laboratory Techniques，也有适合资深研究者的内容，例如旗舰刊Current Protocols in Molecular Biology。

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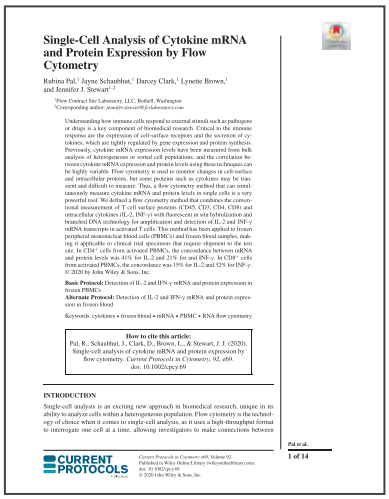

Single-Cell Analysis of Cytokine mRNA and Protein Expression by Flow Cytometry

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Understanding how immune cells respond to external stimuli such as pathogens or drugs is a key component of biomedical research. Critical to the immune response are the expression of cell-surface receptors and the secretion of cytokines, which are tightly regulated by gene expression and protein synthesis. Previously, cytokine mRNA expression levels have been measured from bulk analysis of heterogeneous or sorted cell populations, and the correlation between cytokine mRNA expression and protein levels using these techniques can be highly variable. Flow cytometry is used to monitor changes in cell-surface and intracellular proteins, but some proteins such as cytokines may be transient and difficult to measure. Thus, a flow cytometry method that can simultaneously measure cytokine mRNA and protein levels in single cells is a very powerful tool. We defined a flow cytometry method that combines the conventional measurement of T cell surface proteins (CD45, CD3, CD4, CD8) and intracellular cytokines (IL-2, INF-γ) with fluorescent in situ hybridization and branched DNA technology for amplification and detection of IL-2 and INF-γ mRNA transcripts in activated T cells. This method has been applied to frozen peripheral mononuclear blood cells (PBMCs) and frozen blood samples, making it applicable to clinical trial specimens that require shipment to the test site. In CD4+ cells from activated PBMCs, the concordance between mRNA and protein levels was 41% for IL-2 and 21% for and INF-γ. In CD8+ cells from activated PBMCs, the concordance was 15% for IL-2 and 32% for INF-γ.
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Basic Protocol: Detection of IL-2 and IFN-γ mRNA and protein expression in frozen PBMCs

Alternate Protocol: Detection of IL-2 and IFN-γ mRNA and protein expression in frozen blood

Keywords: cytokines • frozen blood • mRNA • PBMC • RNA flow cytometry

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INTRODUCTION

Single-cell analysis is an exciting new approach in biomedical research, unique in its ability to analyze cells within a heterogeneous population. Flow cytometry is the technology of choice when it comes to single-cell analysis, as it uses a high-throughput format to interrogate one cell at a time, allowing investigators to make connections between

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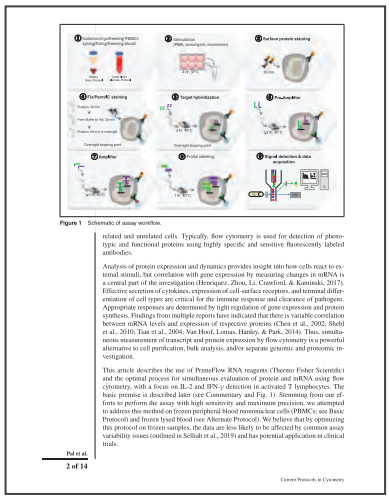

Figure 1 Schematic of assay workflow.

related and unrelated cells. Typically, flow cytometry is used for detection of phenotypic and functional proteins using highly specific and sensitive fluorescently labeled antibodies.

Analysis of protein expression and dynamics provides insight into how cells react to external stimuli, but correlation with gene expression by measuring changes in mRNA is a central part of the investigation (Henriquez, Zhou, Li, Crawford, & Kaminski, 2017). Effective secretion of cytokines, expression of cell-surface receptors, and terminal differentiation of cell types are critical for the immune response and clearance of pathogens. Appropriate responses are determined by tight regulation of gene expression and protein synthesis. Findings from multiple reports have indicated that there is variable correlation between mRNA levels and expression of respective proteins (Chen et al., 2002; Shebl et al., 2010; Tian et al., 2004; Van Hoof, Lomas, Hanley, & Park, 2014). Thus, simultaneous measurement of transcript and protein expression by flow cytometry is a powerful alternative to cell purification, bulk analysis, and/or separate genomic and proteomic investigation.

This article describes the use of PrimeFlow RNA reagents (Thermo Fisher Scientific) and the optimal process for simultaneous evaluation of protein and mRNA using flow cytometry, with a focus on IL-2 and IFN-γ detection in activated T lymphocytes. The basic premise is described later (see Commentary and Fig. 1). Stemming from our efforts to perform the assay with high sensitivity and maximum precision, we attempted to address this method on frozen peripheral blood mononuclear cells (PBMCs; see Basic Protocol) and frozen lysed blood (see Alternate Protocol). We believe that by optimizing this protocol on frozen samples, the data are less likely to be affected by common assay variability issues (outlined in Selliah et al., 2019) and has potential application in clinical trials.

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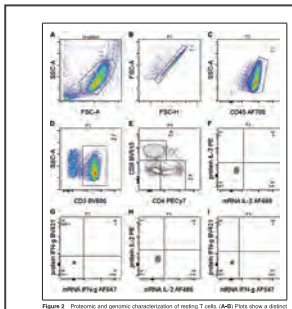

Figure 2 Proteomic and genomic characterization of resting T cells. (**A–B**) Plots show a distinct population of interest. Fewer non-viable cells, debris, and doublets are present. (**C–E**) Surface protein populations are well defined and signal is well preserved. (**F–I**) Intracellular cytokine protein or mRNA is not detected in resting T cells.

Compensation must be performed to eliminate any spectral spillover resulting from overlapping emission spectra between fluorochromes. A combination of automatic compensation and manual compensation should be considered.

51. Ensure FSC-height is active for doublet discrimination.

52. Acquire samples at an optimal flow rate (typically, low to medium works best).

Perform gating and data analysis

The gating strategy is outlined in Table 3. Representative cytograms for resting T cells and stimulated PBMCs are shown in Figures 2 and 3, respectively.

53. Begin by evaluating the scatter parameters (plot A), forward scatter area (FSC-A) versus side scatter area (SSC-A), to ensure that the leukocyte population is within scale. Assess viability and exclude debris.

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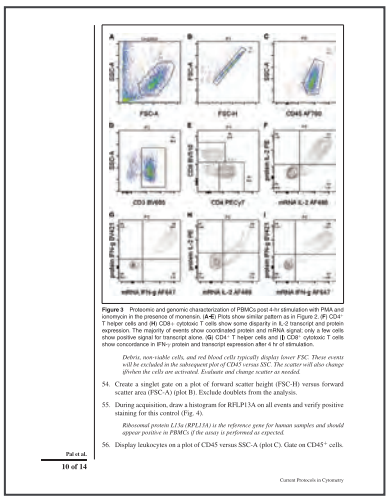

Figure 3 Proteomic and genomic characterization of PBMCs post-4-hr stimulation with PMA and ionomycin in the presence of monensin. (**A–E**) Plots show similar pattern as in Figure 2. (**F**) CD4+ T helper cells and (**H**) CD8+ cytotoxic T cells show some disparity in IL-2 transcript and protein expression. The majority of events show coordinated protein and mRNA signal; only a few cells show positive signal for transcript alone. (**G**) CD4+ T helper cells and (**I**) CD8+ cytotoxic T cells show concordance in IFN-γ protein and transcript expression after 4 hr of stimulation.

Debris, non-viable cells, and red blood cells typically display lower FSC. These events will be excluded in the subsequent plot of CD45 versus SSC. The scatter will also change if/when the cells are activated. Evaluate and change scatter as needed.

54. Create a singlet gate on a plot of forward scatter height (FSC-H) versus forward scatter area (FSC-A) (plot B). Exclude doublets from the analysis.

55. During acquisition, draw a histogram for RFLP13A on all events and verify positive staining for this control (Fig. 4).

Ribosomal protein L13a (RPL13A) is the reference gene for human samples and should appear positive in PBMCs if the assay is performed as expected.

56. Display leukocytes on a plot of CD45 versus SSC-A (plot C). Gate on CD45+ cells.

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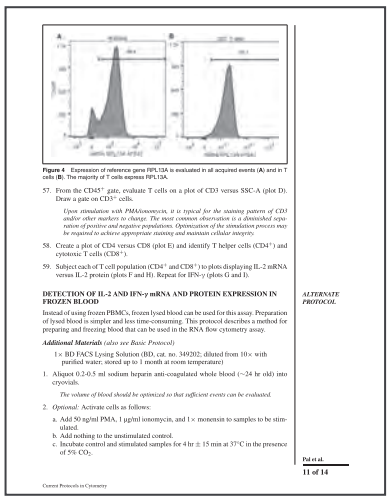

Figure 4 Expression of reference gene RPL13A is evaluated in all acquired events (**A**) and in T cells (**B**). The majority of T cells express RPL13A.

57. From the CD45+ gate, evaluate T cells on a plot of CD3 versus SSC-A (plot D). Draw a gate on CD3+ cells.

Upon stimulation with PMA/ionomycin, it is typical for the staining pattern of CD3 and/or other markers to change. The most common observation is a diminished separation of positive and negative populations. Optimization of the stimulation process may be required to achieve appropriate staining and maintain cellular integrity.

58. Create a plot of CD4 versus CD8 (plot E) and identify T helper cells ($CD4^+$) and cytotoxic T cells ($CD8^+$).

59. Subject each of T cell population ($CD4^+$ and $CD8^+$) to plots displaying IL-2 mRNA versus IL-2 protein (plots F and H). Repeat for IFN-γ (plots G and I).

DETECTION OF IL-2 AND IFN-γ mRNA AND PROTEIN EXPRESSION IN FROZEN BLOOD

ALTERNATE PROTOCOL

Instead of using frozen PBMCs, frozen lysed blood can be used for this assay. Preparation of lysed blood is simpler and less time-consuming. This protocol describes a method for preparing and freezing blood that can be used in the RNA flow cytometry assay.

Additional Materials (also see Basic Protocol)

1× BD FACS Lysing Solution (BD, cat. no. 349202; diluted from 10× with purified water; stored up to 1 month at room temperature)

1. Aliquot 0.2-0.5 ml sodium heparin anti-coagulated whole blood (~24 hr old) into cryovials.

The volume of blood should be optimized to that sufficient events can be evaluated.

2. *Optional:* Activate cells as follows:
   a. Add 50 ng/ml PMA, 1 μg/ml ionomycin, and 1× monensin to samples to be stimulated.
   b. Add nothing to the unstimulated control.
   c. Incubate control and stimulated samples for 4 hr ± 15 min at 37°C in the presence of 5% CO_2.

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3. Add 2-5 ml of 1× BD FACS Lysing Solution (for a 1:10 ratio of blood to lysing solution) and vortex.
4. Incubate 12-15 min in the dark at room temperature.
5. Store samples immediately at −80°C at least overnight.
6. Thaw control and stimulated samples in a 37°C water bath.
7. Centrifuge samples at 600 × g for 5 min at room temperature with brake.
8. Decant, hand mix to resuspend cells in residual volume, and then add 1 ml Stain Buffer FBS.
9. Proceed to surface staining and remainder of protocol (see Basic Protocol, steps 15-59).

COMMENTARY

Background Information

Given the critical importance of cellular heterogeneity, whether due to extrinsic or intrinsic factors (recently reviewed in Goldrath et al., 2019), simultaneous assessment of gene and protein level fluctuations at a single-cell resolution is essential. Flow cytometry has been the gold standard for high-throughput detection of intracellular or surface proteins at the single-cell level, but a lack of reagent availability and discordant data from proteomic and genomic investigations have been common problems.

Historically, analysis of gene expression has been performed using reverse transcriptase polymerase chain reaction (RT-PCR), RNA sequencing (RNA-Seq) and microarray technologies. While these molecular approaches are sequence based and not constrained by reagent availability, the output is a bulk, cell average. Recent advances in next-generation sequencing have led to single-cell RNA sequencing, which combined with cytometry (fluorescence-activated cell sorting) can provide global insights into the proteome and transcriptome of a sorted cell population (Freeman von Bieseiager, Pfeifer, & Fenaeke, 2018). However, this two-step process requires a high level of expertise in cell sorting. Furthermore, it is restricted to sorted cell populations and may not provide the flexibility of multiparametric investigation.

PCR-driven fluorescence in situ hybridization in combination with flow cytometry has been employed for simultaneous measurement of intracellular viral DNA and cell-surface protein expression to determine the number of virus-infected T cells (Patterson, Gioulsby, Hodara, Lohman, & Wolinsky, 1995). This technique opened the avenue for detection of mRNA at single-cell resolution by flow cytometry. Some of the improvements that have been made in recent years include increased sensitivity to detect low-abundance mRNA and less repetitive sequences, reduced autofluorescence caused by higher temperature required for hybridization, and finally the expansion of the technology to enable simultaneous detection of intracellular protein, extracellular protein, and RNA.

This protocol describes the application of the flow cytometry–based PrimeFlow RNA assay by Thermo Fisher Scientific. It combines a conventional flow cytometry cell staining for detection of proteins with fluorescence in situ hybridization and branched DNA technology for amplification and detection of RNA transcripts by flow cytometry (Fig. 1). Cells are initially labeled with surface antibodies, then fixed, permeabilized, and labeled with antibodies for detection of intracellular proteins. Next, a pair of gene-specific oligonucleotide probes, 20-40 bp in length, is hybridized in close proximity to the RNA transcript to create the foundation for branched DNA signal amplification. Presently, four target probes with unique tag sequences can be used together (Alexa Fluor 647-type 1, Alexa Fluor 568-type 10, Alexa Fluor 488-type 4, and Alexa Fluor 750-type 6). Each tag sequence allows hybridization of complementary DNA branches with different excitation/emission spectra. Once the target probes are annealed appropriately, signal amplification is achieved through sequential hybridization with DNA branches (pre-amplifiers and amplifiers). Pre-amplifiers complementarily bind to the anhybridized section of each target probe pair to form the first DNA branch. The signal is further magnified by a second amplification step in which amplifiers hybridize to multiple sites of each pre-amplifier, thus forming the second DNA branch. Finally, fluorochrome-conjugated label probes hybridize to the amplifier and

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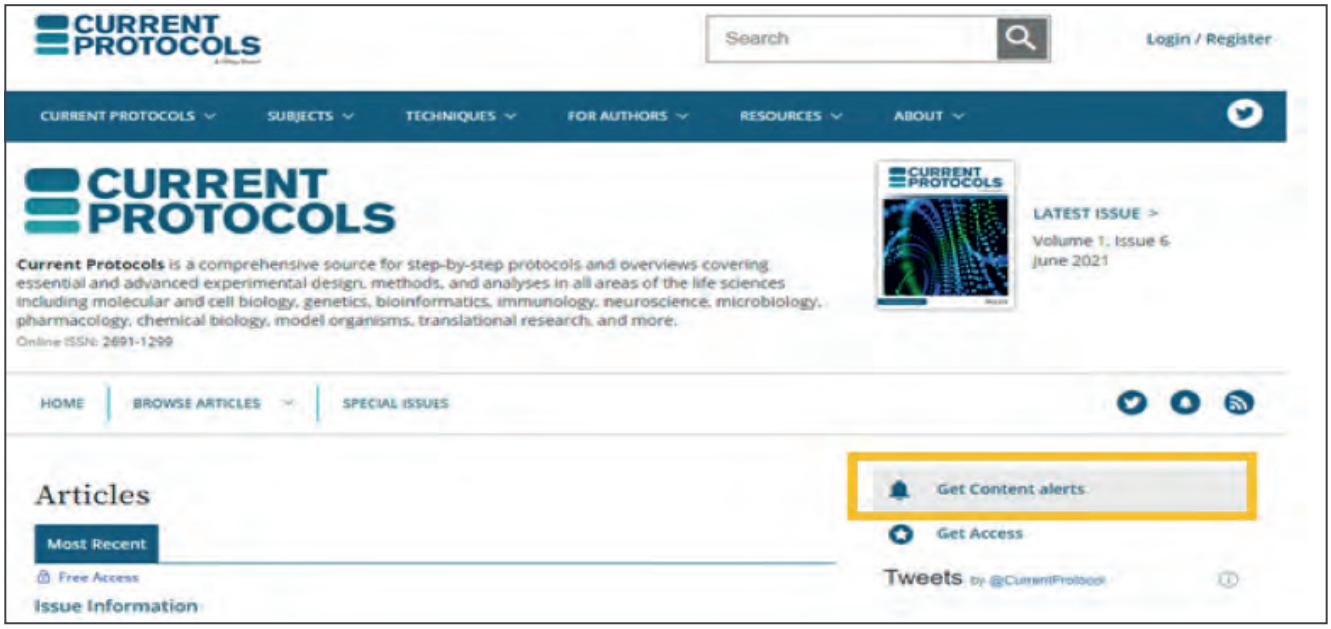

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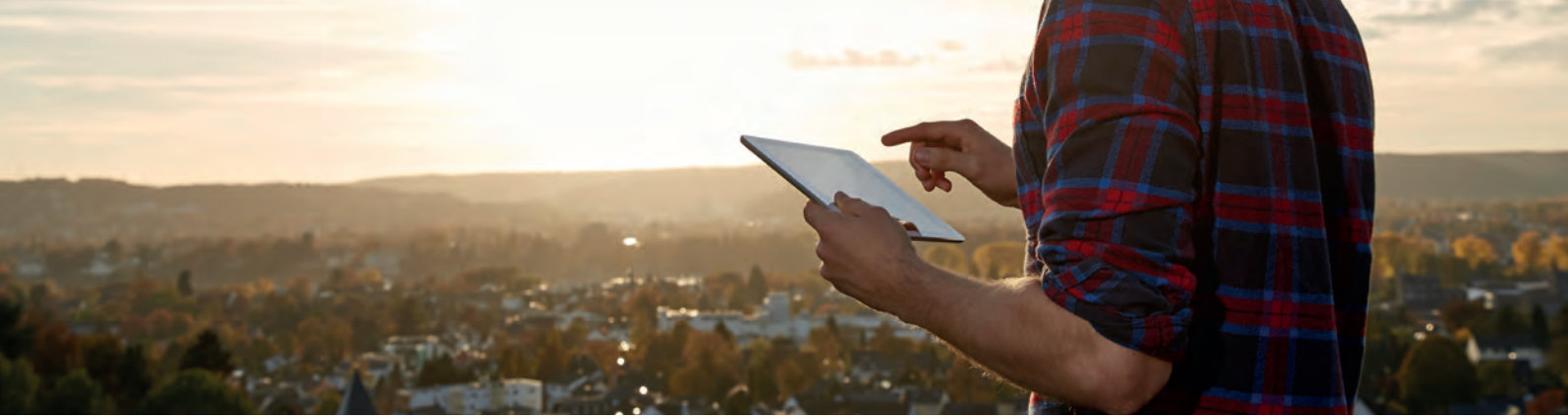

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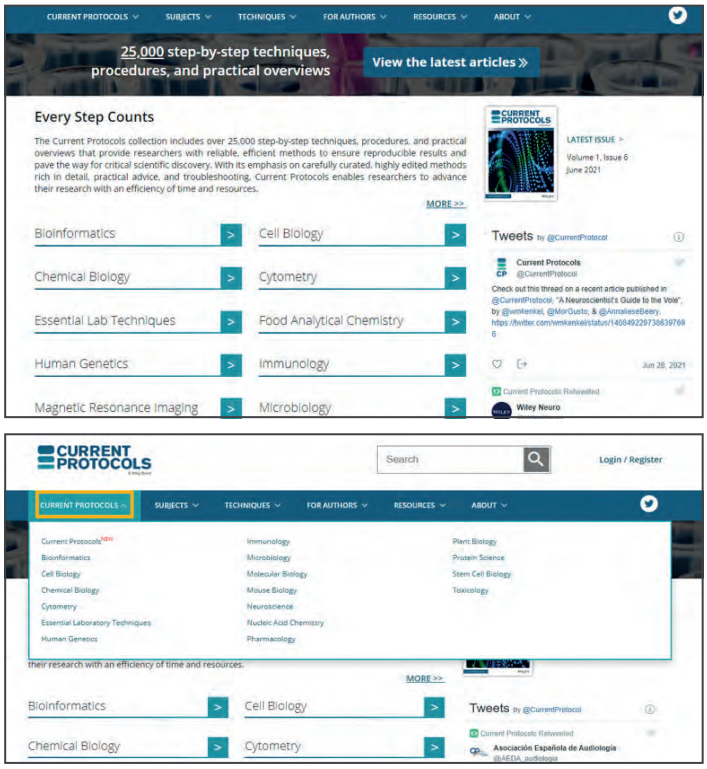

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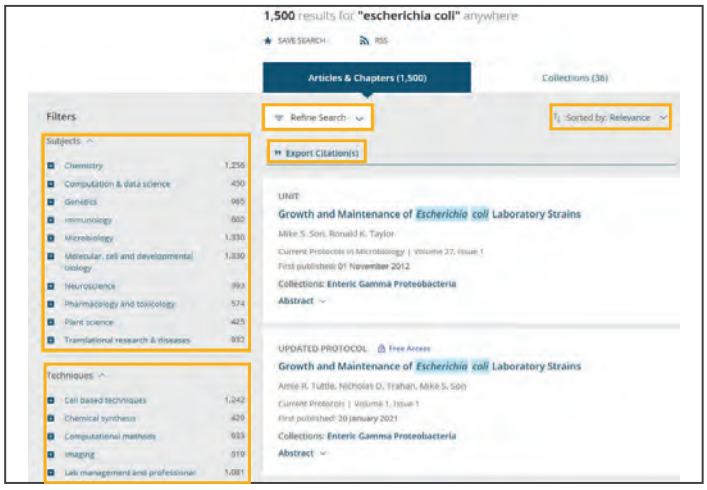

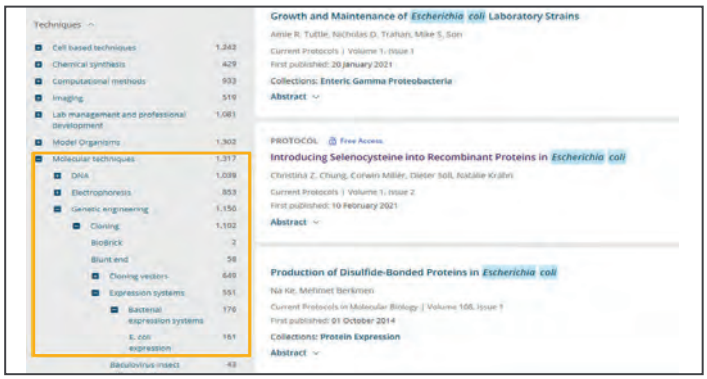

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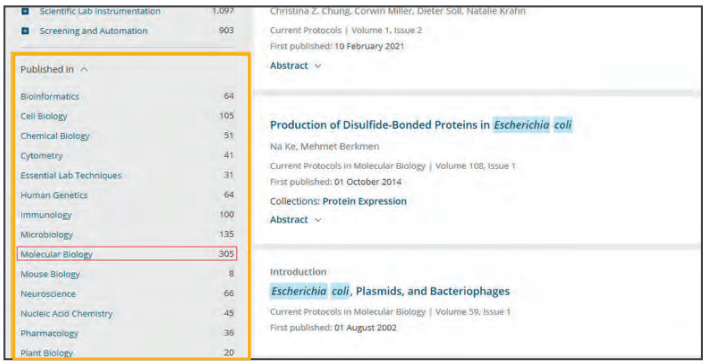

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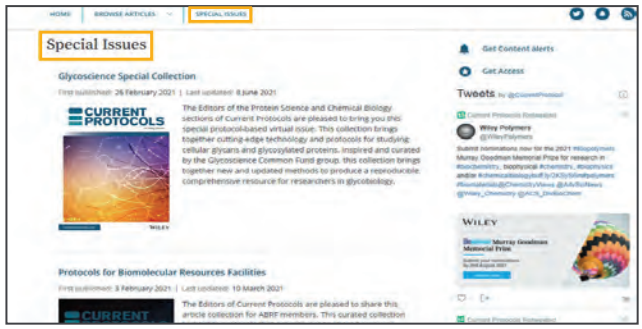

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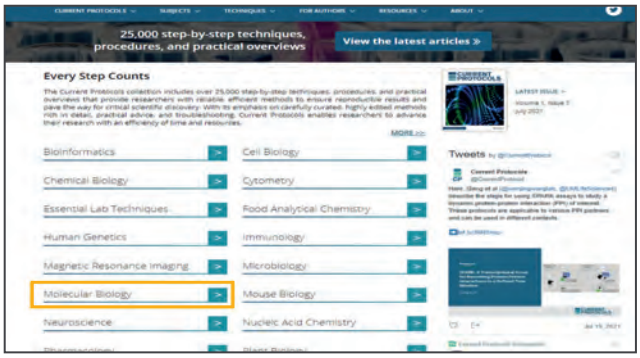

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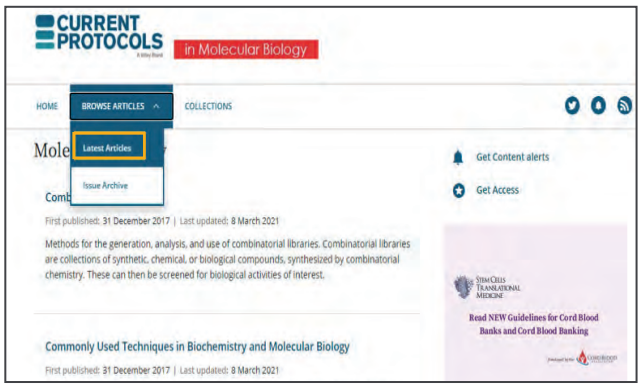

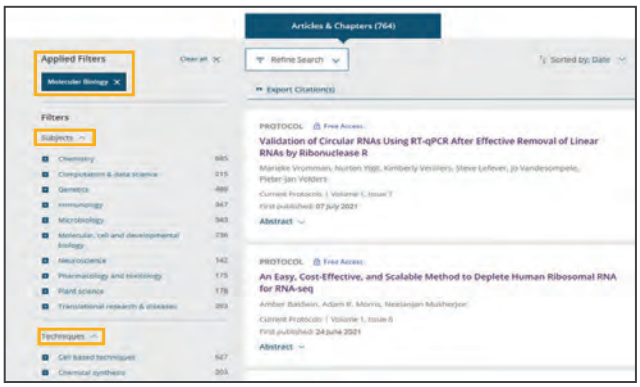

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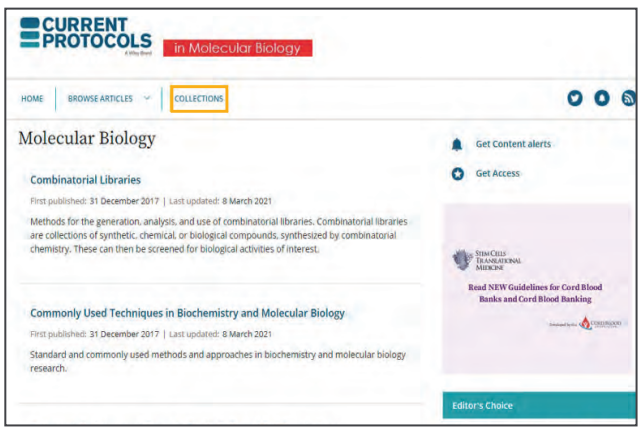

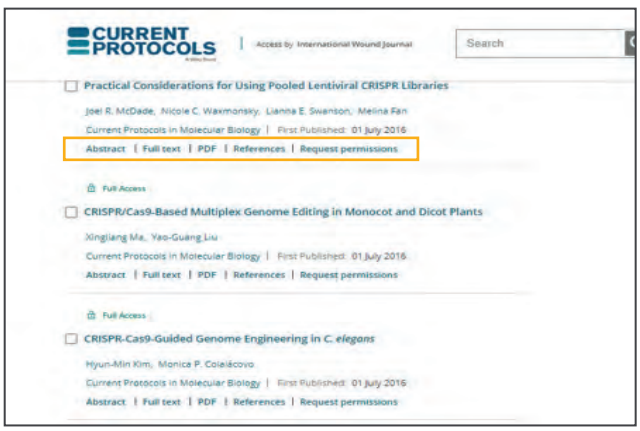

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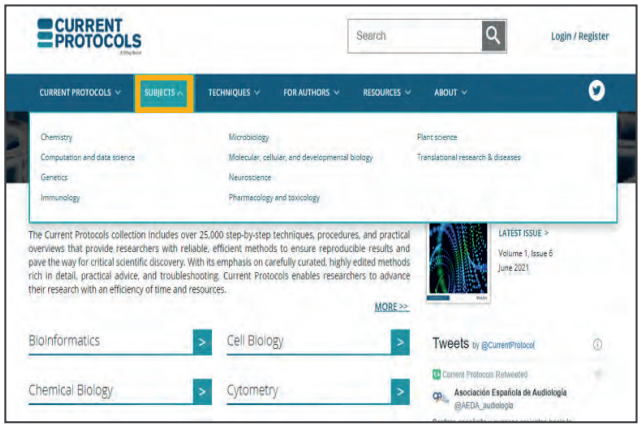
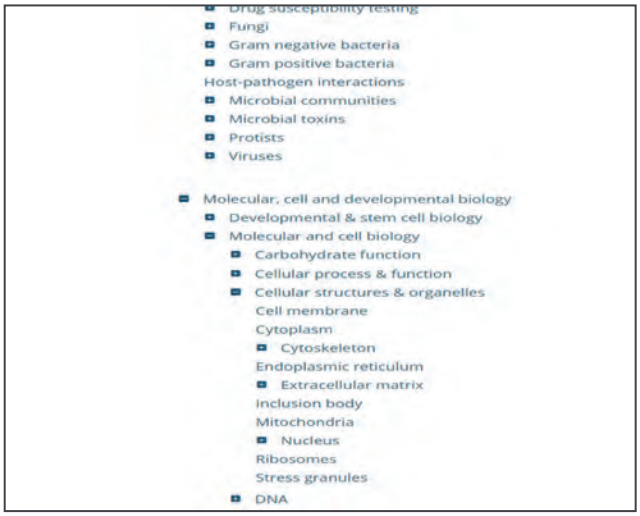
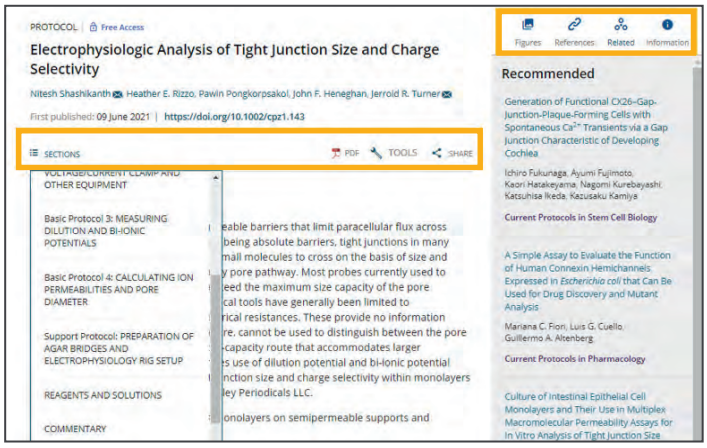
您可以按学科或实验技术浏览Current Protocols完整合集，还可在Current Protocols主页上的下拉菜单中选择。选择您最感兴趣的主题即可进入显示完整Subject（学科）或Technique（技术）的分类页面。还可扩展到您感兴趣的特定领域。单击您感兴趣的类别即可显示有关该主题的CP合集中的全部文章。可以按照上文“Search ”部分中介绍的方法对该列表进行限定。





7 Protocols功能导航

HTML模式下有许多内容和功能。



顶部导航栏

- Sections（章节）：打开下拉菜单，选择您想阅读的部分。
- PDF：获取PDF格式，您可以根据需求选择下载、放大/缩小和打印。
- Tools（工具）：申请权限，添加到收藏夹或跟踪引文。
- Share（分享）：与他人分享内容。

右侧信息栏

- Figures（图表）：指南中所有图表均可查看并可下载png或ppt格式。
- References（参考文献）：可查看该指南引用的一次和二次文献。
- Related（相关）：查看相关指南及与本指南相关的其他文章。
- Information（信息）：查看信息例如metrics指标、相关关键词（带有超链接能直接进行新检索）、出版历史和版权信息。

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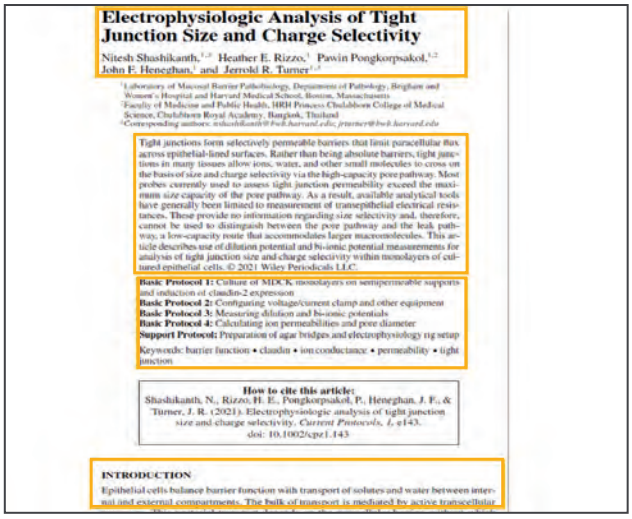
8 Protocols结构

- Title标题
- Author(s)作者
- Abstract摘要，包括了文章中所包含的指南列表
- 文章Introduction（引言）


Electrophysiologic Analysis of Tight Junction Size and Charge Selectivity

Nitesh Shashikanth, Heather E. Rizzo, Pawin Pongkorpsakol, John F. Heneghan, and Jerrold R. Turner

Tight junctions form selectively permeable barriers that limit paracellular flux across epithelial-lined surfaces. Rather than being absolute barriers, tight junctions in many tissues allow ions, water, and other small molecules to cross on the basis of size and charge selectivity via the high-capacity pore pathway. Most probes currently used to assess tight junction permeability exceed the maximum size capacity of the pore pathway. As a result, available analytical tools have generally been limited to measurement of transepithelial electrical resistances. These provide no information regarding size selectivity and, therefore, cannot be used to distinguish between the pore pathway and the leak pathway, a low-capacity route that accommodates larger macromolecules. This article describes use of dilution potential and bi-ionic potential measurements for analysis of tight junction size and charge selectivity within monolayers of cultured epithelial cells. © 2021 Wiley Periodicals LLC

Basic Protocol 1: Culture of MDCK monolayers on semipermeable supports and induction of claudin-2 expression
Basic Protocol 2: Configuring voltage/current clamp and other equipment
Basic Protocol 3: Measuring dilution and bi-ionic potentials
Basic Protocol 4: Calculating ion permeabilities and pore diameter
Support Protocol: Preparation of agar bridges and electrophysiology rig setup

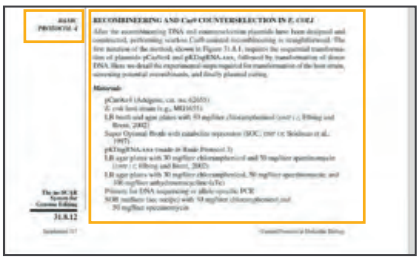
Keywords: barrier function • claudin • ion conductance • permeability • tight junction

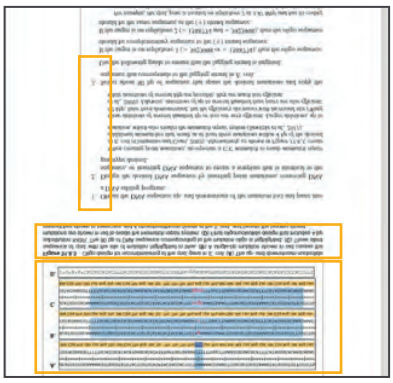
How to cite this article:
Shashikanth, N., Rizzo, H. E., Pongkorpsakol, P., Heneghan, J. F., & Turner, J. R. (2021). Electrophysiologic analysis of tight junction size and charge selectivity. *Current Protocols*, *1*, e143. doi: 10.1002/cpz1.143

INTRODUCTION

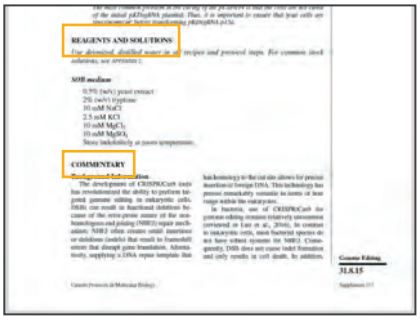
Epithelial cells balance barrier function with transport of solutes and water between internal and external compartments. The bulk of transport is mediated by active transcellular

- Basic, Alternate and Support Protocols（基本、替代和支持实验流程）的指南全部涵盖。文章中的每份指南都有自己的标题、引言和材料清单，包含试剂来源的信息。





- Protocol steps（指南步骤）：所有步骤都有编号，每步都有详细说明。
- 附有提示作用的斜体注释，以及替代方法和附加信息。


REAGENTS AND SOLUTIONS

COMMENTARY

- Reagents and Solutions（试剂和溶液配方）提供了所需要的试剂、溶液和培养基的详细配方。
- Commentary（评论）：背景信息、重要参数、故障排除、预期结果、时间因素、文献引用。

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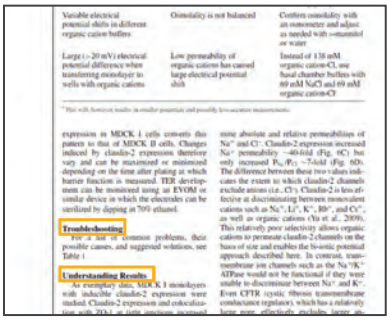
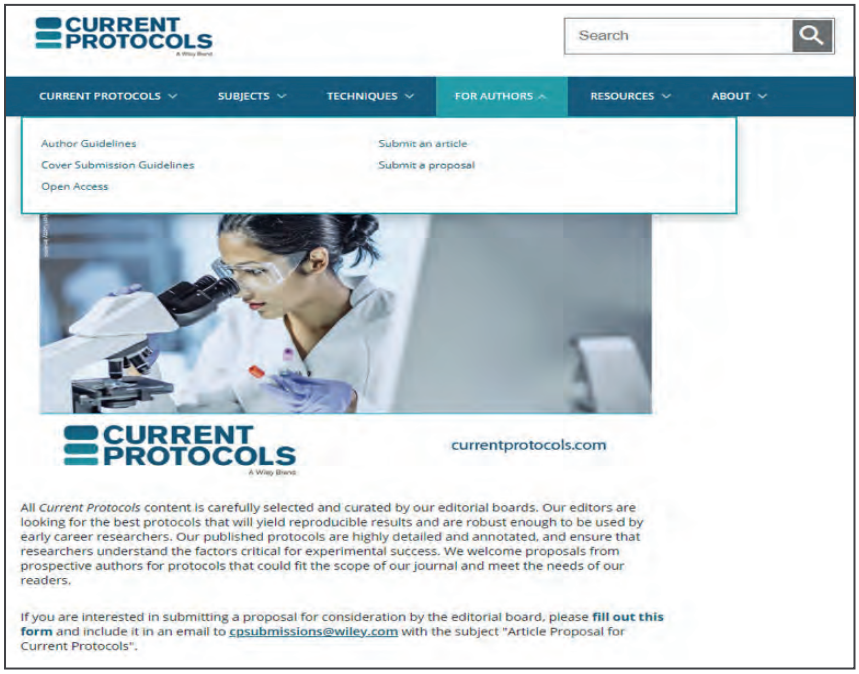
- Troubleshooting（故障排除）：未按预期实验发展的处置方法。
- Understanding Results（预期结果）



Protocols的发表

如您想发表一份指南，请访问您感兴趣的标题。在顶部导航栏中单击For Authors。在下拉菜单中选择Submit a proposal。编辑委员将对您的proposal进行评审，如果合适，将邀请您提交详细描述实验方法的稿件。



可以通过扫描以下二维码提交方案。

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