The Big Picture

Specific Aims

Session Plan

- Discuss the importance and purpose of the Specific Aims page.
- Describe a framework for organizing the Specific Aims page based on its components.
- Dissect examples of Specific Aims pages.

How Important is the Specific Aims Page?

The Specific Aims page…

- **Is the most important part of your application!**
- Outlines the "big picture" of your project.
- Is an executive summary of your plan.
- Is your primary marketing document.

Therefore, the Specific Aims page must…

- Be compelling.
- Excite.
- Move your primary reviewer and, hopefully, all three reviewers to be your advocate.

Create enthusiasm!

It also must touch on all review criteria.

- Significance
- Innovation
- Investigator
- Approach
- Environment

Overall Impact

The likelihood for the project to exert a **sustained, powerful influence** on the research field(s) involved, in consideration of the five core review criteria.

Preparing to Write the Specific Aims Page

Where to Start

- Always consult the guidelines.
  - SF424 (R&R)- Forms Version F.
- You must have well-defined aims!
- The **content** of the Specific Aims page **derives** largely from the **Significance** and **Innovation** sections and from **Preliminary Studies**.
  - **Write the Significance and Innovation sections first.**
- Recognize that refining the aims is an iterative process.

NIH Guideline for Specific Aims Page

- State concisely the **goals** of the proposed research and summarize the **expected outcome(s)**, including the **impact** that the results of the proposed research will exert on the **research field(s) involved**.
- List succinctly the **specific objectives** of the research proposed (e.g., to test a stated hypothesis, create a novel design, solve a specific problem, challenge an existing paradigm or clinical practice, **address a critical barrier to progress in the field**, or develop new technology).

Use the Proposal Worksheet

We have provided a proposal worksheet to help you build the Specific Aims page.

- The worksheet is comprised of questions derived from the NIH review criteria.
  - Significance
  - Innovation
  - Investigator
  - Environment
  - Approach
- In completing the worksheet, you will identify, articulate, and organize the issues that are critical for the review of your proposal.

This must be done in writing!

Main Topics of Proposal Worksheet and Framework for Specific Aims Page

- Overarching problem/big picture and overall goal.
- Context and setting.
- Central hypothesis.
- Specific aims and experimental overview.
- Expected outcomes and impact.
- If possible, develop and include a graphic representation of the project (e.g., flow chart or working model).

Writing the Specific Aims Page

Form of the Specific Aims Page

General, compelling introduction of topic to capture reader (overarching problem). Broad description of what is known about problem and what questions remain unanswered that your overall goal may address. What has been achieved toward that goal (your research and that of others) and specific gaps in knowledge you now plan to address. Hypothesis based on preliminary data. Specific aims to test hypothesis. Experiments that support the aims. IMPACT.

An Organizational Framework

- Overarching problem/big picture and overall goal.
- Context and setting.
- Central hypothesis.
- Specific aims and experimental overview.
- Expected outcomes and impact.

Follows worksheet organization

Specific Aims Page Framework

Overarching Problem and Overall Goal

- In a few interest-grabbing sentences, define the big picture
  - Overarching public health problem/focus of your work.
  - Your goal (long-term or immediate) with respect to the problem.
  - Specific aspect of the problem addressed by your current proposal.

Specific Aims Page Framework

Context and Setting

- Briefly, summarize the current state of knowledge regarding the goal of your current proposal, drawing on/evaluating the contributions of others and your own work/preliminary studies.
- Define the specific gap in knowledge/challenge impeding further progress that your proposal will address.
- Define the importance of addressing this specific gap/challenge to human health and to advancing your field of research.

Specific Aims Page Framework

Central Hypothesis

- State the overall hypothesis, derived from preliminary data, that the studies proposed in your Specific Aims will test, with the objective of filling the gap.
- The overall hypothesis should encompass all aims and should relate directly to the overall goal and public health problem.

Specific Aims Page Framework

Specific Aims/Experimental Overview

- Define at least 2 and no more than 4 specific aims, beginning each with a verb (e.g., determine, evaluate, identify, elucidate, explore).
- For each aim, very briefly describe the general experimental design and/or methods you will use.

Specific Aims Page Framework

Specific Aims…

- Are concrete, well-focused **objectives** that logically flow from your hypothesis and are intended to test its validity.
- Are interconnected, but not interdependent.
- Have clear endpoints — achievable within the time frame of your proposal — that reviewers can easily assess.
- Should demonstrate advancement in your work.

Specific Aims Page Framework

Wording the Aims

Too vague

- Specific Aim: To conduct statistical analysis on already collected data.

Too detailed

- Specific Aim 1. To correlate urinary concentration of X with food consumption patterns available through a questionnaire, thereby identifying foods that are major contributors of X.

Just right

- Specific Aim 1. Identify foods that are major dietary contributors of X.
- Aim 2. Investigate the effects of SON E upregulation on gene expression, cell proliferation, and self-renewal in hematopoietic stem cells.

Specific Aims Page Framework

Expected Outcomes and Impact

- Define the expected impact of your success in achieving the goals of your proposal.
- In other words, what will be possible/known that was not possible/known before, with respect to
  - Human health and disease?
  - Advancement of your field of research?

Relate this to the **overarching problem** stated at the beginning of the Aims page.

Sample Specific Aims Pages

Specific Aims Page: Example 1

- Peter John Myler, PhD, and Marilyn Parsons, PhD "Ribosome profiling of *Trypanosoma brucei*" (R21)

Overarching problem, Gap in knowledge

Preliminary data, Overview of approach

Aims, Experimental design

Outcomes and impact →

2. Specific Aims

Gene expression in trypanosomatids (such as *Trypanosoma brucei* and the various *Leishmania* species) is distinct from other well-studied eukaryotes because the protein-coding genes are transcribed polycistronically. However, co-transcribed mRNAs encode proteins that display dramatic variation in abundance both within and across developmental stages, indicating that post-transcriptional controls provide the major means of regulating expression of individual genes. Our previous microarray study has shown significant differences in mRNA abundance within and across *T. brucei* bloodstream and insect stages (likely reflecting differences in mRNA stability), while other studies have identified considerable changes in the proteome. A recent global analysis of mRNA levels and protein abundances (from the same biological samples) at several time-points during promastigote-to-amastigote differentiation of *L. donovani* (conducted by the Myler lab) showed that the correlation between these is rather low. However, both microarrays and proteomic analysis are limited by a lack resolution in quantitation of lower abundance molecules, leaving the true correlation between mRNA and protein levels open to question. Furthermore, other data suggests that translational and/or post-translational controls also play significant roles. For example, in-depth analysis (by the Parsons lab) of two *T. brucei* genes demonstrated translational control as a key mechanism. We therefore hypothesize that translational controls function both to tune the levels of protein within stages and to change the levels across stages. This project seeks to address this hypothesis by quantitatively assessing the rate at which cellular mRNAs are being actively translated at any particular time. This will be accomplished by adapting and applying a recently-described technology that couples the ability to isolate the specific "footprints" of mRNAs that are occupied by ribosomes (an indicator of translation) with the depth and breadth of next generation sequencing (NGS). To establish the system and test our hypothesis for *T. brucei*, we propose the following Specific Aims.

Aim 1. Establish the ribosome protection assay in *T. brucei* strain 927 cultured procyclic forms. Optimization of footprinting, library construction and informatics will be done using cultured log-phase procyclic forms, which are readily available under standardized conditions. Cell lysates will be treated with RNase I and ribosome-protected RNA fragments will be isolated and used to generate libraries for sequencing *via* Illumina NGS technology. The resulting data will be entered into our RNA-seq pipeline and aligned with the *T. brucei* genome to identify the number and location of ribosomes that are bound to gene-specific mRNA. This data will indicate the level of gene-specific translation for every gene detected, as well as identifying the specific sequences on each mRNA that are translated. Comparison with the profile of total cellular mRNA will establish the translational efficiency of transcripts corresponding to specific genes.

Aim 2. Identify genes that are regulated at the level of translation during *T. brucei* development. We will carry out similar studies on rapidly-dividing, mammalian-infective slender bloodstream forms and non-dividing stumpy bloodstream forms from animals. Comparison of the ribosome profile of mRNAs at these stages and that of procyclic forms (from Aim 1) will identify genes that are regulated at the level of translation.

The proposed work promises to provide an important new tool for studying trypanosomatid gene expression, yielding clues to the mechanism of translational control in trypanosomatids, and new information on the extent of translation of individual gene products. In addition, it should resolve the current debate over the function of the numerous recently identified RNAs that contain only short open-reading frames, and has the potential to identify non-canonical open-reading frames, thus significantly enhancing the ongoing genome annotation. We also anticipate that this technology will be very useful to those researchers wishing to determine which trypanosomatid proteins are likely to be present in infective stages, and thus might serve as drug and vaccine targets.

Specific Aims Page: Example 1

Overarching Problem/"Big Picture"

Gene expression in trypanosomatids (such as *Trypanosoma brucei* and the various *Leishmania* species) is distinct from other well-studied eukaryotes because the protein-coding genes are transcribed polycistronically. However, co-transcribed mRNAs encode proteins that display dramatic variation in abundance both within and across developmental stages, indicating that post-transcriptional controls provide the major means of regulating expression of individual genes.

Specific Aims Page: Example 1

Context and Setting (Preliminary Data)

Gene expression in trypanosomatids (such as *Trypanosoma brucei* and the various *Leishmania* species) is distinct from other well-studied eukaryotes because the protein-coding genes are transcribed polycistronically. However, co-transcribed mRNAs encode proteins that display dramatic variation in abundance both within and across developmental stages, indicating that post-transcriptional controls provide the major means of regulating expression of individual genes. Our previous microarray study has shown significant differences in mRNA abundance within and across *T. brucei* bloodstream and insect stages (likely reflecting differences in mRNA stability), while other studies have identified considerable changes in the proteome. A recent global analysis of mRNA levels and protein abundances (from the same biological samples) at several time-points during promastigote-to-amastigote differentiation of *L. donovani* (conducted by the Myler lab) showed that the correlation between these is rather low.

Specific Aims Page: Example 1

Specific Gap in Knowledge

Gene expression in trypanosomatids (such as *Trypanosoma brucei* and the various *Leishmania* species) is distinct from other well-studied eukaryotes because the protein-coding genes are transcribed polycistronically. However, co-transcribed mRNAs encode proteins that display dramatic variation in abundance both within and across developmental stages, indicating that post-transcriptional controls provide the major means of regulating expression of individual genes. Our previous microarray study has shown significant differences in mRNA abundance within and across *T. brucei* bloodstream and insect stages (likely reflecting differences in mRNA stability), while other studies have identified considerable changes in the proteome. A recent global analysis of mRNA levels and protein abundances (from the same biological samples) at several time-points during promastigote-to-amastigote differentiation of *L. donovani* (conducted by the Myler lab) showed that the correlation between these is rather low. However, both microarrays and proteomic analysis are limited by a lack resolution in quantitation of lower abundance molecules, leaving the true correlation between mRNA and protein levels open to question. Furthermore, other data suggests that translational and/or post- translational controls also play significant roles. For example, in-depth analysis (by the Parsons lab) of two T. brucei genes demonstrated translational control as a key mechanism.

Specific Aims Page: Example 1

Hypothesis/Project's Goal

Gene expression in trypanosomatids (such as *Trypanosoma brucei* and the various *Leishmania* species) is distinct from other well-studied eukaryotes because the protein-coding genes are transcribed polycistronically. However, co-transcribed mRNAs encode proteins that display dramatic variation in abundance both within and across developmental stages, indicating that post-transcriptional controls provide the major means of regulating expression of individual genes. Our previous microarray study has shown significant differences in mRNA abundance within and across *T. brucei* bloodstream and insect stages (likely reflecting differences in mRNA stability), while other studies have identified considerable changes in the proteome. A recent global analysis of mRNA levels and protein abundances (from the same biological samples) at several time-points during promastigote-to-amastigote differentiation of *L. donovani* (conducted by the Myler lab) showed that the correlation between these is rather low. However, both microarrays and proteomic analysis are limited by a lack resolution in quantitation of lower abundance molecules, leaving the true correlation between mRNA and protein levels open to question. Furthermore, other data suggests that translational and/or post- translational controls also play significant roles. For example, in-depth analysis (by the Parsons lab) of two T. brucei genes demonstrated translational control as a key mechanism. We therefore hypothesize that translational controls function both to tune the levels of protein within stages and to change the levels across stages. This project seeks to address this hypothesis by quantitatively assessing the rate at which cellular mRNAs are being actively translated at any particular time.

Specific Aims Page: Example 1

Overview of Approach

Gene expression in trypanosomatids (such as *Trypanosoma brucei* and the various *Leishmania* species) is distinct from other well-studied eukaryotes because the protein-coding genes are transcribed polycistronically. However, co-transcribed mRNAs encode proteins that display dramatic variation in abundance both within and across developmental stages, indicating that post-transcriptional controls provide the major means of regulating expression of individual genes. Our previous microarray study has shown significant differences in mRNA abundance within and across *T. brucei* bloodstream and insect stages (likely reflecting differences in mRNA stability), while other studies have identified considerable changes in the proteome. A recent global analysis of mRNA levels and protein abundances (from the same biological samples) at several time-points during promastigote-to-amastigote differentiation of *L. donovani* (conducted by the Myler lab) showed that the correlation between these is rather low. However, both microarrays and proteomic analysis are limited by a lack resolution in quantitation of lower abundance molecules, leaving the true correlation between mRNA and protein levels open to question. Furthermore, other data suggests that translational and/or post- translational controls also play significant roles. For example, in-depth analysis (by the Parsons lab) of two T. brucei genes demonstrated translational control as a key mechanism. We therefore hypothesize that translational controls function both to tune the levels of protein within stages and to change the levels across stages. This project seeks to address this hypothesis by quantitatively assessing the rate at which cellular mRNAs are being actively translated at any particular time. This will be accomplished by adapting and applying a recently described technology that couples the ability to isolate the specific "footprints" of mRNAs that are occupied by ribosomes (an indicator of translation) with the depth and breadth of next generation sequencing (NGS).

Specific Aims Page: Example 1

Specific Aims/General Experimental Design

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Specific Aims Page: Example 1

Specific Aims/General Experimental Design

Aim 2. Identify genes that are regulated at the level of translation during T. brucei development.

We will carry out similar studies on rapidly dividing, mammalian-infective slender bloodstream forms and non-dividing stumpy bloodstream forms from animals. Comparison of the ribosome profile of mRNAs at these stages and that of procyclic forms (from Aim 1) will identify genes that are regulated at the level of translation.

Specific Aims Page: Example 1

Expected Outcome and Impact

The proposed work promises to provide an important new tool for studying trypanosomatid gene expression, yielding clues to the mechanism of translational control in trypanosomatids, and new information on the extent of translation of individual gene products. In addition, it should resolve the current debate over the function of the numerous recently identified RNAs that contain only short open-reading frames, and it has the potential to identify non-canonical open-reading frames, thus significantly enhancing the ongoing genome annotation. We also anticipate that this technology will be useful to researchers who wish to determine which trypanosomatid proteins are likely to be present in infective stages and, thus, might serve as drug and vaccine targets.

Specific Aims Page: Example 2

- Chad A. Rappleye, PhD

  "Forward genetics-based discovery of Histoplasma virulence genes" (R03)

Overarching problem, Gap in knowledge

Preliminary data, Hypothesis, Overview of approach

Aims, Experimental design

Outcomes and impact

Specific Aims

The fungal pathogen *Histoplasma capsulatum* causes an estimated 100,000 infections annually in the United States. While most infections are self limiting upon activation of adaptive immunity, thousands each year are hospitalized due to acute respiratory disease and life-threatening disseminated histoplasmosis. Unlike opportunistic fungal pathogens, *Histoplasma* causes disease even in immunocompetent individuals. By itself, the innate immune system is unable to control *Histoplasma* yeasts due to *Histoplasma*'s ability to parasitize host phagocytes. The mechanisms that enable *Histoplasma* to survive and replicate with macrophages, ultimately leading to destruction of the phagocyte, are only beginning to be defined.

As the *Histoplasma*-macrophage interaction is key to pathogenesis, our goal is to better understand the factors that enable intracellular growth of *Histoplasma*. Forward genetics is a powerful approach to identify new factors if an efficient mutagen and screen are employed. We have optimized and characterized an insertional mutagen for *Histoplasma* based on *Agrobacterium*-mediated transfer and random integration of T-DNA into fungal chromosomes. In addition, we have developed a high-throughput screen to facilitate identification of mutants unable to persist in the intramacrophage environment. For this, we developed an RFP-fluorescent *Histoplasma* strain and a transgenic *lacZ*-expressing macrophage cell line which permits quantitative monitoring of both intracellular yeast replication and macrophage destruction, respectively. The combination of these mutagenesis and screening advances provides the efficiency necessary for forward genetics-based discovery of new virulence factors that enable *Histoplasma* to overcome innate immune defenses and exploit the macrophage as its host cell.

Aim 1. Screen *Histoplasma* T-DNA insertion mutants for attenuated virulence in macrophages.

Aim 1A. Generate a library of T-DNA insertion mutants in *Histoplasma* yeast.
Agrobacterium-mediated transformation will be used to mutagenize *Histoplasma* yeasts. Individual mutants will be arrayed into 96-well plates to facilitate high-throughput screening and to enable banking of the mutant collection for long term preservation. A library of 40,000 mutants will be generated representing approximately 2.5-fold coverage of the *Histoplasma* genome.

Aim 1B. Identification of mutants deficient in survival and replication within macrophages.
Macrophages will be infected with individual *Histoplasma* mutants and the intramacrophage growth of yeast monitored non-destructively by measurement of yeast-expressed RFP fluorescence. End point macrophage lysis by yeast will be determined by quantifying the remaining macrophage-expressed β-galactosidase activity. *Histoplasma* mutants will be selected that exhibit at least 50% reduction in intramacrophage growth and/or at least 50% decreased ability to lyse macrophages

Aim 2. Determine the identify of genes required for *Histoplasma* virulence in macrophages.

Aim 2A. Map the disrupted loci in attenuated mutants.
Mutants will be tested by PCR to eliminate those with T-DNA disruption of genes known to be required for intramacrophage survival and growth. New virulence genes will be identified by mapping the T-DNA insertions through hemi-specific PCR techniques (e.g., thermal asymmetric interlaced PCR) and sequencing of the amplified regions flanking the T-DNA borders. Disrupted loci will be identified by comparison of sequences flanking the insertion to transcriptome-based gene models (best option) or de novo gene predictions (alternative).

Aim 2B. Classify and prioritize virulence mutants.
Mutants will be classified as: (1) deficient in macrophage entry, (2) impaired survival in macrophages, (3) normal survival but impaired replication in macrophages, and (4) normal replication but deficient ability to cause macrophage lysis. Candidate factors representing each class will be prioritized by the severity of the virulence attenuation, conservation of the factor among intracellular pathogens, and increased expression by pathogenic- compared to non-pathogenic-phase cells.

The virulence genes identified will form the basis of future studies to characterize the factors that promote *Histoplasma* pathogenesis in host macrophages.

Specific Aims Page: Example 2

Overarching Problem/"Big Picture"

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Specific Aims Page: Example 2

Specific Gap/Project's Goal

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Specific Aims Page: Example 2

Context (Preliminary Data)/ Overview of Approach

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Specific Aims Page: Example 2

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Specific Aims Page: Example 2

Expected Outcome and Impact

The virulence genes identified will form the basis of future studies to characterize the factors that promote *Histoplasma* pathogenesis in host macrophages.

Recap

- The Specific Aims page must…
  - Present an overview of the whole project.
  - Create enthusiasm for the project.
  - Touch on all review criteria, including Overall Impact.
- Use the worksheet!
- Continually revise Specific Aims as you develop the proposal.