NUTR 0225: Introduction to Modern Biology Techniques
Fall 2022

Class Meetings: *Tuesday, 1:30-3:00, Jaharis room 105*

Instructor(s): *Martin Obin, PhD, Martin.Obin@tufts.edu*

Instructor Office Hours: Zoom; time to be determined or *by appointment;*

Semester Hour Units: *1.5 SHUs*

Prerequisites: Graduate standing, undergraduate biochemistry or instructor permission

Course Description: This intensive course will familiarize you with techniques employed in nutrition science research at the molecular, cell, tissue, whole organism and population levels. An understanding of why and how these techniques are employed (especially in combination) is essential for reading the nutrition science literature, developing skills as a researcher, and appreciating the goals, current limitations and future potential of modern nutrition science research.

Please note: this course is not a 'hands-on' laboratory course.

At the outset we will use a historical approach to examine the conceptual underpinnings of modern, hypothesis-driven biological research, including the paradigms of "reductionism" vs "holism", the scientific (hypothetico-deductive) method, the use of model organisms, the nature of proof and the challenges of replicability. In weeks 2-15 we will cover nine (often overlapping and complementary) classes of techniques and experimental approaches listed below. The chronological sequence of presentation will not necessarily follow the outline below. Topics marked by * are also presented in BCHM 0223

1. **Separation, Isolation and Identification of Analytes in Complex Biological Samples**
   a) *Separation*: Extraction; Gas and Liquid Chromatography, 1D and 2D Gel Electrophoresis
   b) *Isolation/Purification*: (Co-) Immunoprecipitation, 'Pull-Downs,' Ultrafiltration
   c) *Identification, Quantitation and Structure*:
      i. Single Molecular Targets: Antibodies; ELISA and Western Blot
      ii. Multiple Targets: *Mass Spectrometry, *Nuclear Magnetic Resonance

2. **Cell Culture and Cell/Tissue Fractionation**
   a) *Cell Culture Basics*: Working in a cell culture 'hood'
   b) *Cell/Tissue Constituent Analysis:* Lysis, Subcellular Fractionation; UltraCentrifugation

3. **Flow Cytometry and Cell Sorting**
   a) Fluorescence
   b) *Flow Cytometry*: Flow Cytometer, Fluorophores, Filters, Compensation, Data Analysis
   c) *Bulk and Sequential Cell Sorting:* Beads and Fluorescence Activated Cell Sorting(FACS)

4. **Microscopy and Imaging**
   a) *Light Microscopy: Kohler illumination;* Bright Field, Dark field, Phase contrast
   b) Histology, Immunohistochemistry, In Situ Hybridization: How and Why*;* Limitations
   c) Fluorescence Microscopy: Set-up, Uses and Limitations
   d) Beyond Epifluorescence: Deconvolution, Confocal, Multi-Photon, Super-Resolution
   e) *In Vivo Imaging:* Computed Tomography (CT), Positron Emission Tomography (PET)

5. **Nucleic Acid Techniques**

a) *DNA/RNA Isolation, Quantitation, Integrity assessment, Storage*
b) *Amplification and Quantitation of Target DNA/RNA Sequences*
    i. Cloning (detailed in #6b below)
    ii. PCR, QPCR and Loop Mediated Isothermal Amplification (LAMP)
c) *Gene Expression Analysis:* Northern Blot, RT-PCR, QRT-PCR, Microarrays

6. <u>Manipulating Gene Expression</u>
a) Gene Silencing With Small Noncoding RNAs
b) Transgenesis:
    i. Transformation, Transfection and Transduction: Plasmid and Viral Vectors
    ii. Genome Editing : CRISPR, TALENS, ZN-Fingers
    iii. Transgenic and Cis-Genic Organisms for Research and Agriculture

7. High Throuput Biology I: 'Omics' and High Density Data
a) Role of Advances in Microfluidics, Data Storage and Computational Power
b) Omics Terms and Technologies/Platforms:
    i. Genomics, Functional Genomics, Transcriptomics, Epigenomics, Metagenomics;
    ii. Proteomics, Lipidomics, Glycomics;
    iii. Metabolomics
    iv. Bioinformatics: Data aquisition, storage, retrieval, querying

*c) *Sequencing: Sanger, Next Generation (Illumina), Bisulfite, ChIP-Seq*
d) Nutrigenomics and Metabolomics in Nutrition Research and Dietary Assessment

8. High Throuput Biology II: Systems Biology / Integrating "High Density" Omics Data
a) Systems Biology: Hypothesis-driven vs hypothesis-generating experiments
b) Bioinformatics and Computational Biology: Definitions and Roles in Systems Biology
c) Databases and Knowledgebases: NCBI BLAST, Ingenuity Pathway, Nutrigenome DB
d) *Modeling; Machine Learning; Hypothesis-Free Science*

9. Bioengineering; Synthetic Biology
a) Biomaterials For Boosting Food Security
b) Lab-designed Meat For health and Climate Impact Mitigation

For each technique or approach we will cover the underlying principles/theory, work-flow and examples of data output. Understanding the strengths, limitations, combinatorial use and at times, ethical challenges of these techniques will enhance your development as an experimentalist and success as a grant writer, peer reviewer and colleague.

Course Objectives:

- To develop basic understanding of the theory, technology, methodology and use of techniques and experimental approaches in modern nutrition research.
- Where relevant, to enhance comprehension of concepts presented in Graduate Biochemistry (BCHM 0223-Sackler).
- To develop student appreciation of how these different experimental techniques / approaches can be employed in a complementary and mutually-enforcing manner to address a nutrition research question in depth.
- By so doing, to develop student's ability to read the nutrition science literature and creatively design, conduct and interpret nutrition science experiments during their graduate careers and beyond

- To provide historical perspective to- and an understanding of- different modes of scientific inquiry and how seemingly incompatible approaches (e.g., reductionism vs systems biology, hypothesis-driven vs hypothesis-independent, model testing vs model improvement) can be used in concert to obtain increased experimental power and insight.
- By exposing students to the diversity of research techniques and fields of study, facilitate successful Practicum (NUTR 236) projects, graduate research and career trajectories.

Texts or Materials: All course materials are available on Canvas. A Canvas tutorial is available at https://canvas.tufts.edu/courses/169 . The course material includes lecture slides and PDFs, web-based videos and relevant journal articles to illustrate "real-world" applicability.

Academic Conduct: Each student is responsible for upholding the highest standards of academic integrity, as specified in the Friedman School's Policies and Procedures Handbook and Tufts University policies (http://students.tufts.edu/student-affairs/student-life-policies/academic-integrity-policy). It is the responsibility of each student to understand and comply with these standards, as violations will be sanctioned by penalties ranging from failure on an assignment and the course to dismissal from the school.

Classroom Preparation. During each class I will present a 75-90 minute lecture emphasizing the important concepts for that week. Some of the slides will be directly from the assigned material or home quiz (see below); other slides will be new, but directly related to and explanatory-of the assigned material. **Students are expected to prepare for class by reviewing the lecture slides and answering the home-quiz questions. Attending class without having engaged with the assigned material and completing the "home quiz" is the best recipe for feeling lost and overwhelmed by the material.** Cell phones must be turned "off" during class.

MASK POLICY: I will provide masks to all students entering the classroom. I respectfully request that students put on this 'fresh' mask or a fresh (that day) N95/r KN95 mask of their own before entering the classroom.

Office Hours: Attending office hours is the most effective way to stay on top of the assigned material and avoid becoming overwhelmed. Various concepts and techniques that we will cover are admittedly difficult, so it is expected that each of you may at times be confused or unclear about some of the material. Office hours are your lifeline when this occurs. Please bear in mind that there are no "dumb" questions. **Above all, don't let confusion go unaddressed, because a poor grasp of concepts introduced early in the semester will hamper your ability to understand subsequent lectures and assignments.**

Assessment and Grading: This course is graded S/U**. A "Satisfactory" grade requires an overall average of 80% (B-) or above.** Each week you will be provided with a self-assessment "**home quiz**' to assist mastering the material. The quiz requires short answers (word, phrase or several sentences) and increasingly throughout the course, diagrams/flow charts depicting experimental design, work-flow and data output. The quiz answers will be posted each week after class. Beginning with the unit on Nucleic Acids (November 1), each class will begin with a 15 minute quiz drawn verbatim from the week's home quiz. These in-class quizzes (minus the lowest score) will comprise 40% of your final grade (see below).

How is your final grade score determined?

A midterm exam covering the first 7 weeks of class will count for 40% of your grade. The midterm is comprised of a subset of the "home quiz" questions and lecture slides. An additional 40% of your grade will be the aggregate of ***in-class* quizzes covering lectures from 11/1-12/13.** The quizzes will be comprised of two-four questions from the home quiz for that week. The lowest quiz score can be discarded. The remaining 20% of your grade will be based on your 10 minute oral presentation during the last class (week 15). The presentation will be based on either (or both) of the *Science* articles assigned for that week. More details about the presentation will be forthcoming during the semester.

Summary of Final Grade Criteria:

Midterm (covering 9/6-10/18)	**40% of final grade score**
In-class quizzes (covering 11/1-12/13)	**40% of final grade score**
Week 15 Oral Presentation Class participation	**20% of final grade**

Accommodation of Disabilities: Tufts University is committed to providing equal access and support to all students through the provision of reasonable accommodations so that each student may access their curricula and achieve their personal and academic potential. If you have a disability that requires reasonable accommodations please contact the Friedman School Assistant Dean of Student Affairs (matthew.hast@tufts.edu; 617-636-6719) to make arrangements for determination of appropriate accommodations. Please be aware that accommodations cannot be enacted retroactively, making timeliness a critical aspect for their provision.

Tufts WebEx: Friedman's on-campus courses may be offered by Tufts WebEx (https://it.tufts.edu/webex) on days when the Boston campus is closed due to weather or a temporary cancellation issue. Students should expect to be notified by email in the event that class is cancelled and will be provided with the WebEx link for students to use for any remote class sessions. Also, any relevant course slides or materials will be made available on Canvas. The WebEx will be recorded and posted on Canvas when completed. If an on-campus Examination/Presentation was scheduled on a day when the Boston campus is closed due to weather or a temporary cancellation issue, the exam/presentation will be rescheduled for an alternate on-campus class session date.

Diversity and Inclusion Statement*:* We believe that the diversity of student experiences and perspectives is essential to the deepening of knowledge in this course. We consider it part of our responsibility as instructors to address the learning needs of all of the students in this course. We will present materials that are respectful of diversity: race, color, ethnicity, gender, age, disability, religious beliefs, political preference, sexual orientation, gender identity, socioeconomic status, citizenship, language, or national origin among other personal characteristics.

Course Topics and Assignment Schedule at a Glance: This schedule is subject to modification.

DATE OF CLASS	COURSE TOPIC	LECTURER	'ASSIGNMENTS'
September 6	**The Big Picture** Theory and Practice of Science (Holism vs Reductionism); The Discovery Cycle of Nutrition Research; Model Organisms The Problem of Reproducibility;	Obin	Home Quiz Web Exercises
September 13	**Separation of Compounds in Biological Samples** Electronegativity/Polarity (review) Sample Preparation, Solid Phase Extraction	Obin	Home Quiz

	Gas Chromatography High Performance Liquid Chromatography 1-D & 2-D Protein Electrophoresis		
September 20	**Identity/Structure Determination of Compounds in Biological Samples** Mass Spectrometry Nuclear Magnetic Resonance	Obin	Home Quiz
September 27	**Cell / Tissue Fractionation** Cell Culture, Harvest, Lysis Differential & UltraCentrifugation	Obin	Home Quiz
October 4	**Antibody -Based Techniques I** ELISA, Western Blot Immunoprecipitation and 'Pull-Downs'	Obin	Home Quiz
October 11	**Antibody-Based Techniques II** Principles of Fluorescence Flow Cytometry, Cell Sorting	Obin	Home Quiz
October 18	**Microscopy & Imaging** Light and Fluorescence Microscopy Confocal, 2-photon & Superresolution Immunohistochemistry, *in situ* Hybridization *In vivo* Imaging (CT and PET)	Obin	Home Quiz
October 25	MIDTERM EXAM		
November 1	**Nucleic Acids & PCR-based Techniques** NA Isolation, Analysis, Quantitation Gene Amplification by PCR Gene Expression Analysis by RT-PCR Quantitative PCR and RT-PCR	Obin	Home Quiz In-class quiz

November 8 No Tuesday Class Class Date TBD	**Manipulating Gene Expression I** Transformation & Bacterial Cloning Transfection, Transduction Gene Silencing By Small, Noncoding RNAs	Obin	Home Quiz In-class quiz
November 15	**Manipulating Gene Expression II** CRISPR / TALENS / ZN-Fingers Engineering Transgenic, 'Knockout' and Conditional Gene-Expressing Organisms for Research and Agriculture	Obin	Home Quiz In-class Quiz
November 22	**'Omics' I** Next Generation Sequencing Genome/Transcriptome/Epigenome Analysis GWAS and Functional Genomics Metabolomics Omics in Nutrition Research	Obin	Home Quiz In-class Quiz
November 29	**Omics II: Bioinformatic Tools** Databases and Knowledgebases NCBI BLAST Ingenuity Pathway Analysis Nutrigenome DB	Obin	Home Quiz In-class quiz
December 6	**Systems Biology: Integrating Omics Data** Role of Bioinformatics and Computational Biology	Obin	Home Quiz In-class quiz

	Machine Learning and Modeling Hypothesis-Free Science		
December 13	**Bioengineering & Synthetic Biology** Focus on Food and Agriculture Individual 10 Minute Oral Presentations	Obin	Prepare Presentation

Detailed Course Topics, Assignment Schedule, and the Learning Objectives for Each Class Session:

Assigned materials consist of URLs (web pages, videos, etc), PDFs, MSWord documents and Powerpoint files.

The reading and viewing material for each week will be available on Canvas in "Files >Full Syllabus>week X." The folder for each week will contain a document "Week X "goals and links" that is identical to that in the syllabus. The links to videos and other on-line material should be directly accessible. *Please report broken links immediately via email.* Also in the folder for each week is a subfolder entitled "week X additional." This subfolder contains additional reading (often journal excerpts), lecture slides, the weekly Home Quiz self-assessment tool, and following class, Home Quiz answers.

Week 1 / September 6: Theory & Practice of Science; Model Organisms; Reproducibility in Research

Learning Objectives: At the end of this unit, students should be able to:

1. state what we mean when we say that "science (as opposed to pseudoscience) is 'prohibitive' and 'falsifiable'."

2. define and provide examples of deductive vs inductive reasoning.

3. outline the sequence of steps in hypothesis-driven research

4. define and contrast reductionism vs holism and differences between "top down" and "bottom up" research.

5. discuss the limits of reductionism in nutrition research and suggest an alternative / complementary approach

6. state the difference between "repeatability," "reproducibility" and "robustness."

7. list factors that compromise research reproducibility between labs.

8. state the 'pros' and 'cons' of the following approaches: i) two labs using the same model system and the same techniques, or ii) two labs using different model systems and experimental techniques?

9. list practices that have led one author to state that "many researchers persist in working in a way almost guaranteed not to deliver meaningful results."

10. list 4-5 criteria for selecting a model organism for study, defending your choice(s) of the most important.

11. provide an example of research using a model organism taxonomically distant from mammals that discovered fundamental biology relevant to humans.

Required Reading

A. Modes and Methods of Scientific Inquiry

1. Levels (Stages) of Biological Organization (quick review)

https://biologywise.com/levels-of-biological-organization

2. **"Science vs Pseudoscience; The Scientific Method"**

*Week 1 slides 1-5 (*pdf in Canvas Files folder, "week 1 additional')

http://www.zmescience.com/science/scientific-method-steps/ (also see item below for modified figure)

https://www.thoughtco.com/elements-of-a-good-hypothesis-609096

3. **Holism vs Reductionism ("top down" vs "bottom up" science)**

Week 1 slides 7-12 (pdf in Canvas files folder)

https://explorable.com/scientific-reductionism

"Transcending reductionism in nutrition research" (Folder "week 1 additional ")

"Toward a new philosophy of preventive nutrition: from a reductionist to a holistic paradigm to improve nutritional recommendations", Figures 2 and 3. (Folder "week 1 additional").

4. "Mechanistic" vs "Descriptive Science": Relationship to Holism and Reductionism

"Infect. Immun. 2009, Tables 1 and 2 (folder "week 1 additional"). See also *Week 1 slide #12*

Consider that once a "mechanism" is established, it becomes a "description" that requires a mechanism. The *preferential funding and publishing of mechanistic science* has therefore fostered nutrition research that "drills down" into more biochemical and molecular processes. This encourages researchers to focus on elucidating the next lower level of mechanism (reductionist) without integrating it into the more global research problem (holistic).

B. Model Organisms

Week 1 slides 13-18

*Dietrich MR et al. How to choose your research organism. (*Folder "week 1 additional"). Introduction and Table I; focus on table items #s 1,4,5-7,10-12,18,19).

*Staats S et al. Drosophila melanogaster as a versatile model organism in food and nutrition research. Abstract only. (*folder "week 1 additional")

Hunter, P. The paradox of model organisms. The use of model organisms in research will continue despite their shortcomings (excerpt in folder "week 1 additional")

Model Organisms (PPT slides in folder "week 1 additional")

C. Reproducibility in Science

Week 1 slides 19-24

*The Problem of Reproducibility (*folder "week 1 additional ")

Hard Lessons in Reproducibility: Table from Nature **579**, 190-192 (2020) (PDF in in "week 1 additional")

The Four Horsemen of Irreproducibility (PDF in in "week 1 additional"). Be able to define each of the four.

D. The Big Picture: The 'Discovery Cycle' in Nutrition Research

Week 1 slide 25

Week 2 / September 13: Separation Techniques (Gas & High Performance Liquid Chromatography; 1- & 2-D Protein Gel Electrophoresis, Capillary Electrophoresis)

Learning Objectives: At the end of this unit, students should be able to:

1.State the goal of sample pretreatment and preparation and the three parameters that should be optimized.

2.Compare liquid-liquid extraction to SPE; sketch the general workflow of SPE; list the analyte and sorbent features that distinguish normal phase, reversed phase, ion exchange and size exclusion methods.

3. List the types and features of analytes that can and cannot be analyzed by GC and by HPLC, respectively.

4. Write the formula that calculates the number of "plates" in a column *based on the chromatogram obtained*; discuss column features that you can modify to increase the number of theoretical plates, thereby improving peak resolution.

5. Name / describe the technique that is employed to solve "the general elution problem" in *i*) GC, *ii*) HPLC.

6. Explain the difference between "analytical" and "preparative" chromatography; what parameter is optimized for the latter that is not a consideration in the former?

7. Explain/sketch how external and internal standards are used for qualitative and quantitative analysis, respectively. Explain / sketch the difference between "relative" and "absolute" quantitation.
Explain how chromatographic "fingerprints" can be used in qualitative analysis.

8. Describe *i*) the work-station set-up for separating a protein mixture by gel electrophoresis and *ii*) the visualization (staining) and molecular weight characterization of constituent proteins.

9. Discuss the similarities and differences between (native) PAGE and SDS-PAGE, in particular *i)* the basis upon which proteins are separated, *ii*) the use of detergents in solubilization and negative charge imposition, *iii*) the function of reducing agents, *iv*) the basis by which the gel separates proteins by size/mass, and *v*) the function of the stacking gel.

10. Sketch the work-flow and final gel image of a 2-D PAGE separation of a protein sample. What is the essential feature of proteins that determines their separation in the first dimension?

11. Sketch and label the basic set-up for Capillary Electrophoresis.What are its advantages compared to other separation techniques?

12. State the function(s) and role of "ultrafiltration" and "diafiltration" in sample processing.

Required Reading

A. Chromatography: Gas-Liquid (GC); High Performance Liquid (HPLC); Ion Exchange (IEC); Size Exclusion

A-1. Sample Preparation Methods (liquid-liquid and solid phase extraction)

Week 2 slides 4-19

https://www.youtube.com/watch?v=KNt0IdB_9dI (up to 2:44)

A-2. Desalting, Buffer Exchange and Concentrating Samples for Downstream Applications
Week Two slides 30-32

A-3. Principles and Methods in GC and HPLC
https://www.youtube.com/watch?v=M8d1u7kFZe0 (1:47- end; 12 min, 2s) see also *week 2 slides* 21-26

https://www.youtube.com/watch?v=ZN7euA1fS4Y (7min,37s) see also *week 2 slides* 27-29

http://www.youtube.com/watch?v=kz_egMtdnL4&feature=related (5min, 29s) *week 2 slides* 27-29

https://www.youtube.com/watch?v=MLoitPJQH3g (3min, 23s) see also *week 2 slides* 29,30,46-48

https://www.youtube.com/watch?v=yHQvMNHKxmw (32:40 -37:10; total = 4min, 30s)
see also week 2 slides 33-40; 42-45; 'quiz' question on slide 41.

https://www.youtube.com/watch?v=tAcfJPveWwM (4min, 6 s) see also *week 2 slides* 36-40

https://www.youtube.com/watch?v=fNP3T8SMqJg (18s) see also *week 2 slides* 36-40

https://www.youtube.com/watch?v=3dCl-SLg6yk (47s) see also *week 2 slides* 36-40

"Analytic vs Preparative" (pdf in files week 2 additional) see also *week 2 slide* 45
Note that the text focuses on protein separations, but the **general principles of analytic vs preparative chromatography hold for all types of analytes and all types of chromatography.**

B. Protein and Peptide Separation by RP-HPLC; Preparative Chromatography

"RP-HPLC of Proteins" (pdf in files, week 2 additional) see also week 2 slides 46-49
Key concepts: reversed-phase chemistry, why gradient elution is required for proteins, why are protein digests performed, what and why is peptide mapping.

C. 1-D and 2-D Protein Electrophoresis

"Protein Concentration Assays" (Word Document in files, week 2 additional); see also week 2 slide 50
It is vital to determine the amount of protein in each protein sample in order to load equivalent amounts for comparison of sample. In this table, just note the difference between a **direct assay (i.e., direct measurement in spectrophotometer)** and **indirect assay** (**i.e., spectrophotometer measurement colorimetric assay of sample dilutions compared to a protein standard curve**). Note the disadvantages and limitations of colorimetric assays.

https://www.youtube.com/watch?v=MILiO1XnuqQ (4min, 4s) see also *week 2 slides* 52-56

https://www.youtube.com/watch?v=pnBZeL8nFEo (5min, 18s) see also *week 2 slides* 52-56

https://www.youtube.com/watch?v=i_6y6Z5UvwE&t=5s (5:10 to 7:34; total 2 min, 24a) see *week 2 slide* 54

https://www.youtube.com/watch?v=JqFmnxGMc2g (15 min 53 s) see also *week 2 slides* 57,58

D. Capillary Electrophoresis

"Capillary Electrophoresis" (pdf in files; see also *week 2 slides* 59, 60)

<u>Week 3/ September 20th</u>: Mass Spectrometry; Nuclear Magnetic Resonance (NMR); Non-Nutrient Biomarkers

<u>Learning Objectives</u>: At the end of this unit, students should be able to:

1. Define "M+" and "parent ion." Explain how / why ions with different m/z ratios are differentially deflected by the magnetic field and why "field strength" is adjusted.

2. Interpret 'stick' diagrams of MS output for simple compounds containing carbon, hydrogen and chlorine, indicating fragmentation and relative abundance.

3. State difference between "top down" vs "bottom up" approaches to protein identification in mass spectrometry. Sketch a flow-chart of the "bottom up" approach starting with a protein mixture (same as learning goal #11 from prior week).

4. State why RP-HPLC (or SDS-PAGE) and Mass Spec are "orthogonal." State the additional information provided by the mass spectrogram when a protein mixture or digest is analyzed by RP-HPLC/MS.

5. Describe peptide mass fingerprinting and distinguish it from protein identification by tandem MS (i.e., MS/MS).

6. Briefly describe / sketch a method by which mass spectrometry can be used to determine quantitative differences in protein expression between cells or tissues exposed to different treatments.

7. Name three general classes of bioactive food constituents (in addition to macronutrients and minerals) that are identified in food by mass spectrometry and reported in nutrient databases.

8. Sketch and describe

9. Define "biomarker" and explain its advantage over dietary intake records and food composition data for assessing relationships between dietary exposure and health.

Required Reading/Assignment

A. Mass Spectrometry

http://www.chemguide.co.uk/analysis/masspec/howitworks.html

https://www.youtube.com/watch?v=2oPUyIbPxLo (up until 15:17; 15min, 17s)

https://www.youtube.com/watch?v=qL_N_JLv33I&t=3s (18 min, 36s)

"MS In peptide mapping" (PDF in "week 3 additional"; focus on additional information provided by MS)

https://en.wikipedia.org/wiki/Protein_mass_spectrometry (Read from section "Techniques" through section "Protein Quantitation." Focus on the following: "top down" vs "bottom up" approaches; tandem mass spectroscopy, including the figure "mass spectrometry protocol;" importance of databases; isotope labelling for protein quantitation.

https://en.wikipedia.org/wiki/Tandem_mass_spectrometry (Introduction only)

"*Mass Spectrometry in Nutrition: Understanding Dietary Health Effects At The Molecular Level*" (PDF in "additional readings."; Highlighted text and appreciation of Table 1 only.)

https://www.tofwerk.com/chemical-fingerprinting-of-olive-oil-aroma/ (also in "week 3 additional")

"Week 3 slides" (PDF in "week 3 additional")

B. Nuclear Magnetic Resonance (NMR)

https://www.youtube.com/watch?v=uNM801B9Y84 (8min, 30s)

Week 4/ September 27 Cell/Tissue Culture; Subcellular Fractionation; Ultracentrifugation

Learning Objectives: At the end of this unit, students should be able to:

1. Explain how to use an air-displacement pipette and the common errors in pipetting.

2. Explain the sequence of steps required to aseptically feed and harvest cells in a cell culture hood, including the use of ethanol, gloves, sterile and nonsterile hood areas, laminar flow, ultraviolet light, PBS, trypsin, and incubator settings.

3. Distinguish low speed, high speed, and ultracentrifuges; fixed- angle and swinging bucket rotors.

4. Explain the formula that allows reproducibility of G force among different centrifuges and rotors. Use a nomogram to determine necessary RPM to obtain a desired *g* force and vice versa.

5. Explain in one or two sentences the general principle of differential centrifugation for isolating cell or tissue supernatants and pellets enriched for different cell components.

6. Explain the principles underlying the use of the following in cell/ tissue extractions: hypotonic buffers, ionic detergents, nonionic detergents. State the function of each component of RIPA buffer used to harvest whole cell lysates.

Exercise I: Diagram and label the sequence of steps and reagents for obtaining *enriched fractions of* cytoplasm, nuclei, and lysosomes and *highly purified* ribosomes from the same batch of cells.

Exercise II: The respiratory physiology of exercise training can be studied in isolated skeletal muscle mitochondria. You are handed purified mouse skeletal muscle cells. Outline an approach for obtaining an enriched mitochondrial fraction from these muscle cells *in the fewest steps possible*. Hint: Mitochondria can be pelleted in isotonic buffer at 7,000 x g for 10 minutes.

9. State the basic difference(s) between *rate zonal* and *isopycnic* gradient separation. Name the features of particles that determine their separation by each technique. State why isopycnic gradients can be 'spun' forever without pelleting.

10. Write the equations for the Svedberg that relate it to *i*) terminal velocity and centrifugal acceleration, and *ii*) physical features of a particle and the medium in which it is suspended; state the Svedberg unit of measure; state its importance in sedimentation analysis.

Required Reading

A. Cell Culture

In the videos below in sections ***A*** and ***B*** you will note that different sized centrifuges and centrifuge speeds (RPMs) are used. Ignore these differences until we get to section ***C***, where the relationship between centrifuge speed (RPM), rotor size (*r* = radius of rotation) and centrifugal force (*g*) will be covered.

https://www.youtube.com/watch?v=uEy_NGDfo_8 (8min, 48s)

http://www.youtube.com/watch?v=yJ_acpKglto&feature=related (4min, 23s) (notice 1] the use of vacuum to remove old media. This "house vac" is built into the adjacent wall. The aspirated liquids are collected in the large flask on the right of the researcher. 2] the large hand-held pipettor used for volumes > 1ml to feed, wash and collect cells. An example of this rechargeable device is at the link below:).

https://www.pipette.com/4-000-101-Drummond-Portable-XP-110V-Charger-Included

http://www.promega.com/resources/multimedia/drug-discovery/cell-culture-video/ (4min, 40s) (harvesting cells and plating them for an assay, in this case, response to forskolin; note in this case the use of the protease trypsin to harvest these adherent cells. Some cell types can be harvested without trypsin by scraping into PBS or lysis buffer or by repeatedly pipetting a stream of PBS or lysis buffer onto the plate to dislodge them.

B. Making Whole Cell Lysates

https://www.youtube.com/watch?v=Mwc37prpHU8 (5 min, 21s; shows cell culture techniques and isolation of whole cells for subsequent analysis of whole cell lystates or cell fractions)

"Whole cell lysates" (MSword document in files "additional week 5")

https://en.wikipedia.org/wiki/Lysis_buffer (note function of buffers, salts, detergents and protease inhibitors incell lysis; use of nonionic detergents such as NP-40, Triton X-100 to maintain enzyme activity [i.e., "native state"] in cell lysates; no need to memorize the different buffer 'recipes.'

https://www.youtube.com/watch?v=I3WGAl3oe1E (2min, 4s; in this video, RIPA buffer is used to obtain a whole cell lysate. This buffer us most often used when the 'downstream' cell analysis is focused on total cell protein, as RIPA buffer efficiently extracts /solubilizes cytoplasmic, nuclear and organelle membrane proteins. The proteins are only minimally denatured, hence, the term "native" is used in the video).
Be able to explain the function of each component of the RIPA buffer.

C. Obtaining "Organelle-Enriched" Fractions from Lysates by Differential Centrifugation and Commercial Kits

1. Centrifugation basics

"Centrifugation Basics (pdf in "week 5 additional")

"*Ultrafiltration"* (pdf in "week 5 additional.")

"*RCF, k factor, Svedberg"* (pdf slides in "week 5 additional;" pp 1-5, through k-factor)

2. Differential centrifugation of homogenized tissues or whole cells to obtain "enriched" fractions

https://www.youtube.com/watch?v=esRRS7VLIVo (11min, 15s)

"Cell fractionation- differential vs gradient" (pdf in "week 5 additional")

3. Use of commercial kits to obtain "enriched" fractions

https://www.sigmaaldrich.com/life-science/proteomics/protein-sample-preparation/organelle-isolation/organelle-isolation-analysis-kits.html (commercial kits that facilitate enrichment of one cell compartment; each uses slightly different lysis / extraction buffers and centrifugation steps depending on the target organelle; no need to memorize, just appreciate the variety of available kits for enrichment)

https://www.sigmaaldrich.com/life-science/molecular-biology/molecular-biology-%09products.html?TablePage=9625384&gclid=Cj0KCQjws536BRDTARIsANeUZ5-ZamdHdJDr2GCMnzURv-%09YRzMEQnGt-CXhOZ4_ucXkKArv9sFe6niYaAsicEALw_wcB

D. Obtaining *Highly-Purified*, Intact Organelles by Gradient Centrifugation

"Density gradient centrifugation" (pdf slides in "week 5 additional"; slides 5-15*)*

"meselson-stahl centrifugation" (MSword document in " week 5 additional")

"RCF, k factor, Svedberg" (pdf slides in files "additional week 5") (start at page 6; no need to be able to derive the equations; just understand the initial equation **($S = v_t / r\omega^2$) and the final equation (in red box);** understand the logic of its derivation and what it and the Svedberg tells us about sedimentation rate of particles.)

Week 5/October 4: Antibody Techniques 1: Protein Detection and Isolation (ELISA, Western Blotting, Immunoprecipitation, "Pull-Down" Assays)

Learning Objectives: At the end of this unit, students should be able to:

1. Briefly discuss the source of antibodies and their specificity in response to infection.

2. Diagram an antibody-antigen complex, labeling immunoglobulin (Ig) heavy and light chains, and constant and variable domains. State how Ig isotypes are structurally defined.

3. Define "epitope" and "affinity" and explain the underlying structural molecular features of antibody and antigen that determine them. Diagram Fc, F(ab) and $F(ab')_2$ fragments and explain how they influence target recognition.

4. Describe the key molecular and practical (i.e., experimentation) differences between monoclonal and polyclonal antibodies.

5. Justify / compare the choice of ELISA or Western blotting for detection vs measurement of circulating levels of the proinflammatory cytokine interleukin 1β (IL-1β) in response to dietary intervention. What specific feature / capability of the IL-1β antibody would you need to know to inform your decision about the use of one technique vs the other? (Hint: Laemmli buffer).

6. Explain the advantage of affinity-purified and pre-adsorbed secondary antibodies and the difference between them.

7. Describe the use and detection of the following types of conjugated antibodies: enzyme-linked; fluorescently labelled; biotinylated. Distinguish among colorimetric, ECL and fluorescent detection.

8. Describe the essential steps in Western blotting and be able to sketch the 'gel sandwich" and transfer set-up.

9. Discuss how and why "solid supports" such as Protein A/G Sepharose, Agarose beads, etc. are used.

10. Describe the reagents and steps involved in immunoprecipitation of a protein from human splenocytes (spleen cells). Describe the control(s) you would use and how you would confirm that the IP was a success (i.e., that you selectively immunoprecipitated the target protein).

11. Describe the principle and use of "Pull-Down" assays and state the distinction vs IPs/Co-IPs.

12. List steps might you take to reduce high background staining on a Western blot of an IP.

13. What is the target protein in ChIP? RIP?

Required Reading

Measuring Antigens in Biological Samples (blood, tissue homogenates, cells, etc.)

A. Antibodies

https://www.youtube.com/watch?v=dTb0iEUS1oA (4min, 15s;)

http://www.dnatube.com/video/2220/Antibodies (2min, 39s)

"week 6 slides" (pdf in files >"week 6 additional", slides 1-4)

"secondary antibodies" (pdf in files > "week 6 additional")

B. ELISA

https://www-jove-com.ezproxy.library.tufts.edu/v/5061/the-elisa-method (video up to 9min, 53s and text)

https://www.bio-rad-antibodies.com/blog/deciding-which-elisa-technique-is-right-for-you.html

C. Western Blotting

https://www.youtube.com/watch?v=HqmxLsHKxZE (2min, 15s; (review of SDS-PAGE for those who need it)

https://www.youtube.com/watch?v=CEEekahiqMo (7min, 53 s)

https://advansta.com/wikis/secondary-antibodies/ (same link as in section A).

https://blog.benchsci.com/western-blot-detection-methods

http://assets.thermofisher.com/TFS-Assets/BID/Technical-Notes/fluorescent-western-blotting-guide-multiplexing-tech-note.pdf (Page 2 only; Note that multiplexing allows probing blots with mutliple primary antibodies at the same time. This saves many hours (days) of work. focus on "Selection of Antibodies." Imaging, quantitation and reprobing of a multiplexed fluorescent Western blot requires a dedicated imaging / quantitation system, an example of which is in the link below).

https://www.thermofisher.com/order/catalog/product/A44241 - /A44241
https://www.thermofisher.com/us/en/home/life-science/protein-biology/protein-assays-analysis/western-blotting/detect-proteins-western-blot/western-blot-imaging-analysis/ibright-systems/features.html
(example of gel / blot imager capable of colorimetric, ECL and fluorescent detection).

D. Immunoprecipitation (IP and Co-IP) and Pull-Down Assays

"Immunoprecipitation Overview (pdf in "week 6 additional)

https://www-jove-com.ezproxy.library.tufts.edu/v/5695/co-immunoprecipitation-and-pull-down-assays

Week 6/October 11: Fluorescence, Flow Cytometry, Cell Sorting

Learning Objectives: At the end of this unit, students should be able to:

1. Describe / diagram
   i) the three-step process that results in emission of light from a fluorophore,
   ii) what accounts for the color emitted by a fluorophore,
   iii) the concept of excitation and emission maxima,
   iv) the functional differences between tungsten, laser and light-emitting diode (LED) excitation sources, and
   v) the uses of dichroic mirrors and filters for optimizing excitation and emission in a flow cytometry experiment.

2. Draw a labelled flow cytometry plot showing populations of large cells with agranular cytoplasm and small, granular cells.

3. Briefly outline and state the function of the steps required for labelling cells for i)surface and ii) intracellular staining.

4. Explain and illustrate *spectral overlap* and *compensation* in flow cytometry experiments.

5. Design and diagram the data output from the following flow cytometry experiment:
Peripheral blood mononuclear cells (PBMCs) are a fraction of leukocytes that contains T cells (CD3+ cells) and monocytes (CD14+) cells. Precancerous PBMCs express antigen "Pr" on their surface; 'healthy' PBMCs express antigen "H" but not antigen "Pr" on their surface. You are given a PBMC-enriched fraction of mouse blood cells obtained by density gradient centrifugation. Sketch the flow cytometry biplot data expected from the blood of mice containing i) no precancerous T cells or monocytes, ii) ~20% precancerous T cells and 20% precancerous monocytes, iii) no precancerous T cells, but 20% precancerous monocytes. Specify fluorophores, wavelengths and single-label controls in your experimental design.

6. Describe/diagram the process that enables researchers using a flow cytometer to capture and isolate cells that have for further analysis or experimental manipulation.

Required Reading

A. Fluorescence and Flow Cytometry

https://www.youtube.com/watch?v=SGFlr1jFNBM (8 min, 11s)

https://www.youtube.com/watch?v=oVxpaUfTuXI (3min, 11s)

https://www.youtube.com/watch?v=xJGmARfBasU (4min, 38s)

https://www.youtube.com/watch?v=sfWWxFBltpQ (12 min, 4s)

https://www.youtube.com/watch?v=ccR5snuCE80&t=918s (17 min, 40s)

https://www.youtube.com/watch?v=9UV9KjDoCXE (6 min, 42 s; understand what each step in the protocol accomplishes and appreciate how labor-intensive it can be; what controls should be run for each type of sample?)

https://www.labome.com/method/Flow-Cytometry-A-Survey-and-the-Basics.html (also in files, week 7 additional)

B. Fluorescence-Activated cell Sorting (FACS)

https://www.youtube.com/watch?v=Feg13MB6DWI&t=576s (11min, 13s)

Week7/ October 18: Imaging Techniques (Microscopy and *In Vivo* Imaging)

Learning Objectives: At the end of this unit, students should be able to:

1. State the illumination source and maximal magnification and resolution of light and transmission electron microscopes.

2. Explain the importance of Koehler illumination.

3. Briefly describe the specimens that are best visualized by brightfield, darkfield, Phase Contrast and DIC microscopy.

4. Outline / sketch the steps required to obtain 1) FFPE and 2) frozen tissue sections for immunohistochemistry (IHC).

5. Outline the steps required to visualize a cell surface protein in fixed tissue using IHC and IF. What controls would you use to insure target-specific immunoreactivity? How would you distinguish individual cells in your tissue?

6. Define / state
i) "epifluorescence microscopy" and sketch the relevant feature of the epifluorescent microscope,
ii) advantages and disadvantages of the different light sources used in fluorescence microscopy,
iii) the important features of a specimen that determine the best fluorescent technique to use,
iv) the essential problem of traditional epifluorescence microscopy and the two general classes of solutions,
v) the essential distinction between a fluorescence and confocal microscope,
vi) the optical path difference between scanning and spinning disk confocal microscopes and their (dis)advantages,
vi) the principle of two-photon microscopy and how this differs from confocal microscopy,
vii) superresolution microscopy and the 'problem' that it overcomes,
vii) the goal and methods of *in situ hybridization*

8. Construct a decision matrix of how you would select a type of microscopy for fluorescent imaging of tissue based on *sample thickness, sensitivity and speed of image acquisition.*

9. Describe several important capabilities of image analysis software, including "measurement profiling."

10. Distinguish in vivo optical sectioning from PET and CT; distinguish PET from CT; suggest other techniques that might be used to support / confirm PET-CT data in preclinical research.

Required Reading

A. Microscopy Overview (Magnification in light and electron microscopy; diffraction limit; magnification vs resolution)

https://www.sciencelearn.org.nz/resources/495-magnification-and-resolution (also as pdf in files)

B. Light Microscopy (Koehler illumination, Phase Contrast and DIC)

"Koehler Illumination"(pdf in files)

https://www.youtube.com/watch?v=doxGqBOp5MM (up to 3min, 21s)

https://www.jove.com/v/844/phase-contrast-and-differential-interference-contrast-dic-microscopy (6 min, 50s)

C. Visualizing Cell Structures and Macromolecules: Immunohistochemistry and Immunofluorescence

C-1. Preparing cells and tissues for immunohistochemistry / immunofluorescence microscopy

C-1.1. FFPE tissue
https://www.youtube.com/watch?v=TLm37BbR1mo (up to 9min, 45s; ignore the "decalcification" step)

C-1.2 Cryopreserved (Frozen) tissue
https://www.youtube.com/watch?v=Wiq96lEyu6s (2 min, 11s)
https://www.youtube.com/watch?v=PCj8XrpskPw (2 min, 18s)

https://www.biolegend.com/en-us/videos/frozen-tissue-slide-preparation-and-processing (3 min, 16s)

C-2. "Chromogenic vs. Fluorescent Imaging Methods

https://www.biolegend.com/microscopy; ("Chromogenic vs. Fluorescent Imaging Methods;" "Immunocytochemistry Protocol Step-by-Step" (2 min 56s; immunocytochemistry is simply the visualizing of structures in cultured or dispersed cells as opposed to tissue; this video demonstrates the combined use of chromogenic and immunofluorescence in HeLa cells cultured on glass coverslips.)

C-3. Microscopy Techniques for Visualizing Cell and Tissue Structures and Dynamics

C-3.1 Epifluorescence (Widefield) microscopy
"epifluorescence and TIRF defined" (pdf in files)

https://www.scientifica.uk.com/learning-zone/widefield-fluorescence-microscopy (includes review of fluorescence concepts)

https://www.youtube.com/watch?v=01v2kR8dlnQ (5:58-15:39) (total, 9min, 41 s)

"photobleaching and phototoxicity" (pdf in files)

C-3.2 Optical Sectioning and 3D Reconstruction

C.3.2.1 Deconvolution microscopy
https://www.youtube.com/watch?v=01v2kR8dlnQ (9:08-10:48)

C.3.2.2 Confocal microscopy
"principles of confocal microscopy" (in files "additional")

https://www.youtube.com/watch?v=YRQsjPAx9UU (26min, 40s)

http://micro.magnet.fsu.edu/primer/virtual/confocal/index.html (confocal; practice interactive optical sectioning of mouse intestine and rabbit muscle by modifying axial plane, pinhole size and PMT Channel Gain).

C-3.2.3 Two Photon microscopy
"two photon microscopy" (pdf in files);
see also https://www.youtube.com/watch?v=YRQsjPAx9UU (22:16-24:27)

C-3.2.4 Superresolution microscopy
"superresolution microscopy" (pdf in files; just appreciate the 15-fold increase in resolution vs confocal).

C-4. Fluorescent In Situ hybridization (FISH)
https://www.youtube.com/watch?v=DEIL3KeXL9w (5min, 7s)

D. *In vivo* Optical Imaging and Tomography ("imaging by section through use of penetrating wave")

https://www.fda.gov/radiation-emitting-products/medical-x-ray-imaging/what-computed-tomography

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1126321/ (focus on uses)

http://snmmi.files.cms-plus.com/docs/rpsc_SNMclass1.pdf (also in files; pages 23, 25, 29, 31,32; note the difference between optical imaging (bioluminescence and fluorescence) and tomography.

E. Bioimage Analysis

https://www.youtube.com/watch?v=Odi9pIerT7I&feature=emb_rel_pause (up to 2:44; 3:28- 6:25;)

https://cellprofiler.org/examples (appreciate the various tools for quantifying image parameters such as size, morphology, count, intensity, texture, colocalization and movement; also note tools for different model systems (human, mouse, D*rosophila sp*, C*. elegans*)

Week 8/ October 25th: Mid-Term Exam

Week 9/November 1: Nucleic Acids: Isolation, Electrophoresis, Amplification and Quantitation

Learning Objectives: At the end of this unit, students should be able to:

1.. Describe and justify the steps used in isolating and storing i) DNA and ii) RNA from mammalian cells using phenol-chloroform extraction and commercial kits. Describe the functions of detergents and buffers, the role of phenol, chloroform, isopropanol, guanidinium or other chaotropic agents, enzymes and RNAse inhibitors and the principle and use of solid phase separations such as spin colums.

2. Describe how a "Nanodrop" spectrophotometer assesses the i) purity and ii) concentration of extracted DNA or RNA.

3. Diagram *i*) the set-up and apparatus for separating a mixture of DNA or RNA fragments by gel electrophoresis, *ii*) the visualization and molecular mass estimates of the gel bands using ethidium bromide and a DNA "ladder;" explain why nucleic acid fragments (as opposed to whole DNA/RNA molecules) are analyzed in agarose gel electrophoresis.

4. Be able to draw the cycles of a standard PCR reaction, indicating target sequence and direction of replication for all strands and temperatures, enzymes, dNTPs and primers. What key steps must the PCR hardware be able to perform?

5. Explain *i*) the function of the key enzyme that allows us to adapt PCR for examining gene expression, *ii*) diagram an RT-PCR reaction through RT and first PCR cycles, identifying enzymes and reagents used, iii) explain how we assess mRNA expression levels after RT-PCR, and why these levels are only semi-quantitative (tell us only that a gene is uprfegulated or downregulated vs a control or another treatment).

6. State the key advantage quantitative (real-time) RT-PCR provides vs standard RT-PCR. Explain what the PCR thermocycler is required to do to allow for "real time" measurement of amplicons.

7. Explain/diagram the function and advantages/disadvantages of Cyber/Syber Green vs Taqman-based PCR.

8. Draw and explain in quantitative detail a real-time PCR data plot of standards containing 10 ng, 20ng, 100ng and 1 microgram of hexokinase mRNA, assuming that amplification efficiency {E}=1. . Label the axes and accurately label the Ct differences among the samples. Now sketch the same data assuming that amplification efficiency is 0.75.

9. Explain the method(s) of calculation that is/are used to accurately quantitate the fold-difference in mRNA between two or more samples in a real-time PCR experiment, identifying the role of reference or "housekeeping" or "calibrator" genes. Be able to write out the details of the 'deltas' in the formula, $2^{\Delta\Delta Ct}$. Be able to explain why accuracy of the "delta-delta Ct" method requires that amplification efficiency (E) of the target and calibrator be the same.

10. Explain the basic principle of LAMP and its advantages / disadvantages vs PCR-based techniques.

11. Explain how standard RT-PCR and QPCR are used in high-throuput techniques to assess the expression of thousands of genes at once; sketch a gene chip.

Required Reading

A. Extraction, Quantitation and Electrophoretic Analysis / Purification of Nucleic Acids

A-1. Extraction of DNA and RNA from cells and tissues

DNA

"phenol-chloroform extraction of DNA" (pdf in files: to top of page 3; Fig. 21.4 and legend; compare phenol-chloroform extractions vs solid phase (silica).

"Details of Phenol-Chloroform Method" (pdf in files)

RNA

https://www.ncbi.nlm.nih.gov/pubmed/17406285 (isolating RNA from DNA: acidification with guanidinium!)

https://files.zymoresearch.com/protocols/_r1050_r1051_quick-rna_microprep_kit.pdf (pp. 4-8; focus on RNA from cells; the principle of this kit is that RNA/DNA are insoluble in ethanol, so they remain on the spin filter as impurities are washed away; then after DNAse treatment of the filter, the RNA is eluted with RNAse-free water. "RNAshield" and "RNA*later*" are commercial RNAse-inhibited solutions for storing cells/tissues before RNA isolation; note ability to isolate small (noncoding) vs large (coding) RNAs).

A-2. Quantitation & Purity

https://www.youtube.com/watch?v=y7DLoGxg0k0&t=14s (4min, 36s; compare fluorometer vs spectrophotometer for nucleic acid quantitation (Qbit) vs quantitation and purity (NanoDrop).

A-3. Electrophoretic Analysis of DNA/RNA for integrity, fragment(s) size and excision)

https://www.youtube.com/watch?v=TIZRGt3YAug (5 min, 35 s)
https://www.youtube.com/watch?v=yXkay-R5xvA (3 min, 46 s; note use of MOPS buffer and denaturing agents, formamide and formaldehyde)

B. PCR-based approaches: PCR, RT-PCR, Quantitative (Q)PCR, LAMP.

B-1. Pipetting Small Volumes

https://www.youtube.com/watch?v=54kN88U4Y6w (2min, 19s)

B-2. PCR and Quantitative(Q) PCR

"PCR summarized" (pdf in files; quite dated, but accurate and clear; put simply, **PCR is a method for *amplifying DNA* to obtain sufficient amounts for downstream analysis or procedures)**

http://www.dnalc.org/view/15475-The-cycles-of-the-polymerase-chain-reaction-PCR-3D-_animation-with-no-audio.html (1min, 27s)

(Note that the target sequence is doubled after each cycle, but *it takes 3 cycles to generate the first duplicated amplicon containing only the target sequence defined by the primers*)(see next link)

https://www.youtube.com/watch?v=iQsu3Kz9NYo (3min, 55s) (note formula for # amplicons per cycle: $\mathbf{2^n - 2n}$)

https://www.youtube.com/watch?v=rpLSvEbOmqc (7 min, 8 s; PCR vs QPCR)
Whereas PCR is a method for amplifying DNA to obtain sufficient amounts for downstream analysis or procedures, quantitative (Q)PCR (also known as "real-time" PCR) is a method for determining *relative and absolute amounts of target DNA (or RNA as we will see below) in your samples.*

In PCR, each cycle of amplification increases the amount of product (amplicon) by two-fold until a plateau is reached due to depletion of primers and inhibition of DNA polymerase by the accumulating double-stranded product. Typical PCR reactions are ~30 cycles, generating 1,073,741,824 amplicons for every molecule of template DNA initially present. **In standard PCR**, **the amount of product is estimated by *end point analysis,*** using agarose gel to confirm amplicon size and densitometry to estimate quantity (not very precise).

In contrast, QPCR employs fluorescent dyes or probes to detect how much product is generated *after each PCR cycle.The QPCR thermocycler includes a fluorimeter, digital camera and software to detect and analyze the signal in "real time." **Samples with more target DNA (or RNA) at the initiation of the PCR reaction will generate more signal after fewer cycles than samples containing less target sequence.** For example, two copies of target DNA will generate 1024 amplicons after 10 cycles of PCR, whereas six initial copies will generate 60, 466,176 copies. Working 'backwards' ($2^X = 1024$; $6^X = >60$ million, solve for* x*) the number of copies detected after 10 cycles of doubling in this example allows us to precisely determine the relative amounts of initial (template) DNA or RNA in the two samples- i.e, 1:3(2:6). Absolute quantitation requires a standard curve (see "QPCR Data Analysis" below).*

https://www.youtube.com/watch?v=1kvy17ugI4w (2 min, 44s)

B-3. RT-PCR and Quantitative ("real time") RT-PCR (RT means "reverse transcriptase," NOT "real time.")
In RT-PCR, an mRNA template is used to generate complementary DNA (cDNA), which is then used as a template for the standard PCR reaction. RT-PCR is at the foundation of multiple molecular biology "tools." First, cDNA of expressed gene can be amplified, a first step in cloning. This has led to "cDNA libraries"- i.e., cDNAs of all the total of genes expressed in various tissues / cell types under various types of physiological conditions and or disease. Second, researchers can measure and thus compare the expression of genes of interest in cells and tissues. This function has been adapted for "high throuput" analysis by microarrays (gene chips, see below). In addition, RT- PCR is an essential step in "transcriptomics"- the use of next generation sequencing to examine the entire set of expressed genes in an organism *(the transcriptome*).l

"*RT-PCR and Quantitative RT-PCR*" (in files)
https://www.thermofisher.com/us/en/home/life-science/pcr/reverse-transcription/superscript-iv-one-step-rt-pcr-system/5-steps-rtpcr.html (introductory paragraph and "one step vs two-step" video: 2 min, 32s)

https://www.youtube.com/watch?v=iu4s3Hbc_bw (4min, 37s) See comments above about PCR vs QPCR.

https://www.youtube.com/watch?v=TkCBcL_xUUs (3min, 2s; shows the actual procedure in the lab)

B-4. QPCR Data Analysis
https://www.youtube.com/watch?v=GQOnX1-SUrI (10min, 7s)

https://www.youtube.com/watch?v=tgp4bbnj-ng (8min, 20s)

https://biosistemika.com/blog/qpcr-efficiency-over-100/ (also in files "*Calculating PC Reaction Efficiency*")

C. Loop-Mediated isothermal Amplification (LAMP)

https://www.youtube.com/watch?v=UJE9SBMrh20 (4min, 1s)

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7182526/ (Table I and Figure 2)

D. High(er) Throuput PCR-Based Methods (example: DNA microarrays)

https://www.youtube.com/watch?v=0ATUjAxNf6U (4min, 22s))

https://www.illumina.com/science/technology/next-generation-sequencing/ngs-vs-qpcr.html

Week 10/ TBD: Manipulating Gene Expression I (Transformation/Transfection/Transduction; RNAi)

Learning Objectives: At the end of this unit, students should be able to:

1. Describe the difference between forward and reverse genetics, and state why this topic is part of this week's syllabus.

2.. Sketch a 'basic' plasmid; identify and explain the rationale for the origin of replication, selectable marker, multiple cloning site and LacZ gene.

3. Sketch and describe the work-flow of a bacterial transformation using plasmids and competent bacterial cells and the possible transformation results.

4. Briefly describe three methods of transfecting eukaryotic cells without viral vectors. What is the fundamental problem that each of these approaches seeks to overcome regarding introducing DNA/RNA into cells?

5. List the advantage and disadvantage of viral vectors as compared with plasmids and the major advantage and disadvantage of the following viral vectors relative to each other: Adenoviruses; Adeno-Associated Virus (AAV); Retroviruses (e.g. MMLV); Lentiviruses (e.g. HIV-1, FIV, SIV)

6. State the underlying principle and essential procedures of the Dual Luciferase Assay in transfection.

7. Describe the intra-cellular trafficking, function and distinct functional roles of (a) siRNA and (b) miRNA

8. Clearly diagram and explain the roles of Drosha, Exportin, Dicer, Argonaut, guide strand and RISC complex.

9. Discuss how and why we use RNAi in (a) basic research and (b) agriculture.

Required Reading

A. Forward and Reverse Genetics

https://www.youtube.com/watch?v=Paf5WRMizzQ (5 min, 11s; focus on conceptual difference between forward and reverse genetics and reverse genetics "approaches."

B. Bacterial *Transformation* and Cloning

https://www.youtube.com/watch?v=SdqJFA6mOkI (4 min, 59s)

https://www.addgene.org/protocols/bacterial-transformation/ (video helpful, but optional, 5min, 52s)

https://www.youtube.com/watch?v=cVKsVnE3rYU (5min, 19s)

C. Transfection and Transduction (Introducing DNA, RNA or Oligonucleotides Into Eukaryotic Cells)
https://bitesizebio.com/32346/introducing-foreign-dna-terminology/

C-1. *Transfection* (Introducing nucleic acids into eukaryotic cells by nonviral methods.)
"mammalian plasmid vectors´(pdf in files)

https://www.youtube.com/watch?v=4SjCI1pvyug (up to 4:30- emphasis on figure at 1:58 and how they overcome basic problem of negative charge; then 5:04 – 7:26 with emphasis on electroporation, and biolistic ("gene gun"), viral vectors; note that this video does not distinguish viral-based methods as "transduction." total time, 6 min, 26 s.

C-2. *Transduction* (Introducing nucleic acids into eukaryotic cells by a virus or viral vector.)
http://www.cellbiolabs.com/viral-expression

D. Gene Silencing By Small, Noncoding RNAs (RNA interference [RNAi] using siRNA and miRNA)
https://media.hhmi.org/biointeractive/click/rna_interference/12.html

https://www.nature.com/news/video-animation-rna-interference-1.9673 (5 min, 07 s)

"siRNA and miRNA" (pdf in files)

https://www.youtube.com/watch?v=j-zTy6vOP3M (1min, 48 s)

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1838705/ (Abstract, Intro and details of Fig. 1)

https://www.frontiersin.org/articles/10.3389/fpls.2020.00445/full (through Introduction)

Week 11 / November 15: Manipulating Gene Expression II: Genome Editing

Learning Objectives: At the end of this unit**, students should be able to:**

1. Define genome editing, state the two essential steps required for this technology, and diagram the two types of repair of dsDNA breaks, identifying the one type that is essential for genome editing, not just gene inactivation.

2. Describe the essential similarities and differences among CRSISPR-Cas9, Zn-finger Nucleases, TALENs and RNAi with regard to basic mechanism, ease of experimental design, efficiency, off-target effects and ease of validation.

3. Diagram how the CRISPR locus acts in bacterial adaptive immunity in response to an initial encounter with a novel virus. Include in your diagram the various Cas proteins involved, the generation of proto-spacer(s) and spacer(s) and the PAM sequence.

4. Diagram the CRISPR-Cas9 acts in response to a previously-encountered virus.

5. Explain the functional importance of palindromic sequences in CRISPR-Cas9.

6. Diagram and discuss the modification to the 'original' CRISPR-Cas9 system that produced a "programmable DNA cutting enzyme" and explain how it is programmed. Explain the PAM sequence and howe it limits the applicability of the CRISPR-Cas9 system. What is an important "off target" issue for this technology?

7. Sketch and explain the use of CRISPRa and CRISPRi

8. Outline the specific technique(s) you would employ to evaluate and compare the success of *i*) a gene knockout or *ii*) a gene insertion, both performed in multiple replicates-e.g., six different plant cell lines. You are seeking to select the 'best' one for your next step.

9. Sketch the basic work-flow for generating transgenic, knockout and conditional knockout animal models.

10. Explain *i)* how genome editing technologies reduce but do not remove the potential of novel allergens in genetically-modified food, and *ii*) how Cis-genesis differs from trans-genesis and potentially generates non-GMO "golden rice."

Required Reading

A. Genome Targeting Technologies: ZN Finger Nuclease (ZNF), Talen Effector Nuclease (Talens), CRISPR

https://www.youtube.com/watch?v=iGmq1O6mIy0 (5min, 4s)

https://www.youtube.com/watch?v=U3Z4u0DKbx0 (13 min)

https://www.youtube.com/watch?v=2pp17E4E-O8 (4min, 12s)

https://www.youtube.com/watch?v=MnYppmstxIs (7min, 20s)

https://www.youtube.com/watch?v=iSEEw4Vs_B4 (5min, 04s)

https://www.youtube.com/watch?v=SuAxDVBt7kQ (16min, 41s)

https://wondercollaborative.org/human-nature-documentary-film/ (1 h, 34 min). Not required. A brilliant documentary on CRISPR origins, mechanism and societal implications. Superb animation.

"*confusion about gRNA vs sgRNA*" (pdf in files)

"*CRISPRa and CRISPRi*" (pdf in files)

https://www.sciencemag.org/features/2019/09/beyond-crispr-what-s-current-and-upcoming-genome-editing (also as pdf in files; skip to last section – "Our Genome-Editing Future.")

B. Transgenic Animals for Research and Agriculture
Week 12 lecture slides #17-23

C. Implications for Agriculture and Society
https://www.theguardian.com/us-news/2019/may/30/crispr-gene-edited-food-technology-us-produce

https://www.youtube.com/watch?v=JBp0JoF1utE (up to 7min, 12s)

"transgenic-cisgenic-intragenic and subgenic" (pdf in files)

Week 12 / November 22: "Omics" I

1. Define 'omics' and list the factors driving its development and 'omics' fields that are currently particularly attractive to nutrition researchers.

2. List the technologies (techniques and platforms) that are used in the following: genomics, transcriptomics, epigenomics, proteomics and metabolomics.

3. Explain how 'omics' studies appear to be "reductionist" yet at the same time, not "mechanism-driven."

4. Discuss the methods and use of metabolomic fingerprinting in nutrition research.

5. State the two major research goals of nutritional genomics.

6. Define "functional genomics;" distinguish between 'forward' and 'reverse' genetics with examples.

7. State the goals of genotyping, transcription profiling, epigenetic profiling and meta-analysis. Define genome, transcriptome and epigenome.

8. Sketch how binding sites of DNA binding proteins are identified and discuss what such data tell us.

9. Sketch how DNA methylation is determined and state its importance.

10.Starting with the original DNA sample(s), diagram the process of NGS, emphasizing the four common steps in the workflow of all NGS technologies. Explain what a "library" is and how they are constructed using custom adaptor sequences.

11. Diagram the key difference in nucleotide incorporation between Sanger sequencing and Illumina "sequencing by synthesis." Diagram the key difference in nucleotide incorporation between pyrosequencing and Illumina sequencing.

12. State the role of the following in NGS: adaptor, index, emulsion PCR, bridge PCR, cluster, and describe the raw data generated by NGS.

13. Define "read", "depth" and "coverage" and be able to calculate the latter. Explain why 30x coverage is necessary for accurate sequencing of a whole genome. Discuss analyses that require greater coverage and explain why.

Required Reading

A. 'Omics': Definitions and Technologies

"omics and important terms"(pdf in files)

"omic technologies" (pdf in files; focus on proteomic and metabolomic techniques; data analysis).

B. Deeper Look at Genomic Technologies

B.1 Microarrays

https://www.youtube.com/watch?v=0ATUjAxNf6U **(**4min, 22s**; a** review from week #9).

B.2 Sanger Sequencing
https://www.youtube.com/watch?v=FvHRio1yyhQ (2 min, 51 s)

B.3 Next Generation Sequencing
https://www.youtube.com/watch?v=jFCD8Q6qSTM (9min, 29s)

https://www.youtube.com/watch?v=CZeN-IgjYCo

https://www.youtube.com/watch?v=fCd6B5HRaZ8 (5min, 12s; a deeper dive into Illumina sequencing- by-synthesis) [a note about "index reads." Index primers identify samples. They are added to individual samples in a second thermocycling step, after initial amplification of the target region. This allows you to mix many samples together (up to 96) and sequence them at the same time. Following sequencing, the software identifies these indexes on each sequence read and, because you have already told the machine which pair of index primers was added to each sample, the machine knows which reads 'belong' to which samples.]

B.3.1. NGS Calculations and Bioinformatic Processing
https://www.youtube.com/watch?v=RkttaYc8hfw

https://en.wikipedia.org/wiki/Coverage_(genetics) (focus on Rationale and Calculation)

https://www.illumina.com/science/education/sequencing-coverage.html

https://www.youtube.com/watch?v=l4BAfRekohk&vl=en

C. Omics Approaches in Nutrition Research
"nutrigenomics" (pdf in files)

"Nutritional Metabolomics" (pdf in files; Introduction only)

"Metabolic Assessment of Dietary Intake" (pdf in files)

D. Putting It All Together: Functional Genomics
https://www.ebi.ac.uk/training-beta/online/courses/functional-genomics-i-introduction-and-design/what-is-functional-genomics/

https://www.ebi.ac.uk/training/online/course/functional-genomics-i-introduction-and-designing-e/common-study-types-functional-genomics

https://www.ebi.ac.uk/training/online/course/functional-genomics-i-introduction-and-designing-e/common-study-types-functional-genomics/gen (focus on GWAS)

https://www.ebi.ac.uk/training/online/course/functional-genomics-i-introduction-and-designing-e/common-study-types-functional-genomics/tra (include example 1).

https://www.ebi.ac.uk/training/online/course/functional-genomics-i-introduction-and-designing-e/common-study-types-functional-genomics/epi

https://www.illumina.com/techniques/sequencing/dna-sequencing/chip-seq.html

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3233226/ (Introduction and Figs. 1 and 2)

https://www.illumina.com/areas-of-interest/microbiology/microbial-sequencing-methods.html (focus on 16s vs shotgun metagenomics)

Learn More About 16s and ITS rRNA Sequencing
Learn More About Metagenomic Sequencing

https://www.ebi.ac.uk/training/online/course/functional-genomics-i-introduction-and-designing-e/common-study-types-functional-genomics/met

Week 13 / November 29: Omics II (Bionformatic Tools)

Learning Objectives: At the end of this unit, students should be able to:

1. Define and distinguish "bioinformatics," "computational biology" and "systems biology."

navigate to and understand the differences among the following sites: i)NCBI; ii) the three of the major human genome databases; iii) genomic databases for mice, fruit flies and nematodes;

2. State how omics databases (e.g. NCBI) and "Knowledge Bases" (Qiagen's IPA, NutrigenomeDB) are established.

3. Use BLAST for nucleotide and protein sequence analysis.

4. Describe the various functionalities of Qiagen's IPA package.

5. Explain the overall approach and goals of i) "regulator effects analysis" and ii) "molecule activity prediction"

Required Reading

A. Bioinformatics, Computational Biology and Systems Biology (Definitions / Attributes)

https://www.youtube.com/watch?v=61OvUyzJpY0

"*Bioinformatics and Computational Biology"* (pdf in files)

"Comp. Biol., Models and Machine Learning" (pdf in files; slides #1-4)

B. Bioinformatics

B-1. Databases.

https://guides.library.uncc.edu/c.php?g=173181&p=1143648

i. Navigate to the National Center for Biotechnology Information / NCBI website. The NCBI is one of the essential bioinformatic databases available to you. Note the number and diversity of data and computational tools available.

ii. Navigate the Human Metabolome Database to the "metabocard" for 2-Hydroxybutyric acid (search HMDB0000008). Explore the numerous hyperlinks in this and other bioinformatic databases to get an idea of the informatic power available to you.

iii. Navigate to and appreciate the vast tools available at these three human genome databases: **GenBank**,

U**CSC Genome Browser**, **Ensembl**; Similarly, navigate to these three comparative genome databases: **Mouse Genome Informatics (MGI), FlyBase and WormBase.**

https://www.ncbi.nlm.nih.gov/guide/all/ (appreciate that each of the databases requires human input and curation. The data don't just appear magically. Building a well-curated database that can be queried across multiple fields (data mining and analysis) is a time-intensive undertaking.)

B-2. Examples

B-2.1 Gene Annotation
https://www.youtube.com/watch?v=QLcmEqBayr0

https://www.youtube.com/watch?v=_N3XrrB2u9k

B-2.2 Sequence Analysis
(BLAST): **https://www.youtube.com/watch?v=RzC-V67z5LA (**9 min, 48s**)**
(BLAST https://www.youtube.com/watch?v=HXEpBnUbAMo (7min, 55s)

B-2.3 Differential Expression and Pathway Analysis of RNA-seq Data
"Comp. Biol., Models and Machine Learning" (pdf in files; slides #5,6)

B-3. Software Packages (Coding Not Required)

B-3.1. Qiagen Ingenuity Pathway Analysis
https://www.qiagen.com/us/products/discovery-and-translational-research/next-generation-sequencing/informatics-and-data/interpretation-content-databases/ingenuity-pathway-analysis/

B-3.1.1 Analysis Examples:
https://www.youtube.com/watch?v=XUpbYWdDLhE **(9 min)**

https://digitalinsights.qiagen.com/resources/science/case-studies/feeding-the-world-arkansas-lab-uses-ipa-to- improve-feed-efficiency-in-chickens/

B-3.2 NutrigenomeDB
http://nutrigenomedb.org/ (load and analyze the example datasets).

Week 14/December 6: Systems Biology: Integrating Omics Data Using Computational Biology

Learning Objectives: At the end of this unit, students should be able to:

1. state the role of model building in computational biology.

2. Explain the rationale and work-flow in the assigned article, *"Network analysis and data mining in food science: the emergence of computational gastronomy."* Be able to interpret the graphical network analysis presented.

3. State the function of Systems Biology Markup Language (SBML) and similar formats for other biological systems models.

4. Distinguish "passive" vs "active' machine learning and how the latter offers a solution to the problem of "too many possible experiments."

5. Discuss the rationale for "beyond theory" or "hypothesis-free" science.

Required reading.

A. Computational Biology: Role of Models and Machine Learning

"Comp. Biol., Models and Machine Learning" (pdf in files; slides #7-14)

https://www.the-scientist.com/news-opinion/new-studies-enable-a-clearer-view-inside-cells-69380

Example: https://flavourjournal.biomedcentral.com/track/pdf/10.1186/2044-7248-2-4?site=flavourjournal.biomedcentral.com (also available in files as "computational gastronomy")

B. Systems Biology: Examples

https://irp.nih.gov/catalyst/v19i6/systems-biology-as-defined-by-nih (review from week 2 if necessary)

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4540493/ (focus on Methods and the figures in Results: appreciate the multiple levels of biological hierarchy studied, the different 'omics' approaches used, the commercial and public domain bioinformatic and computational tools used and their output in Figures 2 and 3 and Table 3).

https://www.omicsonline.org/nutrition-research-and-the-impact-of-computational-systems-biology-jcsb.1000122.php?aid=19492 (Introduction and section on the Bio Models Database).

C. Models Give Way to Hypothesis-Free Science

https://www.wired.com/2008/06/pb-theory/ (also available as "The End of Theory" in Files)

Week 15/December 13: Bioengineering and Synthetic Biology (Individual 10 minute Presentations)

"Incorporation of novel foods in European diets can reduce global warming potential, water use and land use by over 80% *Nature Food*, DOI: 10.1038/s43016-022-00489-9 (pdf in files, week 15 additional)

"*Biomaterials For Boosting Food Security" Science, 07 APR 2022: 146-147.*