

GTL P.I. Workshop, Washington, D.C.
Breakout Group: Cultivation Technologies
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This breakout session attracted more than 25 participants at the annual Genomics:GTL Principal Investigator workshop held March 1–3, 2004 (see list of participants). The 1.5-hour session stimulated discussion that targeted issues related to cultivation and production of biological and microbiological samples destined for systems-level analyses. Notes taken during the session comprise this report and will be used to plan and execute a 2-day workshop on this topic.

Background

High-throughput technologies for sequencing entire genomes of microorganisms have emerged during the past two decades. Recent developments in analytical technologies (microarrays, high-throughput proteomics, single-chain antibody production) promise to extend scientific advances in biology beyond the genome to include the proteomes, transcriptomes, and metabolomes of microorganisms. The Genomics:GTL program is charged with further developing, applying, and integrating advanced analytical technologies to expand our understanding of fundamental biological processes. Toward this end, the GTL program is proposing the construction of four facilities with distinct, yet related, objectives.

Each proposed facility will have particular requirements regarding samples to be produced or analyzed; these needs currently are not well defined and are being identified through a series of DOE-sponsored workshops. Generally, each facility embraces a high-throughput production goal targeting thousands or even tens of thousands of samples from single- or multiple-species cultures, so cultivation methodologies must produce sufficient numbers of samples to justify high-throughput facilities. The concept of “garbage in–garbage out” is particularly relevant when discussing high-throughput, high-sensitivity analyses that generate large, complex data sets. High-throughput sample-production requirements cannot compromise sample quality. Because microorganisms respond directly to conditions of environment and culture, traditional and commonly used methods of cultivation such as shake-flask batch cultures, in which environmental conditions are described poorly and continuously changing, should be considered inadequate without confirmation. Cultivation technologies that provide continuous monitoring and control of culture conditions should ensure the production of well-characterized and reproducible samples. Discussion-group issues focused around the production of numerous different biosamples in sufficient amount and reproducible quality for the target analysis.

Introduction and Data Needs

George Michaels, Pacific Northwest National Laboratory, kicked off the session. He correctly pointed out that bacteria very rarely thrive as monocultures and that functionally dense microbial communities are analogous to higher-organism tissues. He illustrated that simultaneous analyses of multiple aspects of biology generate enormous data sets that can challenge integration and interpretation. For example, a single established microorganism can be grown under four different controlled-culture conditions with five time points each. Its proteome could be divided

into four fractions for 2D LC MS/MS analysis. This one experiment would produce 80 MS data files.

High-throughput analytical technologies have distinct computational and quality-control requirements that may not necessarily be needed for more traditional low-throughput approaches. For example, automation may help to meet standard operating procedures for cultivation; appropriate configuration of the equipment that facilitates high-throughput automation, however, must capture and disseminate reliable and accessible data sets. Equipment and procedures must be both flexible and documented. For example, data acquisition and informatics for controlled cultivation will reflect the defined products.

There is a clear need to anticipate research directions and develop appropriate tools to pursue those directions. To define such approaches and technologies, however, desired facility products must be defined.

Biosamples

Of utmost importance is capturing biosamples (proteins and enzymes, metabolites, and mRNA and DNA) that represent conditions under which they were produced. This requires the development and use of constant harvesting procedures that rapidly and effectively stabilize samples. Participants in the breakout session agreed that automated sampling procedures are almost certainly required to remove the human component that contributes to errors and variability.

Defining the “state” of the culture at the time of harvesting may be critical for establishing reliable and reproducible harvesting conditions. Culture descriptors such as cell size and volume, cell numbers, and cell aggregation should be recorded. Digital microscopic images that are amenable to standardized post-imaging analyses may be necessary. Such information is important in establishing QA/QC for sample production. Biological variability, as it relates to growth conditions, should be minimized to reduce variability and hence the cost of downstream analyses.

Growth Medium and Culture Parameters

Components of growth media directly influence microbial metabolism and physiology. Hence, growth media should be defined chemically, not only to address issues of reproducibility but to provide the ability to obtain some element of chemical mass balance that can help to interpret how the culture is processing nutrients. Mass balance in the gas phase also is important.

Culture parameters such as dissolved oxygen, pH, density, and growth rate are important for interpreting metabolic culture responses and for providing another level of quality assurance from one experiment and experimenter to another. Such parameters are tractable using off-the-shelf bioreactors, but issues arise with monitoring and controlling multiple reactors for high-throughput sample production. In addition, cell number, optical density, and dry weight are used to determine a biomass concentration; all are comparable but not linear, even for the same microbe under different conditions.

Parallel continuous-flow reactors with microcontrollers that incorporate 96 well plates are being developed to cultivate in high-throughput mode with control. However, insufficient sample volumes are problematic. Current methods for both MS proteomics and protein over expression

for ultimate crystallization seem to require 1-L cultivation. Improvements in downstream analytical technologies such as proteomics will reduce sample volume requirements; however, the time frame for these improvements is unknown.

Conditions and technologies for monitoring and controlling the cultivation of extremophiles are emerging issues. Conditions of extreme temperature, pressure, pH, metal concentration, and radiation are important for DOE mission-related research. Reactor designs will be necessary to accommodate this research.

Culture Heterogeneity

Microbial physiologists generally agree that defined cultures from continuously stirred tank reactors (chemostats) are the most homogeneous cultures that can be produced. This is not necessarily true, however, since one can demonstrate easily that subpopulations exist on reactor sides and on the surfaces of probes, impeller blades, and baffles. In addition, even in these cultures, individual cells are distributed along the growth and cellular division cycle. Hence, all our assumptions about homogeneous cultures may be wrong and may complicate or invalidate interpretations of downstream analyses. Low numbers should not be considered unimportant. Relative numbers of subpopulations may and almost certainly are important to the system's global biology.

Cultures in continuous-flow reactors may (and often do) express some level of hysteresis when they are operated for long periods of time and under conditions of temporal perturbations. Because cultural drift can directly impact results of downstream analyses, documentation of reactor and culture history is vital for avoiding erroneous conclusions.

We must consider approaches for characterizing the level of culture heterogeneity, especially when moving from relatively homogeneous culture systems such as CSTRs to more poorly mixed systems such as traditional shake-flask cultures, high-throughput microtiter plate reactors, and biofilms. For example, persistent cells survive antibiotic treatment but may represent an important system component. At the very least, heterogeneity should be reproducible. We are just starting to develop techniques for assessing this variability and determining its impact on the biosample's desired analysis. Proteomics appears to indicate some measurable variability during a single "well-controlled" chemostat, while even poor batch conditions have little effect on genomic analysis.

What Next? The Future of Advanced Systems Biology Research

Evolution of systems-biology research will generate cultivation and sampling requirements that are quite different from current ones, so we need to look forward and anticipate future research environments. Analytical and computational capabilities will almost certainly expand and improve. Projecting how this will translate into sample requirements is difficult. Will we be in a position to grow 100 distinct 1-L cultures, conditions, or clones in a week to satisfy facility concepts? Should this target be 1000?

High-throughput production may be completely different from the systems-biology approach. "High throughput" should be better defined so adequate cultivation capabilities can be designed and constructed.

Also, researchers and the organizations that fund them should consider which groups among broad microbial taxa will be considered 5 years from now. We should list the taxa and discuss and design appropriate cultivation capabilities. Big-picture issues such as dealing with “unculturable” microorganisms should be considered. The idea is to go to natural systems and deal with their heterogeneity, which translates into definable requirements for microscopic and spectroscopic interrogations on a cell-to-cell basis. Real-time analysis in terms of imaging gene expression on single cells is an immediate goal for systems-biology research. Correlation of real-time gene expression with real-time (or near real-time) proteomics, transcriptomics, and metabolomics presents a challenge to cultivation technologies.

Single-cell analyses give more information about metabolic regulation and provide more information than bulk-averaging techniques if sufficient sensitivity and quantitation are available. Protocols that still must be improved and captured include standards for taking pictures [e.g., Alliance for Cellular Signaling (AFCS.org)].

Identifiable Cultivation and Biosample Production Tasks for GTL Facilities

- Grow specific biomass under well-characterized states for proteomics,
- Rapidly identify optimal culture conditions for expression of tagged proteins and complexes,
- Rapidly scale up to obtain necessary protein samples,
- Express intact protein complexes,
- Grow microbial cells in controlled mixed cultures and functioning communities,
- Grow them in nonstandard conditions (biofilms, extremes of salt, pH, temperature, light) that may replicate natural environments,
- Develop methods for producing samples of “unculturable” microbes,
- Provide a hospitable environment for the imaging of living cells, and
- Provide bioreactors for “single-cell” analyses.

Crosscutting Needs for Cultivation

- List of target taxa and hosts for the next 5 to 10 years
- Media and methods for this list
- QA/QC baseline for impact of variability on growth and harvest for different biosamples and facilities,
- Automated methods and sensors for growth data and protocol capture,
- Initial multifacility list of target biosamples. In particular, we must estimate the number, amount (mass), and quality for the desired purpose both now and with projected analytical-sensitivity improvements.

This white paper was sent to attendees, who will be asked to help with final documents and future workshops to determine a technology roadmap to meet identified needs.