

The future of small and large molecule bioanalytics — **today**

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ABSTRACT

Traditional mass spectrometry and liquid chromatography have formed the backbone of bioanalytic techniques for small molecules, whereas ligand binding assays have been the backbone for large molecules over the past half century of drug development. As biologic therapies become more common, new analytic approaches have emerged to facilitate the analysis of larger and more complex substances. To truly understand the way these novel therapies function in living biological systems, however, a new dimension of study will be indispensable: spatial bioanalysis. This futuristic analytic technique provides answers regarding molecule function and form that—together with other state-of-the-art analytics—dramatically improves the quantity and quality of information obtainable in drug development studies.



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INTRODUCTION

Applied bioanalysis studies the impact of a drug or compound on biological tissues or fluids. It is meant to characterize how much of the drug is present, where it is present, and what effects it has on the biological systems in which it is present. Over the years, advancements in bioanalysis have repeatedly opened new opportunities for investigation into the effects of a drug on cells and tissue, bringing the science to new frontiers for both drug development and clinical applications. The innovation continues, and novel developments hold significant promise for improved explorations in early discovery efforts as well as the realization of truly personalized medicine.

Historical bioanalysis

Early attempts at laboratory bioanalysis go back to the 1800s, as chemists, biologists, and botanists attempted to study the makeup of their compounds, fluids, or plants of interest. Chromatographic techniques for quantifying the presence of a drug in biological fluid achieved a level of sophistication and practical application in the mid-1900s, and the field of bioanalytics began to expand dramatically beyond forensics and toxicology into pharmacokinetic analysis and therapeutic monitoring. Liquid chromatography (LC) coupled with mass spectrometry (MS) became the mainstay for small molecule analysis as of the late 1980s. It was more sensitive than its predecessors, faster to prepare, and useful in a broad range of analytes and metabolites. Since the 1990s, these approaches have experienced a gradual evolution to systematically improve speed, selectivity, and sensitivity.

Until recently, tandem MS (MS/MS) and LC were primarily used for the study of small molecule drugs, which historically made up the vast majority of therapeutics. For the study of larger molecules, ligand- and cell-binding assays emerged from radioimmunoassays, which were originally developed to quantify insulin in the 1950s. Because of their speed and sensitivity compared to chromatographic techniques of the time and their utility for biologics, binding assays have remained a constant in bioanalytics and are still widely used today.

Revolutionary science

While LC-MS/MS still form a mainstay of bioanalytics for small molecules, numerous incremental advances, such as electrospray ionization, have made MS more precise and easier to use, as well as applicable to a wider range of substances, including large molecules (with specific preparation adaptations to the process). Perhaps the most significant leap forward in bioanalysis since the invention of MS has been the emergence of spatial bioanalysis. This futuristic technology has catapulted the field's ability to analyze molecules both large and small as they interact with living tissue. Spatial analysis can achieve cell-specific and even subcellular studies of the quantity, function, form, and interactions a molecule undergoes in live tissues and fluids. The technique dramatically improves the ability to understand the impact a drug has on gene and RNA expression, as well as offering substantial insight into a drug's biotransformation, metabolism, and on-target efficacy.

As biologics become a growing segment of new drug applications and approvals, understanding the way large molecules behave in biologic systems has become a necessity. The field of large molecule therapeutics continues to expand into novel compounds, including the growing class of mRNA-based vaccines and therapeutics, prompting the U.S. Food and Drug Administration (FDA) to release increasingly detailed guidance for demonstration of the safety and biologic activity of such compounds.

In many cases, the technology is already available to answer the FDA's questions decisively and proactively. In other cases, novel combinations of approaches or new methods for sample preparation may be required to best elucidate a molecule's biological impact. Knowing the available options to achieve the most advanced documentation of a molecule's nature—as well as when to use which method, and what differences exist for large versus small molecules—can smooth the road toward regulatory approval when the techniques are applied appropriately, early, and often.

THE WORLD OF SMALL vs. LARGE MOLECULE BIOANALYTICS

Bioanalysis by molecular size

The difference in chemical complexity accounts for some of the difficulty analyzing the biological processes impacted by small vs. large molecules. The simplicity of small molecules often makes their pharmacokinetic and pharmacodynamic (PK/PD) properties more predictable; likewise, the molecule size and formulation (typically orally bioavailable) result in stable products with expected metabolites and simple dosing regimens.³ Therefore, bioanalysis for small molecule drugs is often faster and more straightforward. In most cases, mass spectrometry is the mainstay for small molecule analysis. Large molecule drugs have the potential for considerable variability from batch to batch, making tight manufacturing controls and routine bioanalytics critical components of drug development and delivery quality. Part of this routine evaluation must ensure that the characteristics of these compounds have not significantly altered from their desired structure, function, and concentration with changes in manufacturing or source materials. Ligand binding assays have predominantly constituted the bioanalytical approach to large molecules.

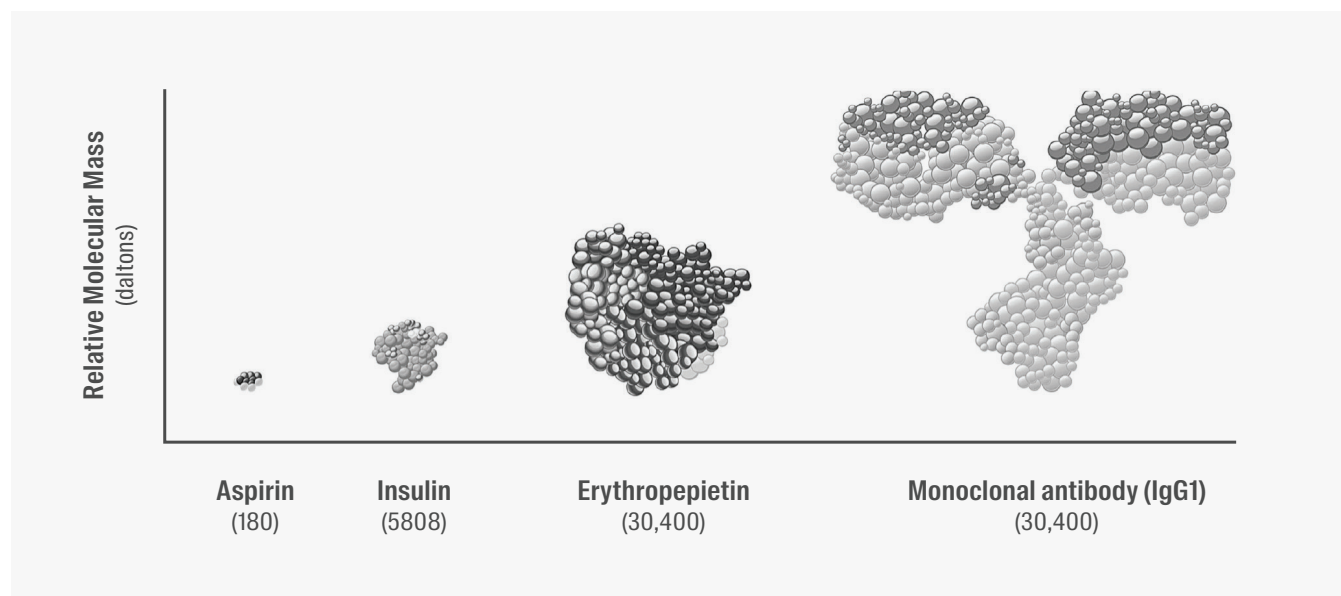
Small molecule drugs

Small molecule drugs are compounds with a low molecular weight that have the ability to impact biochemical processes. These chemical substances formed the foundation of early pharmaceutical development, such as aspirin and penicillin. Although small molecules still dominate most medicine cabinets, they are expected to account for only 3 of the top 10 bestselling drugs globally in 2022.¹ While small molecule drugs are still the predominant class obtaining approval, biologics have experienced steady and substantial increases in annual approvals of drug applications by the FDA.²

Large molecule drugs

Large molecule drugs are both physically much larger than small molecules and typically more complex in terms of chemical structure, manufacturing, storage requirements, regulatory processes, characterization, and replicability.³ As such, they are often more time-consuming and costly to produce, but they may retain some competitive advantage over biosimilars even after patent expiration, unlike most small molecules with generics.

FIGURE 1. Relative Molecular Mass



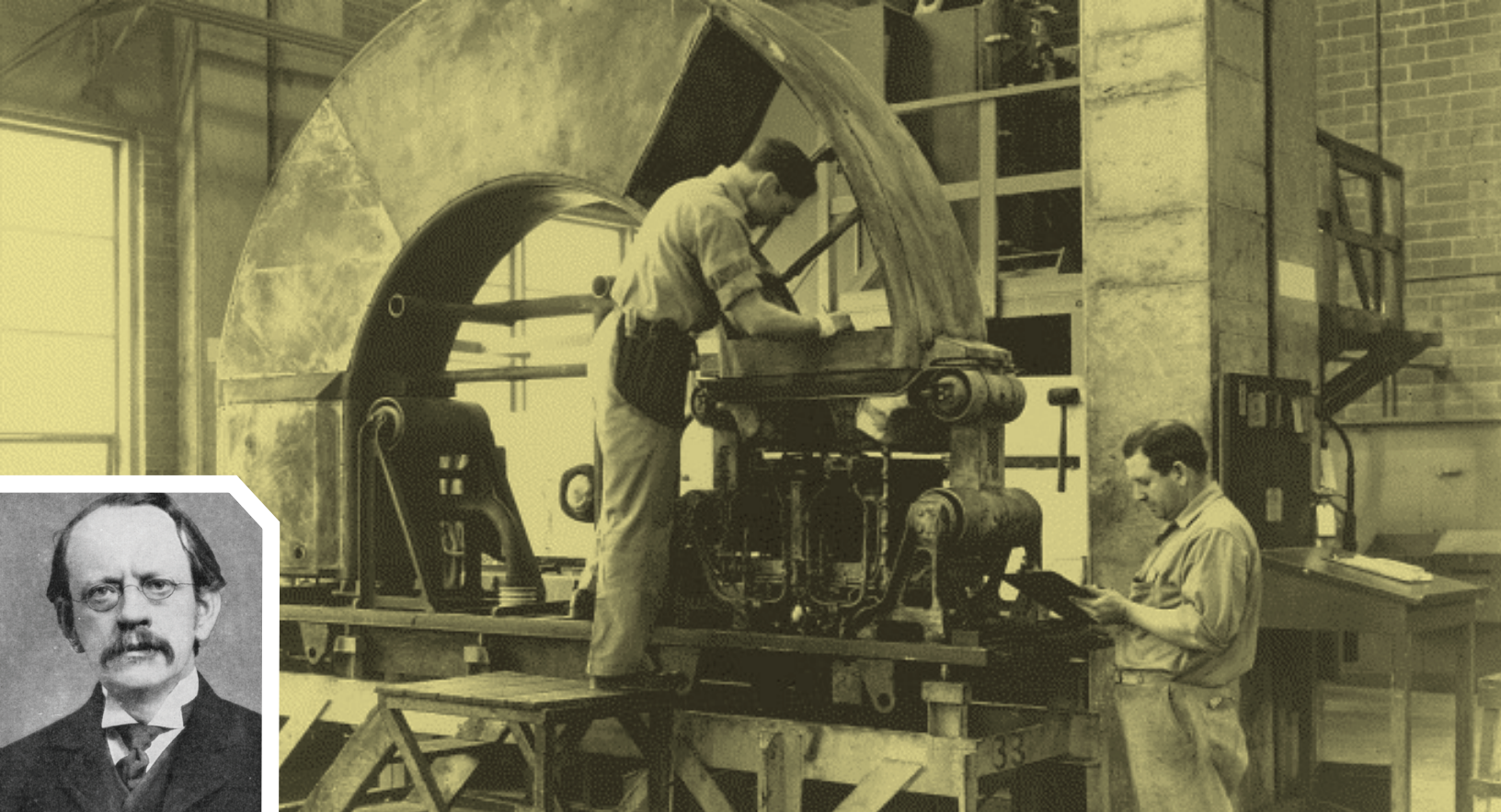


FIGURE 2. Part of the Calutron mass spectrometer first used for preparative MS; inset photo of J.J. Thomson, who discovered the electron in 1897 and built the first mass spectrometer in 1912.⁴

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Methods to aid in initial characterization efforts as well as ongoing monitoring for small molecule drugs typically falls to MS, while large molecules may rely on ligand-binding assays (LBAs) and LC or a combination of these techniques with MS. All of these technologies have suitable applications for specific compounds and pros and cons according to molecule size. However, the continual march toward innovative biologics—many with unique mechanisms of action or highly precise molecular targets—demands an equally innovative set of bioanalytics tools.

Broad biological activity exhibited by large molecule drugs beyond their expected therapeutic effects (such as immunosuppression) has resulted in stringent expectations for regulatory data on PK/PD, toxicity, off-target effects, and potential efficacy. The FDA requires thorough documentation of identity, purity, strength, potency, and quality for biologic drugs just as it does for small molecule drugs, as well as clear delineation

of cell line derivation and origin of source materials. Traditional bioanalytics tools would have fallen short of the FDA's expectations for emerging biologics. However, with custom LBAs, recent advances into spatial bioanalysis, and class-specific improvements in the application of MS technology, emerging technologies can reduce clinical risk and provide better data for regulatory bodies and sponsors alike, offering a level of detail previously unimaginable.

Assays and screening techniques that can anticipate and identify true bioactivity as well as quantify localized concentrations and cellular (and subcellular) interactions pave a path forward for companies exploring biologic therapies. Novel approaches to bioanalytics such as spatial imaging, hybrid immunoaffinity-LC-MS/MS (IA-LC-MS/MS), and other advances offer ways to fill in the knowledge gaps surrounding molecules of any size, creating more accurate pictures of what drugs do in biologic tissue and fluids.

TOMORROW'S TECHNOLOGY TODAY

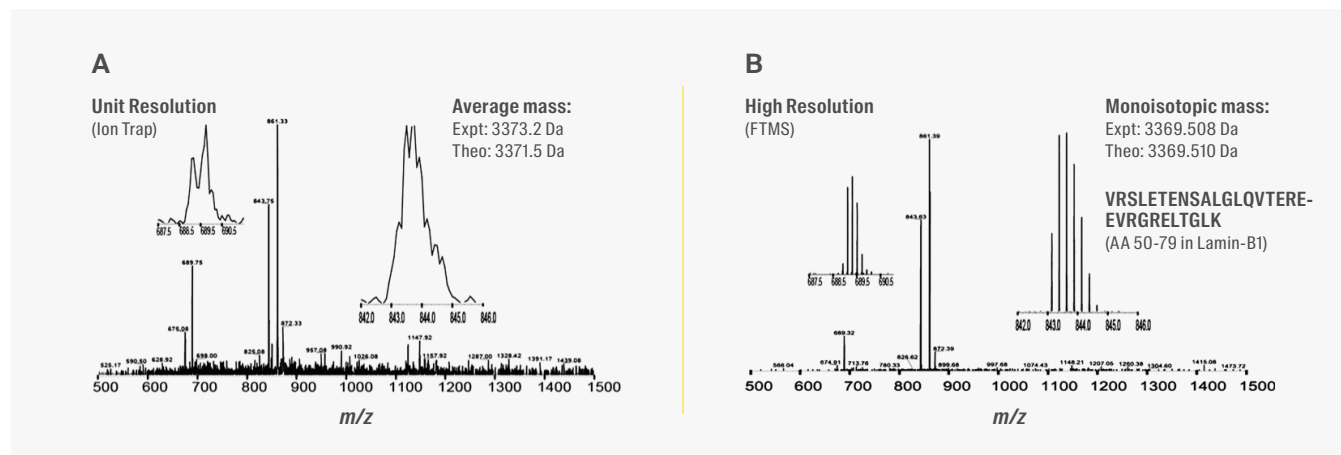
Advances in mass spectrometry

Ever since the advent of MS technology, bioanalysts and chemists have advanced the technology in an effort to improve it, either for unique applications or to address shortcomings in selectivity, speed, precision, or other functions. The past several decades have seen a steady introduction of techniques and add-ons that improve functionality, often for specific applications or narrow fields of study. More recently, a number of broadly applicable advances have resulted in significant impacts on the ability of bioanalysts to study the presence, location, and impact of compounds of interest.

Some of the most significant recent or emerging MS advances include:

- **Hybrid mass spectrometry with time of flight and quadrupole-linear ion trap analyzers.** Hybrid MS involves the sequential arrangement of two different types of mass analyzers between the ion source and the ion detector. Conceptually, the approach aims to use two unique mass spectrometry machines with different performance features so that the output embodies the advantages of both. Early applications of this technology (such as the EB/qQ and the BE/qQ) involved steady but modest improvements in sensitivity and accuracy over prior techniques.⁵ Combined with electrospray ionization, however, hybrid MS took a dramatic leap forward. This much improved ionization process increased efficiency, sensitivity, and performance, enabling improved ion extraction and manipulation of the ion beam. Add to this the time-saving data production rates of time-of-flight (TOF) hybrid mass spectrometer technology and the use of quadrupole-linear ion trap instruments (together creating a triple quadrupole MS with TOF, or QqTOF) to increase selectivity and enable discrete analyses, and modern MS data generation capabilities now far exceed those of machines just a decade ago.
- **High-resolution mass spectrometry (HRMS).** Although HRMS is not particularly new, its cost and level of detail make it a tool not routinely used for samples with known identities or characteristics. However, its higher mass resolution and accuracy than many other MS/MS technologies helps when samples are particularly complex or when interference emerges with difficult samples. HRMS offers a wide mass range of quantification to accurately study biotransformation and biopharmaceutical characterization for large molecules, including intact or digested proteins and peptides.⁶ In most cases, this technique can be saved for large molecules only, when LC-MS does not suffice, assisting with bioanalysis for the most complex molecules or quantification efforts demanding the greatest need for precision. In these cases, HRMS can be used in a multiple reaction monitoring (MRM) mode with high-resolution mass accuracy available for product ion identification. This allows for the greatest selectivity, discrimination, and high-resolution detection of fragments.⁵

FIGURE 3. Image shows the difference in detail for higher resolution MS machines. Low-res you get the big parabolic mountains, high-res offers skinny, precise peaks.





- **Ion mobility spectrometry with differential mobility analyzers (DMA), differential mobility spectrometry (DMS), field asymmetric waveform IMS (FAIMS), or hyphenated differential mobility spectrometry (DMS/FAIMS).** Based on principles initially described in 1896, ion mobility spectrometry (IMS) involves the motion of ions in gases within an electric field. Since its commercial combination with MS as a routine analytical tool in 2006, IMS-MS has evolved to offer unique, if sometimes subtly different, options with application-dependent pros and cons.⁷ IMS offers MS analytics a dimension of molecular separation based on three-dimensional structures. Coupled with DMA devices, IMS enables measurement of very large molecules, including antibodies and viruses; this approach is not often used for small molecule studies.⁶ DMS and FAIMS add a filter to the MS system to use waveforms to periodically separate ions, resulting in increased selectivity and an improved signal-to-noise ratio. These enhancements of the IMS process, as well as recent couplings of IMS with ultra-high-resolution MS, typically shorten analysis timelines and expand potential applications of the technology.
- **Hybrid immunoaffinity (IA) LC-MS or LC-MS with LBA.** While LC-MS has traditionally been used for bioanalytics of small molecules and LBA for large molecules, the increased drive to understand large molecules at multiple stages in production and metabolism—most importantly, the biotransformation of molecules *in vivo*—has spawned the combination of technologies to glean additional insight into biologics. Now, the technologies complement each other in bioanalysis for drug development. Hybrid IA-LCMS/MS uses one binding reagent to capture and enrich a protein and then, after digestions, uses the selectivity of LC-MS/MS to detect signature peptides. Using these complementary techniques, there is low potential for interference, higher selectivity, and a highly multiplexed process.⁸ Hybrid IA-LC MS/MS enables protein detection and precise specificity resulting in a robust assay approach that offers the best from each realm of protein analytics.
- **Advances in high-throughput screening (HTS) and ultra-high-throughput screening.** HTS is used both in drug discovery and in early screening processes to detect potential off-target effects or toxicities of drug candidates. By screening molecular libraries and thousands of compounds and combinations for potential biological mechanisms of interest, researchers can identify new options to pursue for more in-depth discovery efforts. Historically, bottlenecks have occurred either in the stages of sample preparation—the limitation being in how many wells could be accurately filled with different samples at the proper quantity for cost-effective and efficient testing—or at the data analysis stage, where the quantity of information generated often exceeds the capacity of a team to review and interpret. Speed of analysis was another limiting factor, particularly when larger numbers of samples were selected for screening. Now, HTS can screen thousands of compounds per day, with digestible data reports that facilitate interpretation and prioritization of certain candidates for certain purposes. Traditionally used for small molecules, the technique can also be used for large molecules and can help identify likely metabolites or potential targets for specific compounds with known effects.
- **Automated liquid handling** allows precise and minute quantities of samples to be mechanically deposited in well plates with hundreds or even thousands of wells. This can be done using existing laboratory automated fill techniques, or custom-built for facilities without the existing equipment. It dramatically increases the number of samples that can be tested via HTS per day and often enables significant reductions in the quantity of sample used per well, which directly and significantly impacts price.⁹

The era of spatial imaging

Spatial bionalytics integrates imaging techniques to allow the visualization and quantification of biomarkers and drug distribution at the cellular level. In doing so, it enhances the study of a drug's impact on cells and the tissue microenvironment, elucidating the picture of targeted effects, efficacy, metabolism, and toxicity. When used early in development, these technologies allow companies to compare a wide variety of molecules for their various impacts, screening for those with the most probable therapeutic effect. Throughout the development continuum, spatial imaging can provide insight into biodistribution, PK/PD, safety, and potency.

Spatial imaging technologies include:

- **Quantitative mass spectrometry imaging (QMSI).** QMSI offers the ability to quantify the amount of analytes present in tissue samples, opening the door to enhanced PK/PD studies for large and small molecules in tissue, as well as investigations of drug metabolism, target engagement, toxicity, as well as biotransformation and biolocation—identifying exactly where in a tumor or tissue the drug reaches, and in what form.^{10,11}
- **Matrix-assisted laser desorption ionization (MALDI),** also known as molecular histology, is a technique that can simultaneously detect more than 1,000 intact molecules such as amino acids, proteins, lipids, metabolites and drug compounds from a tissue sample. Together with MS, this technique can now be used to generate more than just relative abundance and spatial distribution; absolute quantification is now possible and, when compared to different portions of the same tissue or to other tissues altogether, can offer insight on a drug's pharmacological and toxicological effects.¹² The process can also provide information on molecular turnover, metabolism, disease progression, drug concentration, and PK/PD. This enhanced quantification ability for everything from small molecules to large proteins has resulted from robust improvements in preparation techniques, noise reduction, and data analysis, and holds great promise for the application of QMSI and MALDI for the future of bioanalysis of molecules of any size.
- **Laser ablation inductively coupled plasma (LA-ICP)** instruments combine with MALDI imaging to study and quantify elements that contribute to cell growth, activity and homeostasis, such as copper, iron, calcium, and zinc. Staining images from LA-ICP can then be laid over MALDI imaging to provide combined insight into the bioactivity and microenvironment of various drugs. These approaches have been extensively researched, adjusted, and validated and now represent a rich opportunity for the expansion of QMSI into a broader range of molecules, with enhanced imaging of low ionization efficiency samples.¹³

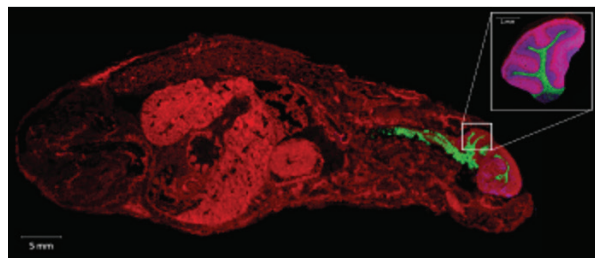


FIGURE 4. MALDI

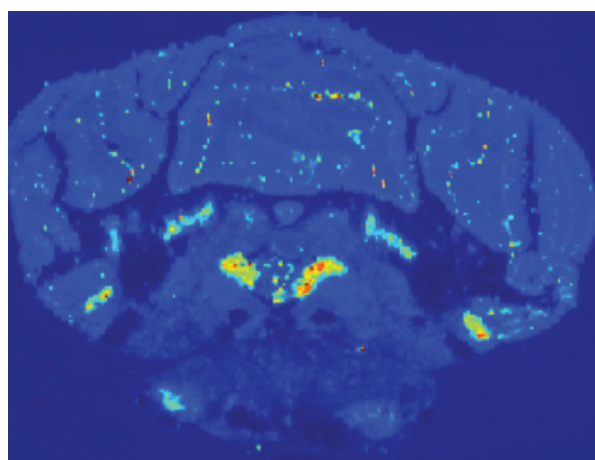


FIGURE 5. LA-ICP

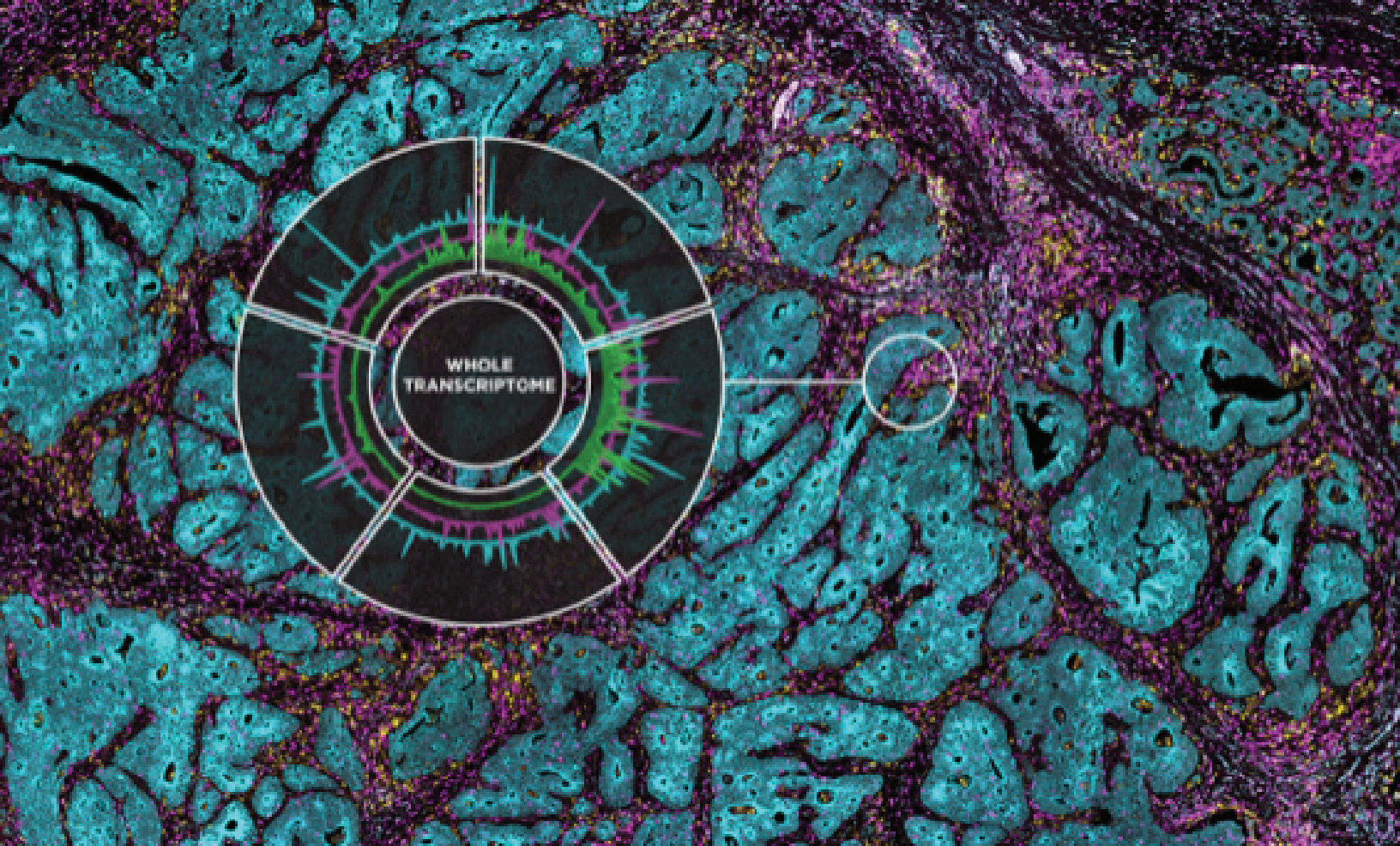


FIGURE 6. GeoMx

- **Imaging mass cytometry/high multiplex immunohistochemistry.** This approach to spatial imaging enhances the quantitative picture of markers of interest (40 or more simultaneously), including proteins, in tissue sections by removing background “noise” so that each metal isotope has a unique detection peak. This creates clear images for analysis. When paired with fluorescently labeled antibody imaging in a region of interest, up to 80 proteins can be quantified, giving a clear picture of tissue morphology, cell-specific protein expression, phenotypes, and pathways. This illuminates the functional impact of drugs or disease on single cells as well as across tissue structures. This provides data on where a drug (small or large molecule) goes and how it impacts cells, signaling, and phenotypic expression in multiple locations.
- **GeoMX single-cell spatial analysis.** Novel applications of laser microdissection to extract RNA, DNA or other subcellular substances of interest enable GeoMx to provide cell-specific transcriptome-based analysis from any target in any tissue. This approach can quantify proteins, viruses, synthetic DNA, non coding RNA, exogenous genes, and—when paired with in situ hybridization (ISH) technology—mRNA, miRNA, and ncRNA as well as cytokines, lowexpressing targets, and transcription factors. This allows analysis of the correlation between a cell’s drug exposure and changes in gene expression. Studies with this type of spatial analysis allow researchers the opportunity to identify precise biological impacts of different therapies, and the technology can be applied to measure responses to any type of drug.



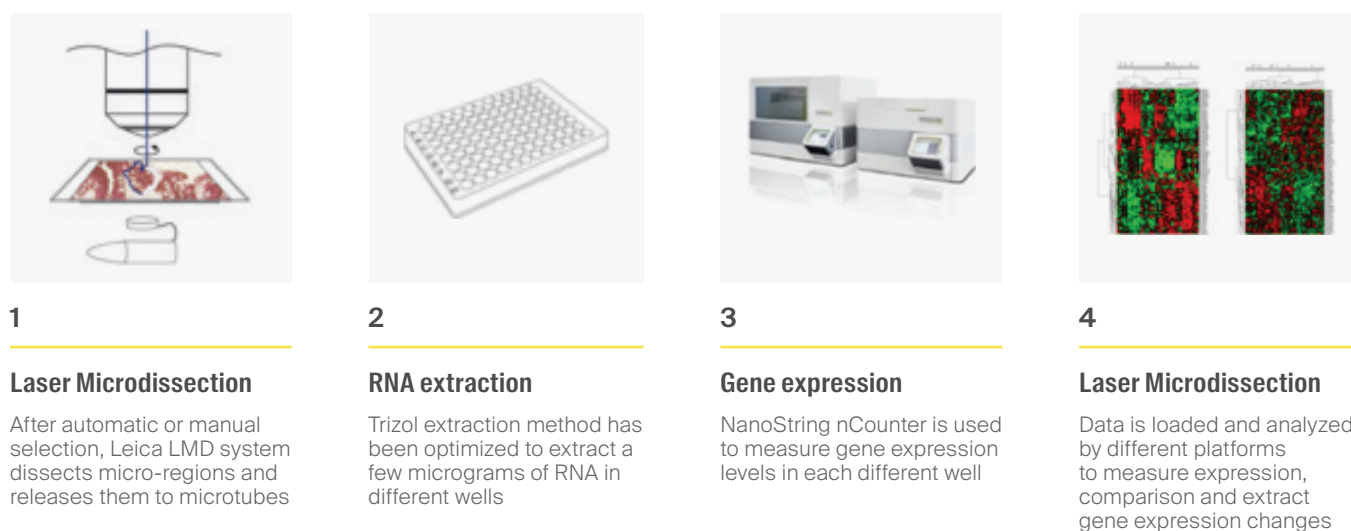
Novel technologies and adaptations

In addition to, and often in partnership with, the primary molecular analysis processes discussed above, new analytic techniques and sample preparation advances have dramatically altered the future of drug discovery, development, and bioanalysis. Especially as biologics become increasingly important in company portfolios, novel techniques to understand their bioavailability, biotransformation, single-cell impacts, and PK/PD become increasingly important. Tools to improve the information available to support development can save time and money as well as increase safety and efficacy data.

Novel additive and emerging technology in bioanalytics includes:

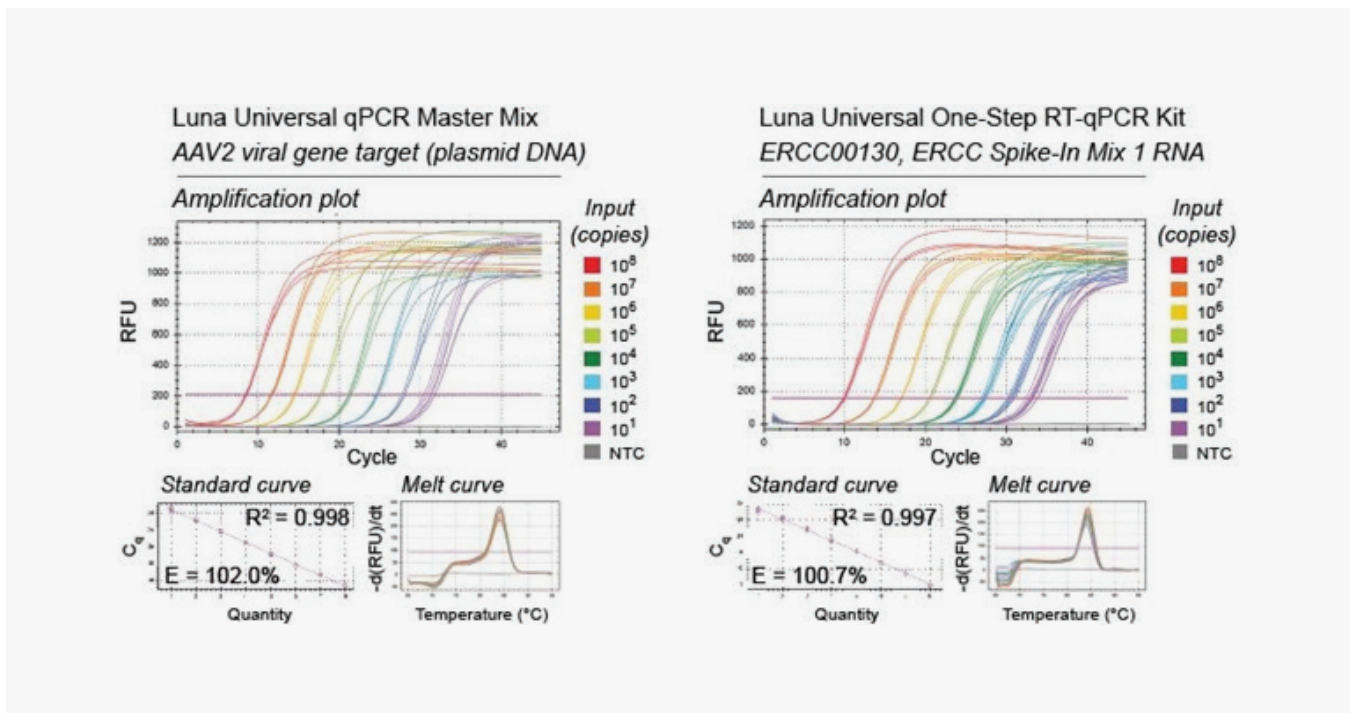
- **Laser capture microdissection (LCM).** LCM enables further study of cells in their native context and unique tissue microenvironment by allowing analysts to microscopically visualize and extract cell subpopulations of interest from tissue. Often paired with spatial imaging studies, single-cell analytics, genotyping, proteomics, pathway analysis, and other complex or advanced innovative studies, LCM offers the opportunity to enhance bioanalytics studies of large or small molecules through direct biodynamic observation of specific cell groups of interest.¹⁴
- **Digital pathology and artificial intelligence (AI).** The increasing digitization of tissue slide images has not only facilitated image sharing and education, it has opened the door to AI applications that could dramatically advance bioanalytics.¹⁵ Machine learning technology enables computer algorithms to study digital images and analyze them for observations and connections that humans cannot make. Digitization and AI can also improve diagnostics and speed up bioanalytics, with the opportunity to use whole slide imaging to inform data-rich research efforts in drug development and clinical trials, especially through integration with clinical data. The approach is agnostic to molecule size.

FIGURE 7. LCM process



- Transcriptomics.** An increasingly robust way to understand cell-specific genetic expression and RNA transcription, transcriptomics is an especially useful tool in bioanalytics when combined with other -omics, such as proteomics, metabolomics, or genomics. These technologies enable studies of circumstance- or cell-specific transcription in response to exposure to a drug of any class or size. By revealing how treatments alter RNA transcription in cell populations of interest, investigators can better understand efficacy, off-target effects, toxicity potential, and potential drug-drug interactions.
- Quantitative polymerase chain reaction (qPCR).** This technology has emerged as a valuable way to study cell and gene therapies as well as gene expression and biomarkers. The application of qPCR assays includes pharmacodynamic and toxicology studies via analysis of biodistribution, persistence and migration. It also enables shedding analysis and, through its gene expression and biomarker detection capabilities, facilitates the study of PD and efficacy endpoints, gene knockout, liver damage, circulating cancer cells, and gene regulation.¹⁶ Although it is typically applied to large molecule assays, qPCR can also be applied for HTS of small molecules using real-time reverse transcription and the proper application of liquid handling techniques. This allows users to better understand a drug's impact on the phenotypes of biological systems.¹⁷
- Additive technologies supporting large molecule bioanalysis.** Techniques to better measure proteins have gradually emerged in an attempt to improve analytic speed and selectivity when using LC-MS rather than LBA or flow cytometry for the study of large molecule compounds. Typically, protease digestion procedures are applied to reduce proteins to their resultant peptides. However, application of immunoaffinity enrichment approaches combine hybrid LBAs with LC-MS to improve selectivity. Furthermore, immunoaffinity enrichment is commonplace to HRMS in order to sensitively study intact proteins. Challenges resulting from analyte ions distributed across multiple charge states are addressed with supercharging agents, allowing HRMS to distinguish between different forms of a single protein.¹⁰ This may be particularly helpful in studying batch-specific variations for biologic drugs, as well as changes that may occur during storage or transport, or even biotransformation in vivo.

FIGURE 7. LCM process



CONCLUSION

Recent advances and emerging technology in the field of large and small molecule bioanalytics have revolutionized the amount of detail and the degree of understanding available to companies interested in documenting the true impact of their compound on biological systems. As practices and equipment continue to evolve, the constant focus on improved selectivity, sensitivity, speed, and in-depth biological effects is undoubtedly poised to enhance screening efforts and improve human safety. The approaches revolutionizing bioanalytics also offer regulatory agencies a more robust understanding of the mechanisms of action of a given drug, as well as its potential for safety and efficacy. Furthermore, these techniques open the door to improved drug candidate selection restricting development efforts to the most promising compounds by better characterizing their molecular behavior and effects on biological systems before they ever enter human trials.

Although large molecules still pose some challenges in the field of bioanalytics due to their variety and uniqueness, the dramatic uptick in biologics entering the market suggests successful, strategically applied bioanalytics processes already offer substantive information supporting their bioactivity. Ongoing efforts to fine-tune or adjust techniques applied to large molecules and continued innovations in mass spectrometry are poised to alleviate any growing pains with MS and assay bioanalysis for biologics.

Companies that are able and willing to adopt the most suitable and advanced techniques available for their molecules of interest stand to benefit from enhanced insights, earlier opportunities for decision-making about advancing a development program, improved opportunities for formulation adjustments, streamlined regulatory conversations, and better preparedness for human trials. Not only can this maximize competitiveness and reduce development costs, but adoption of the right fit-for purpose bioanalytics techniques can dramatically alter the feasibility of successfully bringing a drug to market.

[Connect with us now](#) to learn more about the bioanalytic techniques that are right for your drug.



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