Published in final edited form as:
Mol Pharm. 2011 October 3; 8(5): 1619–1628. doi:10.1021/mp200092v.

The Subcellular Distribution of Small Molecules: from Pharmacokinetics to Synthetic Biology

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Abstract

The systemic pharmacokinetics and pharmacodynamics of small molecules are determined by subcellular transport phenomena. Although approaches used to study the subcellular distribution of small molecules have gradually evolved over the past several decades, experimental analysis and prediction of cellular pharmacokinetics remains a challenge. In this article, we surveyed the progress of subcellular distribution research since the 1960s, with a focus on the advantages, disadvantages and limitations of the various experimental techniques. Critical review of the existing body of knowledge pointed to many opportunities to advance the rational design of organelle-targeted chemical agents. These opportunities include: 1) development of quantitative, nonfluorescence-based, whole cell methods and techniques to measure the subcellular distribution of chemical agents in multiple compartments; 2) exploratory experimentation with nonspecific transport probes that have not been enriched with putative, organelle-targeting features; 3) elaboration of hypothesis-driven, mechanistic and modeling-based approaches to guide experiments aimed at elucidating subcellular distribution and transport; and 4) introduction of revolutionary conceptual approaches borrowed from the field of synthetic biology combined with cutting edge experimental strategies. In our laboratory, state-of-the-art subcellular transport studies are now being aimed at understanding the formation of new intracellular membrane structures in response to drug therapy, exploring the function of drug-membrane complexes as intracellular drug depots, and synthesizing new organelles with extraordinary physical and chemical properties.

Keywords

drug transport; pharmacokinetics; biodistribution; drug targeting; databases; mathematical modeling; drug delivery; drug-membrane aggregates; unnatural organelles; synthetic organelles

Introduction

Many drugs require entrance into specific subcellular organelles to reach their targets, or they have side effects associated with unwanted accumulation in non-target sites within cells. Therefore, novel drug targeting strategies to improve compound efficiency in reaching specific organelles have been sought to increase a molecule's potency and decrease

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Supporting information

An excel database file with all the literature references and information of compounds with reported subcellular localizations or compounds from PubChem and DrugBank databases was deposited at the University of Michigan's Deep Blue data repository and can be accessed through the following URL: http://hdl.handle.net/2027.42/84659

This data has been converted to tabular form and is also provided in the accompanying article [11], under review in Molecular Pharmaceutics.

undesired side effects. For example, small molecules are being targeted to mitochondria by conjugating these molecules to cell-penetrating, lipophilic peptides, oligoguanidinium, or triphenylphosphonium moieties [1–6]. To fulfill this organelle targeting drug design strategy, there have been many efforts aimed at characterizing the physiological properties of the most important intracellular organelles and identifying key physicochemical features that determine the accumulation of exogenous chemical agents inside these organelles [7–10]. More importantly, the mechanisms driving the distribution kinetic of chemical agents within the cell and the dynamic cellular response to these chemical agents are being revealed with the aid of new experimental strategies and conceptual approaches.

To put the state-of-the-art of subcellular transport research in perspective, information about the subcellular distribution of 967 unique compounds was compiled from a total of 448 scientific publications dating back to the 1960s. Current knowledge of the physiological properties and general principles of targeted delivery to the major intracellular organelles were summarized (Table 1). The criteria of article collection and the qualitative description of subcellular localization patterns were described in a separate paper ([11], accepted in Molecular Pharmaceutics). From the outset, it is noteworthy that most compounds were reported to localize within a single organelle, with few reports of compounds localized to multiple sites (Table 2). Based on these references, we review the historical progress of subcellular biodistribution research, focusing on the evolution of experimental, theoretical and conceptual approaches used to analyze the organelle-targeting features of small molecule chemical agents.

Pharmacological effects as evidence for specific organelle accumulation

Eukaryotic cells have highly organized subcellular compartments with distinct structural and functional features. Pharmacological effects, i.e. changes in these features, especially changes in organelle morphology (swelling, rupture, shrinkage, etc.) upon drug treatment have been used in a large number of studies as evidence for localization in specific organelles. Surveying the literature, a large number of subcellular localization reports were based on evidence that exogenous chemicals induced changes in the structure or function of specific organelles (Table 3).

Prior to the widespread adoption of cell-based uptake and transport assays in small molecule drug development, morphological changes were commonly used as evidence for organelle accumulation. From the 1960s to the 1980s, similar numbers of studies were based on observations with light microscopy, fluorescence microscopy and transmission electron microscopy [12–14]. Light microscopy was the preferred tool in detecting expansion in the endolysosomal compartment, visible as a massive, cytoplasmic vacuolation phenomenon. With transmission electron microscopy, morphological changes of the major organelles could be observed directly, with or without the aid of specific organelle tracers. In the early 1980s, fluorescence microscopy became increasingly applied to the detection of organelle swelling and shrinkage using fluorescent probes. In the endolysosomal compartment, the observed morphological changes have been shown to reflect the accumulation of weakly basic compounds inside these organelles, or the inhibitory effects of cations on the activity of lysosomal proteins [15–17].

Morphological changes in lysosomes and mitochondria often coincided with changes in membrane potential, pH gradients or membrane permeability [18–22]. Under some circumstances such changes resulted in the release of a resident, organelle-specific enzyme into the cytosol or into the extracellular compartment. Thus the detection of fluctuation in voltage or pH gradients, or the detection of released organelle components, was used as

evidence for accumulation of exogenous small molecules in specific organelles, from the 1970s [23–26] and continuing to this day[27–30].

Also since the 1970s, analytical measurements using thin layer chromatography and HPLC to detect alterations in organelle composition, including changes in lipid content, protein concentrations and metabolic changes, have been used as evidence to infer accumulation of small molecules in specific organelles [31–33]. For example, significant increases of phospholipids in the renal cortex of gentamicin- or netilmicin-treated rats [34] were ascribed to impaired lysosomal degradation of phospholipids due to inhibition of lysosomal phospholipase C by accumulation of said molecules in lysosomes. Ammonia, amiodarone and some other compounds that interfere with degradation of proteins or phospholipids in lysosomes [35–37] were also associated with inhibition of lysosomal proteases and phospholipases due to the accumulation of these weakly basic compounds in the lysosomes, resulting in intralysosomal pH changes with consequent effects on lysosomal enzyme activities [38–42].

Nevertheless, claims that a molecule “accumulates in” an organelle based on a change in organelle structure (or function) are circumstantial and prone to misinterpretation and experimental artifacts. For example, in the case of toxic compounds, inhibition of organelle function may not require direct interaction, or accumulation within a specific organelle. For instance, apoptosis signal transduction pathways lead to mitochondrial membrane permeabilization, loss of mitochondrial membrane potential and the release of cytochorome c from the mitochondria, as well as nonspecific effects on other organelles. Therefore, induced changes in mitochondrial volume, membrane potential, or permeability do not necessarily reflect a direct interaction with mitochondria. The same is true for other organelles [43].

Chemical analysis as evidence for specific organelle accumulation

Pharmacokinetics gradually became part of drug development from the 1960s through the 1980s. However, only since the 1990s, there has been an increasing recognition of the importance of cellular pharmacokinetics as a determinant of systemic pharmacokinetics. In the process, quantitative measurement of chemical uptake *in vivo* or *in vitro* became increasingly important as direct evidence supporting the actual localization of a molecule in a specific subcellular compartment. Irrespective of the experimental strategy, analytical measurements were increasingly applied in cellular uptake or distribution studies, providing direct evidence for accumulation in specific organelles. However, only a relative small fraction of the molecules whose intracellular localization has been reported in the scientific literature is supported by such evidence (Table 3).

In uptake experiments, researchers measure the mass of exogenous chemical agents accumulating in intact cells or in isolated organelles after *in vitro* or *in vivo* administration of the compound. In some cases, a known, organelle-targeting compound was used to compete for the interaction or otherwise inhibit the organelle-specific accumulation mechanism. For instance, in a report of the subcellular localization sites of weakly basic molecules, the reduced cellular uptake after the disruption of trans-membrane pH gradients was used as evidence for endolysosomal accumulation [44, 45]. Less commonly, ion-selective electrodes have been used to study the uptake of positively charged, lipophilic compounds in isolated mitochondria [25, 46–48]. Binding to resident organelle-specific components including protein, lipids or nucleic acids has also been measured as direct evidence to demonstrate organelle or cytosolic accumulation [49–52].

Starting in the 1990s, there was an increase in the number of investigations looking at the qualitative or semi-qualitative (relative) distribution of a compound in all subcellular

compartments, simultaneously, featuring analytical measurements following cell fractionation (Table 3). In cell fractionation studies, the basic experimental strategy has been to isolate the various organelles by differential centrifugation [53], followed by measurements of the absolute amount of a compound in each organelle fraction [54–56] and/or comparing that amount relative to the total accumulation of the compound in the cell [57–59]. Reliable separation of distinct subcellular organelles is critical to the evaluation of subcellular distribution profile. While the presence or activity of organelle-specific marker proteins in each fraction can be readily determined, effective separation of subcellular compartments requires little overlap in protein markers between the fractions. Then the fractions can be subjected to chemical analysis of organelle associated compound accumulation by means of spectrophotometry [60], HPLC [61–63], LC/MS [64], and most commonly, by scintillation counting of radiolabeled compounds [65–68].

Many significant advances in distribution studies were achieved through the development of cell fractionation techniques. Organelle separation and analysis techniques such as immunoisolation, fluorescence activated sorting and electromigration analysis were developed. However their use in subcellular distribution studies remains infrequent, possibly because they are technically demanding. For organelle immunoisolation, cell homogenates were exposed to organelle-specific antibodies attached to solid supports and the cell fractions of interest were concentrated by binding to antibody [69–71]. Fluorescent activated cell sorting was applied to separate multiple intracellular organelles stained with membrane dyes or labeled with fluorescent antibodies that bind to organelle membrane proteins [72–75]. Since around the early 2000s' electrophoresis has been used to separate different subcellular organelle fractions from cell homogenates [76–84]. Most recently, magnetic chromatography methods were developed to isolate and enrich lysosomes from cells that internalize iron-containing particles [85–87].

As a caveat, organelle isolation procedures are not necessarily free from experimental artifacts: organelle isolation procedures are inherently disruptive [48]. During the multi-step procedures that are necessary to attain organelle purity for further analysis, organelle damage and compound leakage from one or more subcellular organelles are inevitable and difficult to control [53].

Whole cell based microscopic imaging studies as evidence for intracellular localization

Whole cell based microscopic imaging studies using intrinsically fluorescent or fluorescently - tagged molecules accounted for over half of all articles reporting a molecule's subcelllar localization (Table 3) and have been most common over the past decade. Less commonly, electron microscopy combined with immunocytochemical methods was used to obtain high resolution information of intracellular distribution of small molecular weight compounds that precipitated out at their sites of accumulation [88, 89] or that were tagged with a specific epitope for antibody recognition [90, 91] (Table 3).

Compared to pharmacological and chemical analyses which are tedious and prone to artifacts, microscopic imaging has generally been preferred as an efficient and reliable method to obtain real-time intracellular distribution data. Microscopic visualization of intrinsically fluorescent or fluorescently-tagged molecules provided the evidence for establishing subcellular localization in the majority of published, subcellular localization studies (Table 3). While the intracellular accumulation sites of fluorescent compounds can be determined directly based on the characteristic morphology of stained compartments [92–98], the use of resident, reference fluorescent markers [99–103] has enabled determination of subcellular distribution by analysis of colocalization patterns. Following

advances in location proteomics [104–108], machine vision-based quantitative image analysis has been used to establish the degree of co-localization between compounds of interest with an organelle-specific reference marker [109–111]. Furthermore, large combinatorial libraries of fluorescent probes and automated high content screening instruments have facilitated analysis of chemical motifs associated with specific intracellular distribution patterns [110–112]. More recently, fluorescence resonance energy transfer has been used to study the trafficking and distribution of xenobiotics [113].

Although fluorescence-based imaging techniques offer many advantages over other detection methods, evidence for subcellular localization based on a molecule's fluorescence is generally criticized due to well-known artifacts. For example, environmental factors such as binding status [114], ionic strength [115], solvent polarity [116–118], pH [119–121] and temperature [122–124] can affect a molecule's fluorescence spectrum or quantum yield. If fluorescence is dependent on environmental factors, interpretation of subcellular distribution patterns might not be entirely accurate or complete as molecules may not be fluorescent in every compartment they localize to[125]. For non-fluorescent molecules to be detectable using fluorescence microscopy, a fluorescent tag needs to be conjugated to the compound. This tag can alter the distribution of the original compound. Thus, claims about the subcellular localization of a tagged compound are only valid in the context of the entire small molecule-fluorescent probe conjugate.

Over the past decade, more sensitive and general imaging methods, such as the confocal Raman microscopy and secondary ion mass spectroscopy, have been used to monitor the distribution of non-fluorescent compounds in cells [126]. To date, a few pioneering studies based on these techniques have been reported [127, 128] (Table 3). Nevertheless, significant breakthroughs have been achieved. For example, the major challenge in conventional Raman imaging is how to amplify and quantify the weak resonance signal in live cell environment. The application of coherent anti-stokes Raman scattering has led to improvement in signal detection and has allowed tracking the intracellular distribution of endogenous lipids, virus RNA and organelles[129–131].

Yet another recent advance was the application of secondary ion mass spectrometry (SIMS) for analyzing subcellular localization sites of chemical agents. SIMS is a sensitive technique traditionally used in material sciences to analyze the elemental, isotopic or molecular composition of thin films [132–134]. Beginning in the late 1990s' SIMS has been used to monitor the phospholipid composition of biological membranes [135] and map the distribution of isotope labeled chemical agents after *in vitro* or *in vivo* dosing [136–139]. Though still in its infancy, SIMS is garnering attention in subcellular distribution studies because of its high sensitivity and outstanding resolution.

Computational models to frame quantitative hypotheses and predict subcellular distribution patterns

Since the 2000s,cheminformatics and computational modeling-based approaches have become essential to drug discovery and development. In parallel, cheminformatics and computational approaches are increasingly being used to generate and test hypothesis about intracellular distribution patterns and chemical-organelle interactions [7–9, 110, 140–142]. However, evaluation of computational models is inherently dependent on the quantity and quality of subcellular localization measurements.

Computational models for predicting biodistribution can be classified into two categories: statistically-based regression models and mechanism-based physiological models. Typical statistical models use experimental observations of small molecule subcellular localization

as a training dataset. With calculated physicochemical properties as input parameters, regression or multivariate statistical analysis methods can be applied to make qualitative (yes/no) or quantitative (how much) descriptions of the compound distribution pattern in the training set. If the fit between the model and the training set is acceptable, the model can be applied to a different test set of molecules with overlapping physicochemical properties, to make predictions about the molecules' localization. In cheminformatics research [143], this is referred to as a quantitative structure-activity relationship (QSAR) study.

Several published articles have analyzed compound subcellular accumulation sites using decision trees and other QSAR tools [7–9, 144, 145]. However, the success of QSAR-based models depends largely on the accurate calculation of molecular properties and quality of input data. Ideally the observations used for predictive QSAR models should be derived from the same experiments, based on the same mechanism of study, assessed with the same criteria, and performed with similar methods so as to avoid intra-laboratory variations in the manner the measurements are made and defined. QSAR models also benefit from large data sets of compounds. Therefore, QSAR models based on scant published data obtained with different methods and experimental approaches are more descriptive than predictive.

Physiologically-based predictive subcellular localization models have been derived from theoretical computation of mass transfer between different organelles according to Fick's Law of diffusion and Nernst-Plank equations. Physiologically-based models have been developed to calculate the intracellular accumulation and organelle distribution of molecules in cells suspended in homogeneous extracellular concentration of a chemical agent, or in cells exposed to a transcellular concentration gradient [146, 147]. Using combinations of input values, simulations can be performed to mimic the steady state distribution of small molecules in lysosomes, mitochondria and cytosol of millions of virtual molecules differing in molecular properties[10]. To demonstrate the potential of this approach, a predictive, multi-scale, cell-based model was constructed to simulate the distribution properties of pulmonary drugs in different cell types and anatomical regions of the lung [148]. Translated to the *in vivo* realm, validation of whole organ models will require detailed experimental measurements and kinetic analysis of small molecule distribution at multiple scales, in a manner that exceeds the capabilities of state of the art experimental approaches [128] by many orders of magnitude.

From Pharmacokinetics to Synthetic Biology: Conclusion and Future Outlook

Thus far, we have presented a comprehensive survey of the past and present state of the art of subcellular transport knowledge, focused on the evolution of experimental approaches and methods. Our understanding of small molecule distribution inside cells has been shaped (and is being reshaped) by the application and limitations associated of each one of these approaches and methods, and the invention of new ones (Table 4). Analytical measurements following precise cell fractionation can be considered the most quantitative and convincing evidence for the preferential accumulation of chemical agents in specific subcellular compartments. However, fractionation studies are low throughput and labor intensitive. Accordingly, live cell-based imaging with fluorescence microscopes has become the most common method for documenting the subcellular distribution of small molecule chemical agents.

The application of fluorescence-based techniques in subcellular distribution studies has had two major consequences on the current state of knowledge in this field: 1) much of what is presently known about the subcellular localization properties of small molecules is biased towards fluorescent compounds, with either intrinsic fluorescence or fluorescent molecular

tags; and 2) the majority of compounds with reported subcellular localizations are either highly specific, organelle-targeting transport probes or their subcellular distribution has been analyzed only in the context of a specific organelle (Table 2). Thus, the development of methods to quantify the distribution pattern of non-fluorescent, non-targeted molecules at the whole cell level will be necessary to expand our current understanding of the subcellular distribution properties of small molecules. For this reason, in addition to whole cell experimental analysis methods, physiologically-based modeling efforts aimed at predicting cellular pharmacokinetics are contributing positively towards formulating a hypothesis-driven framework for experimental, quantitative analysis of cellular biodistribution phenomena. Although still at its inception phase, whole-cell, mechanism-based computational modeling is a promising tool in terms of providing quantitative hypotheses for guiding the design of experiments aimed at furthering understanding of subcellular distribution and transport phenomena, without focusing on a particular location.

In the future, combinations of experimental methods will be used to study cells loaded with concentrated solutions of small molecules, which should facilitate analyses and provide new insights into the interaction of cells with exogenous chemical agents. For example, by combining computational modeling, Raman confocal microscopy, fluorescence microscopy, electron microscopy and chemical analysis [128], we found that incubating cells with concentrated chloroquine solutions (such as those found in the urine of patients undergoing chloroquine therapy) drives the formation of intralysosomal drug-membrane complexes that bind to other weakly basic molecules (Figure 1) [128]. With clofazimine, combining biochemical, microscopy and molecular imaging techniques, revealed that continuous exposure of cells to supersaturated drug solutions resulted in the synthesis of intracellular, autophagosome-like drug inclusions, new organelle-like cytoplasmic structures formed by condensed drug-membrane aggregates derived from mitochondria and possibly other organelles [149]. While such drug-membrane complexes may form and accumulate inside cells, such complexes may also form at the plasma membrane and can be shed by cells into the extracellular medium[150].

It is important to realize that cells tend to deplete exogenous chemical agents from the extracellular medium. By adding supersaturating solutions of chemical agents to cells and replenishing the chemical agents as they are depleted from the medium, it is possible to drive the formation of drug-membrane aggregates, which in turn could be used to drive the formation of unnatural, semi-synthetic organelles [110, 128, 149, 151–153]. While harnessing the natural subcellular transport pathways, supersaturated solutions of chemical agents (which can be prepared from insoluble molecules pre-solubilized in concentrated DMSO stock solutions before diluting in aqueous media) can drive the formation of atypical supramolecular architectures formed by complexation of chemical agents with cellular membranes. In turn, these supramolecular architectures can aggregate and grow by accretion to form microscopic cytoplasmic inclusions endowed with new, interesting chemical, physical and biological features. The formation of these “unnatural organelles” parallels an emerging interest in the test-tube synthesis of artificial organelles [154–156].

To conclude, traditional approaches aimed at studying the subcellular disposition of small molecules can now be applied towards altering the internal membrane organization of the cell. In our laboratory, this has motivated us to study the synthesis of “unnatural” supramolecular architectures inside cells, derived from complexes of chemical agents with cellular membranes. Continued investigation of subcellular transport phenomena will lead to fundamental insights into the chemistry of life. The ability to predict and optimize the subcellular transport and biodistribution properties of small molecules at the cellular level is now appreciated as a stepping stone towards predicting and optimizing systemic pharmacokinetics and pharmacodynamics. In the future, we envision that today’s research

may culminate in the development of new drug delivery strategies and therapeutic modalities. While accurate, quantitative assessment of the microscopic distribution of small molecules inside cells remains a challenge, subcellular transport research is being re-energized by revolutionary concepts derived from systems and synthetic biology. Progress in this field will continue to accelerate with the development of an increasingly sophisticated combination of methods and analytical techniques.

Acknowledgments

This work was funded by NIH grant 1RO1GM078200-01 to G. Rosania. H.N. Tsai was supported with funds from the Center for Molecular Drug Targeting at the University of Michigan, College of Pharmacy.

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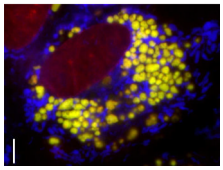

Figure 1.

MDCK cells treated with 50 μM chloroquine concentration for 4 hours prior to staining with Lysotracker Green (yellow, lysosomes), Mitotracker Red (blue, mitochondria) and Hoechst (red, nuclei). Cells exposed to high concentrations of chloroquine undergo profound changes in endolysosomal membrane organization. Continuous accumulation of chloroquine leads to the formation of multilamellar drug-membrane complexes that visibly bind to Lysotracker Green within the lumen of the expanded lysosomes [128]. Scale bar: 5 μm.

Table 1

Features of major subcellular compartments that affect the intracellular distribution pattern of small chemicals.

Major subcellular compartments	Major biological function	Morphological features	Physiological features	General mechanisms of accumulation
Lysosomes & endosomes	Degradation of excessive cellular proteins, lipids and organelles	Membrane-bound, vesicles	Acidic luminal environment (pH<6) Contain unique, lysosomal protease and hydrolyses.	Active transport; ion trapping; interaction with organelle resident proteins; receptor endocytosis; fluid phase pinocytosis; intracellular membrane trafficking.
Mitochondria	Energy conversion and storage of calcium ions	Membrane-bound, with internal membrane structure	Negative membrane potential. Weakly basic luminal pH (~8). Two membranes. Contain DNA.	Active transport; trapping by chemoelectrical potential; interaction with mtDNA andorganelle resident proteins
Nucleus	Storage of genetic materials	Membrane-bound with large protein pores	Composed of phosphate-rich DNA, RNA, and a large variety of proteins	Active transport; partitioning to nuclear envelope; interaction with DNA, RNA (nucleolar) and organelle resident proteins
Plasma membrane	Separation of the cytosol form the outside environment	Membrane protein-rich phospholipid bilayer membrane	Fluid mosaic, lipid bilayer with selective permeability.	Lipophilic partitioning; absorption to phospholipids; interaction with membrane proteins
Endoplasm reticulum	Facilitation of protein folding and transport of synthesized proteins	Interconnected network of membrane tubules.		Intracellular membrane trafficking; interaction with phospholipids or organelle resident proteins
Golgi apparatus	Process and package of macromolecules	Stacks of semicircular or planar membrane-bound compartments		Intracellular membrane trafficking; interaction with phospholipids or organelle resident proteins
Cytosol		Intracellular solution-like matrix		Interaction with cytosolic components such as the cytoskeleton

Table 2

Number and chemical diversity of small drug-like molecules with reported subcellular localization(s)

Chemical structures and reference articles can be found in the supporting information.

Localization	Number of compounds	%	Number of references
Total	967	100	448
Endo-lysosomes	226	23	96
Mitochondria	259	27	136
Nucleus	123	13	67
Plasma membrane	162	17	75
Endoplasmic reticulum & Golgi apparatus	37	4	26
Cytosol	59	6	36
Multiple sites	101	10	71

Table 3
Methods used for subcellular localization studies

Chemical structures and reference articles can be found in the supporting information.

Evidence	Count	% Population
Pharmacological effects	171	18
Chemical analysis		
Uptake/binding studies	67	7
Cell fractionation	54	6
Microscopic imaging studies		
Fluorescence microscopy	633	65
Histochemistry methods	9	< 1
Others	6	< 1
Not mentioned	25	< 3

Table 4

A comparison of experimental methodologies.

Methods	**Experimental systems**	**Instruments**	**Pros. & *Cons*.**	
Pharmacological effect	Dead or living cells	LM; FM; TEM; analytical instruments such as HPLC, LCMS, and GE	*Does not provide sufficient evidence to ascertain localization* *Only provides indirect evidence for effect on a specific organelle.*	
Analytical measurements				
Uptake/binding experiments	Isolated organelles or cell culture	FM; FS; radiometer, or analytical instruments	Provide adequate evidence for localization to a specific organelle. *Cannot assess the accumulation in all subcellular organelles at one time*	
Distribution studies	Dead cells	Centrifuge, FACS, CC, CE, and analytical instruments	Depict the relative distribution pattern in all cellular organelles	*Separation of cellular organelles is difficult* *Not suitable if compound undergoes rapid efflux*
Whole cell based imaging studies				
Immune-/ Histochemistry	Dead cells	TEM		*Sample processing steps may cause redistribution* *Detection of small amounts is challenging.*
Fluorescence microscopy	Live cells	FM		*May miss localization information if fluorescence intensity* *changes with environmental factors*
Raman imaging	Live cells	Raman microscopy		*Signals are weak and require amplification*
Secondary ion mass spectrometry	Dead cells	SIMS device		
Computational predictions	*in silico*	Computers		

Abreactions: LM - light microscopy; FM - fluorescence microscopy; FS - fluorescence spectrometer; FACS - Fluorescence-activated cell sorting; CC – column chromtography; CE - capillary electrophoresis TEM - transmission electron microscopy; HPLC - high performance liquid chromatography; LC/MS - Liquid chromatography-mass spectrometry; GE - gel electrophoresis.