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IROA-based metabolomics protocols for accurate quantitation in metabolomics

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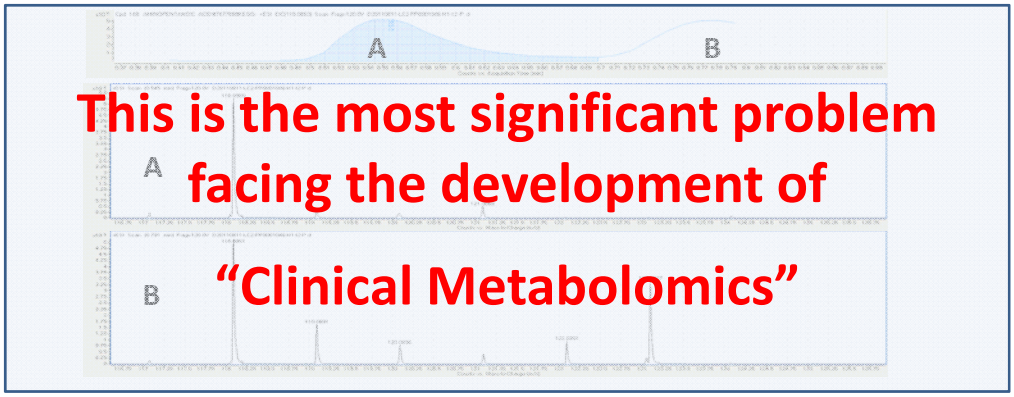
Artifact-based errors are common

The LC-MS retention time of an authentic compound was found to be within the time-range of peak A.

In this run, two peaks A and B are found.

A normal assumption would be that A is the correct peak; however:



IROA identifies **A** as an artifact and **B** as a peak of biological origin, preventing an artifact from creating an error despite a "better time signature" on **A**.

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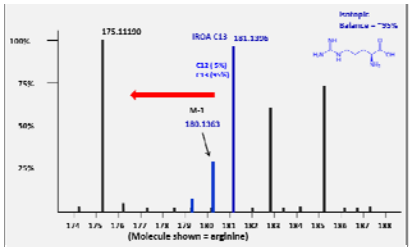
What is IROA?

The IROA (Isotopic Ratio Outlier Analysis) protocols embed specific chemical characteristics into the mass spectral data stream in the form of mathematically definable isotopic patterns.

This information is used to retrieve higher quality data, with reduced error, and lowest possible experimental variance when the IROA peak is a constant or Internal Standard.



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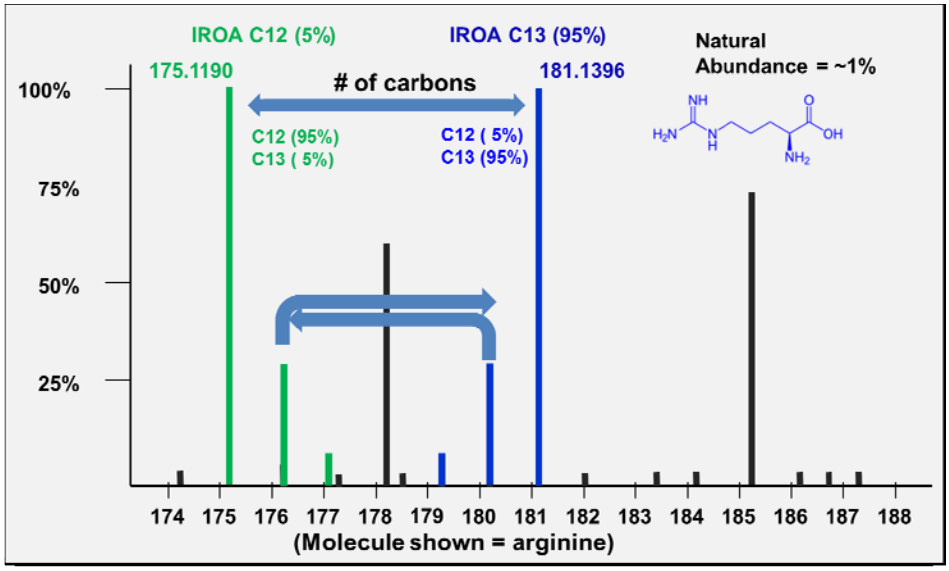
IROA Basic Patterns



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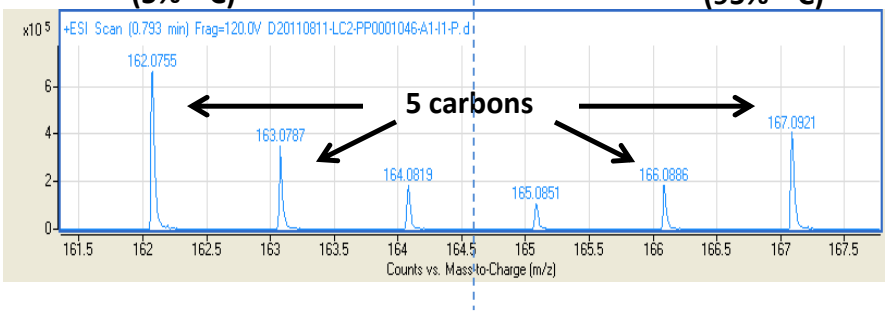
Anatomy of the IROA Signal

C12 side (5% ^{13}C) **C13 side (95% ^{13}C)**



The IROA signal is made up of two halves.
Together they make a complete picture.
<u>Only a molecule of biological origin can show this signal.</u>

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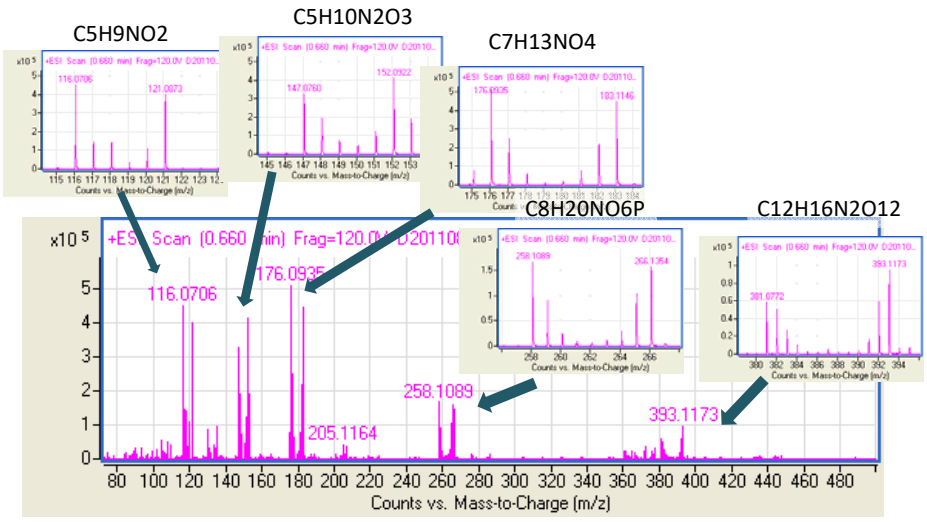
Paired Peaks



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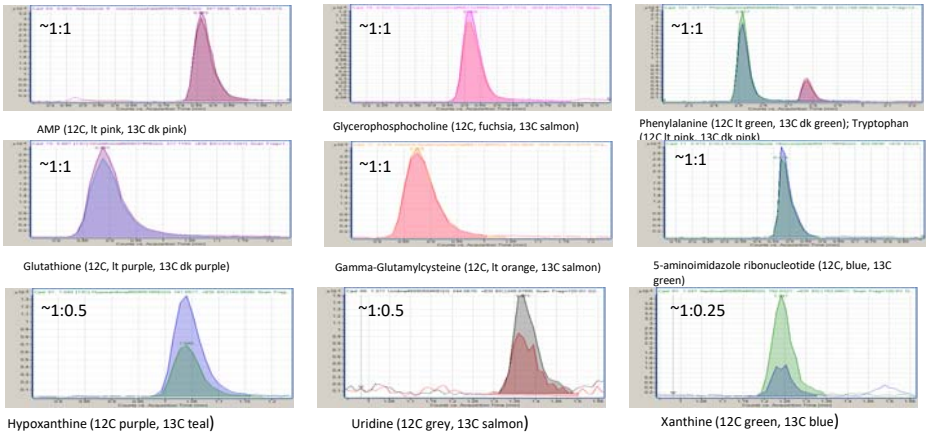
Overlaid C12/C13 Peaks



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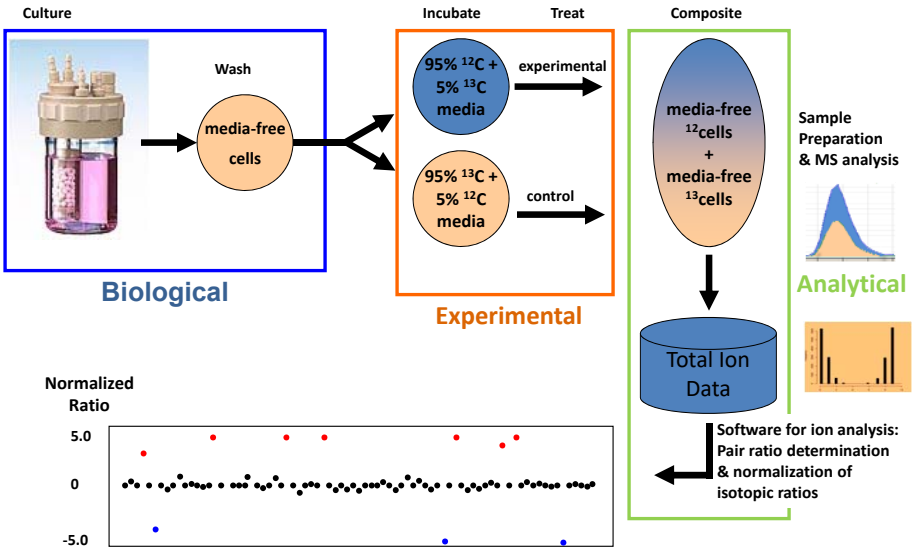
IROA®: Isotopic Ratio Outlier Analysis



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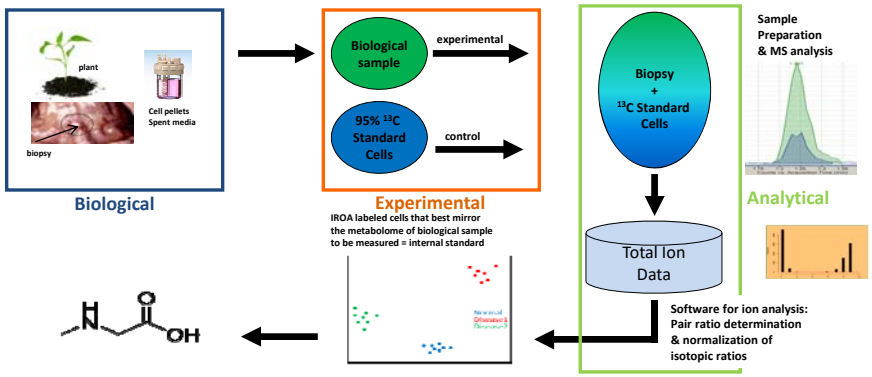
Phenotypic IROA Protocol



The material to be phenotyped is mixed with ^{13}C (IROA) cells and/or standard compounds which allows one to find and pair all peaks. The deviation from the standard is diagnostic of the sample's biochemical phenotype.

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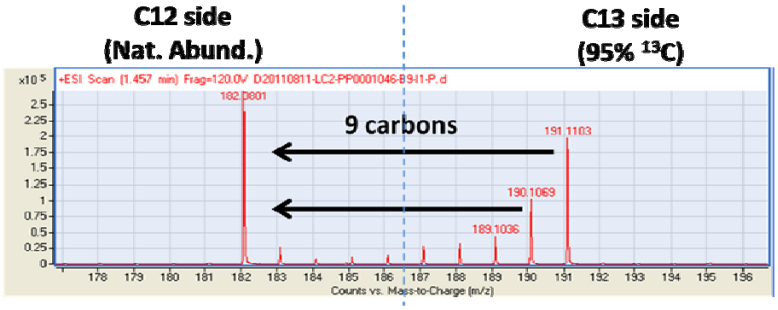
Phenotypic IROA peak pairs



The Phenotypic IROA signal is made up of two halves.
The C13 side tells you where to find the corresponding C12 peak.
The C12 side is unlabeled, i.e. it has natural abundance carbon.
The pairing makes the identity of all peaks measured unambiguous.

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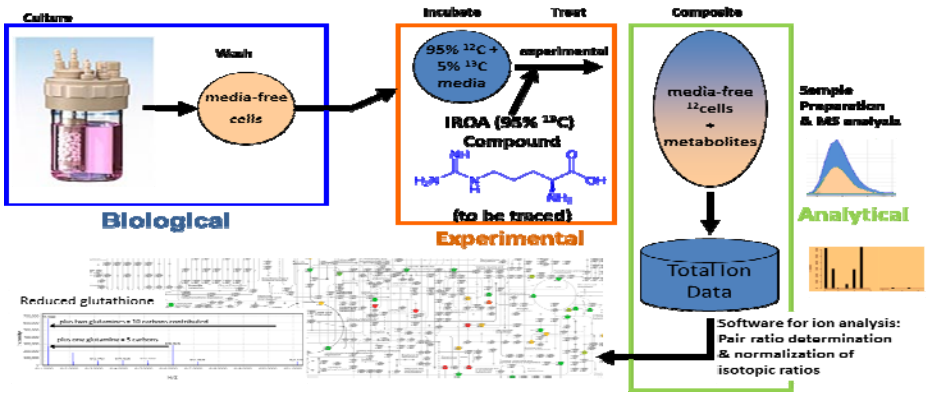
Fluxomic IROA Protocol



Every metabolite that is derivative of the traced compound, and only such metabolites, will have a truncated IROA pattern which indicates the number of carbons transferred and the percent of the overall metabolic pool that is labeled.

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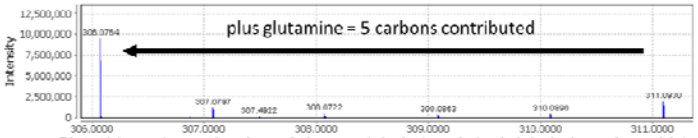
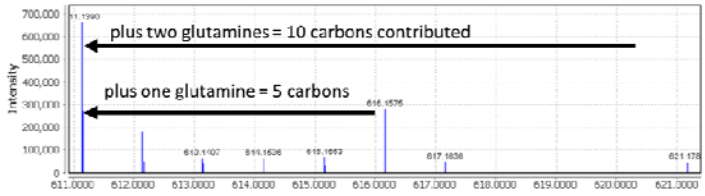
Fluxomic IROA Peak pairs



Glutathione shows the sizes of the non-labeled, and singly labeled species, which inherits a five carbon fragment from glutamine.



Oxidized glutathione shows the sizes of the non-labeled, singly labeled, and doubly labeled species. Each inheriting a five carbon fragment from glutamine.

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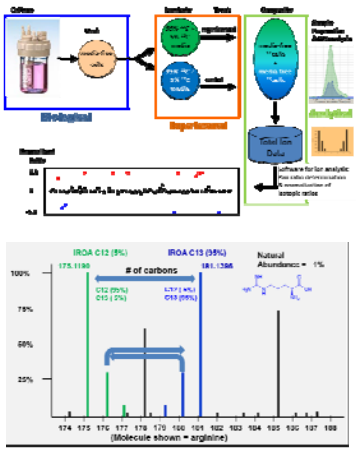
Three IROA Protocols

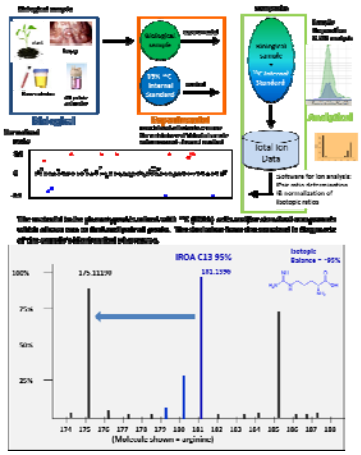
The Basic IROA Protocol

For R&D: labeled isotopomer-paired control and experimental samples; untargeted analysis



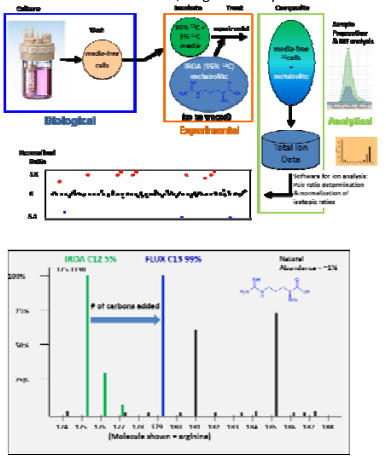
The Phenotypic IROA Protocol

For clinical and molecular diagnostics: labeled Internal Standards; targeted analysis for 100s of compounds



The Fluxomic IROA Protocol

For medical flux studies; labeled experimental samples and tracer metabolites; targeted analysis



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The Three IROA Protocols

- "Basic IROA protocol" - both the full C12 channel and the full C13 channel are used in the experiment.
  - the experiment is a completely **untargeted** analysis
  - **every** biological compound in either the C12 or C13 may be quantitated
- "Phenotypic IROA protocol" only the C13 channel is used in the experiment. Therefore,
  - the experiment is a completely complex **targeted** analysis
  - all compounds in the experimental (NA) sample may be quantitated against **every compound in the control sample (C13)**
- "Fluxomic IROA protocol" - single compound and the C13 channel is used in the experiment.
  - all **derivatives of that compound** will carry a unique signature which indicates the **number of carbons transferred**
  - the **relative size** of the pre and post metabolic pools is measured

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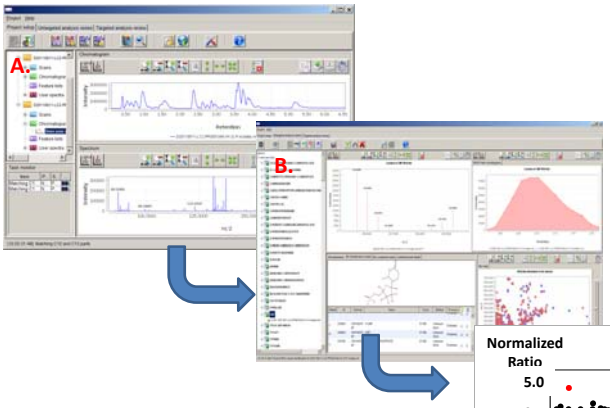
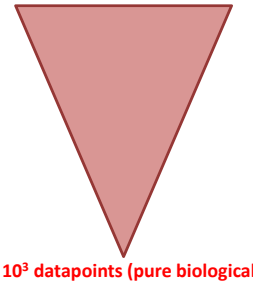
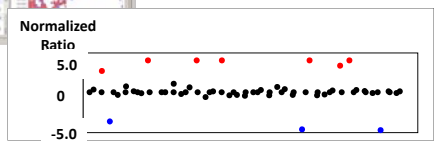
Automated Data Reduction



10^8 datapoints (mostly noise)



1 X 10^3 datapoints (pure biological)



Based on IROA embedded information the ClusterFinder program performs an automated Analysis, including:
Variance control, Data reduction, Noise removal, Data definition, Formula assignment

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THE IROA WORKFLOW

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The IROA Workflow

- The IROA Workflow is an extension of the Phenotypic Protocol in which:
  - a defined IROA-based Internal Standard (IS) is used in any type of experimental or clinical sample, and
  - an equally defined QA/QC sample (Matrix) is analyzed daily.
- Together they make a systematic measurement system that is completely reproducible across sample types, instrument types, and overcomes time-induced variance.

The IROA Workflow is the basis of "Clinical Metabolomics".

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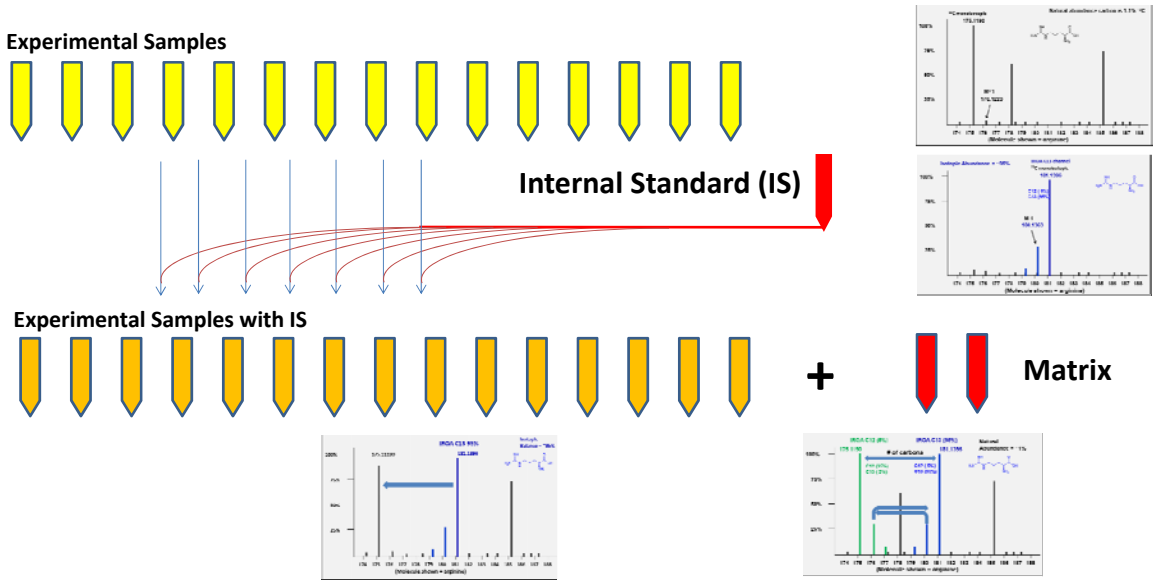
IROA-based Workflow



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What is the IROA-IS

- The IS is a 95% U-^{13}C-labeled complex Internal Standard
- The IS has a standard concentration of 1000+ **identified** and curated compounds for **co-location in an experimental sample.**
- The IS has enough compounds to provide for a Retention Time (RT) ladder that allows **alignment** of all peaks in the chromatogram.
- The IS may be used to **normalize** the samples against one another.
- The IS allows **day-to-day, or even instrument-to-instrument variances to be eliminated.**

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What is Matrix?

- The Matrix sample is a made from the same material as the IS but rather than having a natural abundance partner it is paired with a perfectly matched IROA 5% U-^{13}C sample.
  - The almost perfect balance of the 5% and 95% chemical composition,
  - The completely defined nature of the Matrix sample, and
  - The absolute reproducibility of the Matrix sample.
- Provide a way to compare day-to-day analytical performance on all parts of the analytical process, and
- Provide a daily mapping of all compounds found in the IS so that their complete identification is always assured.

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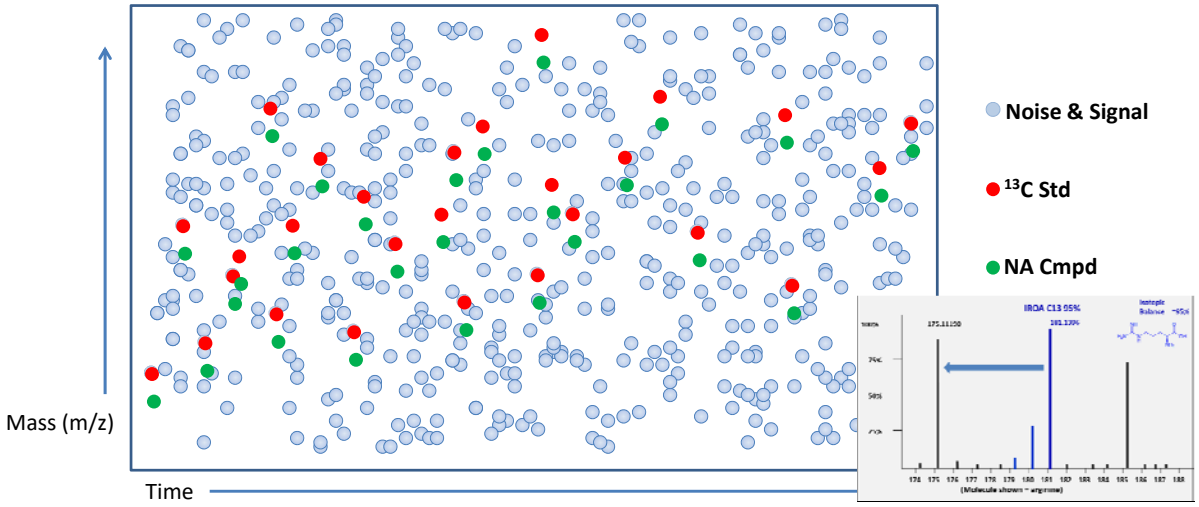
The Experimental Sample Process

Identify, Align, Co-locate, Measure & Normalize



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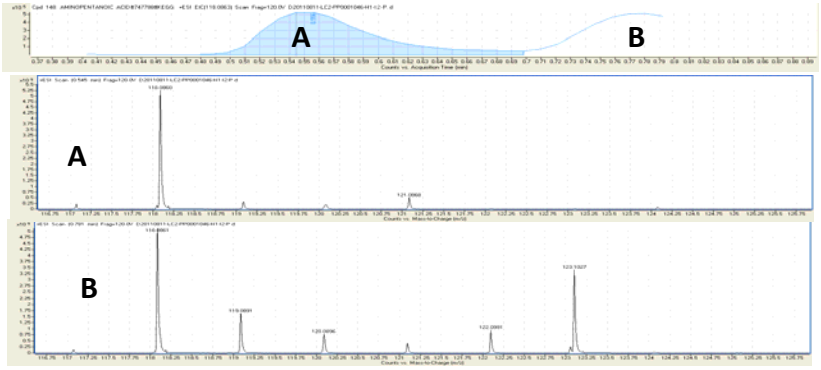
Artifact-based errors are avoided



IROA identifies **A** as an artifact and **B** as a peak of biological origin, preventing an artifact from creating an error despite a "better time signature" on **A**.

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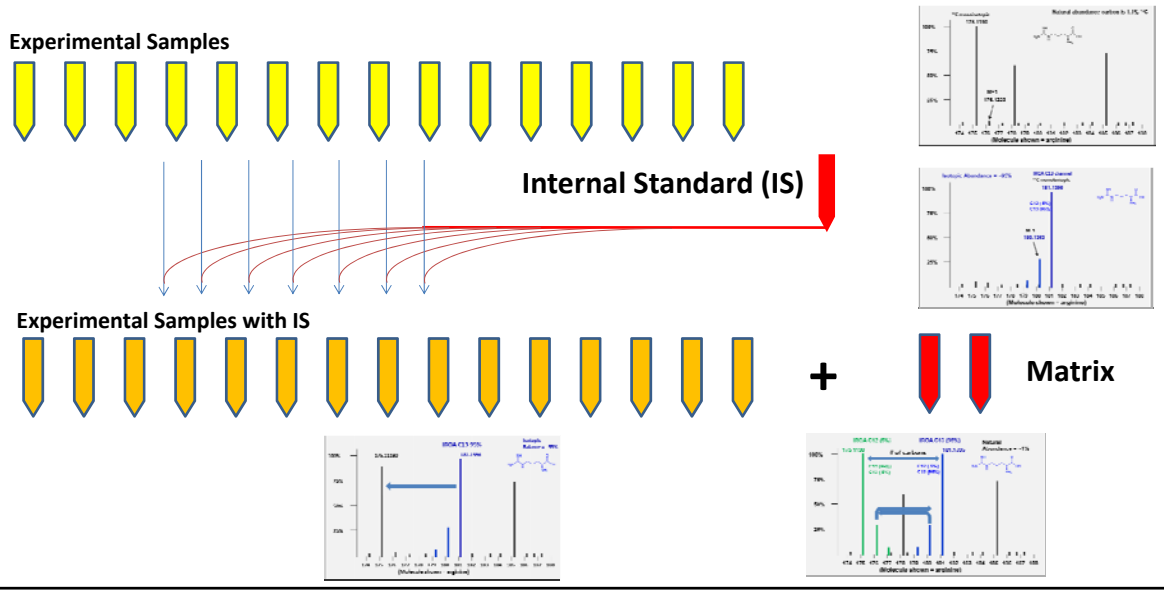
IROA-based Workflow



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Summary of IROA-based Workflow

1) Cost-effective simultaneous quantitative measurement of several hundred biochemicals in a single analytical run through the use of IROA Internal Standards (IS) and Matrix (M);
   i. IS & M provide high level QC for accurate and reproducible results;
   ii. IS enables removal of false data (all noise and artifacts);
   iii. IS enables precise quantitation through complex software algorithms;
   iv. IS allows for normalization of samples to overcome sample-to-sample variation;
   v. Once normalized, IS provides a map that can be used for compounds that are not in the IS.
2) All of the above are completely software automated and may be done with minimal (or even no) human interaction.

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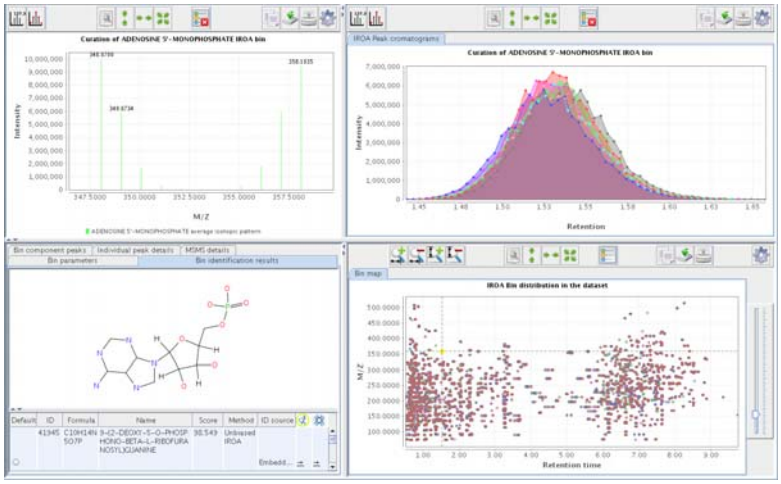
The 1500 peaks in Matrix (pos mode)

While finding them is completely automated, we are currently examining each one, and annotating it

- We have built a database to collect all of this information

- We are using this to directly tackle the problem of the percent of peaks that are “knowns”, and what percent are “unknowns”, i.e. not just fragments, adducts, etc. of “knowns”



*We will be successful because the Matrix is a pure IROA mixture, i.e. we can discriminate between real compounds and artifacts