iFAMS Version 6.3

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General Overview:

iFAMS (Interactive Fourier-Transform Analysis for Mass Spectrometry) software provides a tool for mass spectra white-noise filtering and charge state deconvolution for polydisperse ESI-MS data. Some functions beyond the core implementations of Fourier and Gábor transform (STFT) analysis include macromolecular mass defect analysis (reduction of spectral information to base masses buried within polydisperse signal), analyte quantitation and concentration calibration from "zero-charge" reconstructions/deconvolved spectra, and batch processing capabilities.

Additional features that further simplify use of the software and improve its flexibility include adjustable data domain, automated STFT parameter tuning according to chosen frequency spacing, automated STFT charge state and harmonic selection (after initial selection of two consecutive and well-separated charge state fundamentals), "Gábor Slicer" for rapid ion mass estimation from "slices" of the Gábor Spectrogram, an isotopic distribution calculator for verification of isotope-resolved "zero-charge" reconstructions/deconvolved spectra, and the ability to export and import batch parameters in a human-readable format.

In this document, there are definitions for some shorthand terms, detailed descriptions of each menu and tab in the program (including hard-coded thresholds of background algorithms), brief order of operations for the main analyses, and tips for best program performance. If new to iFAMS, please try the click-through tutorial with the provided demo data, which can be downloaded from the same site as the current release of iFAMS: https://github.com/prellgroup/iFAMS/releases

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iFAMS Terms Key:

TXT = Text file, tab-delimited

CSV = Comma-separated values file, comma-delimited

MS = Mass spectrum

FT = Fourier transform

STFT = Short-time Fourier transform, used in iFAMS to take m/z-localized FT. Various window shapes can be used, but we typically recommend using the Gaussian window shape (Gábor transform)

GT = Gábor transform, specific case of STFT using Gaussian window shapes (which is the default window shape in iFAMS).

IFFT = Inverse fast Fourier transform, refers to any inverse FT or GT/STFT in iFAMS. Typically describes an individual charge state that has not been charge-corrected

Zero-charge IFFT = Charge-corrected inverse fast Fourier transform, contribution of a single charge state to the deconvolved mass spectrum. Also referred to as "individual charge state spectra" sometimes specified as "zero-charge" or "charge-corrected" depending on context

Decon = Deconvolved mass spectrum or deconvolved spectrum, sum of all Zero-charge IFFT, often includes a baseline correction. Although, "zero-charge" reconstruction usually refers to the same thing as Decon in iFAMS, "zero-charge" reconstruction can also refer to Zero-charge IFFT

Peaklist = Integrated peak information from Decons including peak centroids, areas, and heights.

MMD = Macromolecular mass defect (mass of analyte *mod* mass of repeated subunit)

Menu and Tab Descriptions:

- File
    - Load Ordinary FT
        - Loads data from TXT (x, y) or CSV (x, y or index, x, y or iFAMS Decon)
        - Performs FT on data
        - Plots data (x vs. y) as MS and the FT
    - Load STFT
        - Loads data from TXT (x, y) or CSV (x, y or index, x, y or iFAMS Decon)
        - Performs FT on data, estimates appropriate STFT parameters, and performs STFT
        - Plots the GT, the FT, the data as MS, and a "working window"
            - ♦ "Working window" contains the raw MS and populates with IFFTs
    - Plot STFT From Clipboard
        - Performs "Load STFT" for a spectrum list copied to the clipboard (x, y)

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- o Load Deconvolved or IFFT Spectra
  - ▪ Loads data from one or multiple TXT or CSV (x, y) to plot overlaid as "Relative Abundance" vs. "Mass (Da)"
  - ▪ Alternatively, loads iFAMS-generated Decon file(s) (CSV)
    - ♦ Contains integration bounds and Zero-charge IFFT spectra
    - ♦ Additional information is only displayed when a single file is loaded
    - ♦ Multiple files loaded will overlay Decons only
  - ▪ Alternatively, loads iFAMS-generated IFFT file(s) (CSV)
    - ♦ IFFT files contain the MS with IFFT from a single charge state series
    - ♦ Plots MS with IFFTs overlaid
    - ♦ Although multiple IFFT files can be loaded, only the MS from the first file will be plotted, so this is only recommended for IFFT files generated from the same MS file
- o Load Batch Parameters
  - ▪ Loads iFAMS-generated "batch_params" file (CSV)
  - ▪ Populates necessary variables for complete iFAMS analysis with stored values
- o Batch Data Files
  - ▪ Loads multiple TXT (x, y) or CSV (x, y or index, x, y or iFAMS Decon)
  - ▪ Gives options for data to be saved for each file processed
    - ♦ Always saved with batching: batch parameters, Decons, and Peaklists
    - ♦ Option 1: Ordinary FT spectrum
    - ♦ Option 2: IFFT spectra
    - ♦ Option 3: "charge-state zero-charge spectra" (Zero-charge IFFTs)
    - ♦ Option 4: individual ion series deconvolution (Only applies to MS processed for deconvolution of multiple charge state series. If unchecked, all Decons will be combined to one Decon for each MS. If checked multiple Decons can be produced from each MS)
  - ▪ Creates a batch folder in the location of the files loaded where all data is saved
    - ♦ Named as YYMMDD_BatchX, where X is a counter to avoid overwriting data
    - ♦ Dialog confirming the save of batch parameters reports folder name
  - ▪ Saves stored or loaded variables as a batch parameter file
  - ▪ Saves any processed spectra or data (unless batch parameter file was loaded)
  - ▪ Performs batch processing of loaded files using identical parameters
- o Batch Deconvolved Spectra
  - ▪ Loads multiple TXT (x, y) or CSV (x, y or index, x, y or iFAMS Decon)
  - ▪ Saves any stored Decon and Peaklist data
  - ▪ Integrates loaded spectra with stored parameters
  - ▪ Saves new Decons and Peaklists (does not save a batch parameters file)
- o Export Data
  - ▪ Gives options for data to be saved for the file processed

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- ♦ Saved by default: processing parameters, Decon, and Peaklist
- ♦ Option 1: Ordinary FT spectrum
- ♦ Option 2: IFFT spectra
- ♦ Option 3: "charge-state zero-charge spectra" (Zero-charge IFFTs)
- ♦ Option 4: individual ion series deconvolution (Only applies to MS processed for deconvolution of multiple charge state series. If unchecked, only one Decon will be saved)
- ♦ Option to change save location: "Directory Name" given will be used as the location for a new folder with the name of the "File Name" given plus a counter to avoid overwriting data
    - o Export Batch Parameters
        - ▪ If not yet created, creates a batch folder in the location of the last file loaded
            - ♦ Named as YYMMDD_BatchX, where X is a counter to avoid overwriting data
            - ♦ Dialog confirming the save of batch parameters reports folder name
        - ▪ Saves stored processing parameters (CSV with labeled values) to batch folder

- ● Settings
    - o Positive Ion Mode
        - ▪ Toggle for positive/negative ion mode processing parameter
        - ▪ Active mode is displayed above the "Home" button
        - ▪ Checked indicates positive ion mode, unchecked indicates negative ion mode
        - ▪ Ion mode only affects the charge-correction step when adjusting masses for the charge carrier
            - ♦ Positive ion mode: Mass = $(m/z - \text{mass}_{\text{proton}}) * z$
            - ♦ Negative ion mode: Mass = $(m/z + \text{mass}_{\text{proton}}) * z$
    - o Adjust Data Domain
        - ▪ Opens dialog for MS pre-processing after loading file for FT or STFT
        - ▪ Input min. and max. m/z to be included in data processing
            - ♦ Default values are raw data "x" min. and max. from loaded file
        - ▪ Also takes input specified interpolation value (m/z-spacing per MS point)
            - ♦ Default value is based on the total number of points from the raw data evenly spaced between the raw data min. and max.
            - ♦ Increasing the value can be useful to speed up processing if high frequency information is not needed (max. frequency = 2 / input value)
        - ▪ "Replot" makes new x-, y-lists for values between min. and max., interpolates lists to match input interpolation value, and replots FT or STFT (replotted STFT may require STFT parameter adjustments)
            - ♦ This step should be done after loading data but before any other data processing steps

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- ♦ Only min. and max. x-values are tracked through batch parameters and the adjusted *m/z* range will be applied to every file in a batch (only default interpolation values will be used in batching)
  - ▪ Saving options for "truncated" and "interpolated" data
    - ♦ Option 1 (truncated): saves CSV of data from input min. and max. x-values but with default interpolation value
    - ♦ Option 2 (interpolated): saves CSV of data from input min. and max. x-values and input interpolation value
  - ▪ "Restore Spectra" rewrites x- and y-lists to stored lists generated when dialog box was opened, replaces input values based on the state of the stored lists, and replots FT or STFT
  - o Show Interpolated MS
    - ▪ Display Setting: toggles between displaying raw MS and interpolated MS
      - ♦ Affects MS window for FT and STFT
  - o Normalize Abundances
    - ▪ Display Setting: toggles between displaying Decon raw relative abundances and Decon percentages normalized to most abundant feature
      - ♦ Affects all loaded Decons
      - ♦ Removes any displayed integration results
      - ♦ Does not affect integration values
  - o Smooth Isotope Resolution
    - ▪ Display Setting: toggles between displaying Decon with default resolution and Decon with resolution smoothing for average masses
      - ♦ Smoothing is done by convolution with a Gaussian of full width at half max. of 1.8 Da
      - ♦ Affects all loaded Decons
      - ♦ Removes any displayed integration results
      - ♦ Does not affect integration values

- ● Fourier Analysis Tools
  - o Plot Absolute Data
    - ▪ Takes the absolute value of the IFFTs and reruns iFAMS analysis
    - ▪ Gives a better visual sense of average mass
    - ▪ Plots peak valleys as artificial secondary peaks at half the true peak spacing
  - o Plot Real Data
    - ▪ Takes the real valued component of the IFFTs and reruns iFAMS analysis
    - ▪ Peak valleys are below 0 relative abundance
  - o Open iFAMS Maxima Peak Finder
    - ▪ Opens dialog for finding peaks in the FT using minimum frequency, amplitude, and peak spacing (by number of data points) parameters.
    - ▪ Returns subunit mass and charge range calculated from centroids of the first frequency peak distribution found in range with even spacing.

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- o Manually Enter Charge States and Submass
  - ▪ Opens dialog for selecting peaks in the FT based on charge state range and subunit mass inputs.
  - ▪ Adds blue data points to the plotted FT distribution at the frequency values corresponding to the charge state and subunit mass inputs.
- o Run iFAMS Analysis
  - ▪ Opens dialog for inputting the highest harmonic to include and finishing deconvolution.
    - ♦ Harmonics are selected for every charge state from the fundamental (1st harmonic) up to and including the number entered.
  - ▪ iFAMS Analysis corrects each IFFT for the charge state and adds the zero-charge IFFTs to make a Decon.
    - ♦ Charge correction is applied to the x-coordinates by $(x - mass_{proton}) * z$
    - ♦ $mass_{proton} = \pm 1.00727647$ Da
    - ♦ Each zero-charge IFFT is interpolated to a data density equal to the density of the MS multiplied by the lowest charge state +20%
    - ♦ The abundances of the IFFTs are summed to make the zerofull (Decon)
  - ▪ Updates Main Window plots with the "working window" on the left with IFFTs and the Decon on the right with zero-charge IFFTs.
- o Run Mass Defect Analysis
  - ▪ Opens dialog for running MMD on FT charge state selections.
  - ▪ Repeated subunit mass and charge states must be specified (see *Open iFAMS Maxima Peak Finder* or *Manually Enter Charge States and Submass*)
  - ▪ "Highest Harmonic to Include" must be specified. Harmonics are selected for every charge state from the fundamental (1st harmonic) up to and including the number entered.
  - ▪ "Baseline Correction", when checked, applies the "Minima Baseline Correction" which corrects for negative values by subtracting a composite baseline made from lines between negative local minima.

- ● STFT Analysis Tools
  - o Change STFT Parameters
    - ▪ Opens dialog for manual and automated STFT and Gábor spectrogram parameter adjustment
    - ▪ "Window Type" is an STFT parameter that defines the shape of the window function. Gaussian is the default and generally recommended for all cases
      - ♦ The window function is finite and only spans ten standard deviations
    - ▪ "Number of FFT Channels" is an STFT parameter that defines the size of the window function. Specifically, the window function has a standard deviation equal to one tenth of the number of FFT channels

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- ▪ "Color Scale Relative Maximum" is a Gábor spectrogram parameter that adjusts the display of the heat map
  - ♦ Higher values increase the relative blue content
  - ♦ Lower values increase the relative red content
  - ♦ This parameter does not affect the STFT values, only the image
- ▪ "Oversampling Factor" is an STFT parameter that affects the shift of the window function and the total number of points or "pixels" in the STFT
  - ♦ The number of "pixels" is defined as the total number of MS points multiplied by the oversampling factor
  - ♦ The oversampling factor only affects the number of pixels in the m/z direction
  - ♦ Default value is ten and is generally recommended for all cases
- ▪ "Auto Adjust" re-estimates STFT parameters based on an input frequency
  - ♦ Input frequency should be near the middle of the target signal
  - ♦ If "High Mass Resolution" is checked, the number of FFT channels will be one third of the value initially estimated
- ○ Open Gábor Slicer
  - ▪ Opens dialog for exploring signal in the Gábor spectrogram and instant mass estimation
  - ▪ Plots GT, GT slice, MS, and Quick Decon windows. Orange "slice" plotted on GT follows $y = 1/x * y_{input}*x_{input}$ based on the cursor x, y coordinate input
  - ▪ Moving slice across GT will instantly update GT slice plot which plots the GT magnitude vs. m/z but the y-axis scale will remain constant
  - ▪ Releasing the slice will update the GT slice plot y-axis and estimations for ion mass and (adduct mass/harmonic number)
  - ▪ Ion mass estimation is based on peak spacing in the GT slice and prioritizes high abundance across several charge states to tolerate moderate noise and high-abundance contaminant signal
    - ♦ Algorithm performs four searches using different starting pairs of the top four most abundant peaks (1st & 2nd, 1st & 3rd, 2nd & 3rd, 3rd & 4th)
    - ♦ Each starting pair is used to calculate mass and charge assuming they are consecutive charge states
    - ♦ Based on the calculated mass and charge, abundances from the expected positions of the six charge states centered about the initial two are combined for a search score
    - ♦ The search with the highest score is used for reported mass estimations
    - ♦ (adduct mass/harmonic number) is then calculated based on the cursor x, y coordinate input: (adduct mass/harmonic number) = ion mass / $(y_{input}*x_{input})$. Which is derived from the equation for GT frequency: frequency = $n(m/\Delta m) \times (m/z)^{-1}$, where n = harmonic number, m = ion mass, Δm = adduct mass, and z = charge state.

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- ▪ "Quick Deconvolve" is a tool designed to quickly check the accuracy of the mass estimation, and it prioritizes computational time over optimal GT selections.
    - ♦ Currently, results from "Quick Deconvolve" cannot be saved or exported.
    - ♦ GT selections are based on the ion mass estimation.
    - ♦ Every theoretical charge state position is checked in the mass spectrum, and the most abundant charge state is identified.
    - ♦ The charge state range is quickly adjusted based on the most abundant charge state.
        - o For a most-abundant charge state between 10 and 20, the charge state range is limited to 5.
        - o Between 20 and 35, the range is limited to 9.
        - o 35 or greater, the range is limited to 13.
    - ♦ For each charge state, frequencies from 0-1.8 are selected and those corresponding to isotope frequencies (at multiples of integer values equal to the charge state) are selected with frequency ranges ±0.75 about the integer frequency.
- o Run Guided Search
    - ▪ Opens dialog for automated STFT signal search and selection tools
    - ▪ Signal can be searched based on box selection input of fundamental signal from two consecutive charge states or based on text input of an average mass estimate (within ~10 Da).
        - ♦ Alternatively, if the box selections contain frequency 0, signal will be searched for in the near-zero frequencies only based on m/z separation and not on frequency separation.
        - ♦ Searching by input mass will result in the program searching expected charge state positions in slices of the Gábor spectrogram to select fundamental signal from two consecutive charge states. The search is then refined based on the box selections. Note that this method prioritizes frequencies from isotope resolution. For reliable results for lower frequency signal (< 3), data may need to be downsampled to remove higher frequencies (e.g. adjusting m/z sampling to > 0.05).
    - ▪ Before searching, two additional settings are considered: box and range thresholds
        - ♦ By default, boxes are adjusted to the fundamentals of each charge state to include the top 90% of the peak (after estimated baseline adjustment).
            - o Box shapes are kept identical for higher harmonics.
            - o Toggling this threshold off will result in selections of each charge state using an average box shape of the input selections. The box shape is adjusted to accommodate the

expected stretching/compressing of the mass distribution with changing charge state. This is especially useful for deconvolving multiple GT peaks of the same charge state with a single selection.

- ♦ By default, the charge state range is adjusted to include consecutive charge states with abundances within 6% of the most abundant charge state (abundances are first corrected by an estimated baseline).
  - o Additionally, the charge state range is assumed to follow a normal distribution. Each charge state is screened moving out from the center of the distribution (the most abundant charge state), and, if one of the charge state abundances increases by 25% of the previous charge state's, then the range is cut off to avoid including interferences.
  - o Toggling this threshold off will result in the search including all charge states that could fit in the m/z range with the given mass.
- ▪ The search uses the input selections' IFFTs maxima to calculate the charge states and most abundant mass of the ions assuming that they are consecutive charge states.
- ▪ Then the change in mass/adduct mass (Δm) is calculated from the average charge-corrected frequency from the input selections.
- ▪ The search then explores the expected charge state positions along the GT slice corresponding to the calculated mass and Δm to find the most abundant charge state.
- ▪ After the search, the GT spectrogram is updated with the fundamental selections labeled with assigned charge states and an orange highlighted region indicating the pixels making up the searched GT slice. The GT slice is also plotted in the top right plot as relative magnitude vs. m/z with charge state peaks labeled with charge assignments. The "working window" is also updated with the selections' IFFTs.
- ▪ Each charge state can be toggled and the number of harmonics specified.
  - ♦ Harmonics are selected for every charge state from the fundamental (1st harmonic) up to and including the number entered.
  - ♦ The recommended value is input by default and is to include harmonics that have a maximum within 1% of the fundamental maximum (after baseline correction). This value is calculated as the average across the charge states.
  - ♦ The option to include the near-zero frequency (0th harmonic) is offered as a separate toggle and is generally recommended unless there is overlapping signal in the MS that can be separated by frequency.
    - o If toggled off, the decon will likely include negative values which can be corrected using the "minima baseline correction"

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- ▪ "Run iFAMS Deconvolution" finishes the search with the final charge state and harmonic information and performs iFAMS Analysis to generate decon (see additional details on "iFAMS Analysis" below).
- ▪ "Deconvolve and Select Additional Series" finishes the current search results with the final charge state and harmonic information, stores the selections, and resets the GT spectrogram to allow for another search. Any stored selections are faintly displayed on the spectrogram. If another search is not needed, "Finish Search" processes all stored selections by performing iFAMS Analysis to generate decon (see additional details on "iFAMS Analysis" below).
- ▪ "Adjust Assigned Charges" performs a quick deconvolution of the current selections and opens the Charge Adjuster menu (see Adjust Charge State Assignments below).
- ▪ "Remove Selections" deletes search results and resets the GT spectrogram.
  - o Add Gábor Selection (Ctrl+E)
    - ▪ Stores drawn GT rectangle selection
    - ▪ Added rectangles appear with thin red borders
    - ▪ Multiple rectangles can be "added" for a single charge state before saved
    - ▪ If Harmonic Finder or MMD is going to be used, only add fundamentals
    - ▪ Adds/updates selection IFFT in the "working window"
  - o Save Gábor Selection (Ctrl+S)
    - ▪ Saves all "added" rectangles as a single selection and charge state
    - ▪ Rectangle borders change to thicker borders with a matching color
    - ▪ Rectangles for next charge state can now be "added" to a new selection
  - o Delete Previous Gábor Selection (Ctrl+Del)
    - ▪ Removes last saved selection
    - ▪ Updates Gábor spectrogram and IFFT "working window"
  - o Open Harmonic Finder
    - ▪ Opens dialog for expanding all "added" selections to include specified number of harmonics
    - ▪ Harmonics are added using identical rectangle dimensions as the initial fundamental rectangle and positioned at multiples of the center coordinate of the initial fundamental rectangle
    - ▪ Every rectangle is treated as a fundamental (1st harmonic) and harmonics are added up to and including the input value
    - ▪ A checkbox allows near-zero frequency (0th harmonic) to be included and is recommended unless there is overlapping signal in the MS
  - o Run iFAMS Analysis
    - ▪ Opens dialog for assigning charge states to each selection and finishing deconvolution
    - ▪ iFAMS Analysis corrects each IFFT for the charge state, adds the zero-charge IFFTs to make a Decon, and subtracts the simulated baseline.
      - ♦ Charge correction is applied to the x-coordinates by $(x - mass_{proton}) * z$

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- ♦ $\text{mass}_{proton} = \pm 1.00727647$ Da
- ♦ Each zero-charge IFFT is interpolated to a data density equal to the density of the MS multiplied by the lowest charge state +20%
- ♦ The abundances of the IFFTs are summed to make the zerofull (Decon)
- ♦ Note, a charge state of "0" can be entered if GT filtering is desired without charge correction, such as in Deconvolution Refinement.
- ▪ The baseline is simulated from a MS of constant value (y = 1) and performing iFAMS Analysis using identical parameters.
    - ♦ The resulting "Decon" is the shape of the baseline due to the combination of Gaussian windowing, hard-edge box selections, charge-dependent x-axis transformation.
    - ♦ The scale of the simulated baseline is then fit to the edges of the Decon to avoid signal. The edges are defined from the simulated baseline as abundances lower than 25%
    - ♦ Note, the center of the simulated baseline is often flat which means the signal in the middle is only affected by a constant offset. Wide GT selections can help decrease errors arising from estimating the scale of the baseline.
- ▪ Updates Main Window plots with the "working window" on the left with IFFTs and the Decon on the right with zero-charge IFFTs, simulated baseline, and some of the top peak centroids.
- o Adjust Charge State Assignments
    - ▪ Opens dialog for assigning charge states to each selection before finishing deconvolution with any stored charge state assignments pre-filled. Also, gives the option to open the Charge Adjuster.
    - ▪ The Charge Adjuster is used to assess agreement between charge states with the current assignments and allows easy adjustment to the range of assignments.
        - ♦ The dialog displays the "working window" with IFFTs (top) and a quick Decon (bottom) and reports a score.
        - ♦ The quick Decon runs the iFAMS Analysis on whatever selections are made currently but skips the baseline correction step.
        - ♦ The score is calculated as the natural logarithm of the integrated area of the product of the zero-charge IFFTs. Larger scores indicate greater agreement between charge states and greater likelihood of correct charge assignments.
        - ♦ The "+1" and "−1" buttons shift the assigned charge range up or down by 1, respectively, regenerate the Decon and score, and update the plots.

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- ♦ "Finish" closes the Charge Adjuster and updates the dialog for assigning charge states with the last charge range assignment. Updates any relevant plots in the Main Window.
  - ▪ "Run iFAMS Analysis" performs iFAMS Analysis as usual
- o Open Quantitative Peak Integrator
  - ▪ Opens dialog for integration options and Decon baseline correction and resolution adjustment.
  - ▪ "Minimum x" and "Maximum x" define the search range for Peak Integration and the bounds for Simple Integration.
    - ♦ 2nd range is only used in Simple Integration
  - ▪ "Minimum y" defines the abundance threshold for the Peak Integration search
    - ♦ Input is in normalized abundance (Decon maximum normalized to 1)
  - ▪ "Minimum Distance Between Peaks" defines a peak width threshold (in Da) for the Peak Integration search
    - ♦ If two peak centroids are closer than the input, the peak with a lower maximum will be skipped
  - ▪ "Noise Tolerance for Peaks" defines a threshold (normalized abundance) for determining peak boundaries for the Peak Integration Search
    - ♦ Peak widths are initially determined by adjacent local minima, then made symmetric about the centroid based on the narrower side.
    - ♦ With noise tolerance, local minima from peaks smaller than the input will be ignored (normalized abundance after minimum subtraction).
  - ▪ "Perform Peak Integration" uses input parameters to search for and integrate peaks.
    - ♦ Updates Main Window by plotting the Decon with the integration results shaded and labeled with peak centroids
    - ♦ Opens dialog with integration table including "Mass (Da)", "Peak Area", and "Peak Height"
  - ▪ "Perform Simple Integration" integrates over input x range and 2nd range if input
    - ♦ Reports integration results for each range separately
    - ♦ Updates Main Window by plotting the Decon with the integration results shaded and labeled with peak centroids
    - ♦ Opens dialog with integration table including "Mass (Da)", "Peak Area", and "Peak Height"
  - ▪ "Smooth Isotope Resolution" smooths Decon by convolution with a Gaussian of full width at half max. of 1.8 Da
    - ♦ Useful for calculating average mass centroids or reducing number of peaks, if isotopic peaks are not of interest
  - ▪ "Minima Baseline Correction" adjusts baseline that extends below zero.
    - ♦ Subtracts a composite baseline made from lines between negative local minima

- ◆ Recommended for Decons generated without near-zero signal
  - ▪ "Fourier Baseline Correction" adjusts the baseline by subtracting half of the near-zero frequency contribution.
    - ◆ Only recommended when near-zero frequencies are included
  - ▪ "Segmented Baseline Correction" adjusts Peak Integration results. For each peak, the integrated area is adjusted by subtracting the line between the peak boundaries.
    - ◆ Updates shaded peaks plotted on Decon
  - ▪ "Undo Adjustments" restores all baseline and resolution adjustments.
  - ▪ "Toggle Charge-state Spectra" hides/shows the zero-charge IFFTs on the Decon plot.
  - ▪ "Batch Integration Type" is used for applying the Peak Integration parameters in batch processing.
    - ◆ "Bounds Match" (default) stores the bounds for each peak and integrates batched Decons using identical bounds.
    - ◆ "Parameters Match" uses the stored search parameters to search for peaks to integrate in each Decon
- o Run Mass Defect Analysis
  - ▪ Opens dialog for running MMD on STFT charge state selections.
    - ◆ This step should be performed **after** selecting fundamentals, but **before** adding harmonics.
  - ▪ Each selection must be assigned a charge state
  - ▪ "Repeat Subunit Mass" must be specified. This can be identified a priori or from the reciprocal of the frequency difference between consecutive charge states in the STFT
  - ▪ "Highest Harmonic to Include" must be specified. Harmonics are selected for every charge state from the fundamental (1st harmonic) up to and including the number entered.
  - ▪ "Baseline Correction", when checked, applies the "Minima Baseline Correction" which corrects for negative values by subtracting a composite baseline made from lines between negative local minima.
- o Show Only Spectrogram
  - ▪ Removes all Main Window figures and replots the GT spectrogram to fill the whole window. Used for visualization only. Selections cannot be made from this window.
- o Open Noise Calculator
  - ▪ Opens dialog for adjusting noise baseline estimation and MS baseline subtraction
    - ◆ The noise calculation assumes the noise in the FT is uniformly distributed across the MS. First, the RMSD is calculated from a stretch of frequencies in the FT. Then, the FT RMSD is divided by the square root of the total number of MS data points.

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- ◆ The range of frequencies sampled for calculating the FT RMSD should be in a region free of signal but near the frequencies containing the analyte signal.
- ◆ MS baseline subtraction is generally not recommended. Baseline subtraction is best done after deconvolution.
  - ▪ "Replot Fourier" adjusts the zoom of the Fourier Spectrum in the dialog box to the input frequency range.
  - ▪ "Update Noise Calculation" uses the input frequency range to calculate the "Frequency Noise RMSD" (FT RMSD) and the "Mass Noise RMSD" (MS RMSD).
    - ◆ This button will also store the noise calculation in the background to be carried through to the batch parameters.
  - ▪ "Multiples of Noise RMSD" is used in the "Subtract Average Noise" option. The extremes of the noise likely will extend 3-5 RMSDs past the baseline (depending on the total number of points). By subtracting the minima from the MS, then subtracting 3-5× the MS RMSD, the noise should be centered about an abundance of 0.
  - ▪ "Linear Baseline Subtraction" is the first option for subtracting the minima. Subtracts the line between the minima at either end of the MS (first 5% of points and last 5% of points). Updates figures in the Main Window
  - ▪ "Segmented Baseline Subtraction" is the second option for subtracting the minima. Subtracts lines between minima at 30 evenly spaced intervals. Updates figures in the Main Window
  - ▪ "Subtract Average Noise" subtracts the input multiples of the MS RMSD. This step should be taken after Linear or Segmented Baseline Subtraction. Updates figures in the Main Window
  - ▪ "Undo Subtraction" will undo any baseline subtraction steps taken in this instance of the Noise Calculator dialog. Updates figures in the Main Window

- Calibration Curve Tools
  - ○ Create New Curve
    - ▪ Starts series of dialogs for defining parameters to generate a calibration curve from peaklist files.
    - ▪ Loads iFAMS peaklist CSVs
    - ▪ Calibration Setup Menu 1:
      - ◆ "Number of Calibrant Masses" indicates the number of peaks summed together for the analyte signal.
        - ○ Peaks are selected by mass centroids (within a defined tolerance) which are entered in the next step
      - ◆ "Number of Internal Standard Masses" indicates the number of peaks summed together for the internal standard signal
        - ○ Total analyte signal is divided by the total internal standard signal for the calibration

- o If samples have no internal standard, leave input as "0"
  - o Peaks are selected by mass centroids (within a defined tolerance) which are entered in the next step
- ♦ "Concentration Units" is an optional parameter that only affects the x-axis label.
- ♦ Each peaklist file is displayed with a checkbox and a text box.
  - o Concentrations need to be entered in each text box before continuing to the next step (unless the file is unchecked)
  - o Files that are unchecked at this step will be removed from the calibrant list after proceeding to the next step. Calibrant files can also be toggled after initial calibration setup.
- ♦ "Load File Label List" allows a CSV to be loaded to autofill the calibrant concentrations. The labels must be in the same order and the CSV must only contain the labels in a single column
- ▪ Calibration Setup Menu 2:
  - ♦ "Tolerance" is used for selecting calibrant and internal standard peaks from peaklists. For each entered mass centroid, the peak with the smallest mass difference is selected from the peaklist as long as the peak centroid is within the input centroid ± tolerance.
  - ♦ Tolerance can be defined in units of Da or ppm
  - ♦ "Signal Type", "Curve Type", and "Weighting" can be specified at this step, but can also be adjusted after initial calibration setup.
  - ♦ "Signal Type" can be set to "Integration", which uses peak areas, or "Height" which uses peak heights. Both signal types are stored in peaklist files
  - ♦ "Curve Type" can be set to "Linear", "Quadratic", or "Logistic" to adjust the regression function used to fit the data.
    - o Linear: $y = Ax + B$
    - o Quadratic: $y = Ax^2 + Bx + C$
    - o Logistic: $y = A/(1 + e^{-Bx}) - C$
  - ♦ "Weighting" can be set to "no weighting", "1/x", "1/x^2", "x", or "x^2" which weights the residuals **before** squaring.
    - o (Note that weightings will be changed in future versions of iFAMS to be applied **to the squared residuals**. This change will be made to more closely match the language used in the biopharma industry).
  - ♦ Calibrant and internal standard mass centroids need to be entered for every text box.
  - ♦ "Load Calibrant Mass List" and "Load Standard Mass List" allow CSVs to be loaded to autofill the mass centroids. The CSV must only contain the centroids in a single column.

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- ▪ Calibration Setup Menu 3:
  - ♦ Calibrant peaks, standard peaks, and calibrant peaklist files can be toggled individually.
  - ♦ Tolerance, tolerance units, signal type, curve type, and weighting can also be adjusted at this step.
  - ♦ Calibration curves are automatically saved and will overwrite the previous curve in this instance of dialogs unless the "Overwrite previous curve file" is unchecked. Unchecking this checkbox will cause each calibration curve file name to iterate to avoid overwriting.
  - ♦ "Calculate Concentrations" opens the Concentration Calculator dialog (described below).
  - ♦ If multiple identical concentrations are loaded consecutively, the option to fit the average signal is given.
    - o Files with identical concentrations must be loaded consecutively in order to be averaged.
    - o Signal is summed, corrected for internal standard if applicable, then averaged before regression.
- o Load Existing Curve
  - ▪ Loads iFAMS calibration curve CSV file
  - ▪ Calibration CSVs store concentration labels, total calibrant signal at each concentration, total internal standard signal at each concentration, and various parameters such as mass centroids, tolerance, tolerance units, signal type, curve type, weighting, and whether signal was averaged, if applicable.
  - ▪ Calibration curve is regenerated from loaded data and displayed in the Main Window.
- o Save Calibration Curve
  - ▪ Manual option to save calibration curve, although calibration curves are automatically saved
- o Load Files for Concentration Calculation
  - ▪ Opens the Concentration Calculator dialog
  - ▪ Loads iFAMS peaklist CSV files to calculate concentration using the calibration curve equation. Signal is pulled from the new peaklist files using the same parameters used in generating the loaded calibration curve (such as number of calibrant peaks, mass centroids, and tolerance).
  - ▪ Each peaklist file is displayed with a checkbox and a text box.
    - ♦ Expected concentrations can be entered in each text box for % Bias calculations or files can be given alphanumeric labels.
    - ♦ If a text box is left blank, the file name will be used as a label.
    - ♦ Files can be toggled from calculation using the checkbox.
    - ♦ "Load Label List" allows a CSV to be loaded to autofill the file labels. The labels must be in the same order and the CSV must only contain the labels in a single column

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- ▪ "Plot Expected Concentrations" (if checked) will add vertical dashed lines at concentrations corresponding to any numeric labels.
- ▪ "Average Signal" (if checked) will average the total corrected signal of any consecutive files with identical numeric labels before calculating the concentration.
- ▪ "Export Concentrations" will generate a CSV with sample labels, total corrected signal, and calculated concentrations.

- ● Isotopic Distribution
  Inputs are deconstructed from neutral pH amino acid/ribonucleic base residues and chemical formulas into a tally of each element. FFTs of each element's natural stable isotope distribution are multiplied together, then converted back to a theoretical mass distribution.
  - ○ Calculate Distribution
    - ▪ Opens dialog for calculating theoretical isotope distributions from protein sequences, nucleotide sequences, or chemical formulas
    - ▪ Uses 2 Python dictionaries:
      - ♦ Elemental compositions of neutral pH amino acid and nucleotide base residues
      - ♦ Natural abundances and atomic masses of stable isotopes
    - ▪ "Protein/Nucleotide Sequence" must be entered with no spaces or other symbols
      - ♦ Protein sequences must be entered in uppercase
      - ♦ RNA sequences must be entered in lowercase with an 'r' at the beginning
      - ♦ DNA sequences must be entered in lowercase with a 'd' at the beginning
      - ♦ An 'X'or 'x' can be used for an unknown amino acid or nucleotide, respectively, and an average subunit mass will be applied.
    - ▪ "Oligomeric State" input can be used to add multiples of the input sequence.
    - ▪ "Adduct Composition" input adds a chemical formula to the input sequence.
      - ♦ Use chemical symbols followed by the number included in the formula
      - ♦ Spaces need to be included between every symbol and number
    - ▪ "Adduct Density" input can be used to add multiples of the input formula
      - ♦ This value is per monomer, so the total adducts applied to the final distribution will equal the "Adduct Density" input multiplied by the "Oligomeric State" input.
      - ♦ If "Protein/Nucleotide Sequence" is left blank, the isotope distribution is calculated solely from "Adduct Composition" and "Adduct Density"
    - ▪ "Set Resolution Width" input defines a target full-width at half-max for the theoretical isotope peaks.
      - ♦ The calculated isotope-fine structure distribution is convolved with a Gaussian to broaden the isotope peaks

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- ♦ This can be helpful for comparing the theoretical distribution to an empirical distribution
  - ▪ Plots the theoretical mass distribution both with resolved isotopic fine structure and with broadened isotope peaks for comparison to deconvolved MS data (see "Set Resolution Width")
- o Overlay Data Reconstruction
  - ▪ Loads a Decon or a CSV (x vs. y) and plots the normalized data on top of the calculated isotope distribution.
- o Export Calculated Reconstruction
  - ▪ Exports the broadened theoretical isotope distribution as a CSV.
  - ▪ The peak widths or broadening is defined by the "Set Resolution Width" parameter.

Analysis Procedures:

- ● FT deconvolution
  Works best for low-resolution m/z data of high charge state ions with high mass polydispersity (i.e. high frequency resolution in FT)
  - o Load MS data
    - ▪ *File > Load Ordinary FT >* select MS file
  - o Check Ion Mode
    - ▪ Current mode is displayed in the bottom left of the window (above the "home" icon)
    - ▪ If Ion Mode needs to be switched: *Settings > Positive Ion Mode*
  - o Trim *m/z* range (Optional)
    - ▪ *Settings > Adjust Data Domain*
    - ▪ Adjust minimum and maximum x-values and click "Replot"
    - ▪ Window can be closed after clicking "Replot"
  - o Select fundamental frequency peaks from FT Spectrum
    - ▪ Mass Defect (MMD) analysis can be performed as part of Options 1 and 2
    - ▪ Option 1: Use the Peak Finder Tool
      - ♦ *Fourier Analysis Tools > Open Maxima Peak Finder*
      - ♦ Enter minimum frequency, minimum amplitude, and minimum peak spacing to search for fundamental frequencies
      - ♦ If the noise tolerance of the search function results in partial selection of observable peak distributions, see Option 2
      - ♦ Perform MMD Analysis (Optional)
        - o *Fourier Analysis Tools > Run Mass Defect Analysis*
        - o Enter "Repeated Subunit Mass" and "Harmonics to Include", then click "Run Mass Defect Analysis"
      - ♦ *Fourier Analysis Tools > Run iFAMS Analysis*
      - ♦ Enter "Highest Harmonic to Include", avoiding overlapped harmonics
    - ▪ Option 2: Manually specify charge state range and subunit mass of interest

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- ♦ *Fourier Analysis Tools > Manually Enter Charge State and Subunit Mass*
- ♦ Enter minimum charge, maximum charge, and empirical average subunit mass (can use outputs of Option 1 to identify parameters)
- ♦ Perform MMD Analysis (Optional)
  - ○ *STFT Analysis Tools > Run Mass Defect Analysis*
  - ○ Enter "Repeated Subunit Mass" and "Harmonics to Include", then click "Run Mass Defect Analysis"
- ♦ *Fourier Analysis Tools > Run iFAMS Analysis*
- ♦ Enter "Highest Harmonic to Include", avoiding overlapped harmonics
- ○ Choose to plot the deconvolved mass spectrum as magnitude or real-valued data
  - ▪ *Fourier Analysis Tools > Plot Absolute Data*
    - ♦ Gives a better visual sense of average mass
    - ♦ Artificial secondary peaks arise at half the true mass peak spacing
  - ▪ *Fourier Analysis Tools > Plot Real Data*
    - ♦ Peak valleys are below 0 relative abundance
- ○ If selecting overlapped harmonics is **not avoidable** (e.g., 3rd harmonic of 4+ occurs at same frequency as 2nd harmonic of 6+) see "STFT deconvolution" protocol
  - ▪ Harmonic overlap can be observed 2 ways:
    - ♦ FT Spectrum: frequency peaks with additional modulation
    - ♦ Deconvolved Mass Spectrum: smaller mass distributions at rational fractions of the main mass distribution, observed in only 2 or 3 of the individual charge state IFFTs
- ○ Save data or continue on to another analysis
  - ▪ To save: *File > Export Data*

- STFT deconvolution
  - ○ Load MS data
    - ▪ *File > Load STFT >* select MS file
  - ○ Check Ion Mode
    - ▪ Current mode is displayed in the bottom left of the window (above the "home" icon)
    - ▪ If Ion Mode needs to be switched: *Settings > Positive Ion Mode*
  - ○ Trim *m/z* range (Optional)
    - ▪ *Settings > Adjust Data Domain*
    - ▪ Adjust minimum and maximum x-values and click "Replot"
    - ▪ Window can be closed after clicking "Replot"
  - ○ Adjust STFT parameters (Optional, recommended after trimming *m/z* range)
    - ▪ *STFT Analysis Tools > Change STFT Parameters*
    - ▪ Option 1: Enter target frequency on the right and click "Auto Adjust"
    - ▪ Option 2: Increase "Number of FFT Channels" for higher frequency resolution or decrease "Number of FFT Channels" for higher *m/z* resolution.

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- ♦ Adjust "Color Scale Relative Maximum" to achieve proper peak contrast
- ♦ Click "Replot Gabor Spectrogram" to apply changes.
  - ▪ Window can be closed after completing Option 1 or Option 2
- o Use Gabor Slicer to identify related signal in the STFT (Optional)
  - ▪ *STFT Analysis Tools > Open Gabor Slicer*
  - ▪ Drag orange trace across the spectrogram to select related signal
  - ▪ Release trace to estimate ion mass and adduct mass
- o Select signal from the Gabor Spectrogram
  - ▪ Mass Defect (MMD) analysis can be performed as part of Options 1 and 2
  - ▪ Option 1: Use the Guided Search tool
    - ♦ *STFT Analysis Tools > Run Guided Search*
    - ♦ Enter ion mass or select two adjacent fundamental signals
    - ♦ For selecting signals, click and drag on the spectrogram to draw a box
      - o *ctrl + e* to add the box
      - o *ctrl + s* to save the box
      - o repeat for an adjacent charge state fundamental signal
    - ♦ Click "Go"
    - ♦ Deselect any unwanted charge states in the "iFAMS Guided Search" window
    - ♦ Perform MMD Analysis (Optional)
      - o *STFT Analysis Tools > Run Mass Defect Analysis*
      - o enter "Repeated Subunit Mass" and "Harmonics to Include", then click "Run Mass Defect Analysis"
    - ♦ In "iFAMS Guided Search", adjust "Harmonics to Include" if necessary and click "Run iFAMS Deconvolution".
      - o If multiple series are to be deconvolved together, click "Deconvolve and Select Additional Series" instead.
  - ▪ Option 2: Manually select fundamentals
    - ♦ Click and drag on the spectrogram to draw a box
      - o *ctrl + e* to add the box
      - o *ctrl + s* to save the box
      - o repeat for the rest of the charge state fundamental signals
    - ♦ Keep track of the order of selections for assigning charge states
    - ♦ Perform MMD Analysis (Optional)
      - o *STFT Analysis Tools > Run Mass Defect Analysis*
      - o enter "Repeated Subunit Mass" and "Harmonics to Include", then click "Run Mass Defect Analysis"

    - ♦ Add harmonics to the STFT selections
      - o *STFT Analysis Tools > Open Harmonic Finder*
      - o Enter "Highest Harmonic to Include*"*

- o Click "Add Harmonics to Plot"
- o Click "Start iFAMS Analysis"
  - ▪ or *STFT Analysis Tools > Run iFAMS Analysis*
- o Assign charge states to each selection
- o Click "Run iFAMS Analysis"
  - ▪ Option 3: Manually select all signal
    - ♦ Click and drag on the spectrogram to draw a box
      - o *ctrl + e* to add the box
      - o Add boxes for all signal included for a charge state before saving selections, i.e., add boxes for fundamentals and harmonics
      - o *ctrl + s* to save the box
      - o repeat for the rest of the charge state signals
    - ♦ Keep track of the order of selections for assigning charge states
    - ♦ *STFT Analysis Tools > Run iFAMS Analysis*
      - o Assign charge states to each selection
      - o Click "Run iFAMS Analysis"
  - ▪ In addition to the keyboard shortcuts, boxes can be added and saved from the "STFT Analysis Tools" menu
- o Save data or continue on to another analysis
  - ▪ To save: *File > Export Data*

- Quantitation and Calibration
  - o After generating a deconvolved mass spectrum, integrate peaks
    - ▪ *STFT Analysis Tools > Open Quantitative Peak Integrator*
    - ▪ Adjust parameters for Peak Integration or enter one or two sets of min. and max. x-values for Simple Integration
    - ▪ Apply any desired alternative baseline correction (some need to be applied after integration).
      - ♦ Details and recommendations for the different baseline corrections are described and illustrated in a separate guide.
  - o Batch deconvolve and quantitate
    - ▪ *File > Batch Data Files > *select MS files
    - ▪ If the Decon was loaded from a saved file, batch quantitate deconvolved spectra instead: *File > Batch Deconvolved Spectra > *select Decon files
  - o Generate calibration curve
    - ▪ *Calibration Curve Tools > Create New Curve > *select calibrant Peaklist files
    - ▪ Enter "Number of Calibrant Masses" and "Number of Internal Standard Masses"
    - ▪ Enter calibrant concentration labels
    - ▪ Click "Next"
    - ▪ Enter a tolerance for selecting peaks from the Peaklists for each input mass
    - ▪ Enter "Calibrant Masses"

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- ▪ Enter "Standard Masses"
- ▪ Click "Create Calibration Curve"
  - o Optimize calibration curve
    - ▪ Deselect any undesired calibrant masses, standard masses, or calibrant concentrations
    - ▪ Adjust signal type between integrated area and peak height
    - ▪ Adjust curve type and weighting
    - ▪ Click "Update Calibration" to apply changes
  - o Calculate concentrations using the calibration curve
    - ▪ Click "Calculate Concentrations" in the Calibration Curve Menu
      - ♦ or *Calibration Curve Tools > Load Files for Concentration Calculation*
    - ▪ Add expected concentration labels (optional)
    - ▪ Click "Calculate Concentrations"
    - ▪ Click "Export Concentrations" to save a CSV of the calculation results

- ● Isotopic Distribution Mass Accuracy
  - o Calculate the theoretical mass distribution of your sample
    - ▪ *Isotopic Distribution > Calculate Distribution*
    - ▪ Enter the amino acid sequence, nucleotide sequence, or chemical formula for your sample
    - ▪ Adjust the "Oligomeric State" and add the chemical formula of adducts if necessary
    - ▪ Click "Calculate Mass Distribution"
  - o Load a Decon or Zero-Charge IFFT CSV file
    - ▪ *Isotopic Distribution > Overlay Data Reconstruction*
  - o Adjust the "Set Resolution Width" in the "Molecular System Parameters" window to match the full width at half max of your data
  - o Export the adjusted theoretical isotope distribution
    - ▪ Click "Export Mass Distribution"
      - ♦ or *Isotopic Distribution > Export Calculated Distribution*

- ● Deconvolution Refinement
  - o Load deconvolved mass spectrum for STFT
    - ▪ *File > Load STFT > select Decon file*
  - o Adjust STFT parameters
    - ▪ *STFT Analysis Tools > Change STFT Parameters*
    - ▪ The signal of interest will be centered about a frequency of 1 with significant deviations as small as 0.05. Generally, this approach achieves good enough frequency resolution to detect such deviations:
      - ♦ Enter "1" for frequency to resolve and click "Auto Adjust"
      - ♦ Double the updated "Number of FFT Channels" and click "Replot Gabor Spectrogram"

- Select signal that is symmetric about a frequency of 1 or is consistent with being a harmonic of such an identified signal (i.e., same shape but centered about a higher integer frequency).
  - Signal needs to be selected manually
  - Since all signals are already charge corrected, all boxes can be added to a single saved selection. But it can be useful to periodically save selections as long as all boxes at the same "*m/z*" (mass) are saved to the same selection.
  - The Harmonic Finder tool can also be useful, but it is important to note that contaminant signals can appear at higher integer frequencies while being absent from a frequency of 1. Also, the Harmonic Finder tool cannot be used if some selections already contain higher harmonics.
  - Do not include the "near-zero frequency" signal
- "Deconvolve" to finish refinement
  - *STFT Analysis Tools > Run iFAMS Analysis*
    - ♦ Enter "0" for all selections
    - ♦ Click "Run iFAMS Analysis"
  - Recommended, baseline correct to adjust negative values
    - ♦ *STFT Analysis Tools > Open Quantitative Peak Integrator > Minima Baseline Correction*
    - ♦ Recommended, re-integrate with any parameters
- Save data or continue on to another analysis
  - To save: *File > Export Data*

Tips for Resolving the Gábor Spectrogram:

- The number of FFT channels directly affects the apparent frequency and *m*/*z* resolution in the Gábor spectrogram. A greater number of FFT channels will result in greater frequency resolution. Because the number of "pixels" in the Gábor spectrogram is fixed as the number of MS data points multiplied by the "oversampling factor," the spectrogram resolution is a tradeoff between frequency and *m*/*z* resolution, i.e., increasing the number of FFT channels (and the frequency resolution) decreases the *m*/*z* resolution.
- Optimizing the number of FFT channels can be tedious, but a wide range of values will produce accurate deconvolutions. The default value is based on an initial guess from frequency abundance and tends to prioritize high frequencies. If the initial guess is off, the Auto Adjust tool does an excellent job of estimating the number of FFT channels within the desired range. Just input a frequency that seems to have a lot of signal. The ordinary FT can be useful for identifying frequencies that have peaks.
- A typical target for fine-tuning is to resolve signals in the Gábor spectrogram so that a peak spans 3-5 pixels in the *m*/*z* direction.

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To download the latest version of iFAMS, go to https://github.com/prellgroup/iFAMS/releases

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