Questions:	Answers (Written or time location in video)
Sorry for the perhaps basic question due to lack of familiarity with timsTOFs, but could you explain a bit more about the 3 quadrupole isolation schemes for each IMS "cycle"? What is going on sequentially in the instrument?	Live answered - 1:10:58 AM
What criteria does Skyline use to pick 15 endogenous iRT peptides out of 81? Is it just RT span or also other parameters?	Live answered - 1:21:14 AM
When selecting decoys for DIA data why is "shuffle sequence" preferred over "reverse sequence"?	Live answered - 1:14:33 AM
What is the differernce between repeated and duplicated peptides?	Duplicate completely removes peptides that show up in multiple proteins. Repeated will leave these peptides in one protein, but it is just the first protein it appeared in the FASTA file. We hope we can improve this, but if you don't want to lose any peptides, you would choose "repeated". If you only want peptides that appear in only one protein, you would choose "duplicates".
Ques on building Spectral Library: which is a better input between DDA_search vs. DIA_search (directDIA)?	Live answered - 1:18:29 AM
For Decoy DB, shuffle or reverse, any reason to select shuffle?	Live answered - 1:14:33 AM
Is it necessary to correct mis-ID retention marker?	Live answered - 1:16:55 AM
A question to Ben regarding the chromatography used.. Amazing RT reproducibility between runs.. Could you provide details on the LC, column, and chromatogrpahy methods used here.	Live answered - 1:22:50 AM
How does the setup change if I have a control sample and I am planning to compare 2 experimental samples? Do the experimental values get adjusted based on the control?	BC: I'm not sure if I follow the question exactly. If you want to make quantitative comparisons to this control then this can be defined but there would not be any adustment made to the experimental samples, rather just ratios and statistical tests. When you refer to adjustments maybe you are talking about normalization. There are different options for this in Skyline but I don't think any of them will make use of external control runs. For normalization and batch effects in general discussion you would take a look here https://www.embopress.org/doi/full/10.15252/msb.202110240
If there is no standard peptide for retention time calibration, how could we pick the home peptides for this intention? how many point should we need for this calibration? thank you	Live answered - 1:23:35 AM
Is there any news on support for DIA-NN Speclib v3 libraries? That program is just so convenient, especially for Library-free searches.	Yes. That will be supported in our next Skyline-daily release and in Skyline version 21.2
Can you have more than 3 quadrupole positions per tims cycle? Also, what's the reasoning behind the extended IM range on the lower/higher mass range?	Live answered - 1:25:46 AM
I noticed that you selected 20 ppm in the "centroided" transition setting. Is that preferred over setting the TOF resolution?	Bruker TOF data is so dense that they don't even really support our retrieving these spectra in profile mode. Bruker has always pushed us to used centroided spectra for their data. In the MacCoss lab, we have derived ourselves that Thermo centroided spectra work better for our data processing tha profile mode spectra. Only with SCIEX, is the centroiding algorithm not always beneficial. So, for SCIEX TripleTOF data we recommend you use the TOF setting.
Question to Brendan about iRT import on automatic mode if there weren't any spike-in standards.. I might have missed this.. Does it need to have a predefined set of endogenous peptides provided as a iRT library for this? Follow up question.. You have selected 15 out of 81 peptides in this case.. How does Skyline choose those 15? Any criteria to define these?? Thanks..	Automatic will search for all know iRT standards mixes. Failing finding one of them, it will fall back to 100 or so endogenous peptides conserved in eukaryotes, and failing that it will simply fall back the entire library. It will then prioritize the highest scoring peptide spectrum matches most consistently matched over any mutliple runs and spanning the gradient. I usually would choose toward 15-20, but I ran a successful published data set with 60 or so endogenous iRT standard peptides, and I was very happy with the scoring performance of the Retention time difference score.
Is there a reason why you use the import wizard for PASEF analysis, or it could be equally good by changing the settings directly in the Transsition and Peptide settings tabs?	1:27:47
I'm not sure if I understood. Since Ben used reverse sequence decoys in the DDA search for building the library and it's in the FASTA file already do you have to use shuffeled decoys for analyzing the DIA data?	From what I can see in the Targets, none of the reversed sequences come through in the search results. What you need to be careful of is a DDA search pipeline that uses reversed decoys which end up in your Targets view. Then if you use reversed decoys in Skyline, it will end up with true peptides labeled as decoys, which will confuse the scoring. That was not the case with Ben's search method. So, it would have been okay to use reversed decoys.

So my experience with IMS comes from Synapts. And what I have seen is that the arrival time distribution of the ions varies with abundance, which requires different IMS resolving power to encapsulate it without overextending. Is a similar effect observed in data from timsTOFs? Would there be any way of implementing in Skyline for each precursor "ideal" IMS filters?	It is possible to set an "Explicit" IMS filter for each peptide. This is somewhat laborious, but might make sense in a more targeted method. We have not yet implemented a feature that would set this automatically based on observed data for each molecule.
Still about decoys, isn't there a "shuffle + a fixed Da mass shift" available? This might help with peptides with repetitive sequences	Skyline always applies a +10 m/z (adjusted to fit optimal m/z frame shifts) to its precursors to avoid decoys having matching scores with Targets for precursor scores like idotp. All testing of Skyline for DIA data has been performed with this feature. It also frequently shifts a peptide to a different DIA/SWATH window from its matching target.
Have you thought about checking the correlation of precursor+fragments in ion mobility or is that maybe already part of the scoring?	Live answered - 1:29:25 AM
How would Skyline deal with actual isomeric species? how would that quantification work?	I am guessing this refers to isomers that are resolvable in the IM or RT dimesion. I am not aware that there is a way to deal with this specifically in Skyline. Isomers resolved in RT would be observable in XICs but the IM dimension is a filtered based on the library value with some tolerance so I think will not be dealt with in a sensible way.
Still about decoy DB, any difference you see using shuffle vs reverse?	In my use, I have never seen a meaningful difference between the two. So, I tend to prefer shuffled, because it is more flexible.
Based on your experience, do you know how much difference for the identified peptides using DDA library and other standard download database library?	Others have written papers on this, and it depends on the libraries in question. I leave it to the reader to seak out the papers and make your own conclusions about the libraries you have available, which now also include predicted libraries from trained neural networks, like Prosit.
Has your group(s) investigated how well PTMs can be investigated by DIA-PASEF?	This is something we are starting to work on but not much experience yet. I am not aware of any publications yet. Our expectation is that we might be able to resolve some isobaric species (phospho positional isomers) and some data have been presented in ddaPASEF mode (Stefan Tenzer). It may also be possible that larger PTMs could move these peptides into a different space in the m/z vs IM map such that they cuold be targeted directly (e.g. shown crosslinked peptides in ddaPASEF mode shown by Richard Scheltema https://pubmed.ncbi.nlm.nih.gov/32694122/). Still much to be explored here.
In the diaPASEF paper, you use overlapped windows for one particular method. However, its unclear what those overlaps are and how they vary across the m/z spectrum. Is there any guidance for selecting overlapping windows?	Here I think you refer to overlap in the IM dimension. In the 'standard diaPASEF' method described in the paper do use overlap in the IM dimesion. The point here is that for a given mass (i.e. quadrople position) there is a spread for precursors in the IM dimension. So, this is basically an effort to cover those precursors. If you are going to have 2 IM ranges for a given quadrupole position then it is a good idea to have overlap that is at least as large as the expected peak width in the IM dimension will be in one or other of the IM windows. As mentioned there are a lot of options here and I would suggest to take a look at the windows design tool in TimsControl if you have access.