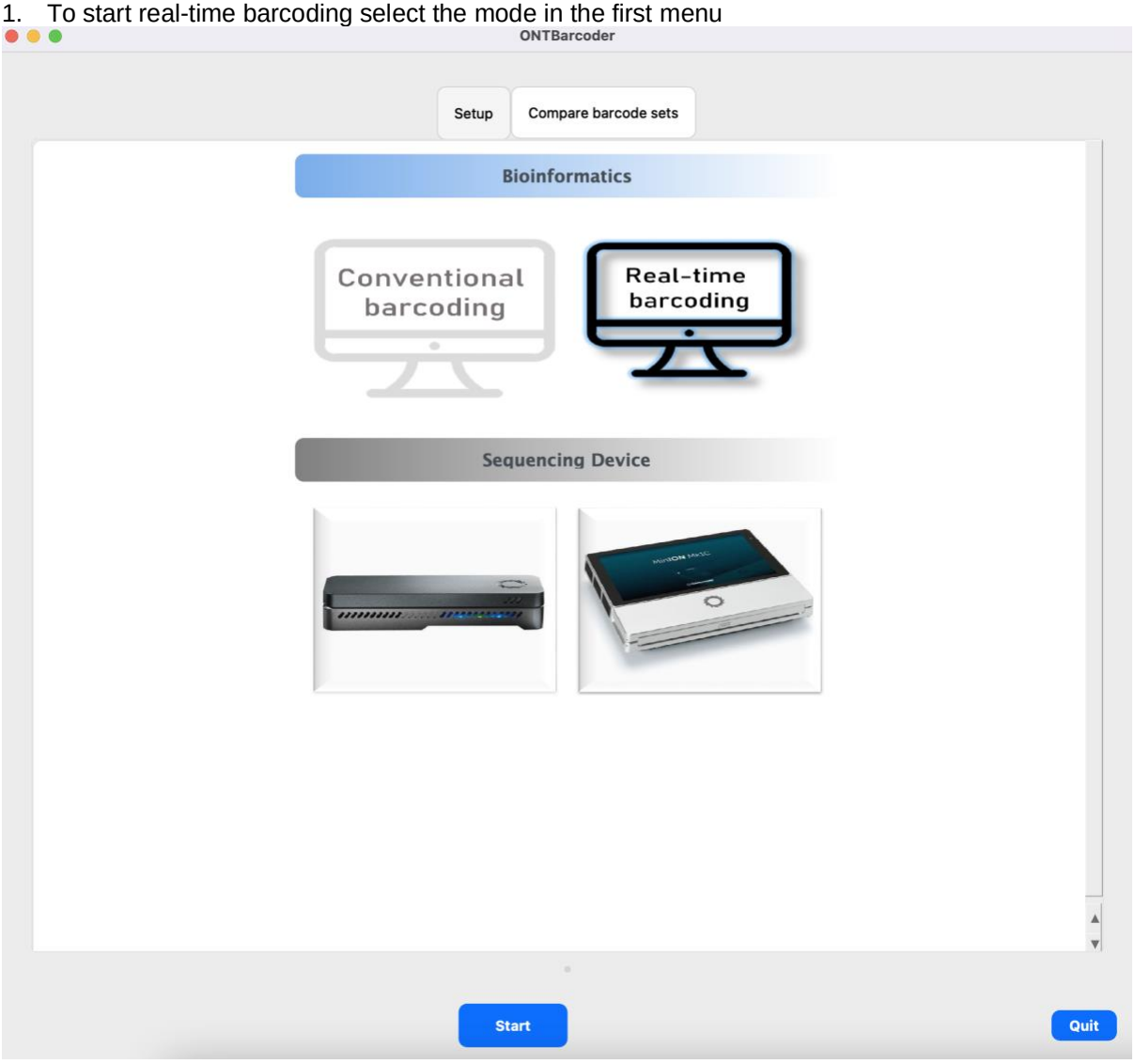
REAL-TIME BARCODING

ONTbarcoder (available from https://github.com/asrivathsan/ONTbarcoder) is installed by unzipping the folder with the version of the program that supports the operating system on your computer. The folders are available from releases in Github. For MacOS, an .app bundle has been created.

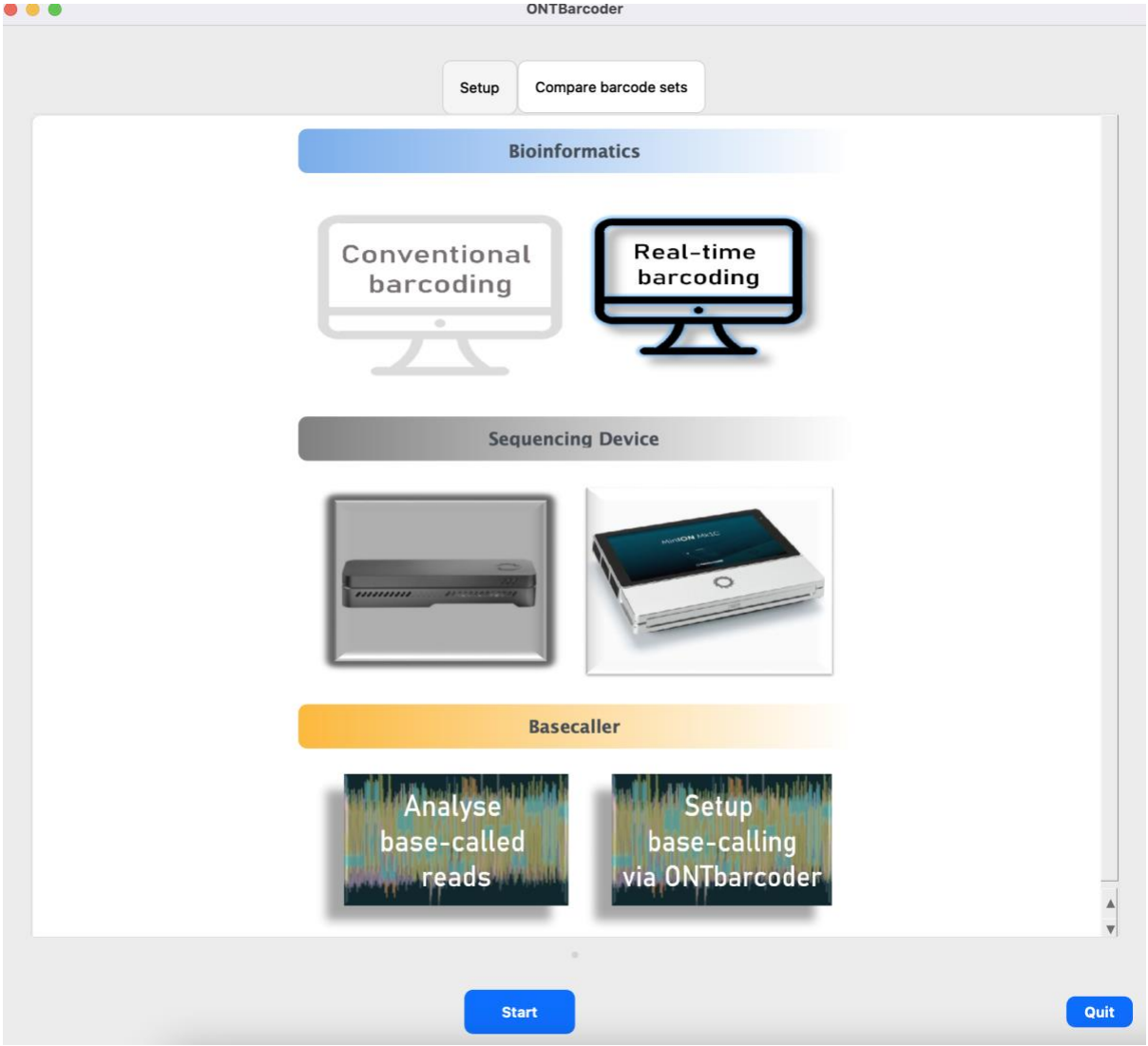
We recommend the video tutorial for real time barcoding to understand the overall set up.

Real-time barcoding module of ONTbarcoder accepts is compatible with both Mk1B and Mk1C and accepts input in the form of either a folder where base-called fastq files are stored or a folder containing fast5 are stored

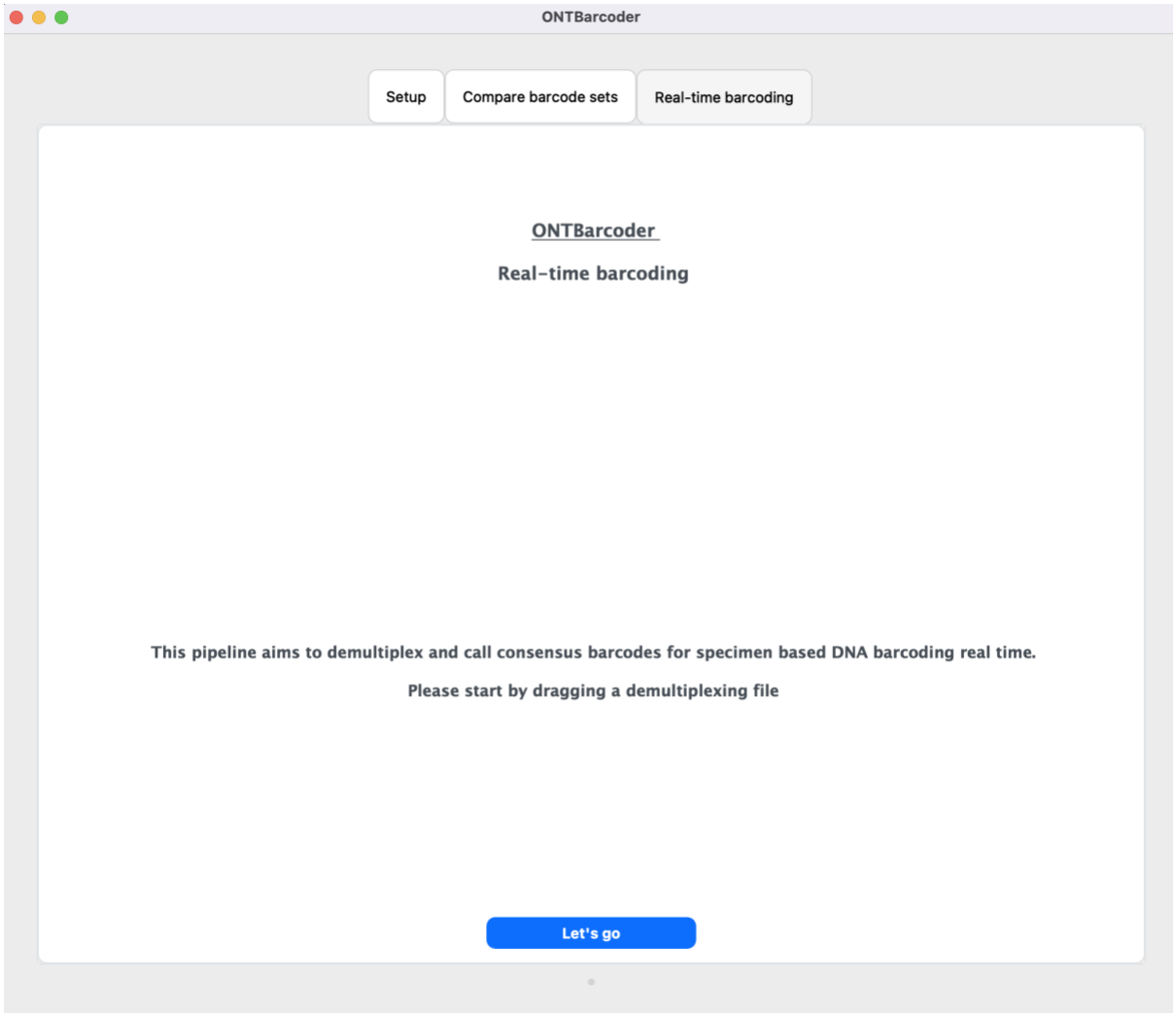
1. To start real-time barcoding select the mode in the first menu



2. Next select the sequencing device of interest

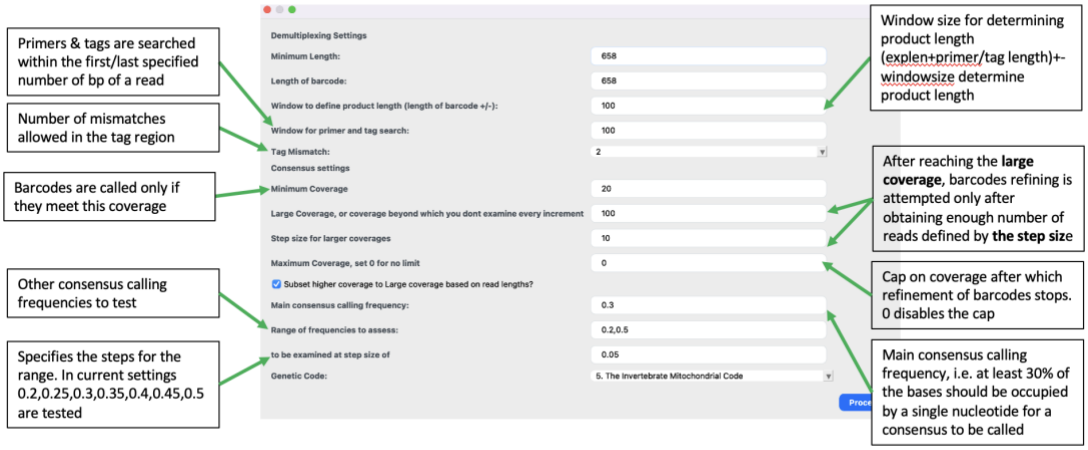


3. Finally select the type of reads you would be inputting, folder containing base-called fastq files, or folder containing fast5 files for basecalling and press “Start”.
4. The next screen will ask you to drag in a demultiplexing file. Drag your demultiplexing file and press the “Let’s go” button that appears



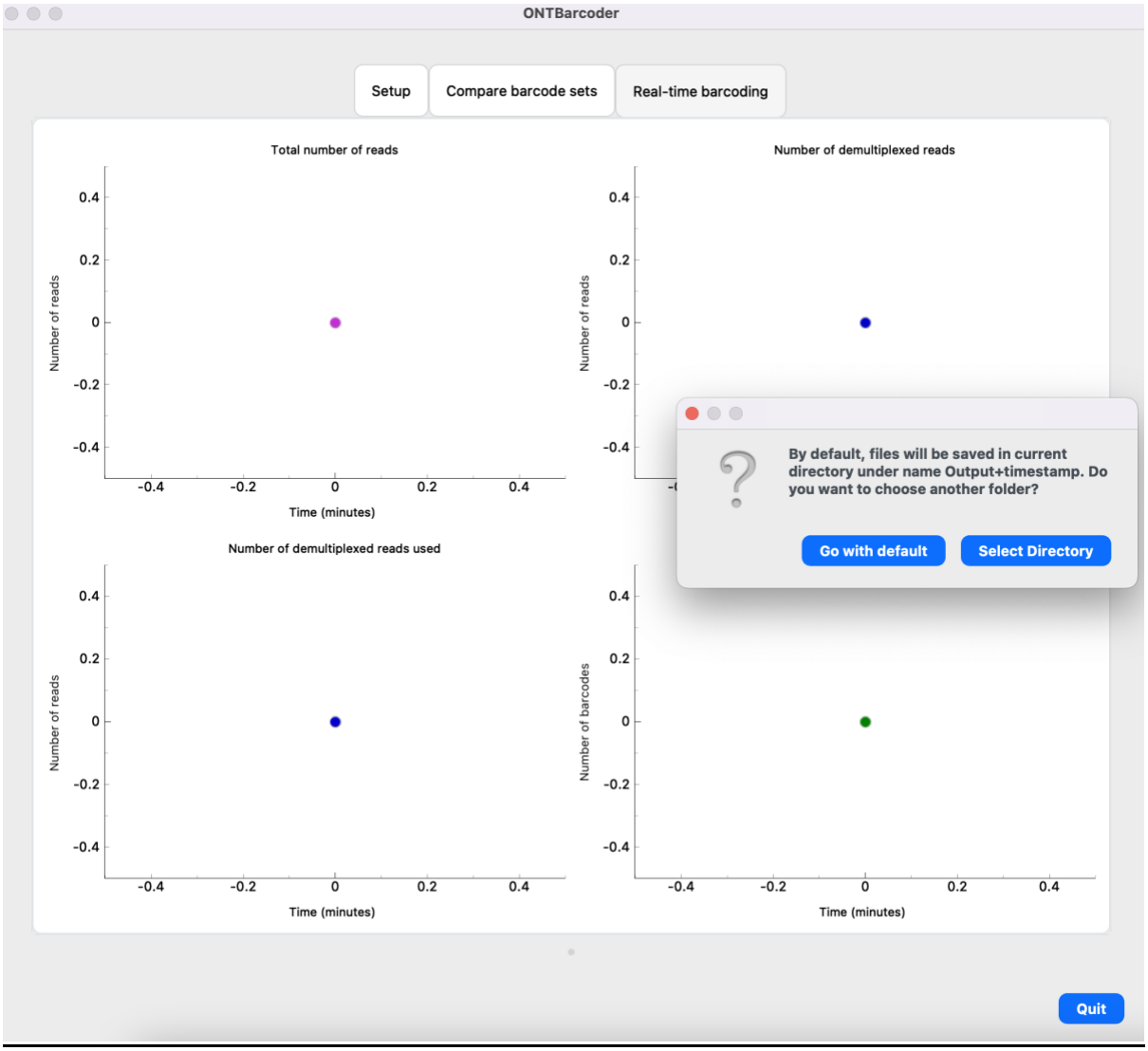
5. The four modes (Mk1b+analysis of basecalled reads, Mk1b+analysis of fast5, Mk1c+analysis of basecalled reads, Mk1c+analysis of fast5),will give you different option menus. Common to all is the basic set up for the barcode calling.

Advanced features can be edited if you want to test if they improve your yield:



6. At this point we recommend you start your sequencing run.

7. Select where you want the output to be stored.



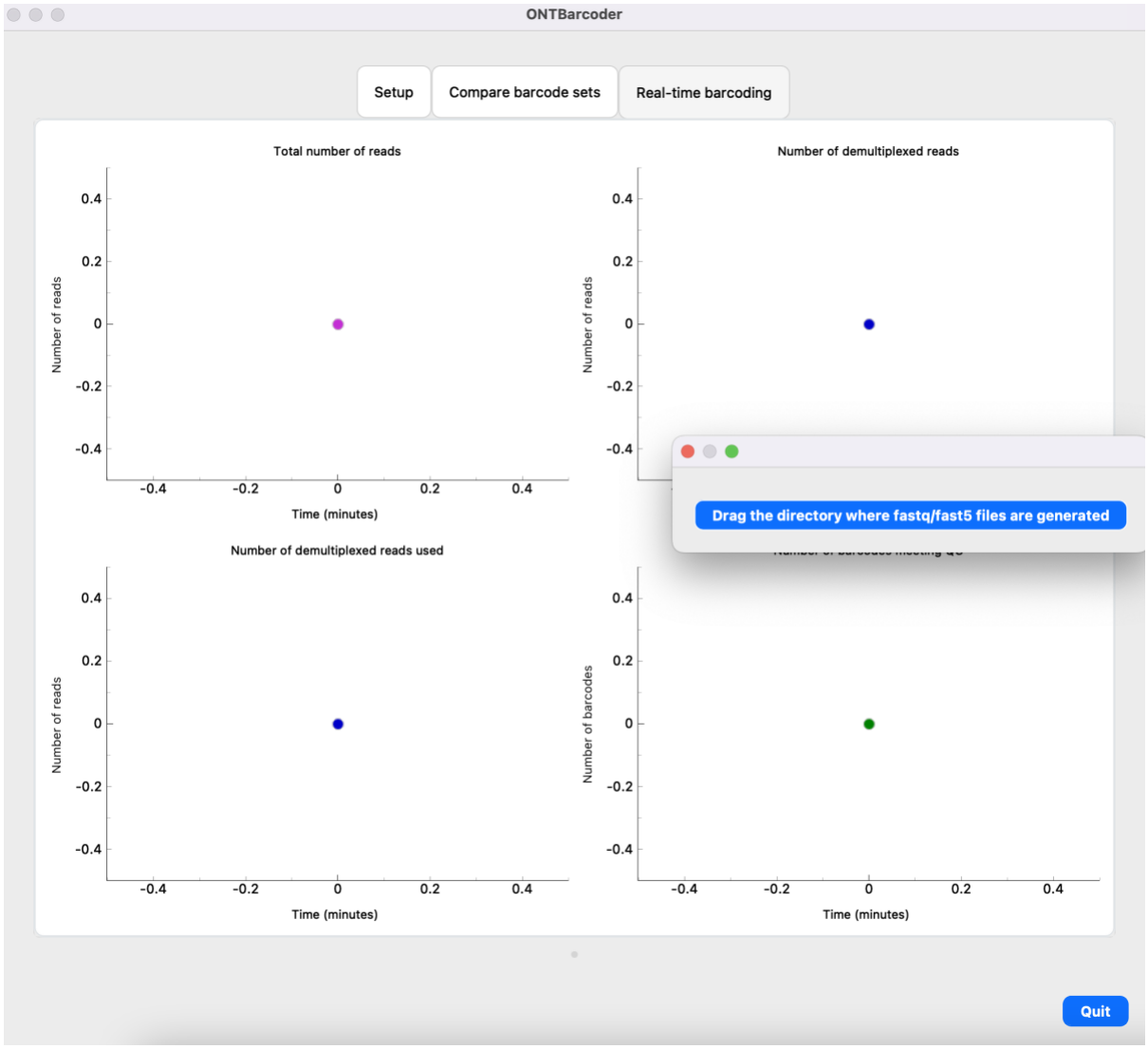
Next we detail the four modes that can be run in this software

Mode 1: Mk1B + Analysis of base-called reads

Here ONTbarcoder is run in the same computer as the one sequencing. Basecalling is set up by MinKnow.

What is needed:

1. Demultiplexing file (already used in set up)
2. Path to folder where fastq files are stored. It is important that the path contains the fastq files themselves. MinKNOW if set up for filtering based on qscore- saves reads in folders called **pass** or **fail**. In this case use the pass folder. However, overall we recommend that MinKNOW is not set up filtering.



At this point a pop up menu will appear asking the User to drag in the directory where the fastq files are being generated. Drag in this folder, and press “Click to proceed”

The run should start

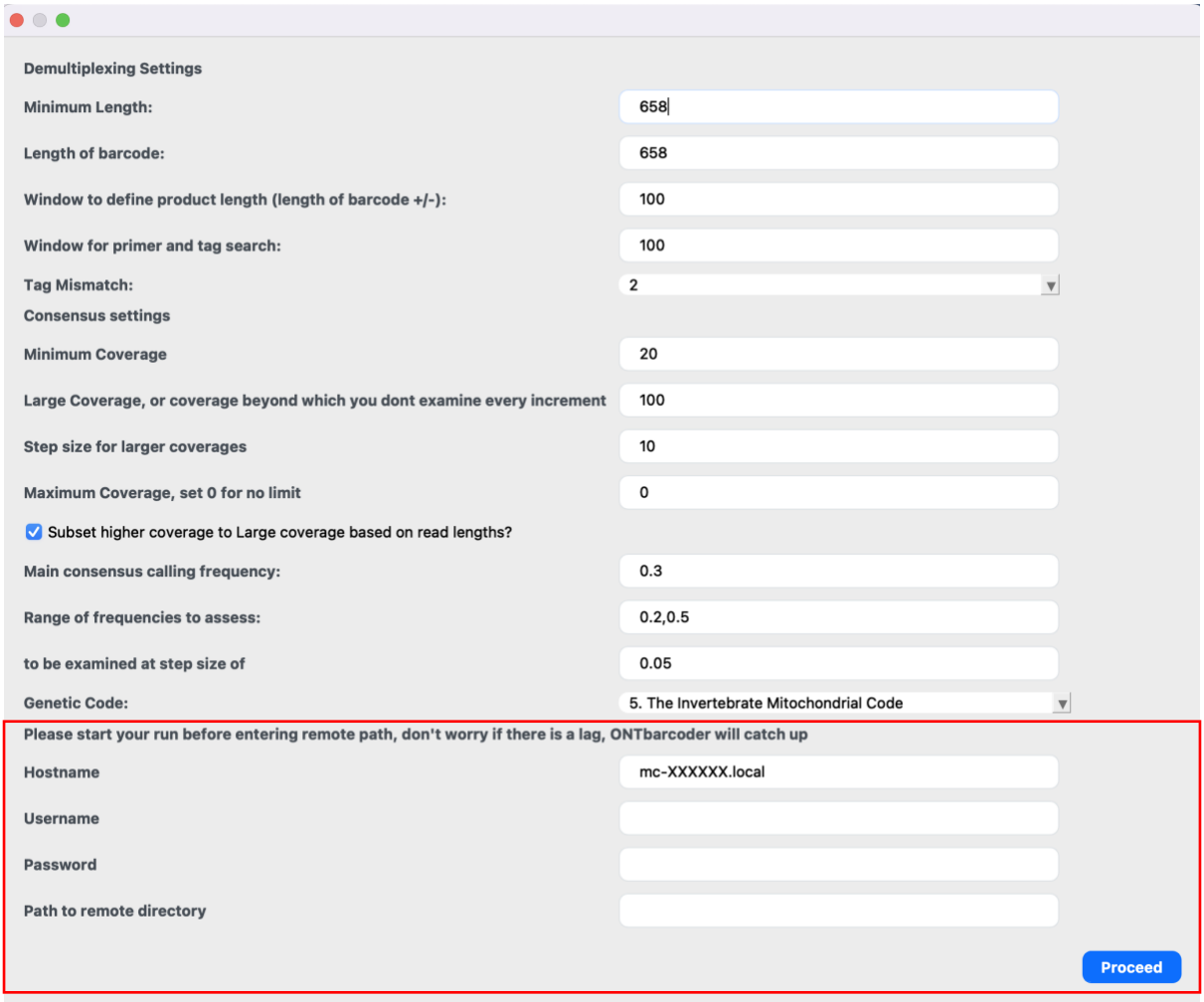
Mode 2: Mk1C + Analysis of base-called reads

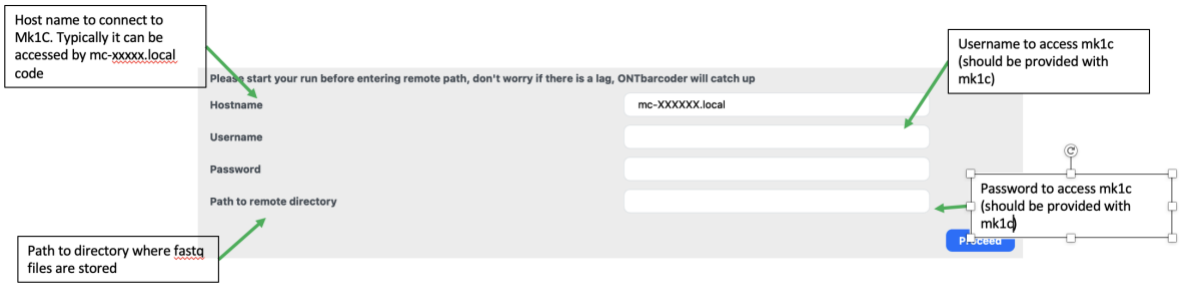
In this mode sequencing is in a remote device like Mk1C, and you can connect to it either over ethernet (best practice) or hotspot.

What is needed:

1. Demultiplexing file (already used in set up)
2. Path to folder where fastq files are stored in the remote device. You can connect over ssh to the remote device and copy the text of the path , either via WinSCP/Putty in Windows or terminal in Mac/Linux. It is important that the path contains the fastq files themselves. MinKNOW if set up for filtering based on qscore- saves reads in folders called **pass** or **fail**. In this case use the pass folder. However, overall we recommend that MinKNOW is not set up filtering.
3. Details to login to mk1c (host name, username, password)

The big initial menu that pops up contains a section in the end





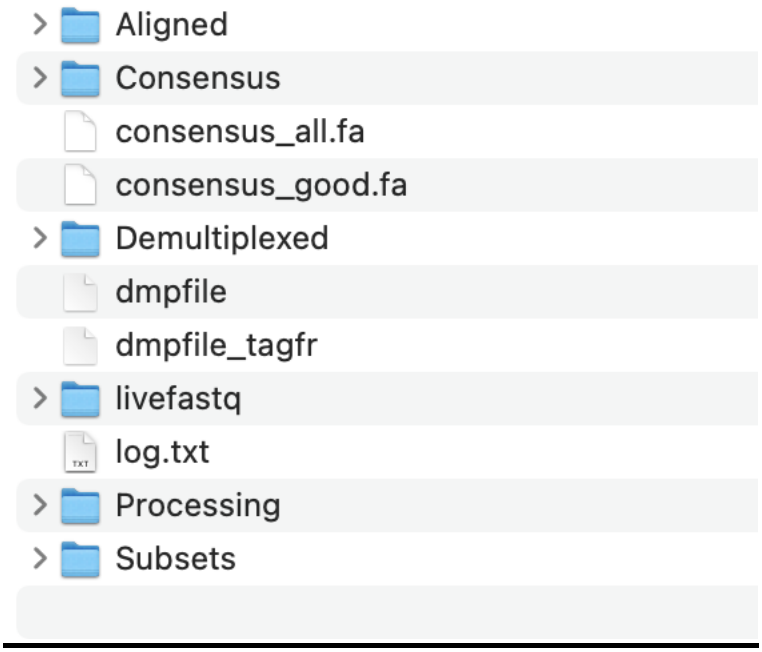
Mode 3 and 4: Mk1B/Mk1C + Basecalling is set up via ONTbarcoder

In these modes, you can configure ONTbarcoder to do basecalling via guppy. Depending on whether you use Mk1B and Mk1C, Mode 1 and Mode 2 menus will appear. However an additional menu will appear for configuring Guppy

What is needed:

1. Demultiplexing file (already used in set up)
2. Path to folder containing fast5 (either locally, same as Mode1) or remotely (same as Mode 2)
3. Guppy (GPU mode) installed in the computer and its path.
4. Path to guppy basecalling model cfg file

Outputs



“consensus_all.fa” stores the final iteration consensus barcodes for all dataset irrespective of quality, while “**consensus_good.fa”** stores the QC filtered barcodes and is the recommended barcode set

Summary

Expected input and output

1. Conventional barcoding	Input required	Results
Mode 1: Mk1B + Basecalled reads	• Demultiplexing file • Local directory where fastq files are stored, can be dragged in	consensus_good.fa (QC filtered) consensus_all.fa (all barcodes)
Mode 2: Mk1C+ Basecalled reads	• Demultiplexing file • Path to remote directory where fastq files are stored, has to be pasted in text menu	
Mode 3: Mk1B + Fast5	• Demultiplexing file • Local directory where fastq files are stored, can be dragged in • Path to guppy executable • Path to guppy config file	
Mode 3: Mk1C + Fast5	• Demultiplexing file • Path to remote directory where fastq files are stored, has to be pasted in text menu • Path to guppy executable • Path to guppy config file	

Format Specifications

File	
1. FASTQ file	Standard fastq, generated after basecalling with ONT software
2. Demultiplexing File	A 5-column csv file with the following headers: SpecimenID, TagFsequence, TagRsequence, PrimerF, PrimerR You can only demultiplex one one primer pair at a time. FASTQ files with data for multiple pairs, have to processed sequentially. **Please avoid unusual characters in Specimen ID (e.g. characters like “(){}[]\/.,;*$” will lead to crashes)**

3. Directory containing fastq file	Folder containing fastq files generated by ONT can be compressed or not compressed
4 Directory containing fast5 files	Folder containing fast5 files generated by ONT