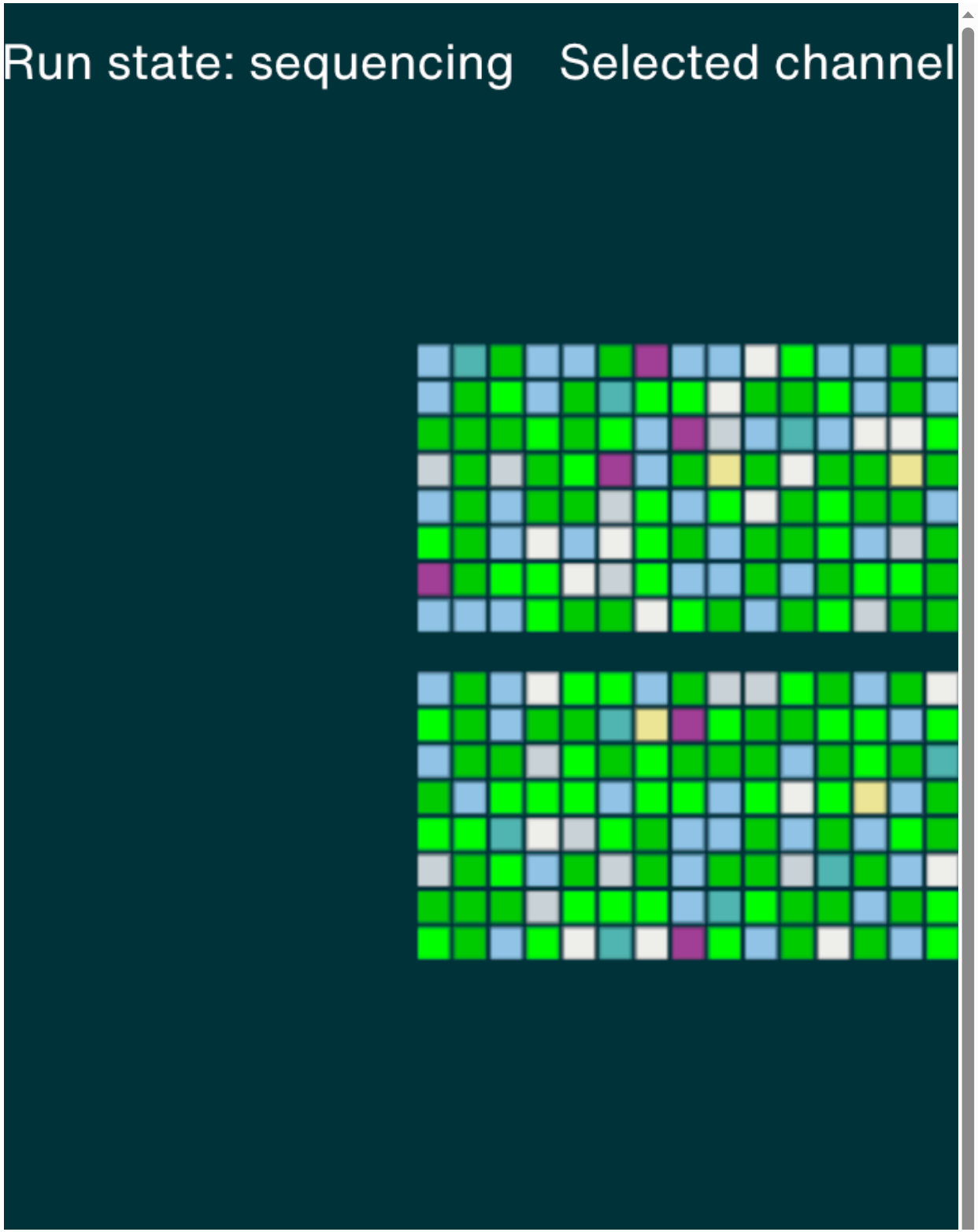
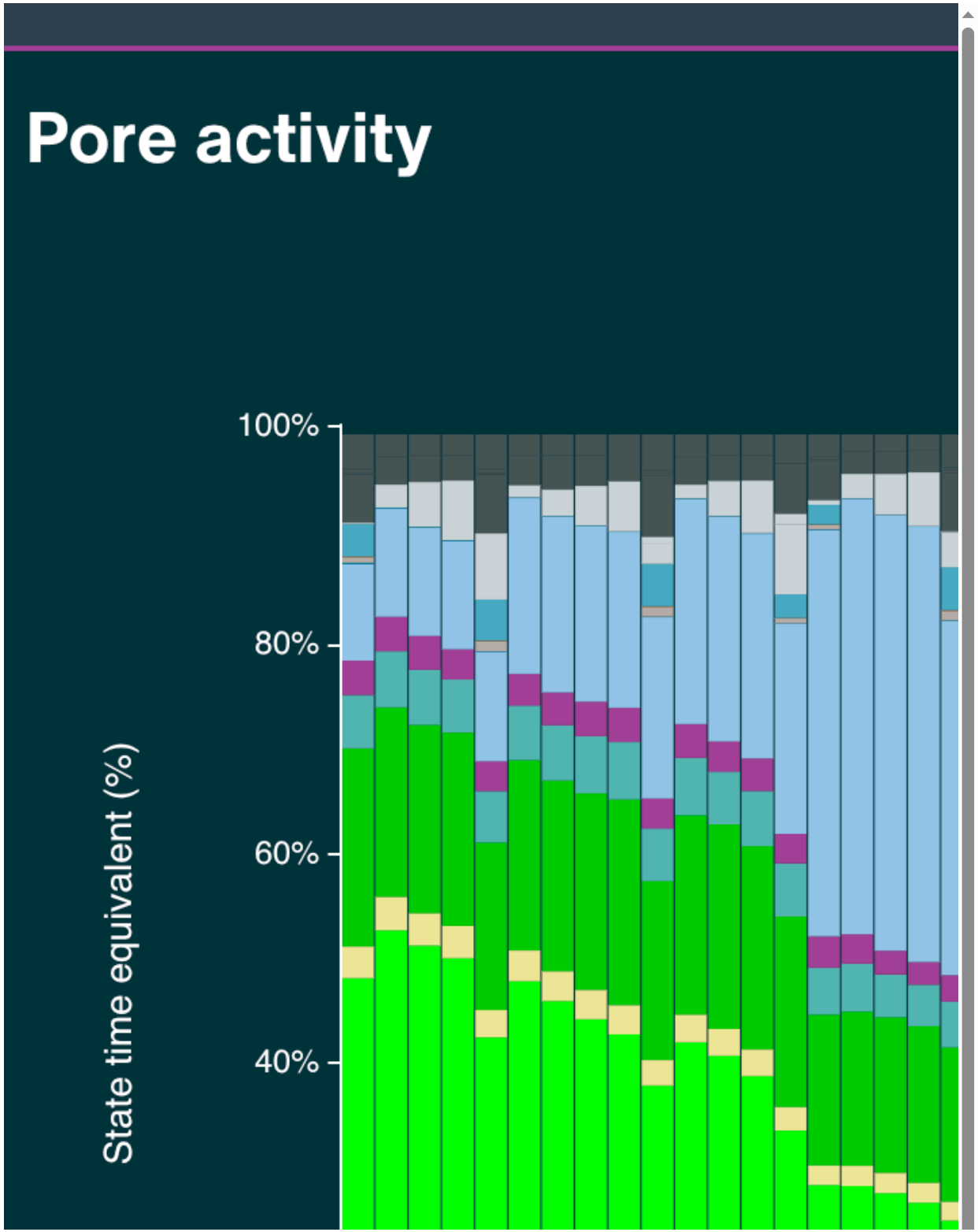
Wildflower Flow Cell Patterns

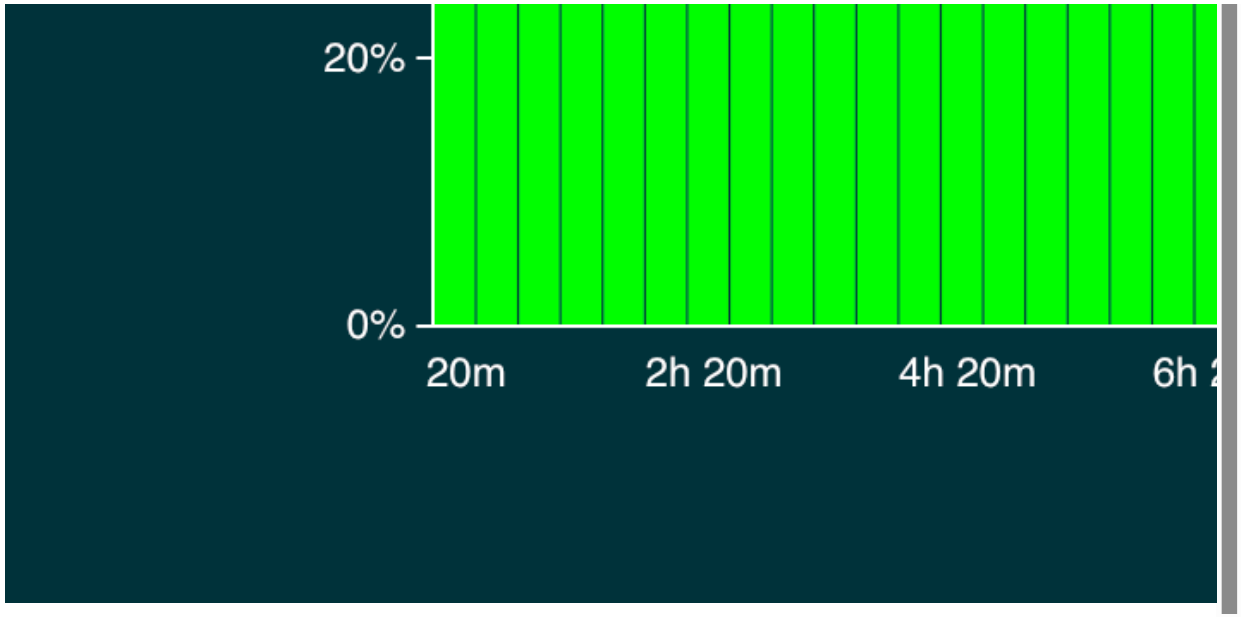
8th March 2023 | 1044 Views | 31 31 Replies | 15 Users

As I've reported briefly in the past, we've been seeing a lot of R9.4.1 flow cells with what I like to call a "wildflowe pattern, because the detailed channel plot looks a bit like a field of wildflowers, with a large mix of different types channel classification (including purple "Active Feedback" pores) and no obvious pattern:


Run state: sequencing
Selected channel

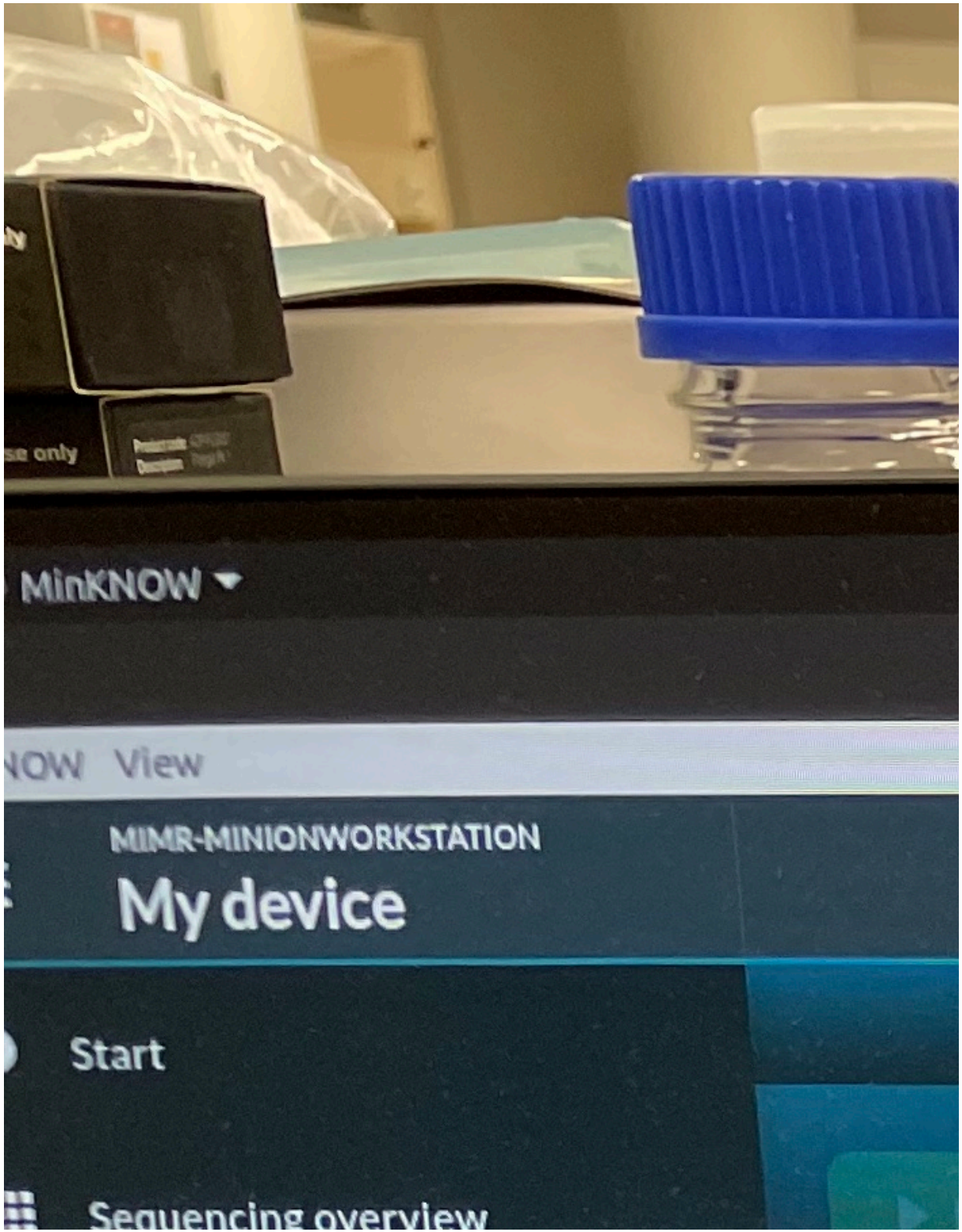
What happens with these flow cells is that they tend to crash quickly. We see steep decreases in sequencing capacity, this most recent case losing about 50% of sequencing capacity overnight, despite having a well-loaded flow cell (tl was with a rapid adapter cDNA kit; it's quite rare for us to see loading over about 75%). I've noticed that this sha decrease is commonly linked to the "active feedback" and "channel disabled" pore states:

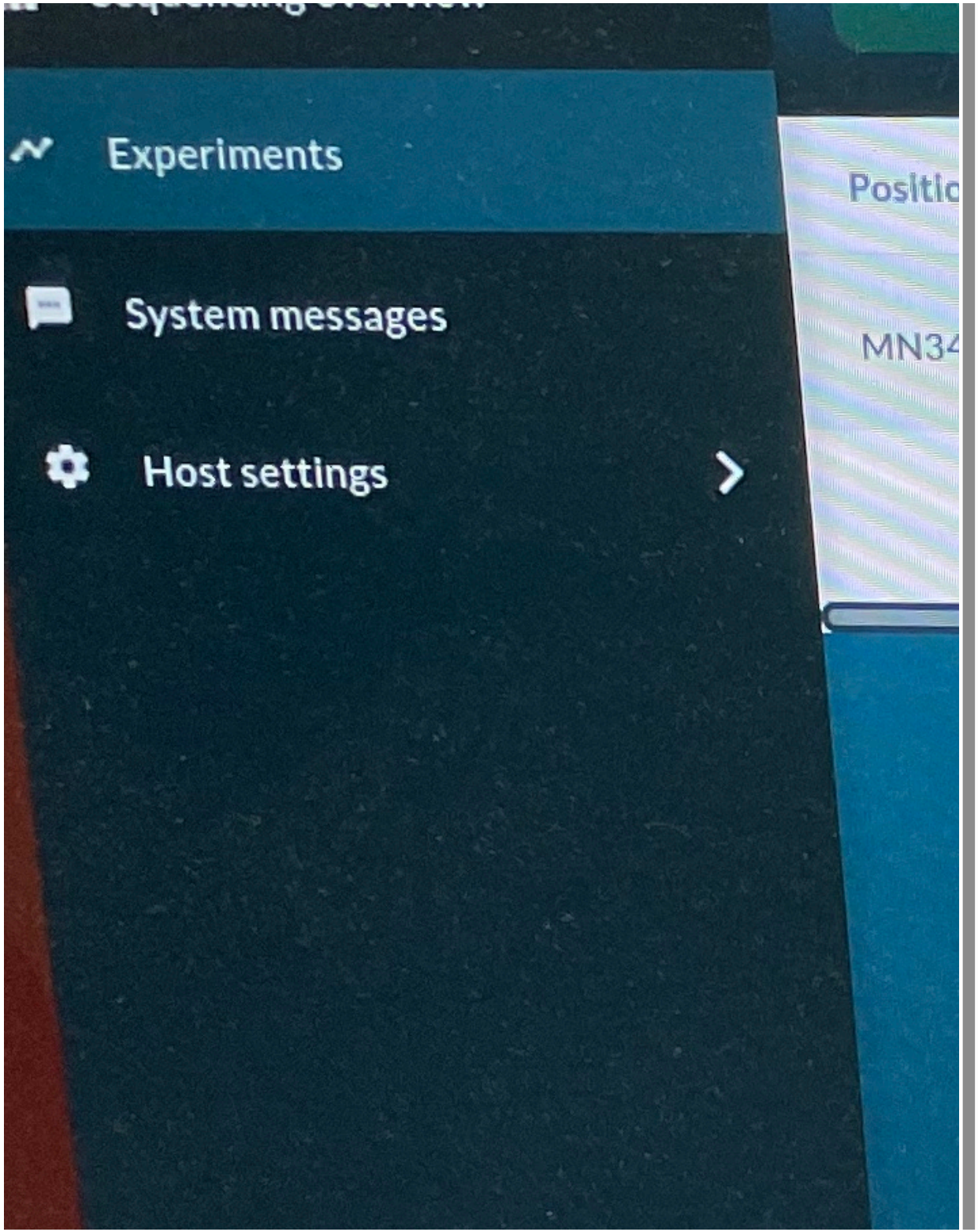

Pore activity
100%
80%
60%
40%
State time equivalent (%)

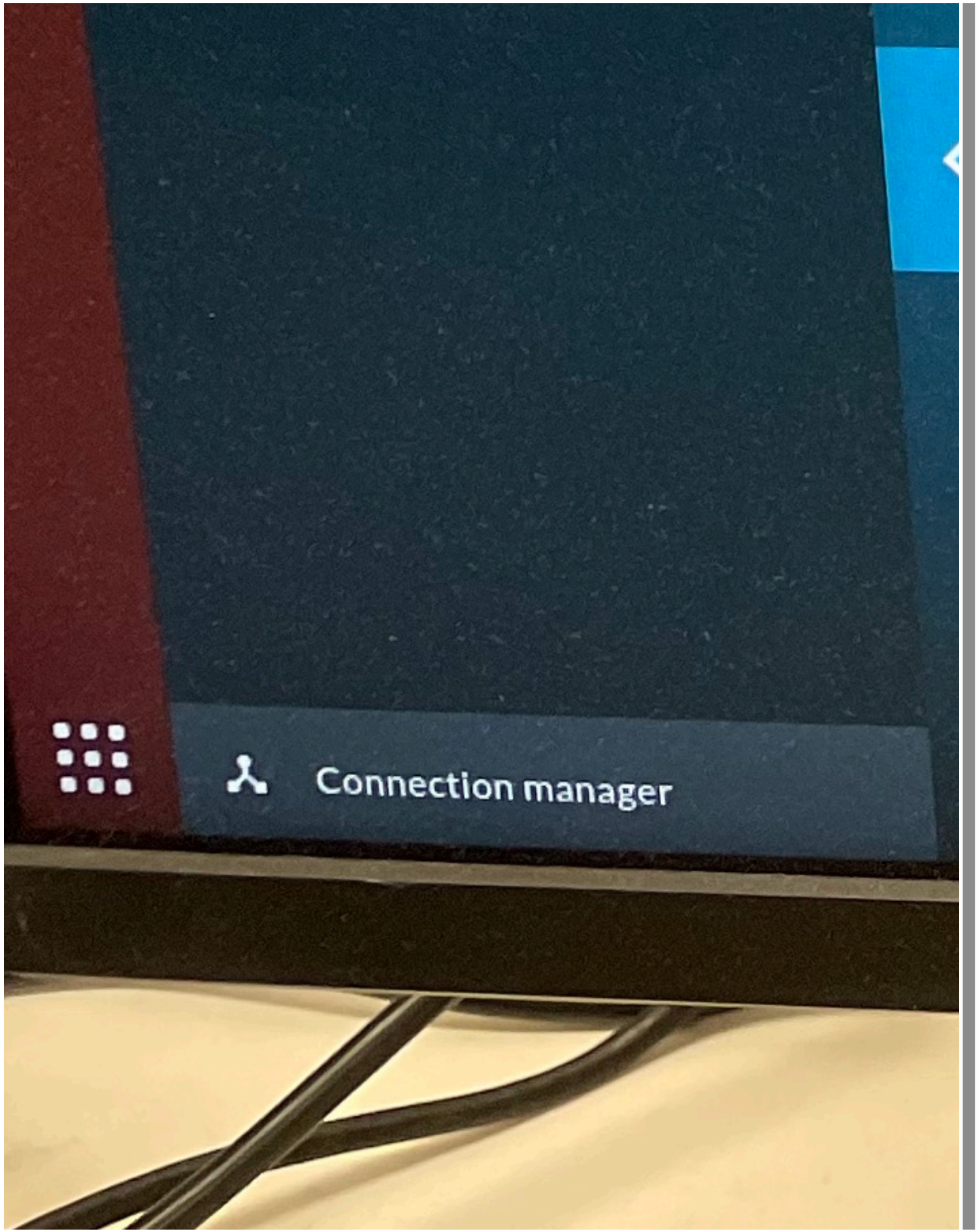


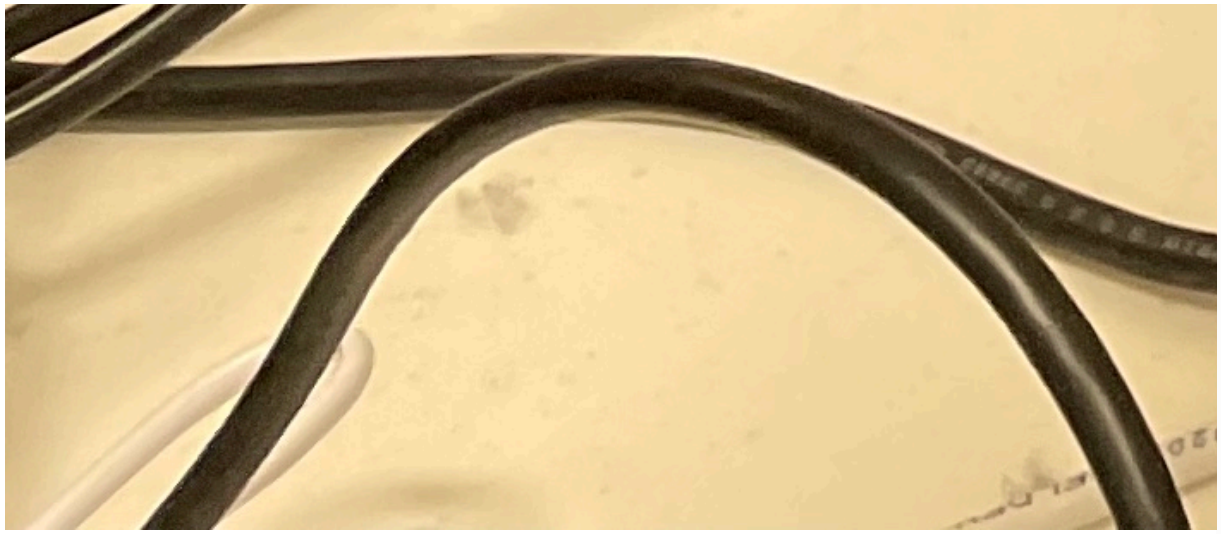
This looks quite different from the more homogeneous channel state that we expect to see on flow cells (here shown a reasonably good channel state image; I think this was prior to loading anything on the flow cell, which is why t total read count is so low):

Dashboard
Getting started
Posts
Groups
Nanopore Learning
Documentation
Highlights
Updates
Notifications
Profile




Experiments
System messages
Host settings
Positio
MN34


Connection manager



Unfortunately, the wildflower pattern isn't recognised as bad in the initial flow cell QC check (the QC message for t first flow cell was "Flow Cell FAT13376 has 1059 pores available for sequencing"), and it's only once we st sequencing that we notice this bad patterning.

I haven't yet worked out *why* this is happening, but it's not to do with sample prep. In the past, we have seen the sar effect from flow cells when there's nothing loaded on them, and also prior to loading any priming mix (i.e. when doi a 5 minute sequencing run before doing any priming). This does mean that we can do an additional channel state C prior to priming to identify these cells... but we need to remember to do that.

It also doesn't seem to be related to storage time (we've had this happen on flow cells that were checked the day af they arrived) or shipping conditions (about a year ago we had two flow cells that had a long stopover in Australia, o of which exhibited this problem, one which didn't).

It has been challenging discussing this issue over email with ONT in the past, because every support email seems end up with a different person. They aren't generally aware of this problem, so almost always fall back on it being sample loading issue (which is certainly not the case in situations where we haven't done priming or loading). Tl means it can be a challenge getting replacements for these flow cells: they're showing fine on the initial count-bas QC, so don't count as poor quality as part of the standard "fewer questions asked" warranty replacement.

This does seem to be more of an issue currently with R9.4.1 flow cells, but I thought I'd do a more full writeup just case it ends up as an issue with R10.4.1 flow cells later on.

Has anyone else seen this in their flow cells? Can anyone offer an explanation about what might be going on?

31 REPLIES

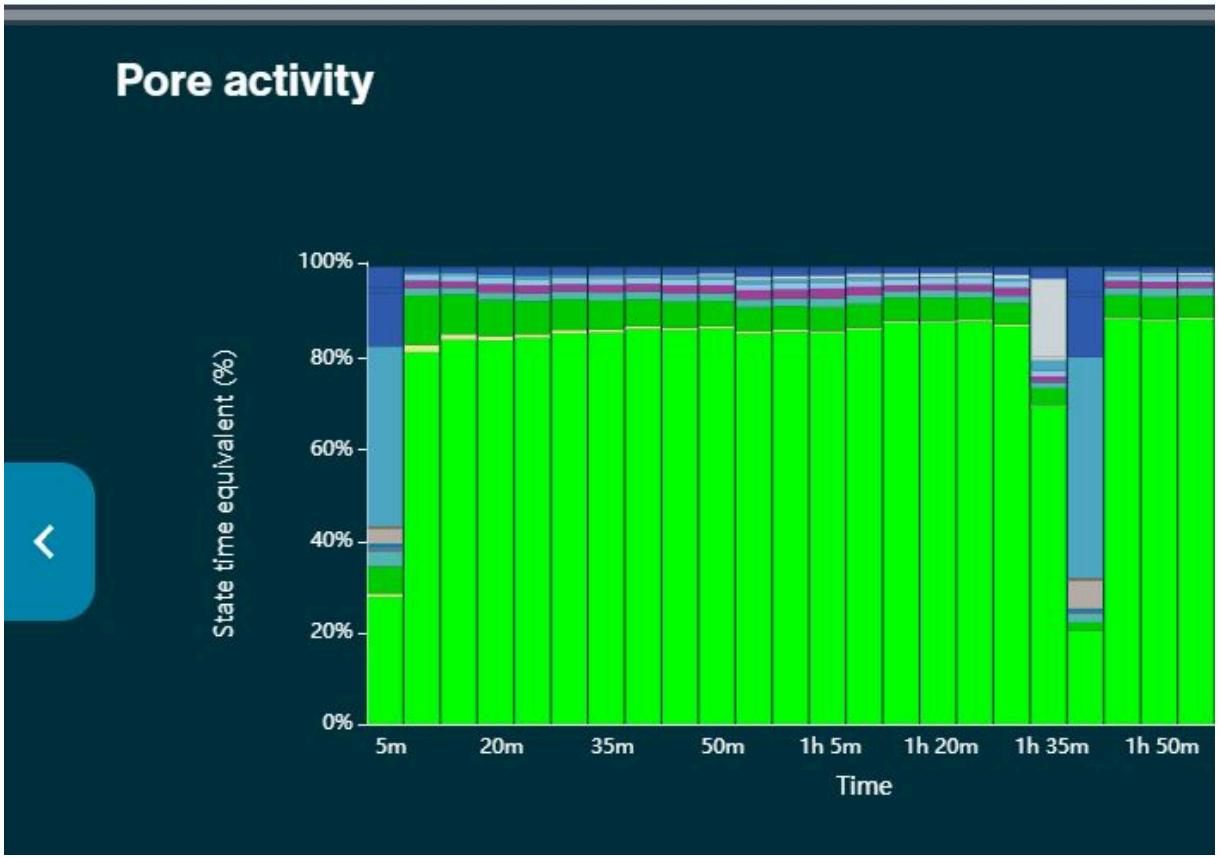
I used to notice something similar with Flongles. It got to the point where I could look at the actual flow cell and, based on the way the light bounced off the pore area, I could guess if it would be a mediocre one.

The good flow cells seem to have a high number of pores at initial check (~1600) whereas flow cells just above the warranty tend to fall off quickly. I saw it with flongles too, those with >80 pores were great, those starting around ~50 died quickly. It seems to be a quality control thing.

Alexandra J. Weisberg | 2 years ago | Kudos | Reply

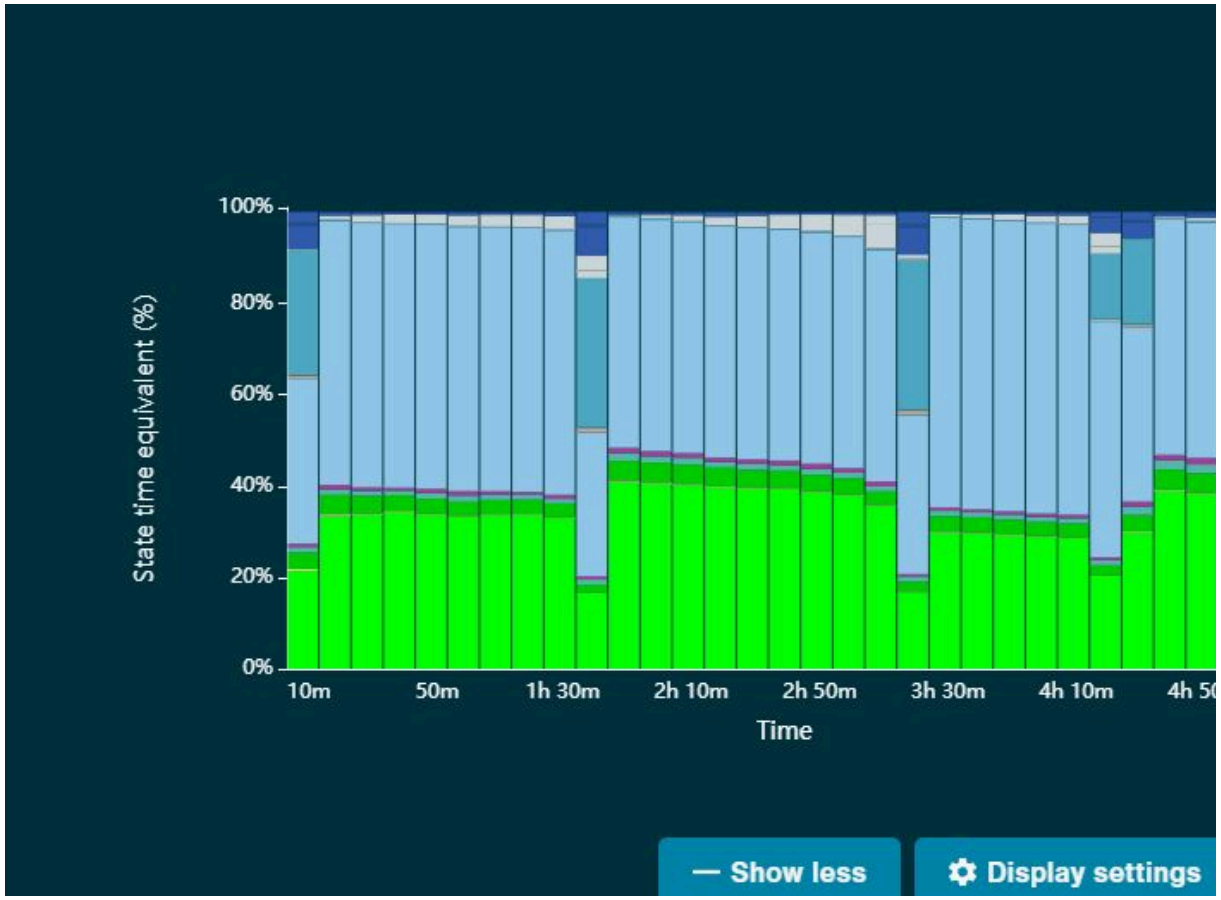
Hi @David Eccles , I cannot recall if the "wildflower" pattern was visible in our flow cells, but we do recently experience unexpected rapid decline in pore numbers in R10.4.1 flow cells when using these for amplicon sequencing. So far we have seen this with several amplicon libraries with fragment lengths between 250-500bp, and not with whole genome sequencing. A nuclease flush cannot recover these pores. I will share a rather puzzling case here:

Position —	Flow cell ID —	Sample ID —	Health	Run time
MC-112940	FAW03601	1_91	—	2 h 13 m



Very nice run, but it stopped with an error after 2h13 minutes. We restarted the Mk1C, and started the run again (same flow cell with the library still loaded). In our case the available pores started dropping even faster then in your example, see this image:

We though there could be some blockage, but it does not seem physical pore blockage by the DNA has anything to do with this, since the same library on the same flow cell showed again different behaviour after a nuclease flush, see below (again, same flowcell, same library, only difference is nuclease flush in between)



This makes me think this is most likely some software issue, or, less plausible, that these flow cells somehow have large set of poor pores, and that after the flush+reload only the good, sturdy pores are left. Curious to learn what your take is on this.

Reindert Nijland | 2 years ago | Kudos | Reply

How old are your flow cells? We have noticed (on R10.4 though) that after a couple of months, pores decay much more rapidly even if the initial pore scan shows >1500 pores.

Another reason may be your DNA fragment size. It was pointed out to us that shorter DNA fragments seem to be degrading the pores quicker because there is more docking on and off the pore than there would be with long fragments. We are also sequencing very short cfDNA fragments (N50 is 200bp) which is similar to your amplicons and we are seeing this, particularly with older flow cells.

Thomas Erblich | 2 years ago | Kudos | Reply

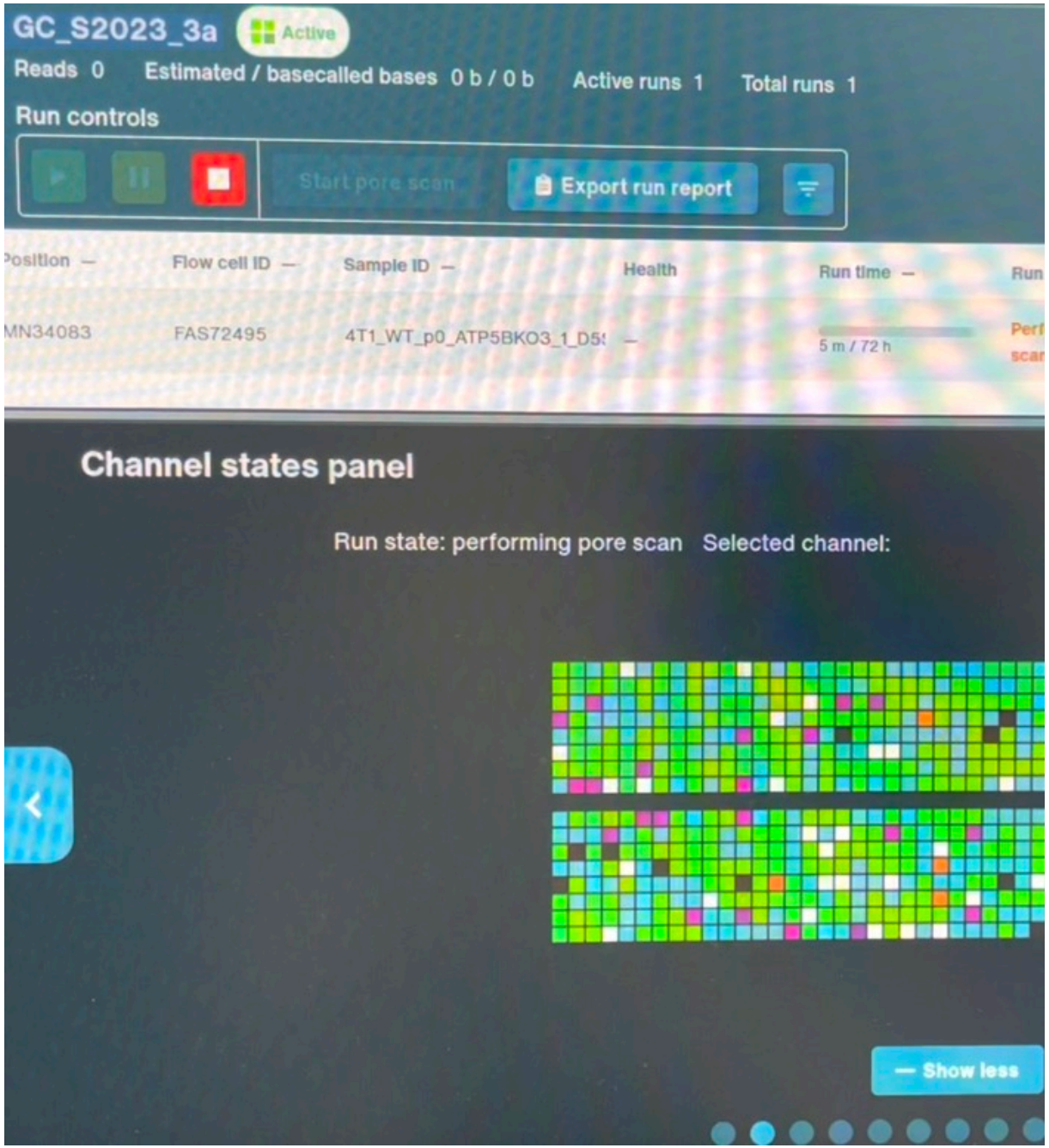
> As I mentioned in my post, this has happened with flow cells that are a couple of days old (post shipment), so I don't think it has much to do with age at our end. It also happens on unloaded, unprimed flow cells, so there's no way

sample prep has anything to do with the patterning.

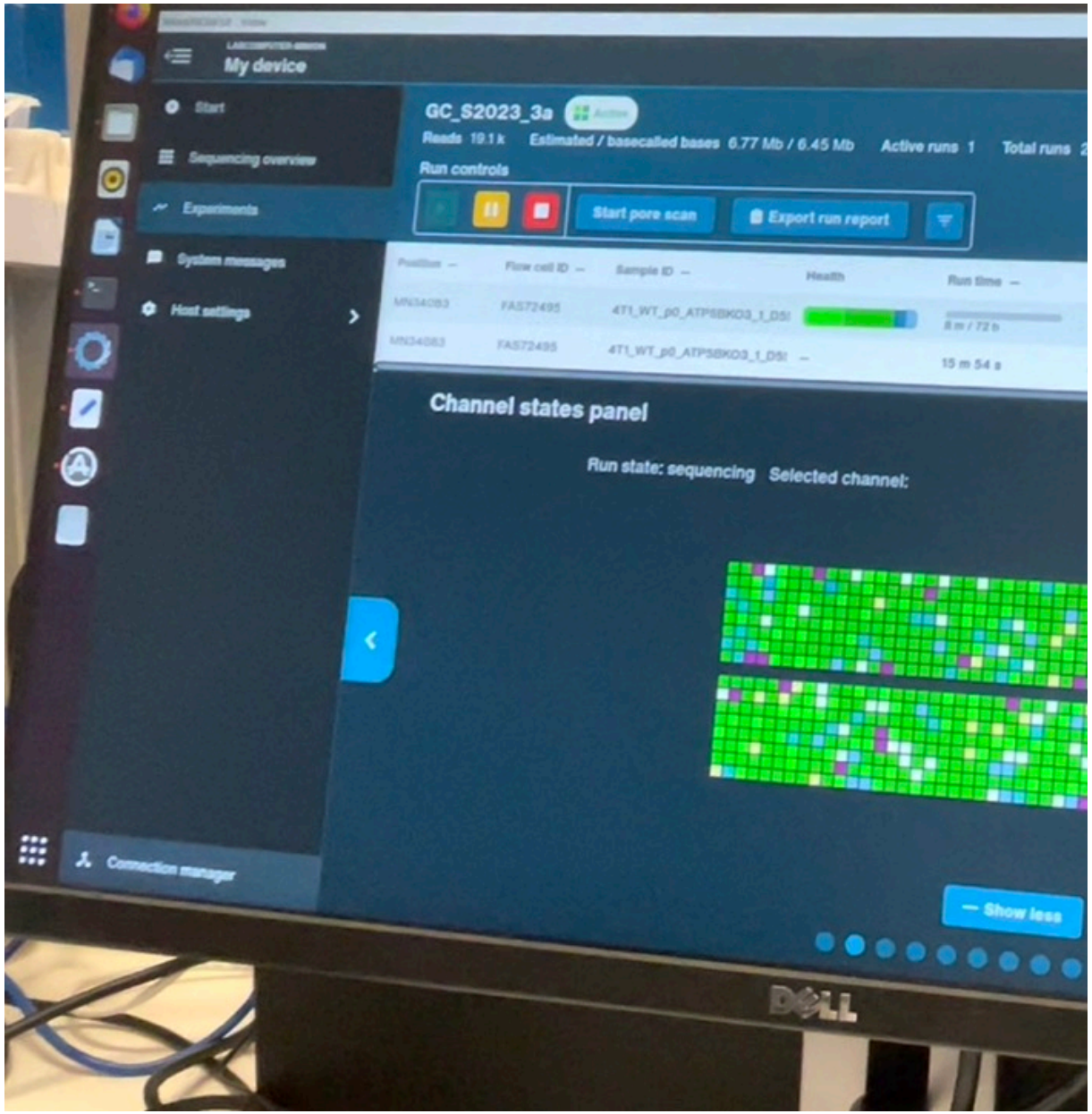
It could have something to do with storage time on ONT's side of things, but there's no way for us to know that.

David Eccles | 2 years ago | AUTHOR | Kudos

Got another one today. Here's a screenshot of what the flow cell looked like during QC before anything was loaded on it (QC was 1267 pores):

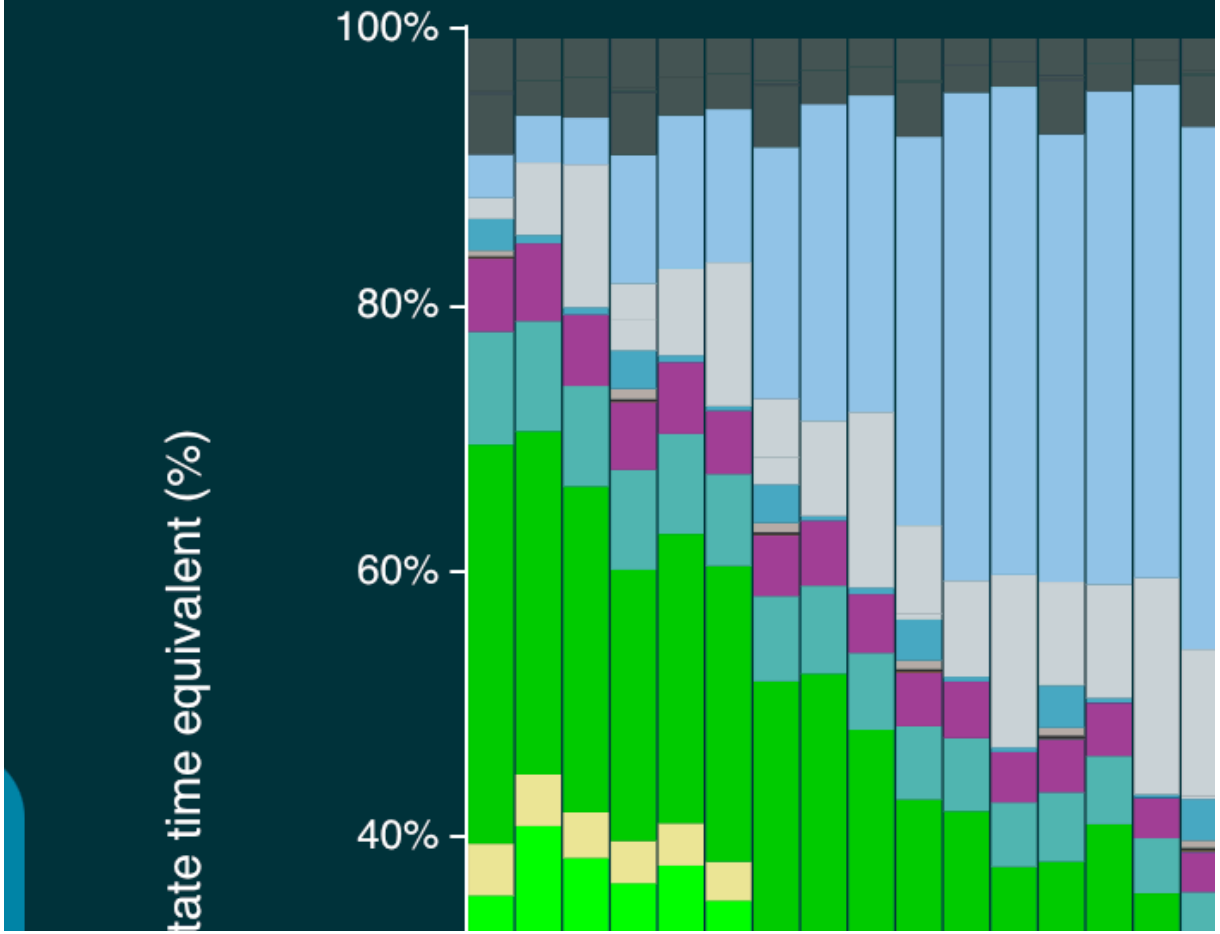


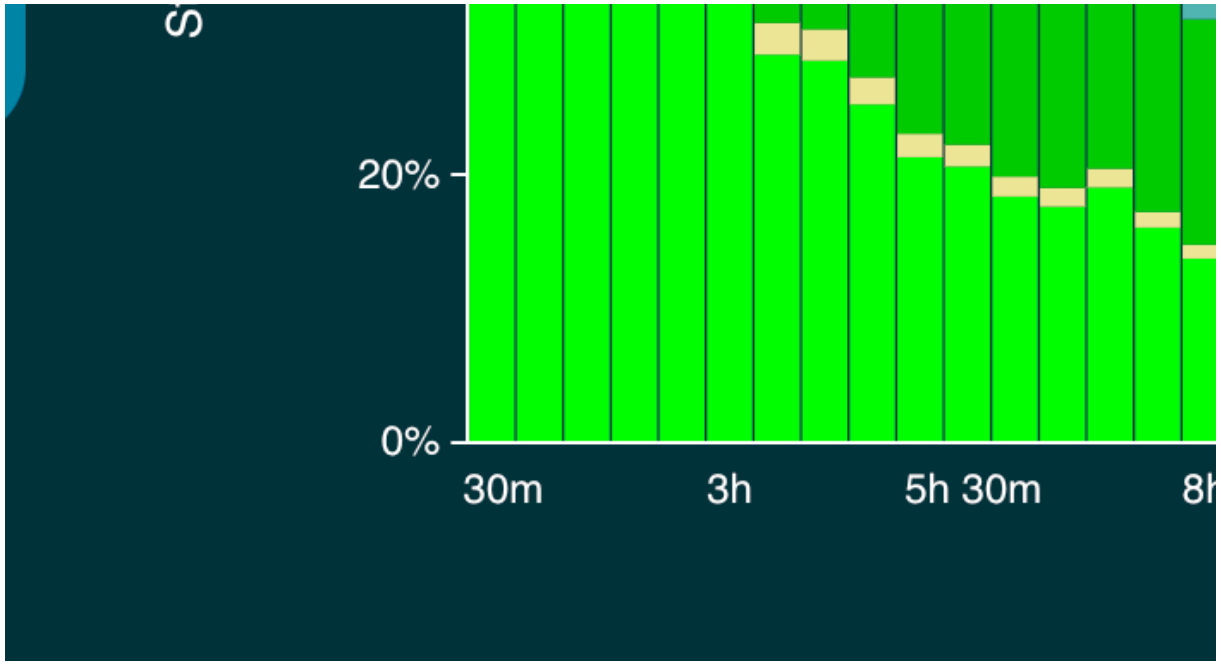
And here's what it looked like just after the library was loaded:



Here's the Pore activity plot from the run (the bit at the right hand side is from pausing and waiting to reload):

Pore activity

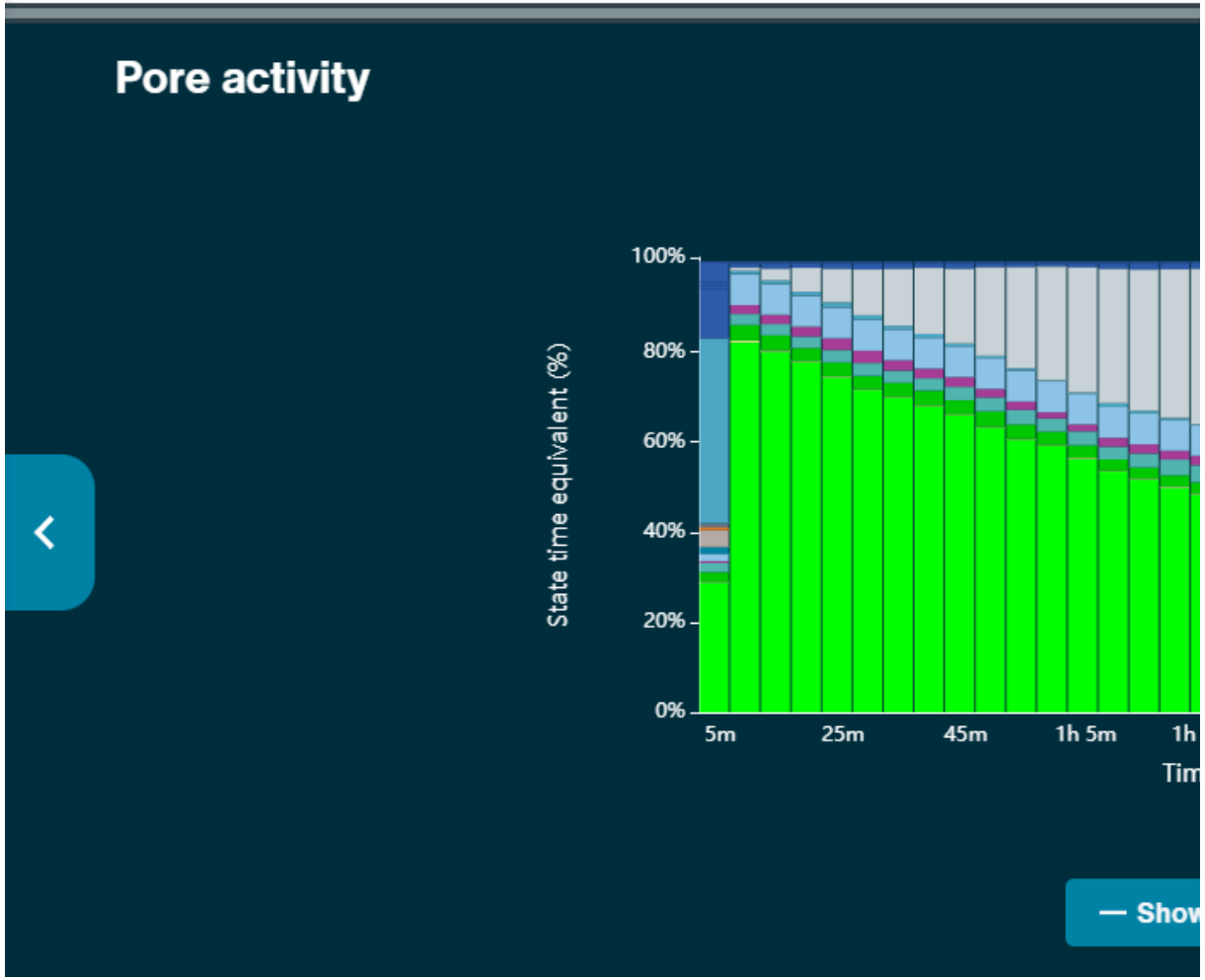




David Eccles | 2 years ago | AUTHOR | Kudos | Reply

We are experiencing the same issue as @Reindert Nijlande. We are sequencing amplicons of arround 400bp in a R10.4.1 flow cell with the 260bps mode in a MK1C. As you can see in the following pictures the number of Channel disabled increased rapidly. The flow cell had almost no active pores before 4-5h and we had to use a new one to obtain enough data.

Position	Flow cell ID	Sample ID	Health	Run time
MC-112257	FAW78930	no_sample		2.6 h / 100



PORE ACTIVITY

The Pore activity graph shows the performance of your sample as it is being sequenced during a r

Legend

Sequencing
Pore currently sequencing

Adapter
Pore currently sequencing adapter

Pore available
Pore available for sequencing

Unavailable
Pore unavailable for sequencing

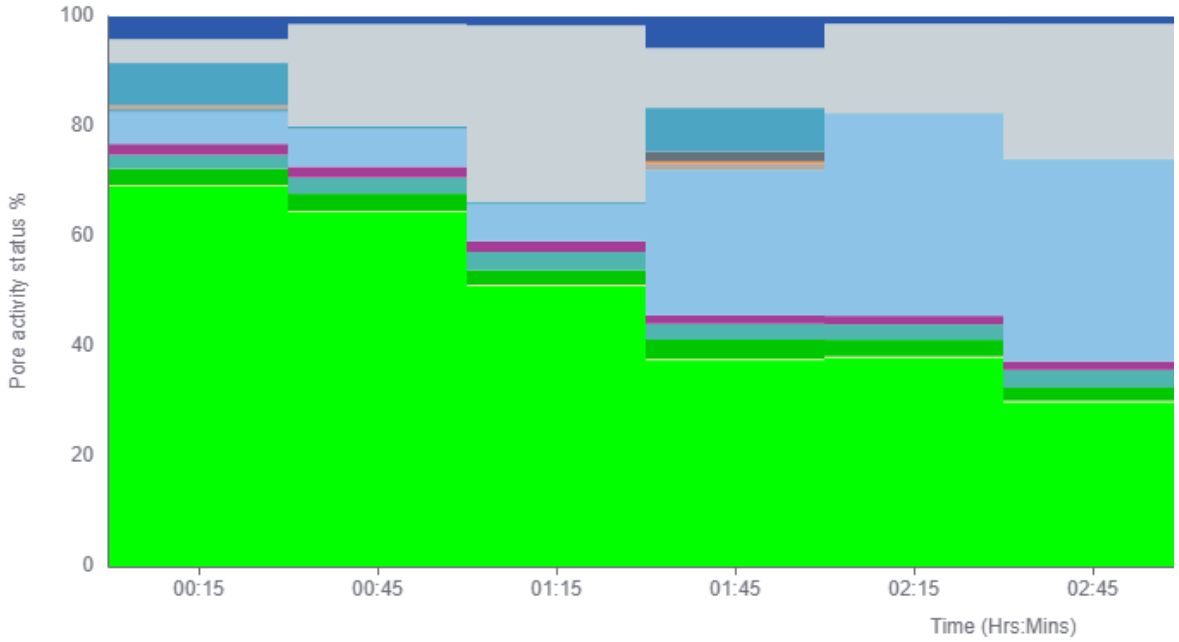
Out of range-high
Current is positive but unavailable for sequencing

Out of range-low
Current is negative but unavailable for sequencing

Multiple
Multiple pores detected. Unavailable for sequencing.

Saturated
The channel has switched off as current levels exceed hardware limitations

Unclassified
Pore status unknown



A very similar issue happend with the R9.4.1 which was solved with a software update. We have contacted with support explaining the issue.

Albert Carcereny | 2 years ago | Kudos | Reply

> "Channel disabled" is clearly a software problem. It'd be good to have some more clarity from ONT as to *why* the channels are being disabled until the next pore scan.
>
> David Eccles | 2 years ago | AUTHOR | Kudos

That's good to know,

@David Eccles I have a question related to this ***"In the past, we have seen the same effect from flow cells when there's nothing loaded on them, and also prior to loading any priming mix"***

How do you do that? "Just connect the flow cell and start running without anything there?" and what do you expect to get as results from this?

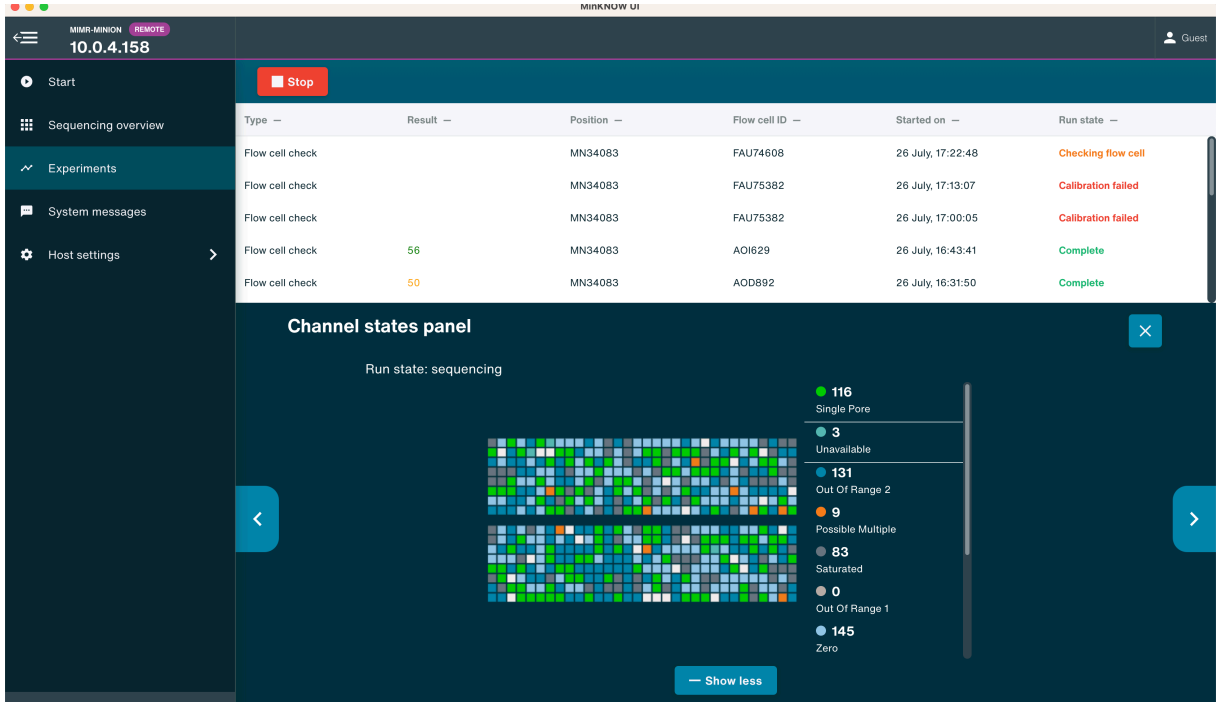
thank you,

Amro Hashish | 2 years ago | Kudos | Reply

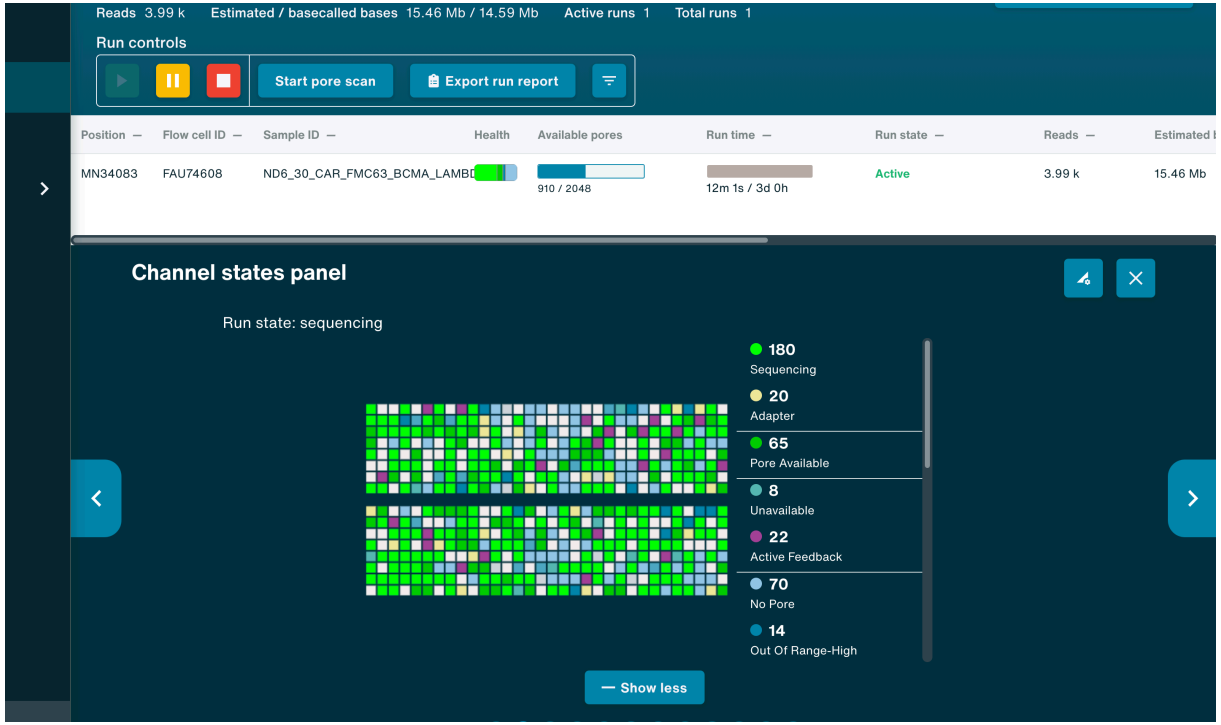
> Yes, start a sequencing run prior to loading anything, and take a screenshot of the channel states panel in "Show More" mode after about 5 minutes of sequencing post-QC.
>
> If it's a good flow cell, there should be a field of almost entirely green. If it's a wildflower flow cell, there will be a random scattering of lots of different pore states.
>
> The main purpose of doing this is to make it really obvious *to ONT support* that the problem has nothing to do with sample prep or loading, almost entirety excluding user error as a factor.
>
> David Eccles | 2 years ago | AUTHOR | Kudos

> That's very interesting, Thank you Very much, @David Eccles
>
> Amro Hashish | 2 years ago | Kudos

Had another one today, this time on an R10.4.1 flow cell. Here's what the flow cell looked like during flow cell QC:



And here it is after a few minutes of sequencing:



The MinKNOW flow cell QC reported 910 pores, with 442 available for immediate sequencing, but after the run began, the available pore count is closer to 300 (including the active feedback pores).

David Eccles | 2 years ago | AUTHOR | Kudos | Reply

> We expected that this issue would have been fixed with the R10.4.1. However, we have come across several flow cells still displaying this pattern. Fortunately, for the time being, it seems to be confined to a specific LOT that we had.
>
> Albert Carcereny | 2 years ago | Kudos

> Thanks for sharing this - I've been having similar problems and suspected it was my gDNA (from maize leaf tissue). I'm sure it might still be something to do with the DNA but I'd like to try this pre-loading check.
> Can you clarify, are you starting a run with an untouched flow-cell that still contains the storage buffer, or are you doing all the priming and starting a short check just before the library is loaded?
>
> Andrew read | 2 years ago | Kudos

> My colleague did the flow cell check prior to loading anything (and my first screenshot is from my remote monitoring of that flow cell check). The second screenshot is after loading a sample onto the flow cell.
>
> It would be helpful if ONT could create a mini report of the flow cell checks showing the full classifications for each sequencing well when the flow cell voltages go down to around -200 mV.
>
> The second screenshot looks better (but still pretty bad) because it combines the best available wells from all sequencing channels, whereas the first one is a single MUX selection across all channels.
>
> David Eccles | 2 years ago | AUTHOR | Kudos

Hi everyone,

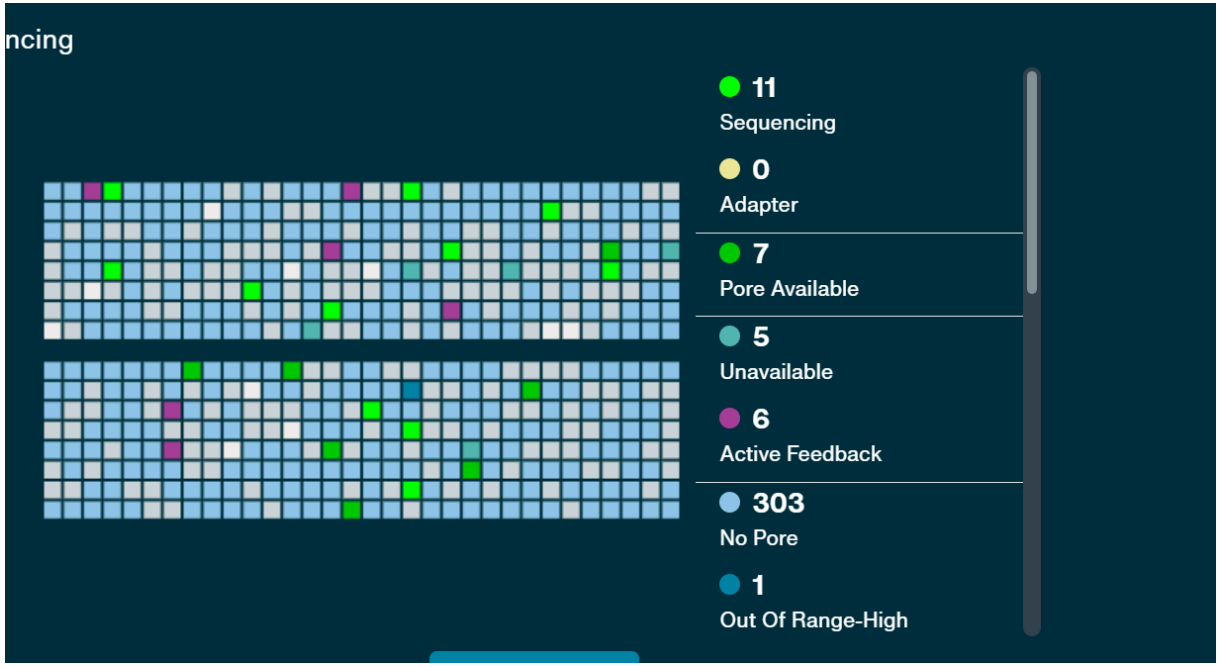
I just experienced the worst loss of pores I've ever had.

The only change I made is the input DNA was isolated from FFPE tissue. I used the SQK-LSK114 Kit and protocol including the FFPE repair mix and buffer. As per protocol there are two cleaning steps with beads before the library is ready to load, so I did not expect FFPE contaminants.

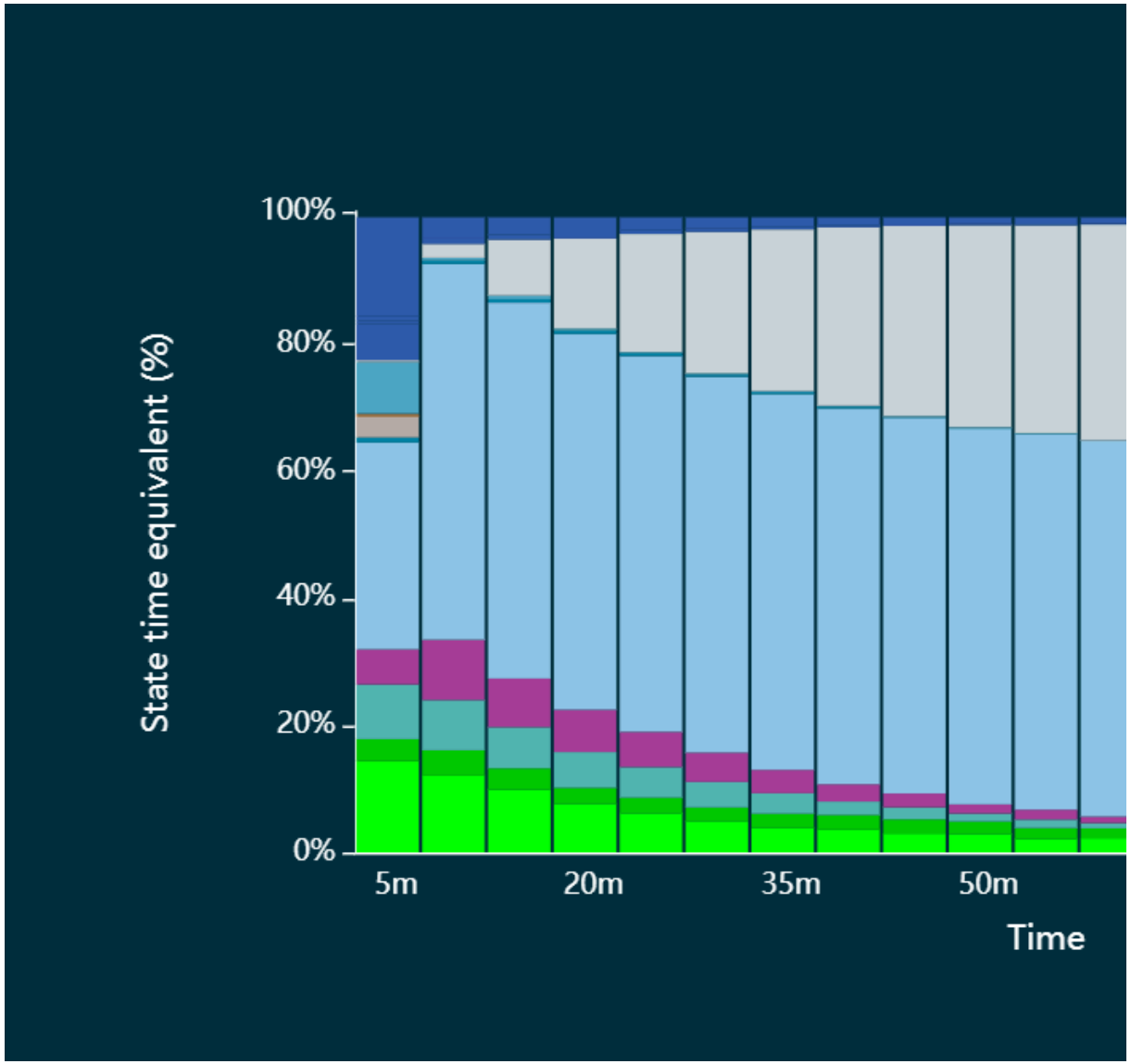
I used a R 10.4.1 flow-cell. The flow cell scan showed 1545 available pores.

After loading only 60 pores started to sequence, without a spatial pattern (I don't think there were air bubbles, the array looked fine and the pattern is not an air-bubble-pattern).

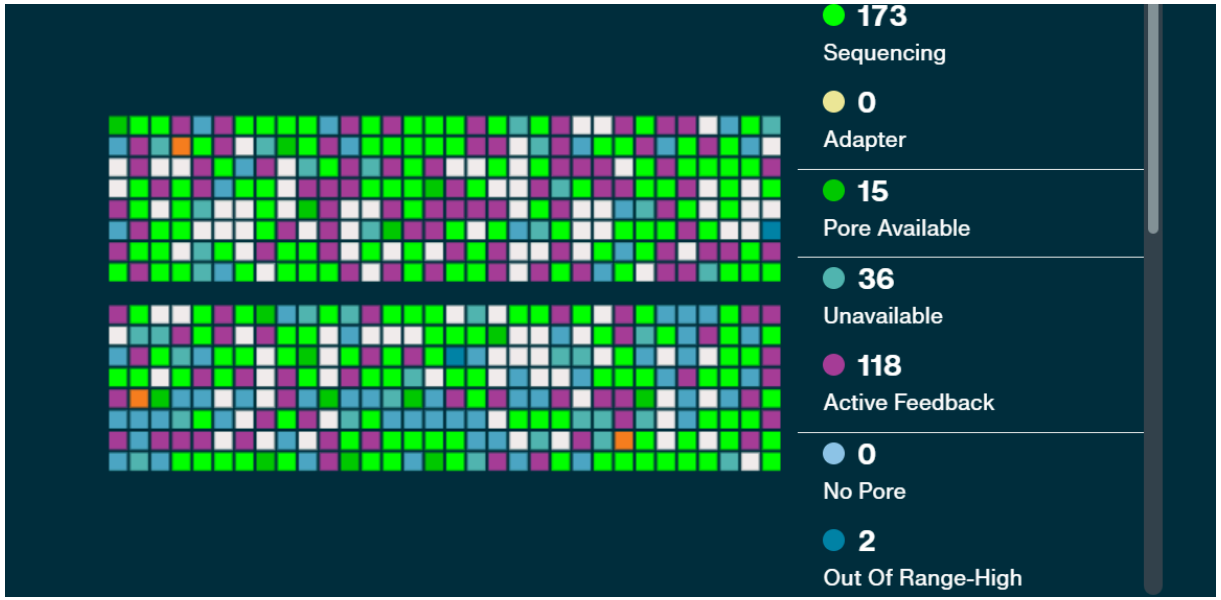
That is after about an hour of sequencing:

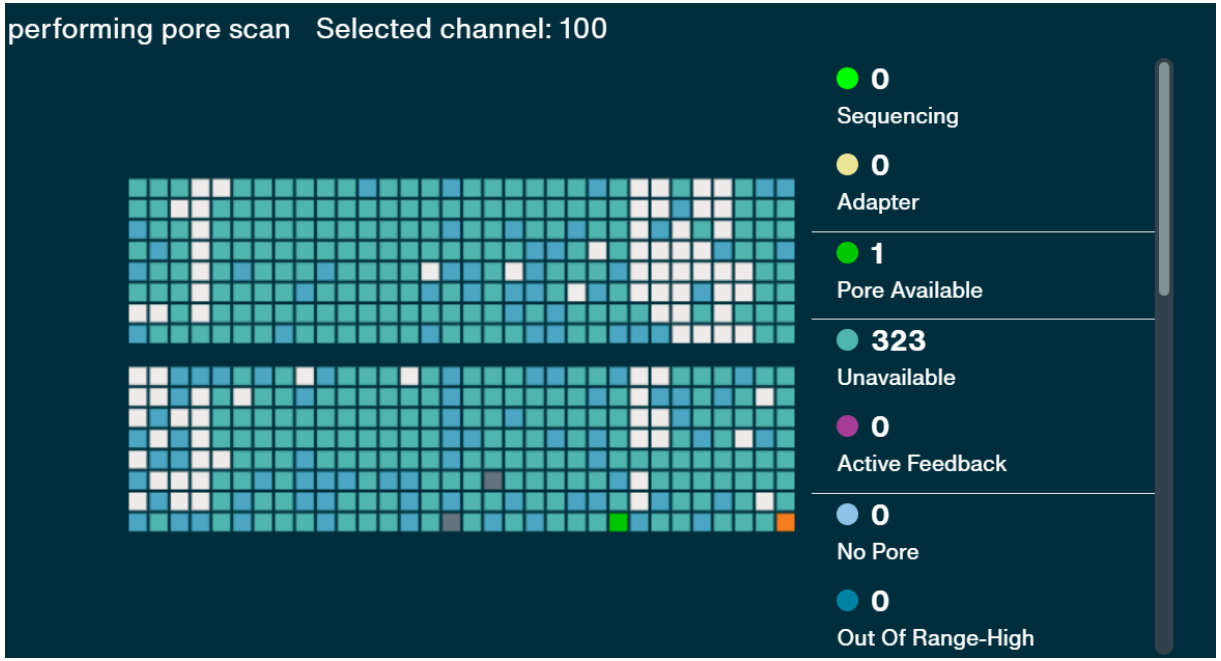


Interestingly, after the first mux scan the number of sequencing pores increased a bit:



And this is how it looks during the scan:





@David Eccles do you think it's the wild-flower pattern again?

@everybody

Any suggestions how to screen for relevant contaminants?

I have about 10fmol library left, I'm thinking of either using it on another flow cell asuming that it's a flow-cell problem; or using it (4,5µL) to check for contaminants...

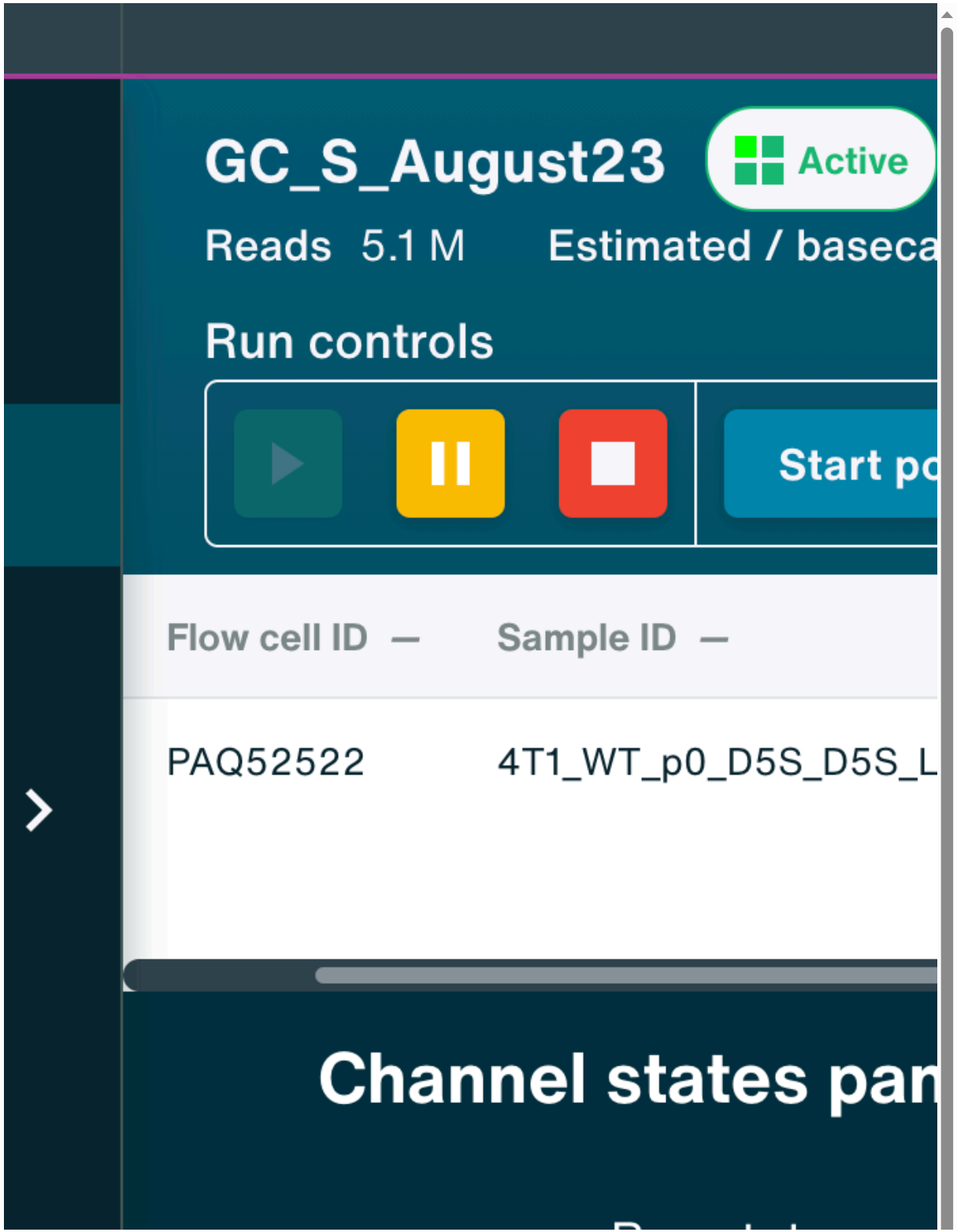
Natalia Stepien | 2 years ago | Kudos | Reply

> do you think it's the wild-flower pattern again?

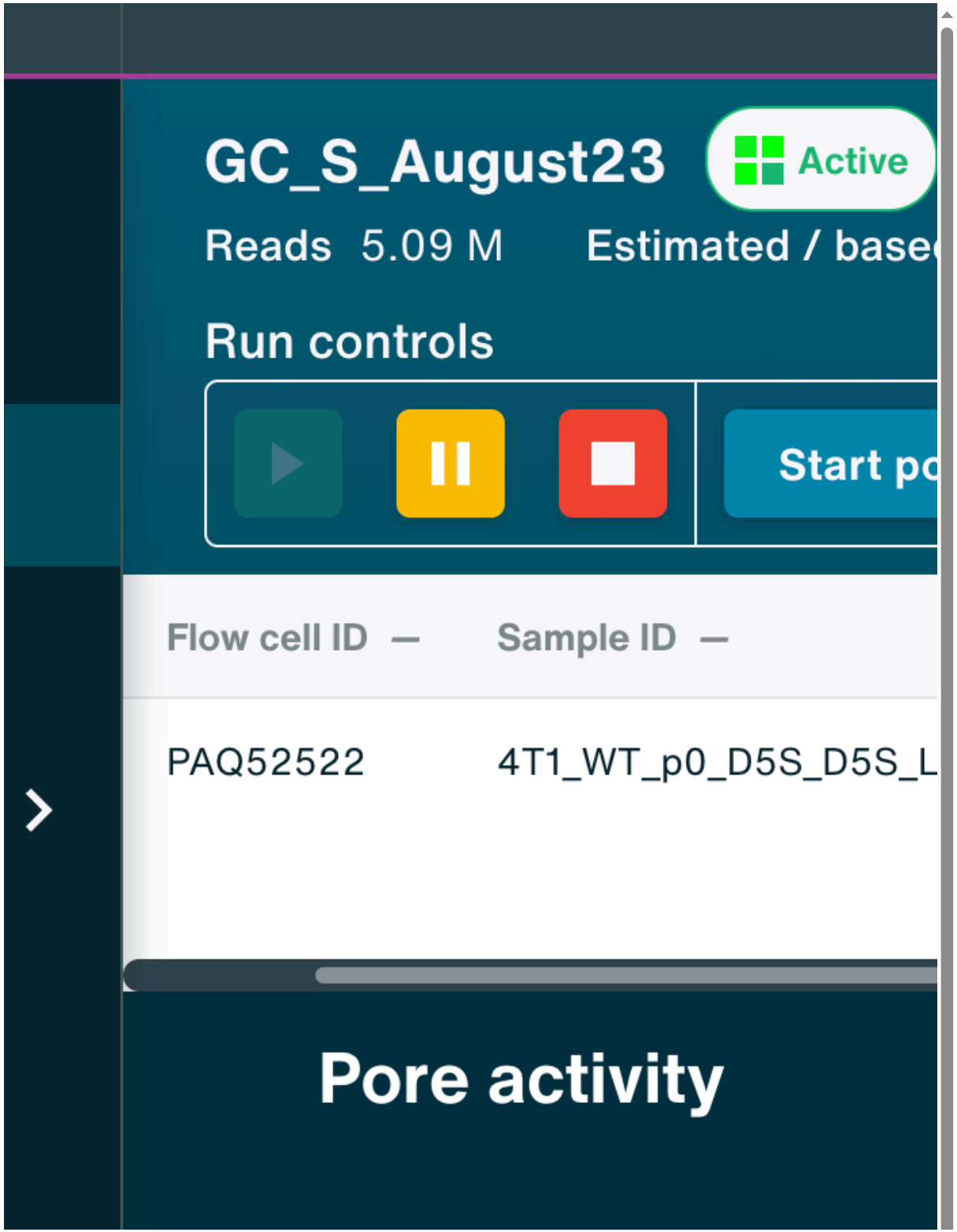
Yes

David Eccles | 2 years ago | AUTHOR | Kudos

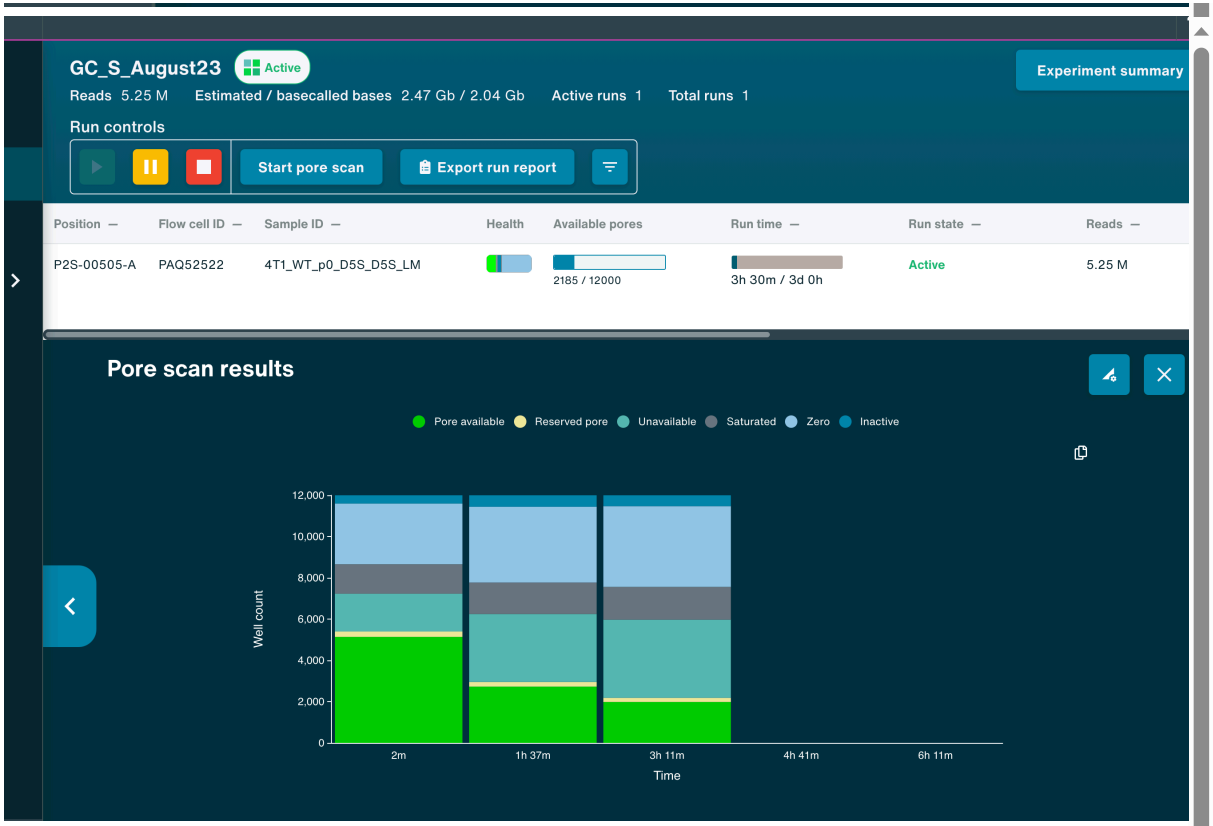
Here's what it looks like on a PromethION R10.4.1 flow cell. This flow cell reported 7195 at QC, which dropped down 5397 pores [2424 available] after sample loading. After 3.5 hours of sequencing, it's down to 2185 [1430 available].


GC_S_August23
Active
Reads 5.1 M
Estimated / baseca
Run controls
Start po
Flow cell ID —
Sample ID —
PAQ52522
4T1_WT_p0_D5S_D5S_L
Channel states pan

Run state: seq


GC_S_August23
Active
Reads 5.09 M
Estimated / base
Run controls
Start po
Flow cell ID —
Sample ID —
PAQ52522
4T1_WT_p0_D5S_D5S_L
Pore activity

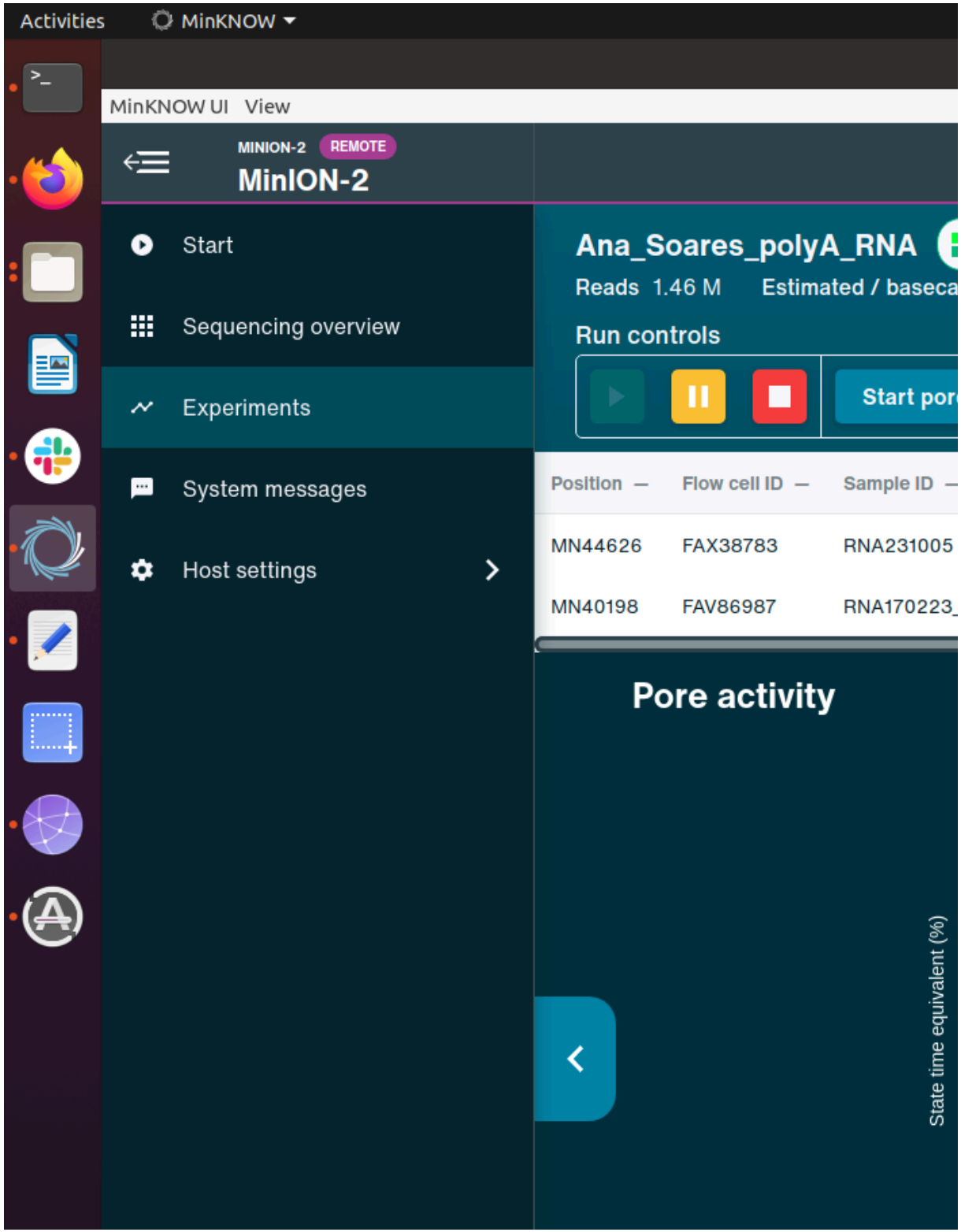
State time equivalent (%)
100%
80%
60%
40%
20%
0%



David Eccles | 2 years ago | AUTHOR | Kudos | Reply

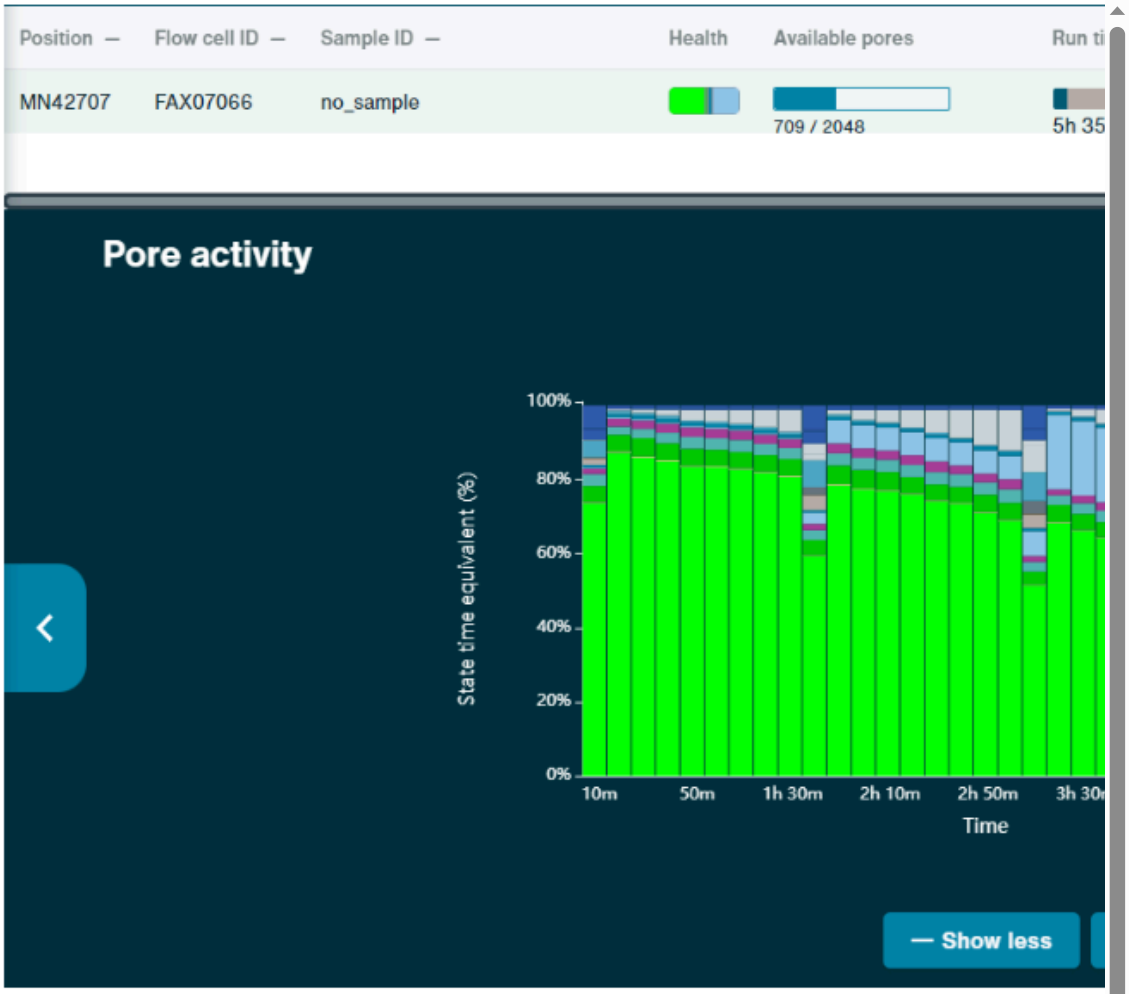
We have had the same exact thing. Twice in a month. We hadn't seen this in the past, as far as I remember.... The worst drops in pores happen with the mux scans- It seems that the pores die after the mux scan... This behaviour suggests that the issue isn't sample-related, but software or device-related. While it is nice if at least we can get flowcell replacements, it would be

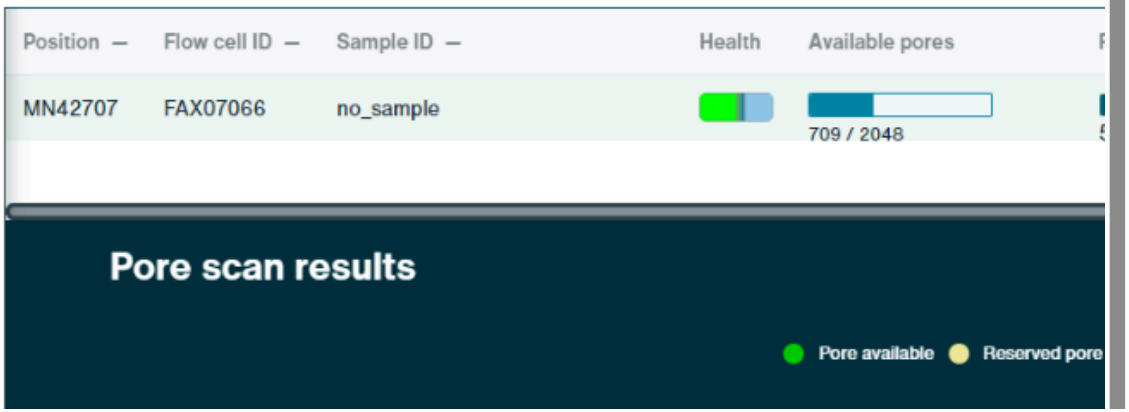
good to know the source of this problem, as the samples are not always easily replaceable...

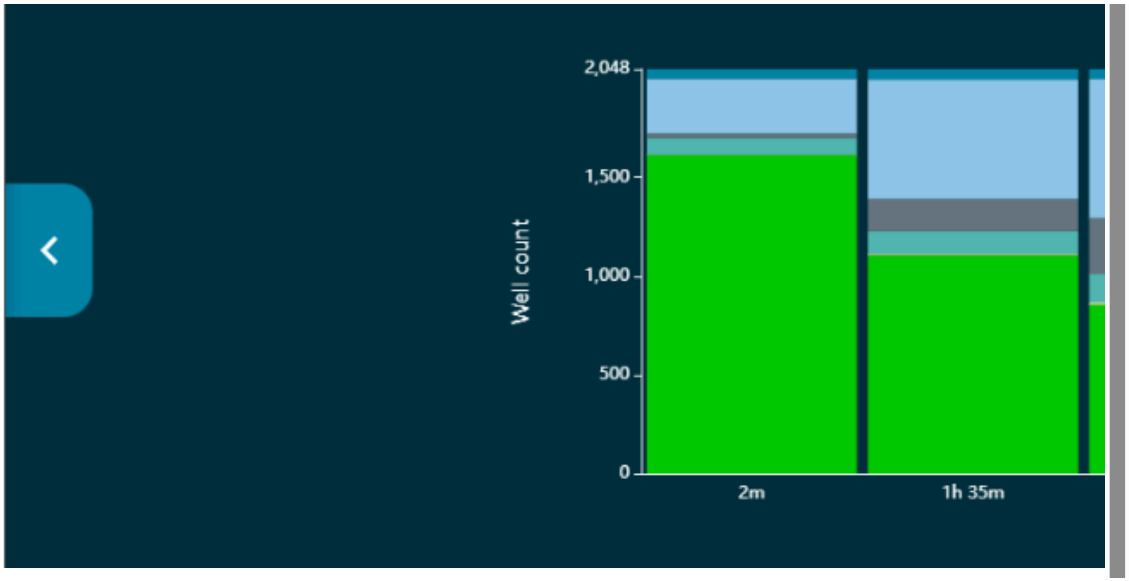

Activities
MinKNOW
MinKNOW UI View
MINION-2
REMOTE
MinION-2
Start
Sequencing overview
Experiments
System messages
Host settings
Ana_Soares_polyA_RNA
Reads 1.46 M
Estimated / baseca
Run controls
Start por
Position
Flow cell ID
Sample ID
MN44626
FAX38783
RNA231005
MN40198
FAV86987
RNA170223_
Pore activity
State time equivalent (%)

Eva Maria Novoa Pardo | 2 years ago | Kudos | Reply

We have encountered this flow cell issue intermittently. Just this week, we observed this behavior with an R10.4 flow cell.


Position
Flow cell ID
Sample ID
Health
Available pores
Run ti
MN42707
FAX07066
no_sample
709 / 2048
5h 35
Pore activity
State time equivalent (%)
100%
80%
60%
40%
20%
0%
10m
50m
1h 30m
2h 10m
2h 50m
3h 30
Time
— Show less


Position
Flow cell ID
Sample ID
Health
Available pores
MN42707
FAX07066
no_sample
709 / 2048
Pore scan results
Pore available
Reserved pore



The pattern is always the same, so there must be some software or flow cell issue that should be addressed to avo this problem. It happended with R9.4.1 and it is also happening with R10.4.1.

Thankfully, ONT already ordered a replacement.

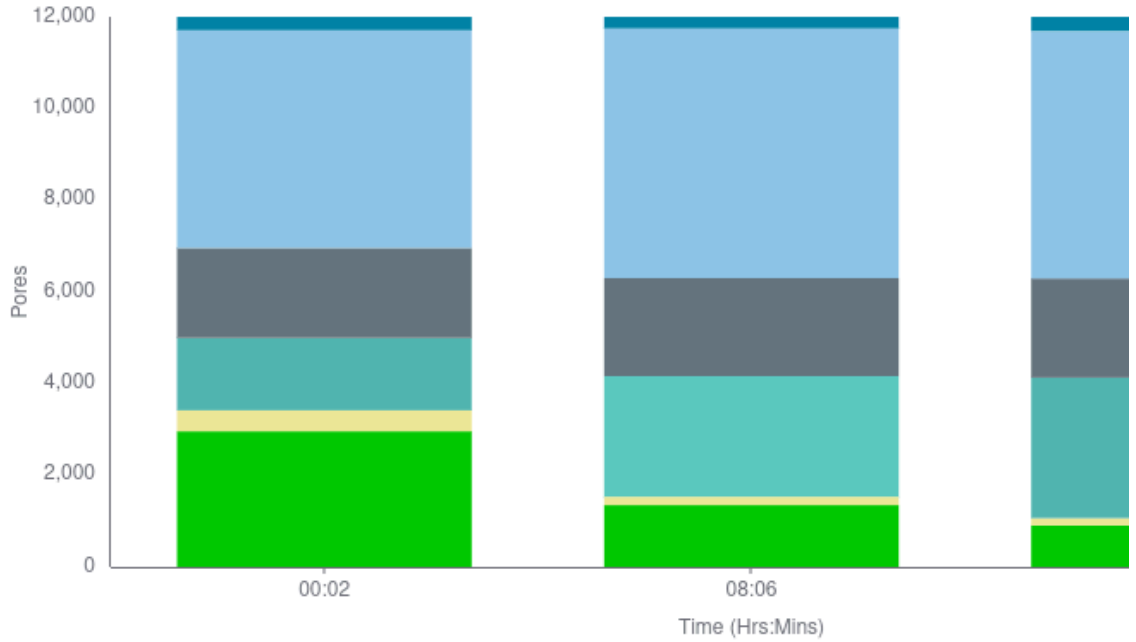
Albert Carcereny | 2 years ago | Kudos

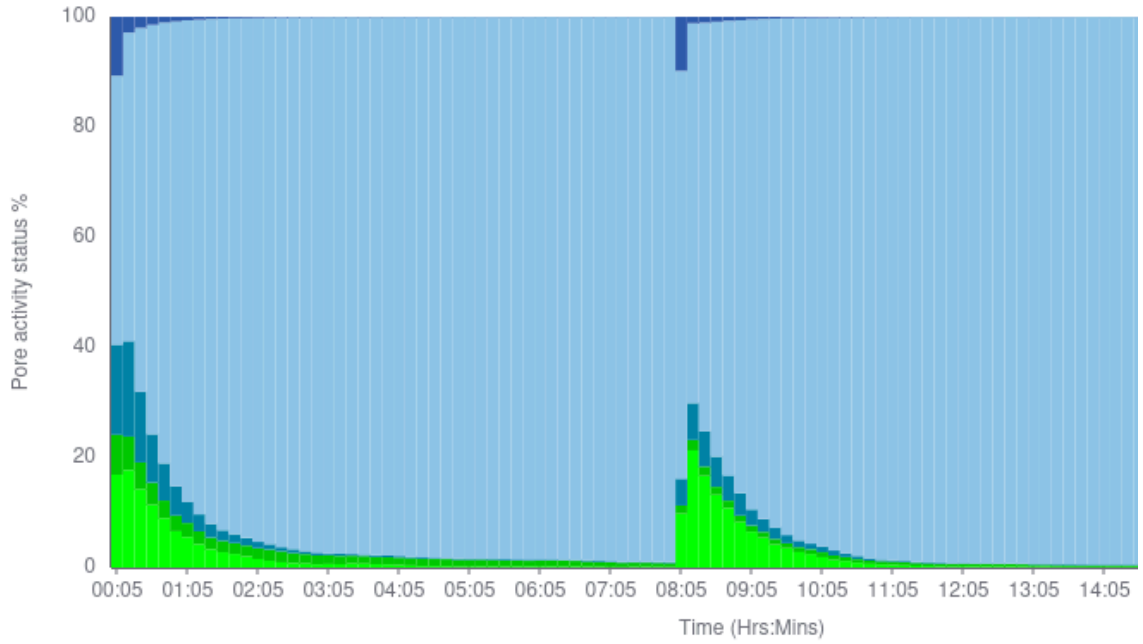
I am also seeing this in two out of the three flow cells I have used from our latest delivery. We have PromethION R10.4.1 running SQK-ULK114. Due to the long reads we have longer intervals between pore scans, which normally works well in a good flow cell.

At flow cell check there were over 7000 pores, but immediately halved at first pore scan. This was the "wildflower" pattern I saw after only 46 minutes. When I came back to see how it went over night, unsurprisingly it has not performed at all. Hoping for a replacement.

Available pores	Run time	Run state	Reads	Estimated bases	Basecalled bases	Basecalled %	Started on
3416 / 12000	46m 27s / 3d 0h	Active	12.21 k	268.18 Mb	199.04 Mb	95%	24 October, 16:16:40

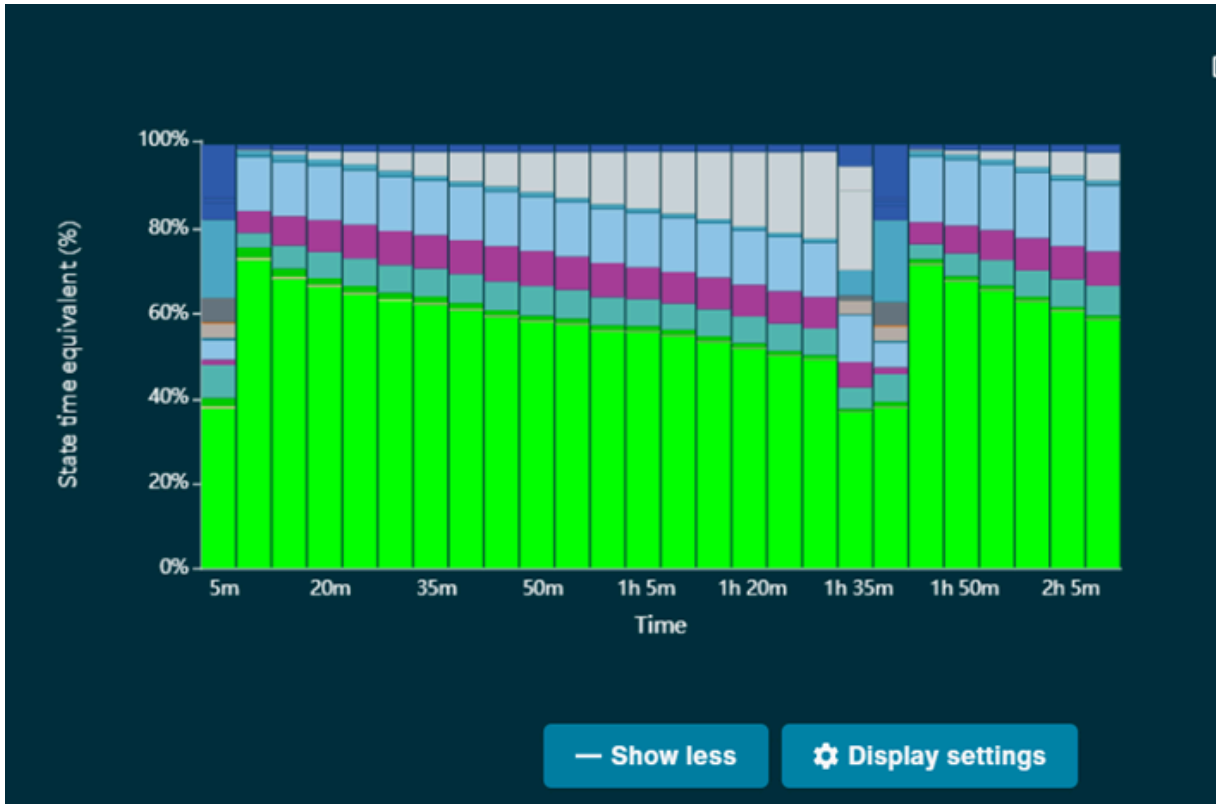
possibly due to bubbles
on the membrane



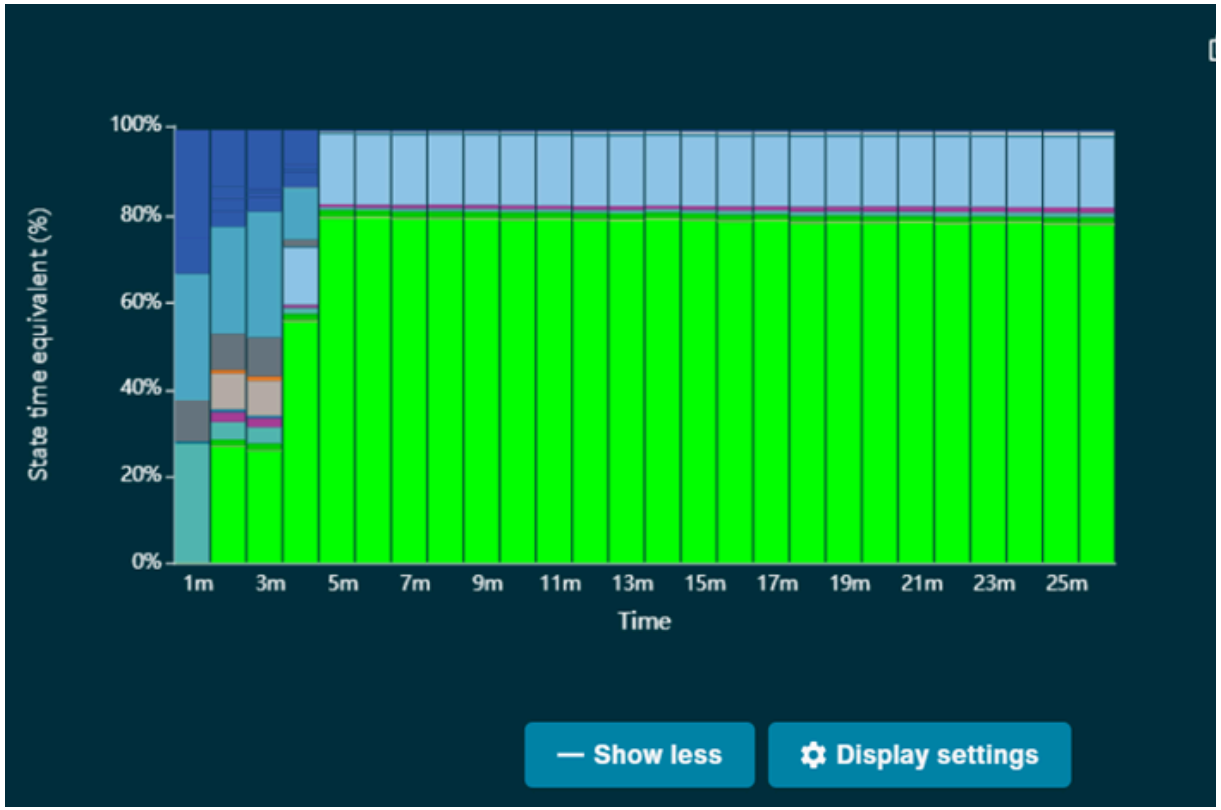


Mari Gornitzka | 2 years ago | Kudos

I have also seen this recently and it points to a software issue.



We have a P24 so I stopped this run after 2hrs and moved the flow cell to another position and restarted the run.



I didn't do any flow cell washes or reloads. Just moved it to another position on the machine. I've seen this in at least 3 flow cells recently and it seems random where it happens.

Jasmaine Lee | 2 years ago | Kudos | Reply

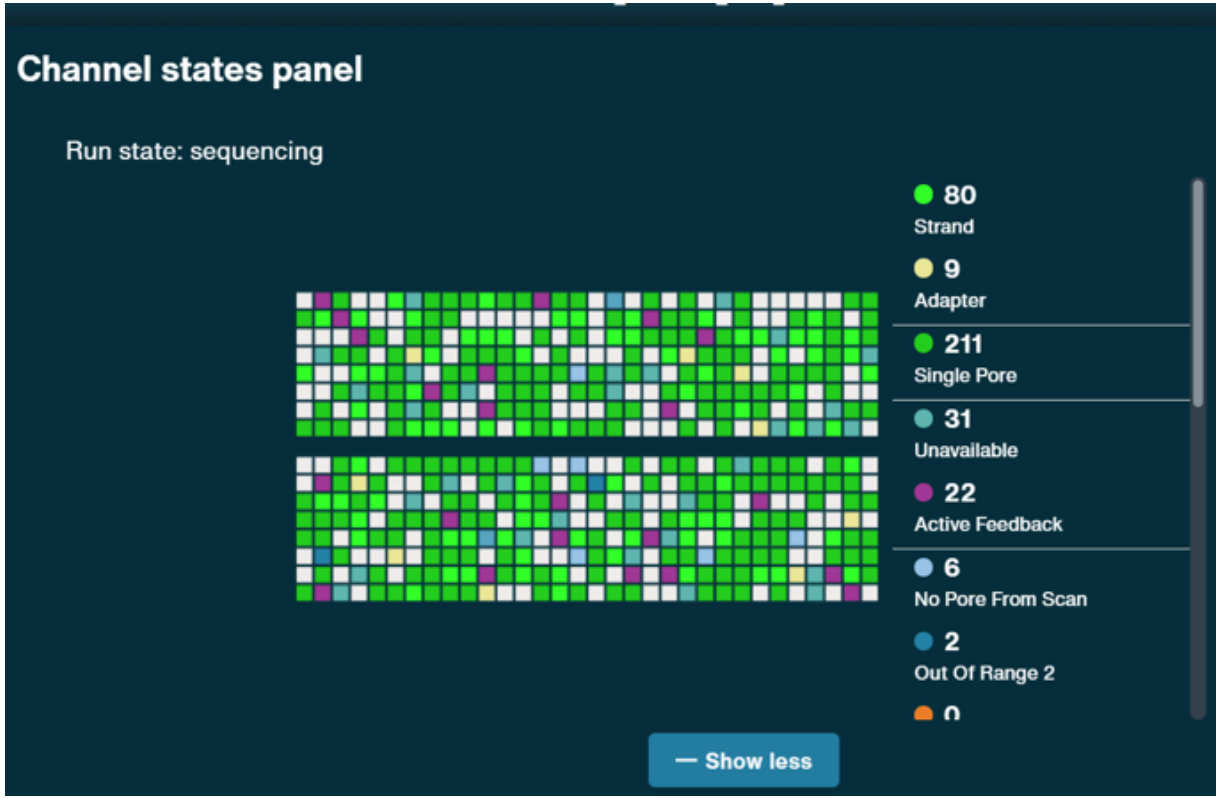
Dear David, Jasmaine and all-your comments on the "Wildflower" phenomenon have been a life-saver and I can't thank you enough.

I've been struggling with this issue since August and felt like I'm in the twilight zone. I've gone through many flow cells, not knowing what to make of it. ONT replaced 2 free of charge and said they haven't rec'd complaints from other customers.

My wildflower pattern contains a preponderance of the white petal (unclassified pores), which results in poor data accumulation (50% from prior peak performance).

Out of frustration, I tried the same experiment Jasmaine just described (prior to seeing her post). By moving my R9.4.1 flow cell to another Mk1B and restarting the run, 5 min. later the pore occupancy is normal??? I repeated this the following day with 2 Mk1Bs, one of which is new. By moving the flow cell repeatedly between devices, the pore occupancy returned to

normal. Thanks again



Kevin Pirelli | 2 years ago | Kudos | Reply

Dear @Kevin Pirelli and @Jasmaine Lee

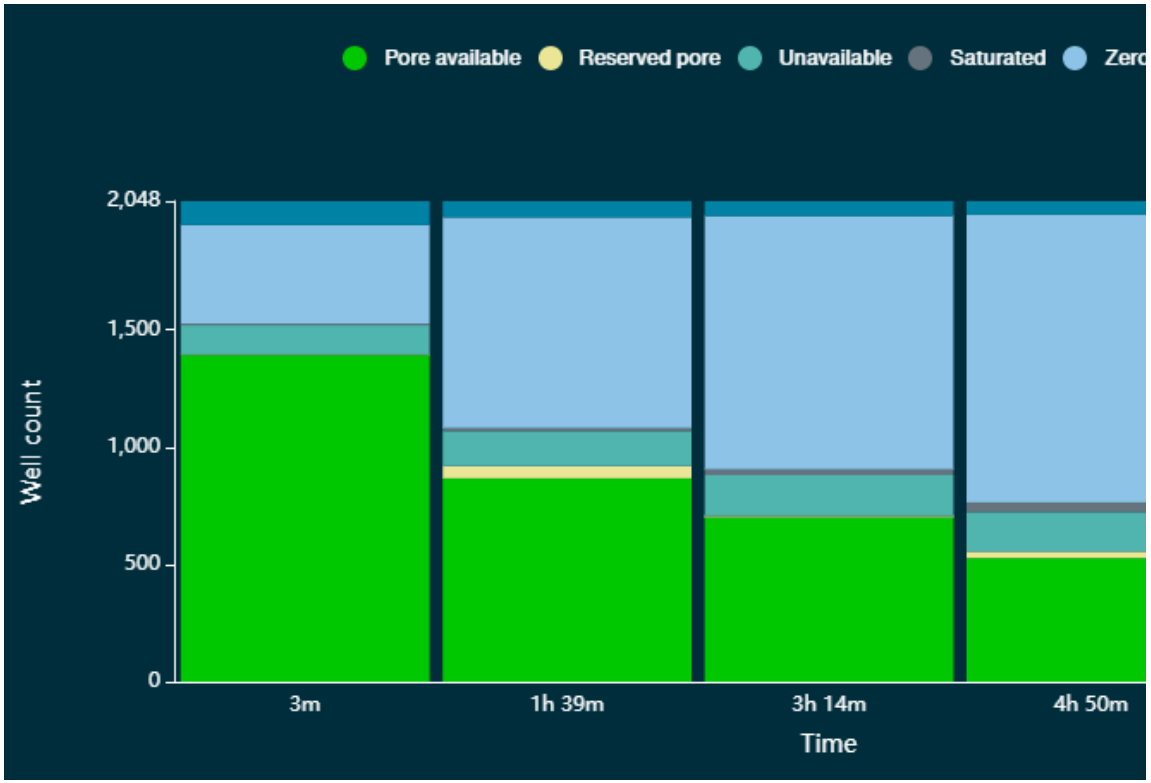
We have and Mk1B and had some "wildflower" flow cells. When it happens again, I will try to connect and disconnect the flow cell and see if it works. I hope that it works with the same device/position.

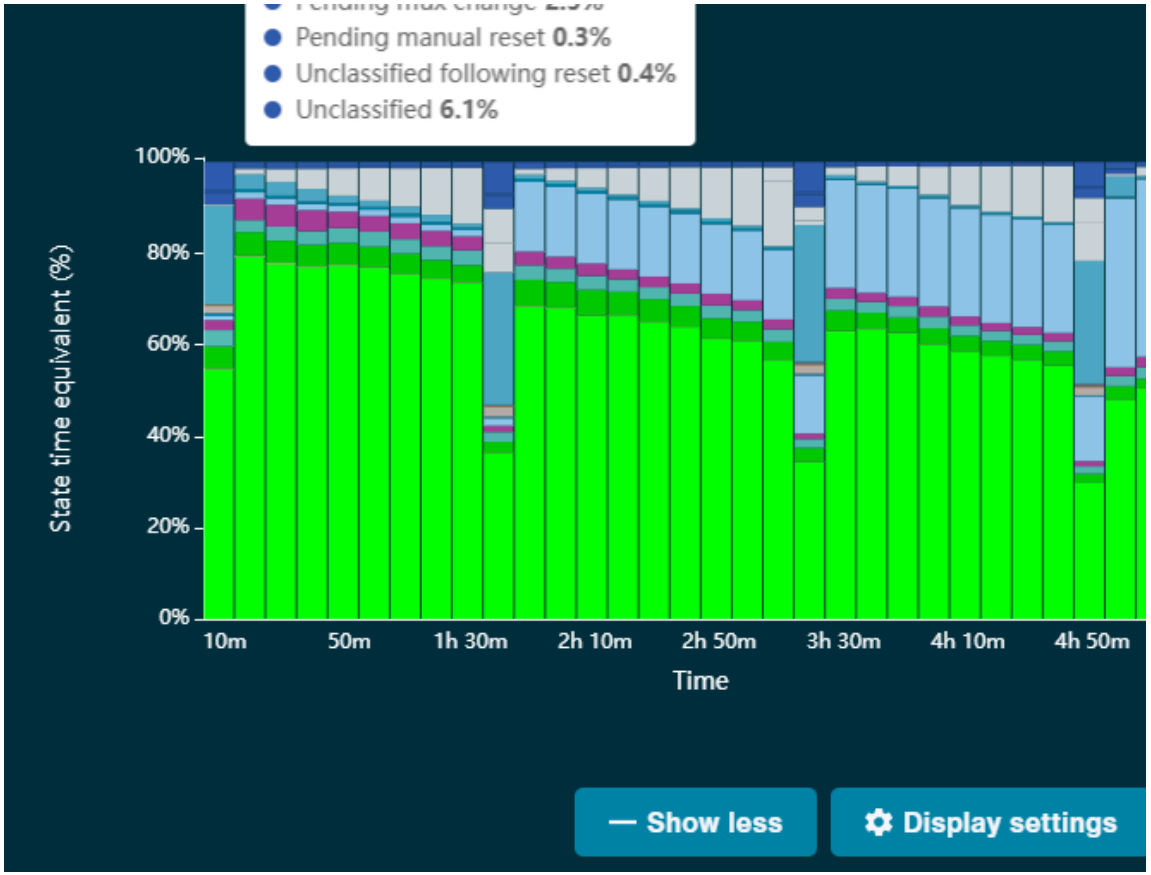
Thank you very much for sharing this!

Best,

Albert Carcereny | 2 years ago | Kudos | Reply

> Dear @Kevin Pirelli and @Jasmaine Lee
>
> Last week we performed a run in a new R10.4.1 flow cell and it clearly showed the wildflower pattern as you can see in the following screenshots:


Pore available
Reserved pore
Unavailable
Saturated
Zero
2,048
1,500
1,000
500
0
Well count
3m
1h 39m
3h 14m
4h 50m
Time



When I saw that the flow cell was showing this behaviour, I changed it from one Mk1C device to another to see if there was an improvement. Unfortunately, the flow cell did not show any signs of improvement. I changed it from one Mk1C to the other several times without success. In fact, I ended up loosing pores in every initial pore scan.

Could it be that this trick does not work with Mk1C? Or it depends on the flow cell or maybe on the time that flow cell has been running before changing it to a different device/position (I should have change it after the first MUX scan)?

Any help would be much appreciated because we have seen the wildflower pattern in the last 4 R10.4.1 flow cells and we still have to more which very likely will show the same behaviour.

Thank you very much,

Has there been any resolution to this problem? I am trying to transition a small amplicon (200-400 bp) sequencing method from R9 to R10 and having similar issues of losing pores rapidly during the first 4hrs. I am using the cDNA-PCR barcoding kit so thought it was an issue with the V14 RAP but after reading these posts, it looks like it could be a R10 flow cell issue. For example on the R9 using the V11 SQK-PCB111.24 I was getting ~12 million reads, with SQK-PCB114.24, im lucky to get a 1 million reads before I lose all the pores.

Michael Wiley | a year ago | Kudos | Reply

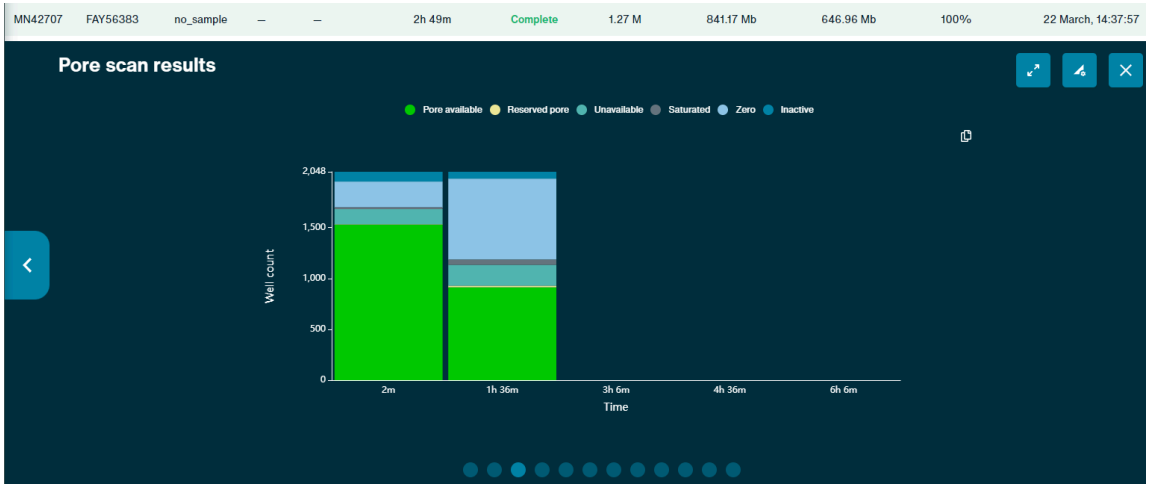
Dear @Michael Wiley

In our lab we have been struggling with this issue in our last 4 runs.

We sequence SARS-CoV-2 400bp amplicons using the SQK-NBD114.24 kit. We have been using the exact same protocol for the last 3 years (changing the flow cells and kits from R9 and V12 to R10 and V14) with no major issues (some wildflower flow cells, though) usually getting between 10 and 20 million reads.

However, the last 4 R10.4.1 flow cells have been a complete disaster outputting at max 2 million reads per flow cell. We have tried everything we could, from washing and reloading the flow cell, changing between devices and connecting and disconnecting the flow cell. Anything worked. It is truly frustrating to spend a day or more preparing your samples and the corresponding library to see how the flow cell dies in a few hours.

The following screenshots are just an example of one of the 4 runs:



That run barely reached 2 million reads. As you see, in the first MUX scan we lost more than 500 pores. I have seen better flow cells after 48h running than these ones in an hour or two.

We do not understand why this is happening. As said, our samples are the same kind as always, the procedure has never changed and no mistakes were made in neither library prep nor in library loading.

Is this problem a software issue? Should we install a previous version of MinKNOW and see if the problem persists?

Best,

Albert Carcereny | a year ago | Kudos

Active feedback means something is blocking pores (and software tries voltage reversal to eject it).

Also check the light sensiticity for R10.4.1 adapters/pores, especially with shorter amplicons. (Some bit of adapter/helicase comples probably has conjugated double bonds, and forms a radical when excited by photon above some energy threshold.) Than this radical would form a covalent bond with whatever it comes across, and this complex would get stuck in the pore...

I would load an R10.4.1 flowcell in by the **dim red light** (like the lamp used in the darkroom with b/w photography), and close the Mk1b lid immidiatelly after loading... (use light shields + black cloth cover for GridIon/PromethIon ...

An in the typical lab settings, brightly lit by cool whilte LED lights (plenty of photons in 380 - 450nm range) provides great enviroment for various photon induced reactions...

Markiyan Samborskyy | 9 months ago | Kudos | Reply

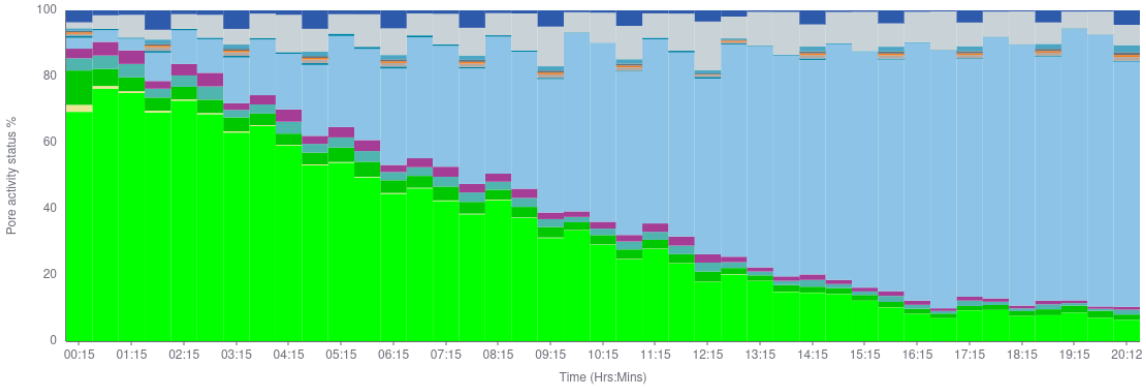
This is not a library-related pore blocking issue. I've loaded the same library on two flow cells (R9.4.1) that arrived in the same shipping batch, one had this problem, and one didn't.

David Eccles | 9 months ago | AUTHOR | Kudos

Dear community,

Do you think the screenshots I am posting are related tot this wildflowers pattern ?

I am seeing this kind of stuff many times in the last few months on most of my sequencing.

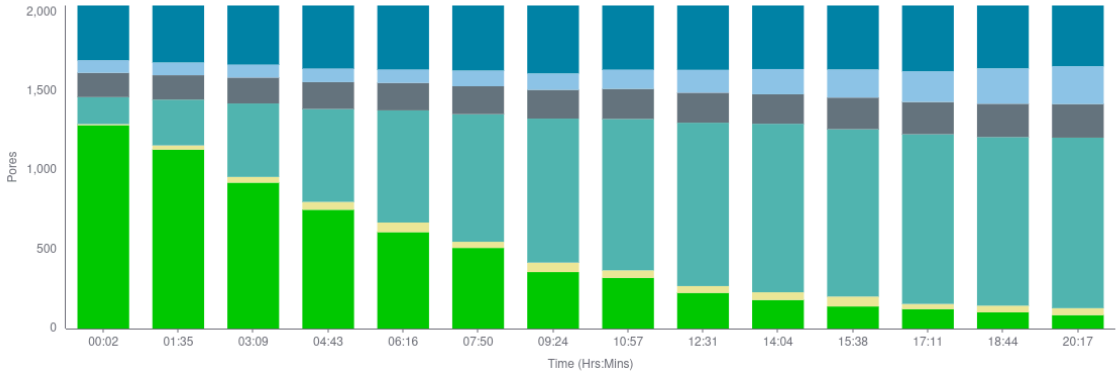


PORE SCAN

A Pore scan is performed at configurable time intervals to determine the current status of pores within channels on a Flow Cell. For this run a Pore scan is performed every 1.5 hrs.

Legend

Pore available	Reserved pore	Unavailable	Saturated	Zero	Inactive
Pore in channel available for sequencing	Pore in reserve, will return to available when required	Pore inhibited from sequencing	Possible contamination in the sample	No current is passing through this pore, possibly due to bubbles on the membrane	Pore no longer suitable for further sequencing



Looking for your comments,

Best

Maxime Lambert | 8 months ago | Kudos | Reply

> Yes, the pore activity plots look similar, with lots of pores in the 'feedback' state.
>
> David Eccles | 8 months ago | AUTHOR | Kudos

A Field Application Scientist in Nanopore told me the "feedback" state is explained because my gDNA was too long (more than 60kb on TapeStation). So I tried to fragmente my gDNA (29kb on TapeStation) but I observed the same pattern in my flowcell. The only thing the fragmentation changed is my N50 (from 20kb to 8kb) but I still have the purple part on the pore activity plot.

As I am using a Gridion I will try to change my flowcell position if I have the same pattern again as mentioned by @Jasmaine Lee

Maxime Lambert | 8 months ago | Kudos

Add a comment

Start typing...

Kathleen Durkin
Profile 44% complete
2670Ku

Smithsonian Institution; NMNH
Full Member

0 Following
0 Followers
0 Mentions
1 Interesting

Create new post

Author

David Eccles
Gringene Bioinformatics
View profile

Interesting 27

Subscribe 46

DNA CDNA FLOW CELL

- Privacy Policy