SINGLE-NUCLEI RNA-SEQ ANALYSIS USING AMAZON WEB SERVICES (AWS)

Izar Lab — October 2020

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Overview

1. Course preparation
2. AWS introduction
3. Launching an instance
4. SSH into your instance
5. Updating the instance and installing AWS CLI
6. Installing Cell Ranger
7. Creating a snRNA-seq reference
8. Creating, attaching and mounting a volume
9. Downloading raw data
10. Running Cell Ranger
11. Setting up an alarm for your instance
12. Running CellBender
13. Running Seurat

COURSE PREPARATION

Course preparation: Installing OSXfuse

FUSE allows you to mount and interact with directories and files located on a remote server over a normal ssh connection

- Go to https://osxfuse.github.io/
- Download FUSE and SSHFS
- Open the Terminal and check if it's working:

```
sshfs --version
```

- If you can see the version number, the installation was successful

- Windows users can install MobaXterm to access remote servers using SSH; a graphical SFTP browser is included (https://mobaxterm.mobatek.net)

Course preparation:
Setting up R and installing libraries

- Follow steps 1-4 on this web page to install R, RStudio and the Swirl R-package:

  https://swirlstats.com/students.html

- Please work through the following Swirl courses before the beginning of the workshop (depending on your familiarity with R):
  - 1: R Programming: The basics of programming in R
  - 4: Exploratory Data Analysis: The basics of exploring data in R

- Install the following libraries before the beginning of the workshop and make sure you can access them by calling library(*nameoflibrary*) without any errors:
  - install.packages(c('dplyr', 'BiocManager', 'httr', 'plotly', 'Seurat', 'cowplot', 'ggplot2', 'gplots', 'ggthemes', 'stringr', 'pheatmap', 'Matrix'))
  - BiocManager::install(c('SingleR', 'SingleCellExperiment', 'scater', 'purrr', 'celldex'))

Setting up Python and associated libraries

- Install Python 3 by running:
  - sudo apt-get install python3
- Install pip, a package manager that helps with installing libraries on python, by running:
  - sudo apt-get install python3-pip
- Install scrublet, a python package for detecting doublets, by running:
  - pip3 install scrublet
- While running the above command, you may encounter an error about a library called llvm missing. To fix this, run the below commands, and then rerun pip3:
  - sudo bash -c "$(wget -O - https://apt.llvm.org/llvm.sh)"
  - sudo ln -s /usr/bin/llvm-config-10 /usr/bin/llvm-config
  - LLVM_CONFIG=/usr/bin

Course preparation: Command line tutorials

- Work through any of these free command line tutorials (as you see fit):

  https://blog.teamtreehouse.com/introduction-to-the-mac-os-x-command-line

  https://www.udacity.com/course/linux-command-line-basics--ud595

  https://www.udemy.com/course/command-line/

  https://ubuntu.com/tutorials/command-line-for-beginners#1-overview

- You should be familiar with basic commands such as pwd, ls, cd, mv, cp, mkdir etc.

Course preparation: Sign in to AWS

- When your AWS account is created you'll receive a credentials files that should be kept in a hidden folder
- Try signing in to AWS to make sure it's working before the beginning of the course
- You'll find your one time-use password in the credentials file

https://us-east-2.signin.aws.amazon.com

Course preparation: Reading list

- Please read the following papers and pre-prints before the beginning of the course:
  - Current best practices in single-cell RNA-seq analysis: a tutorial
    - 10.15252/msb.20188746
  - CellBender remove-background: a deep generative model for unsupervised removal of background noise from scRNA-seq datasets
    - https://doi.org/10.1101/791699
  - Scrublet: Computational Identification of Cell Doublets in Single-Cell Transcriptomic Data
    - 10.1016/j.cels.2018.11.005

Important points

- You can find your password in the credentials file
- Keep the credentials file save in a hidden folder
- S3 is in most cases the only copy of our data so make sure not to delete or alter anything
- Most people in the lab have access to S3 so you can direct them to your analysis output
- Downloading data from AWS is very expensive so please don't download large files but instead analyze them on AWS instances until you reach a smaller format that can be downloaded
- The volume storage associated with instances is also very expensive so try to keep it small and store data on S3
- Please only use your own instances, volumes and images generated with your own key
- If you don't need an instance anymore you can terminate it
- You can check the budget in the 'Cost Explorer' menu and filter for costs generated with your key. This will only include instance-related costs and not the costs generated through S3 and volumes.

INTRODUCTION TO AWS

Cloud computing with AWS

- On-demand availability of computer system resources, especially data storage and computing power
- Based on pay-as-you-go concept
- AWS technology is implemented at server farms throughout the world
- Amazon Elastic Compute Cloud (EC2):
  - Called an "instance"
  - Rent virtual computers to run your own applications
  - Boot Amazon Machine Image (AMI) to configure a virtual machine containing specific software
  - Create, launch, and terminate server-instances as needed
- Emulates most attributes of real computers:
  - Hardware central processing units (CPUs)
  - Graphics processing units (GPUs)
  - Local/RAM memory
  - Hard-disk/SSD storage
  - Choice of operating systems
  - Networking etc.

AWS Global Infrastructure

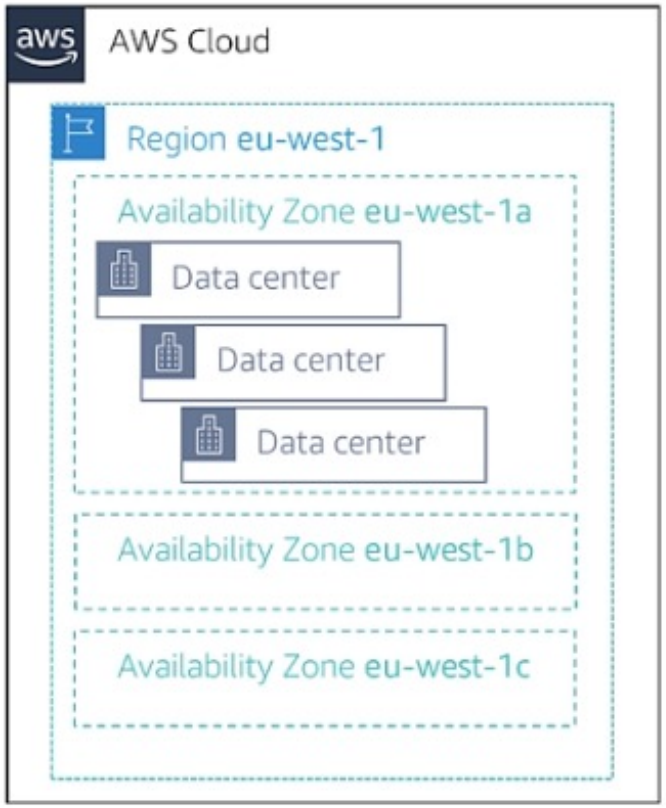
aws training and certification

- The AWS Global Infrastructure is designed and built to deliver a flexible, reliable, scalable, and secure cloud computing environment with high-quality global network performance.
- This map shows the current AWS Regions and more that are coming soon.

Availability Zones

aws training and certification

- Each Region has multiple Availability Zones.
- Each Availability Zone is a fully isolated partition of the AWS infrastructure.
  - There are currently 69 Availability Zones worldwide
  - Availability Zones consist of discrete data centers
  - They are designed for fault isolation
  - They are interconnected with other Availability Zones by using high-speed private networking
  - You choose your Availability Zones.
  - AWS recommends replicating data and resources across Availability Zones for resiliency.



- You need to stay within the region us-east-2 (Ohio)
- Availability Zones can be freely chosen

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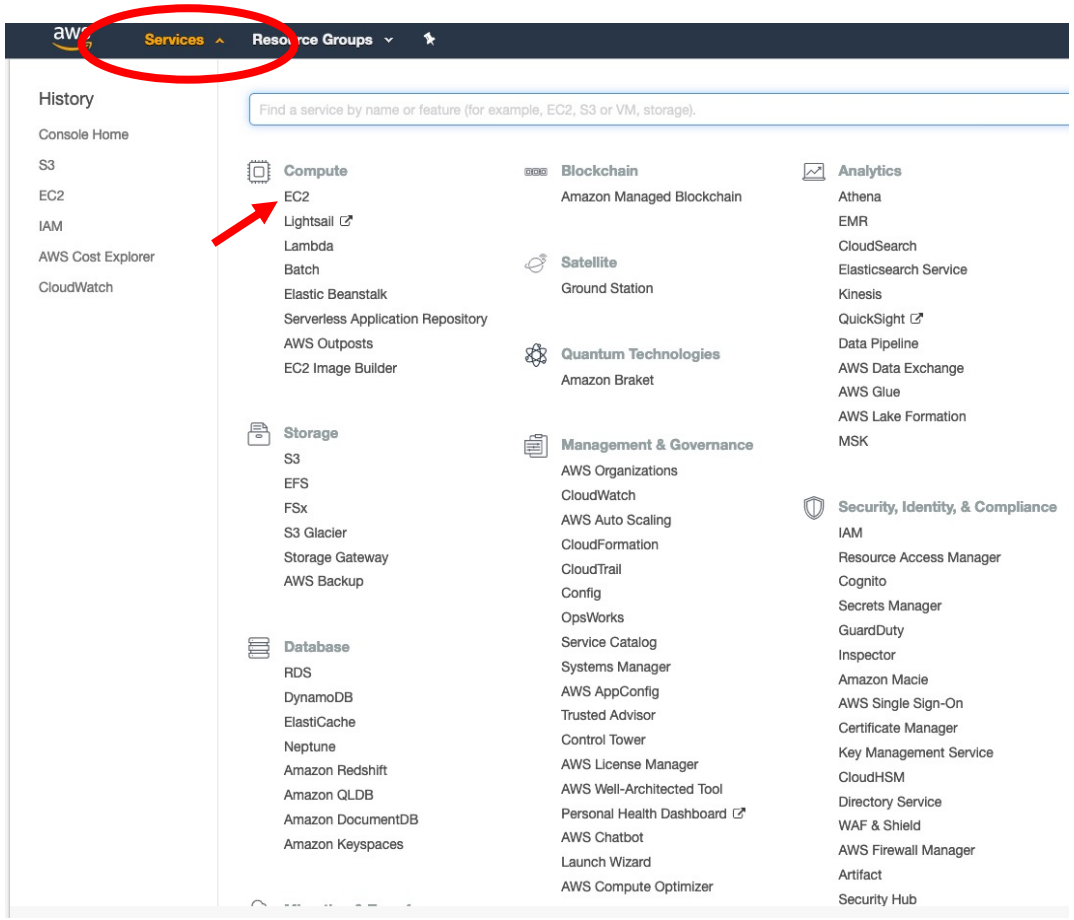
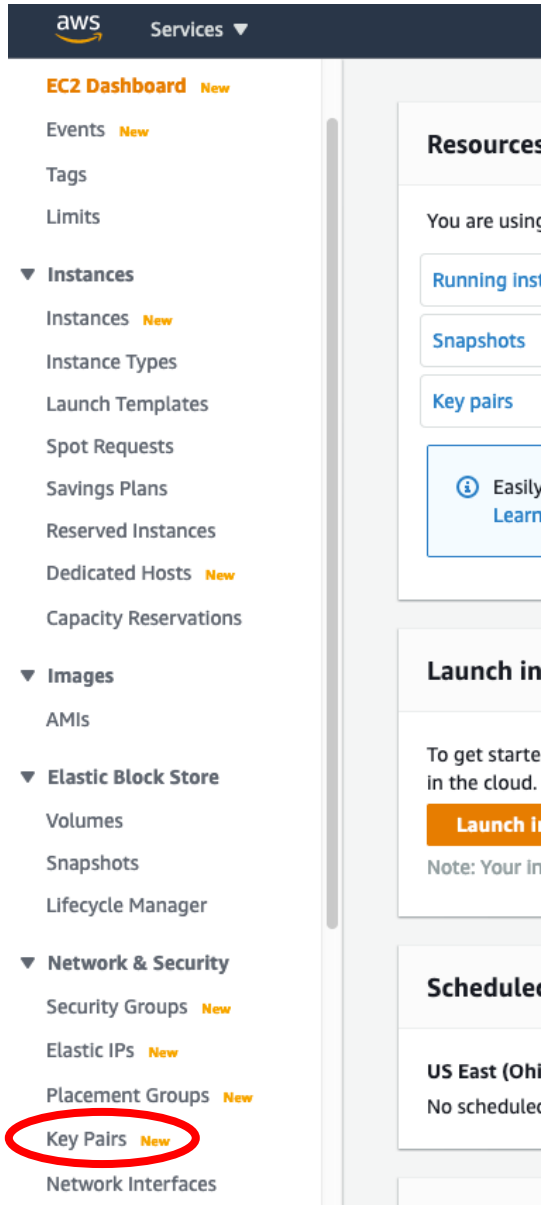
Sign in

https://us-east-2.signin.aws.amazon.com

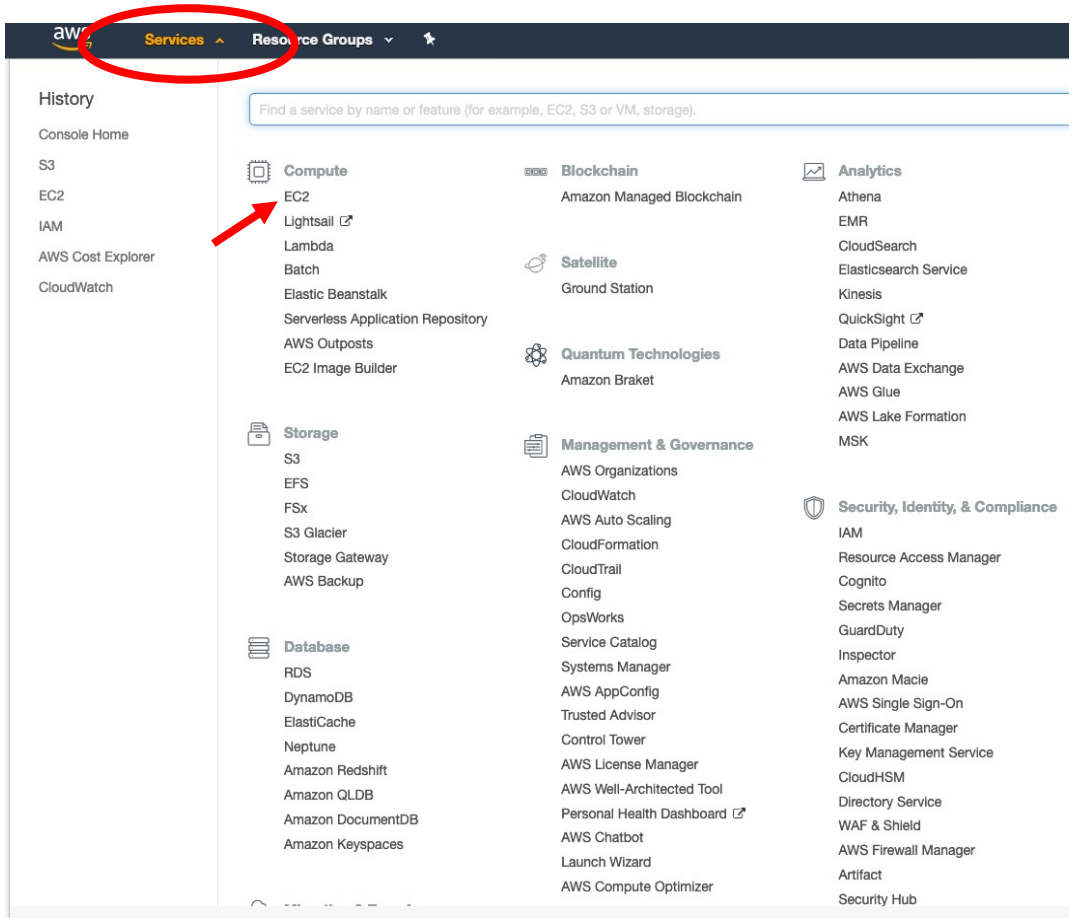
Navigate to Key Pairs and create your key

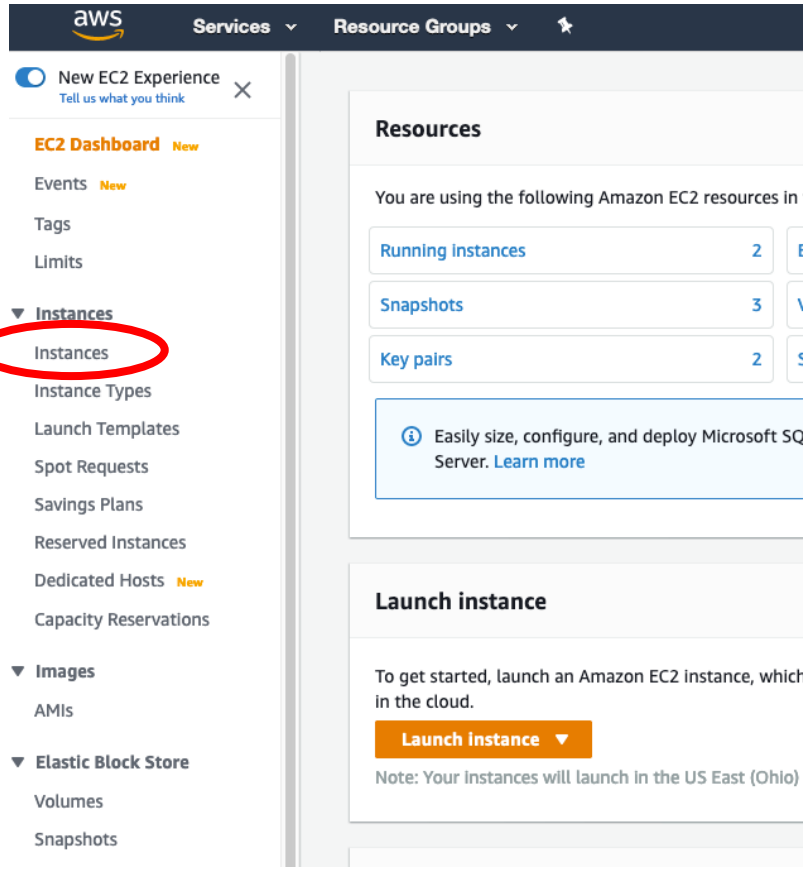


- Name your key starting with your name_key
- E.g. ‘yiping_key’

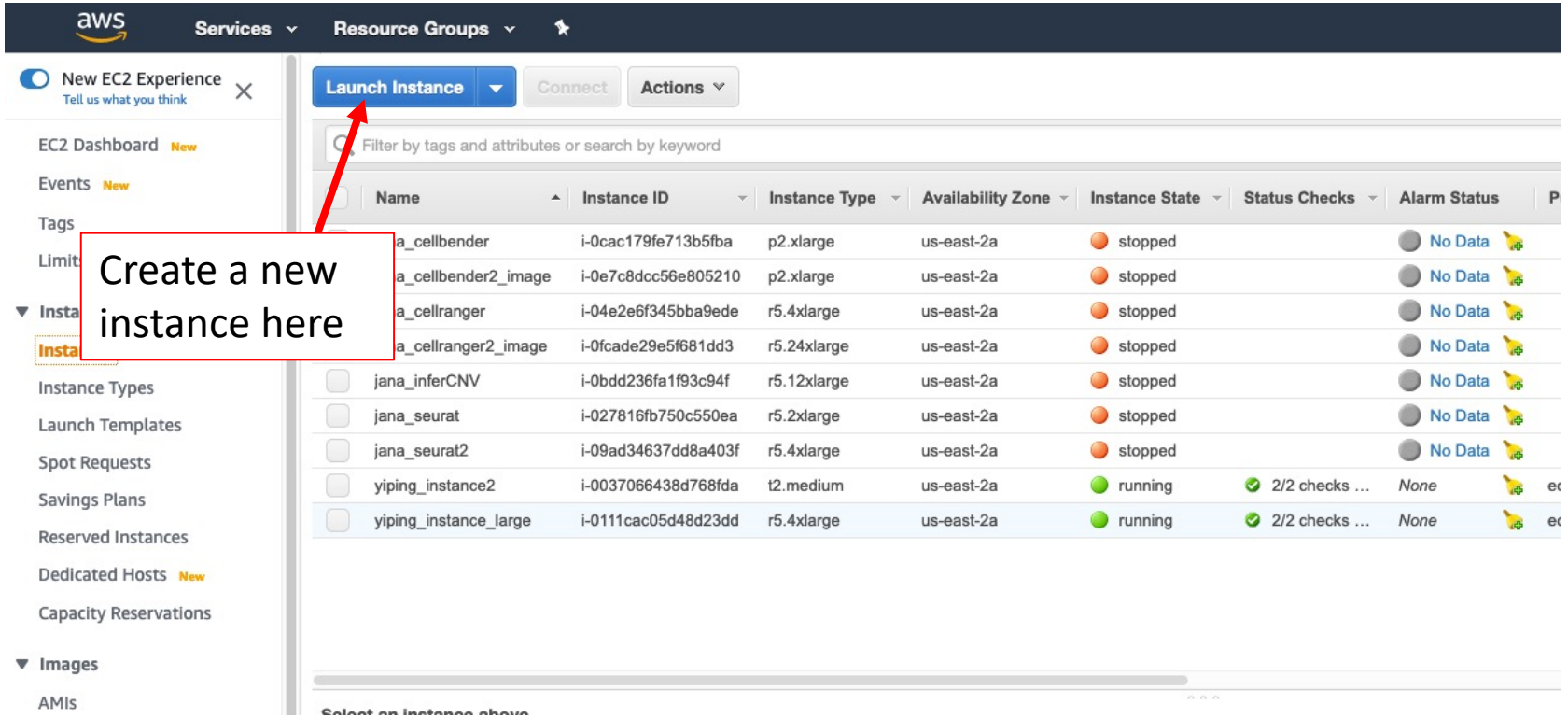


Navigate to E2/Instances



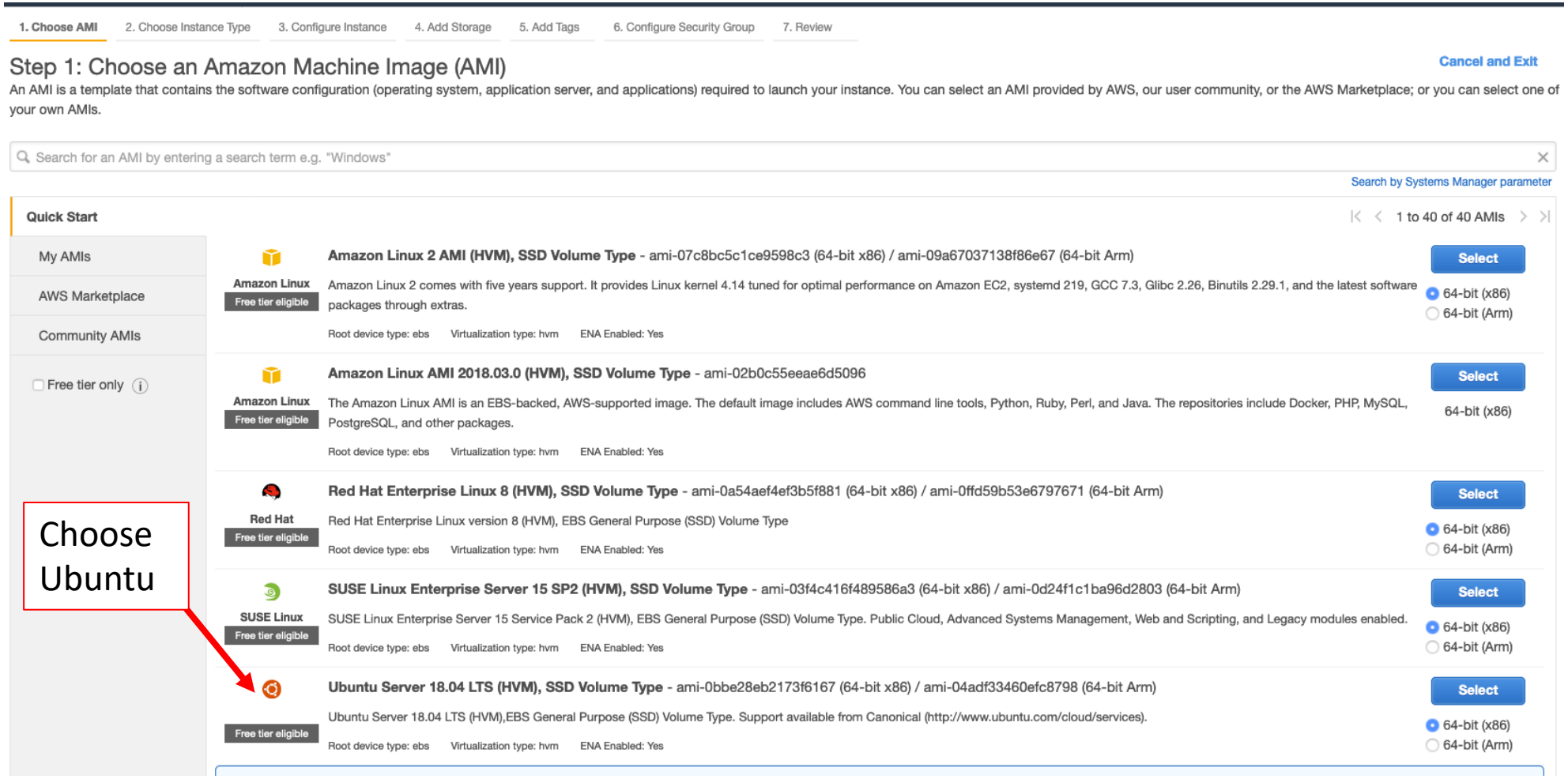


Launching an instance for CellRanger

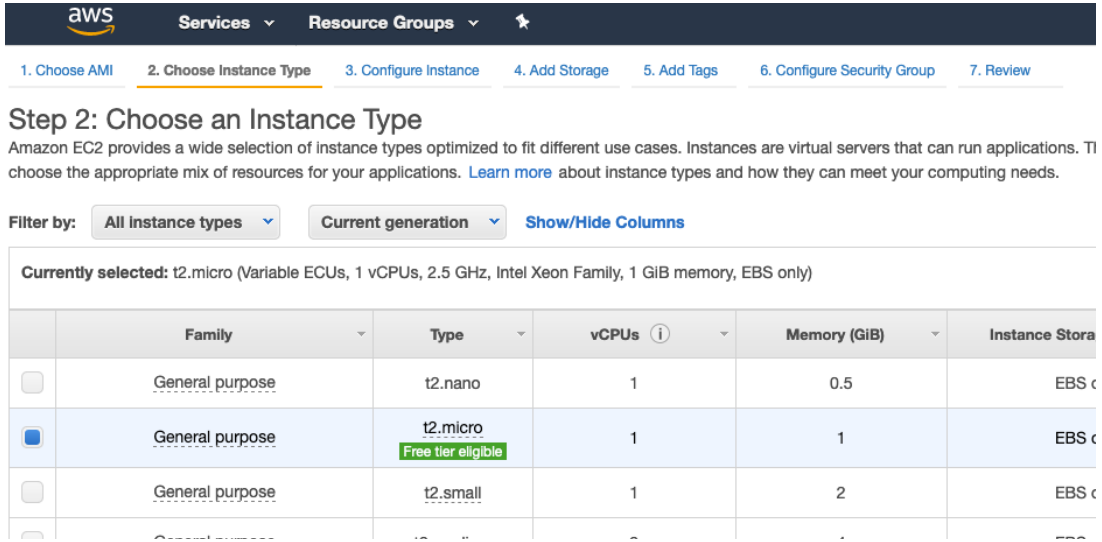


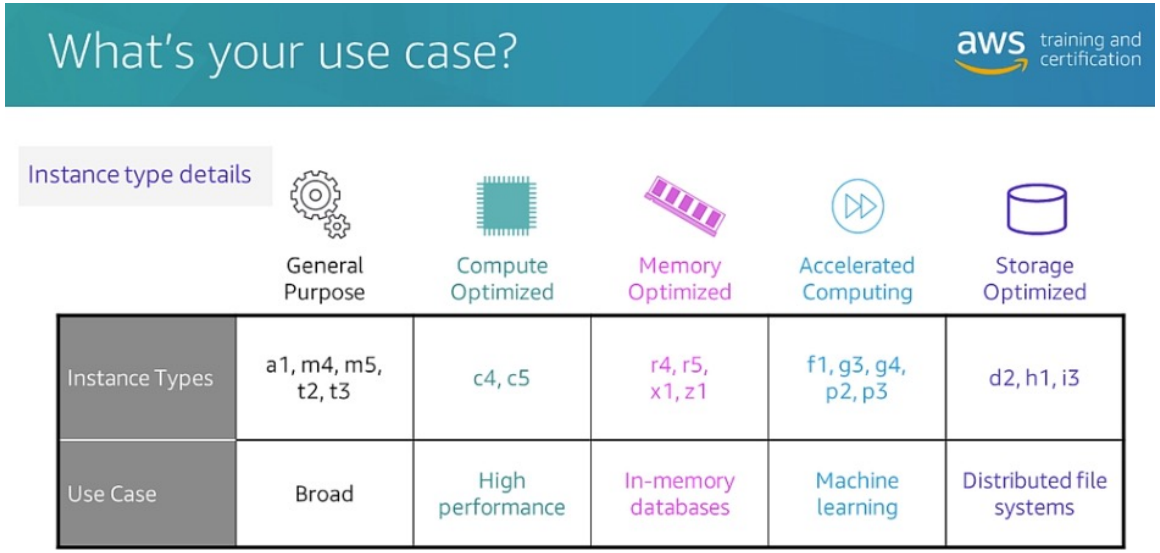
- Name your instances and volumes starting with your name
- E.g. ‘yiping_instance_large’

Launching an instance — Step 1

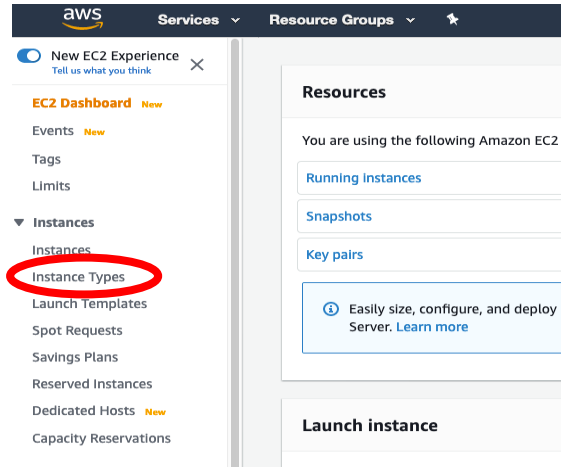


Launching an instance — Step 2

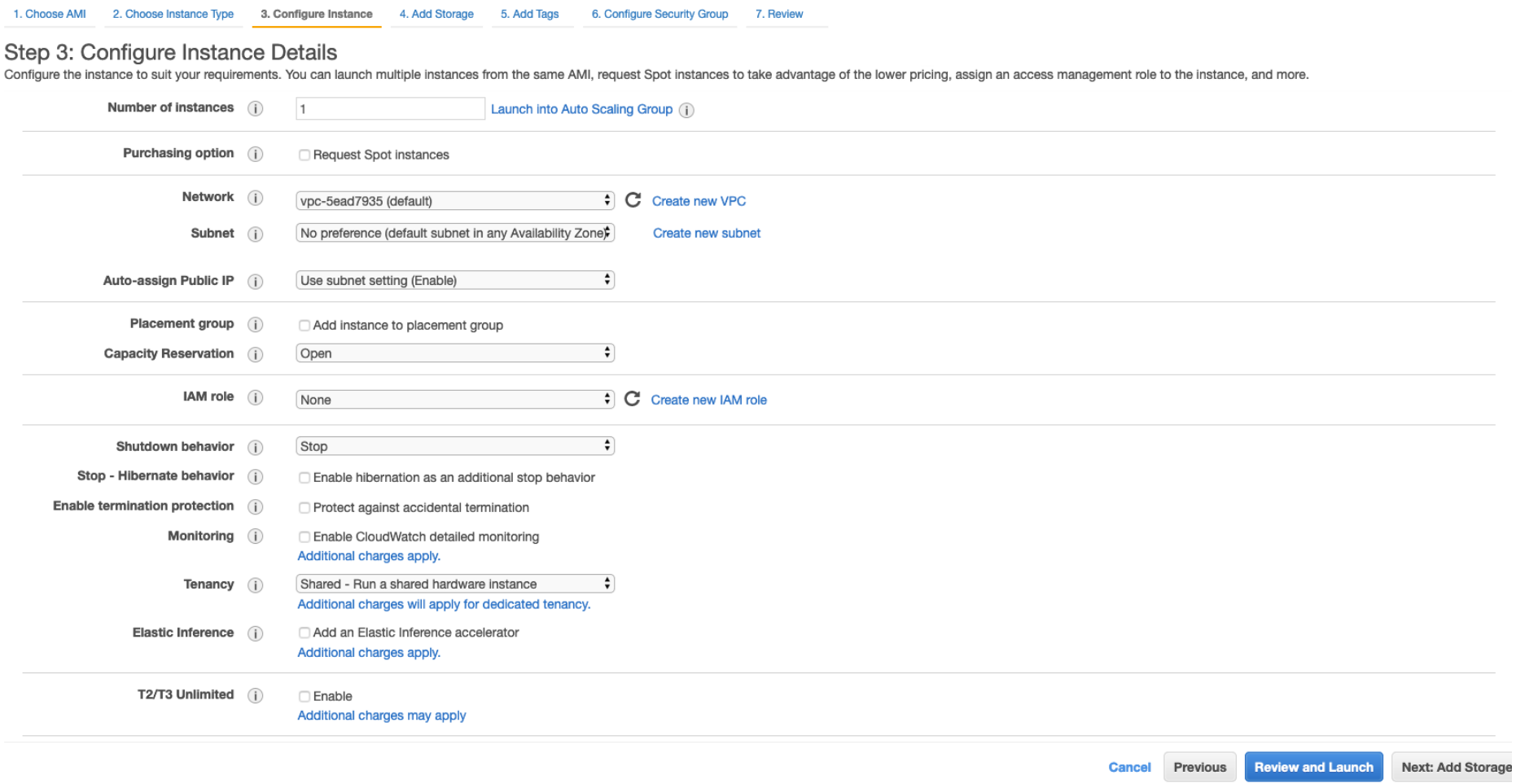




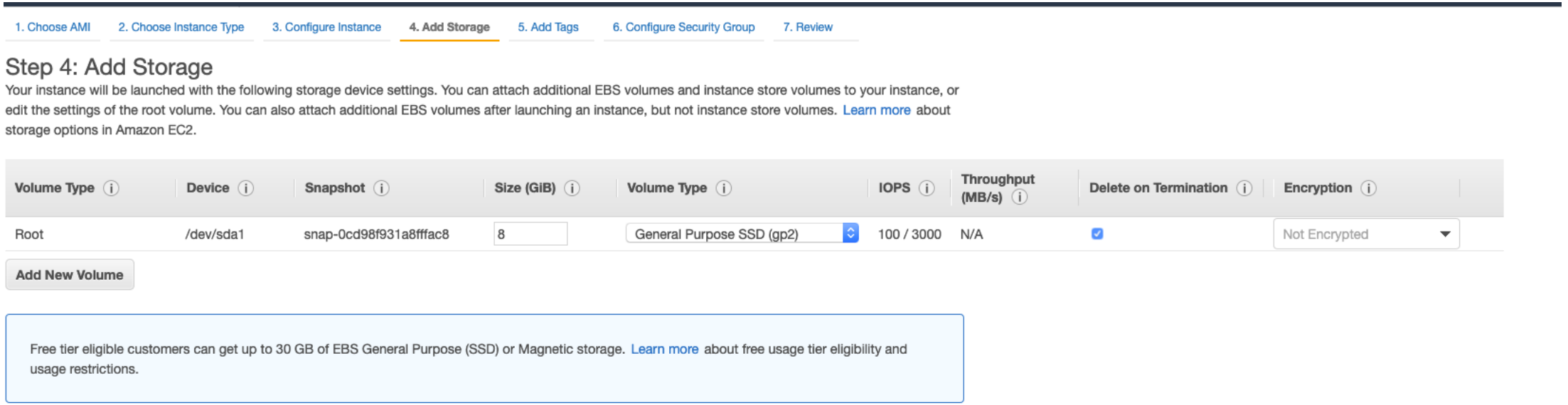
- Choose an instance based on use case
- Check properties of different instances in the 'Instance Types' menu
- Requirements for Cell Ranger (one sample):
  - 16 cores
  - 128 GB
  - → Select instance r5.4xlarge here



Launching an instance — Step 3



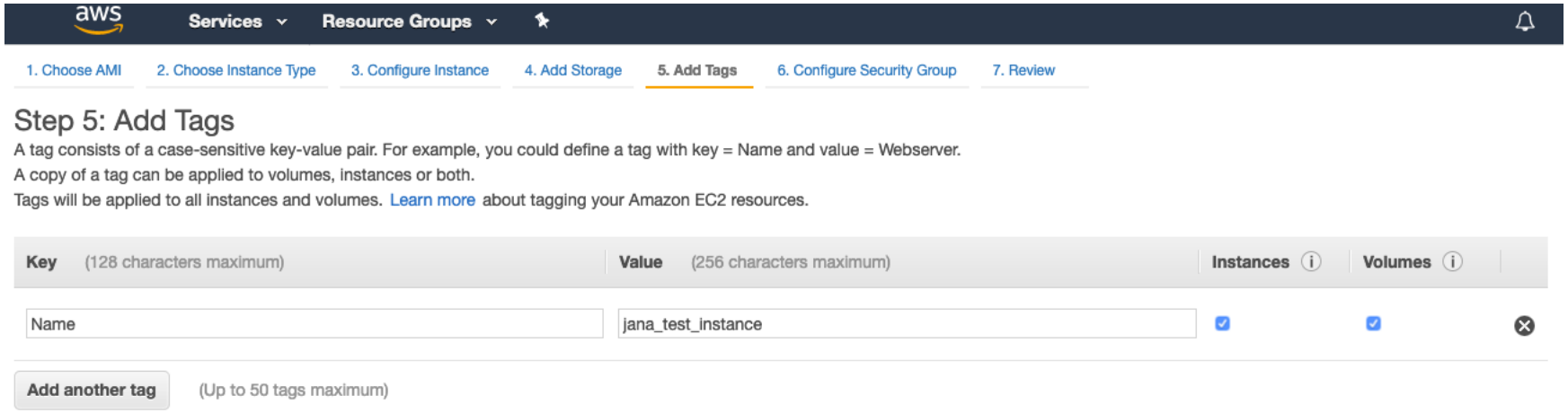
Launching an instance — Step 4



- ❖ Choose the size of the root volume
- ❖ The size can also be changed later in the ‘Volumes’ menu (only to increase it and it takes some time)
- ❖ You can add additional volumes to your instance (need to be attached and then mounted)
- ❖ This type of storage is expensive

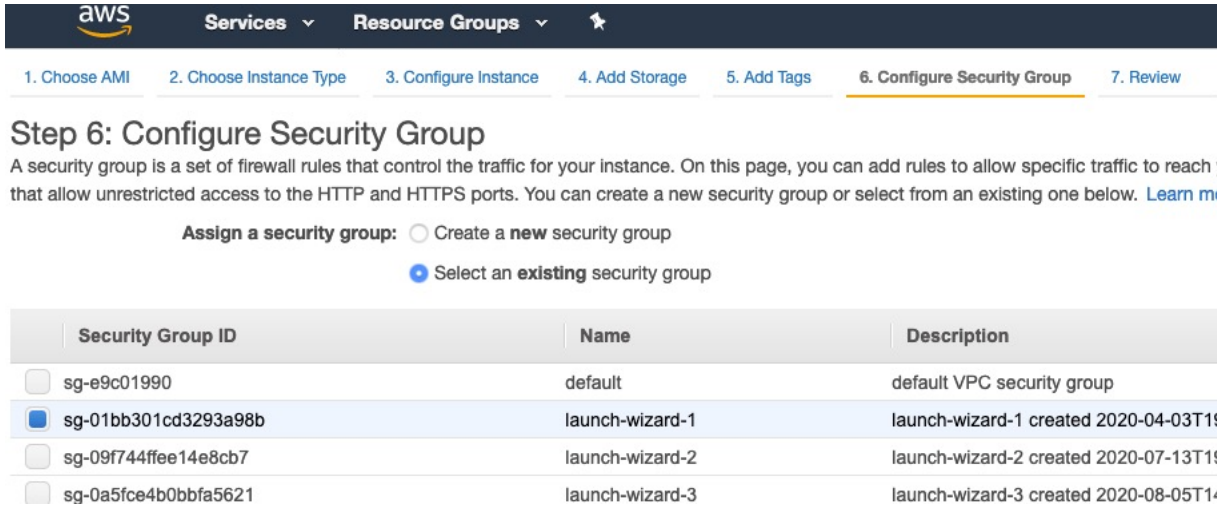
→ Choose 50 GiB today

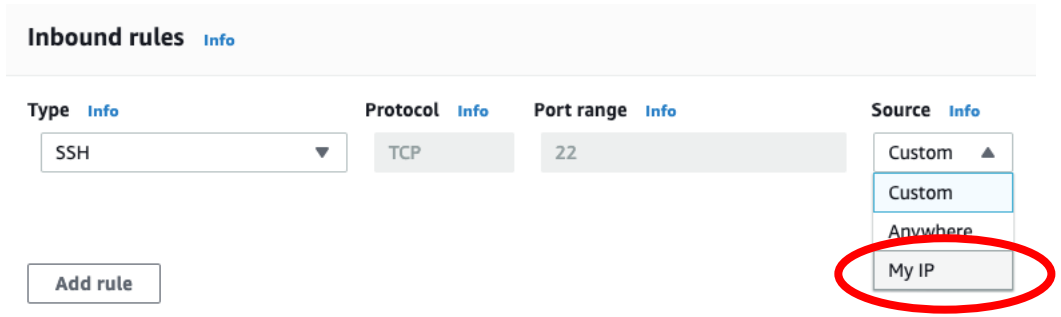
Launching an instance — Step 5



- Name your instances and volumes starting with your name
- E.g. ‘yiping_cellranger’

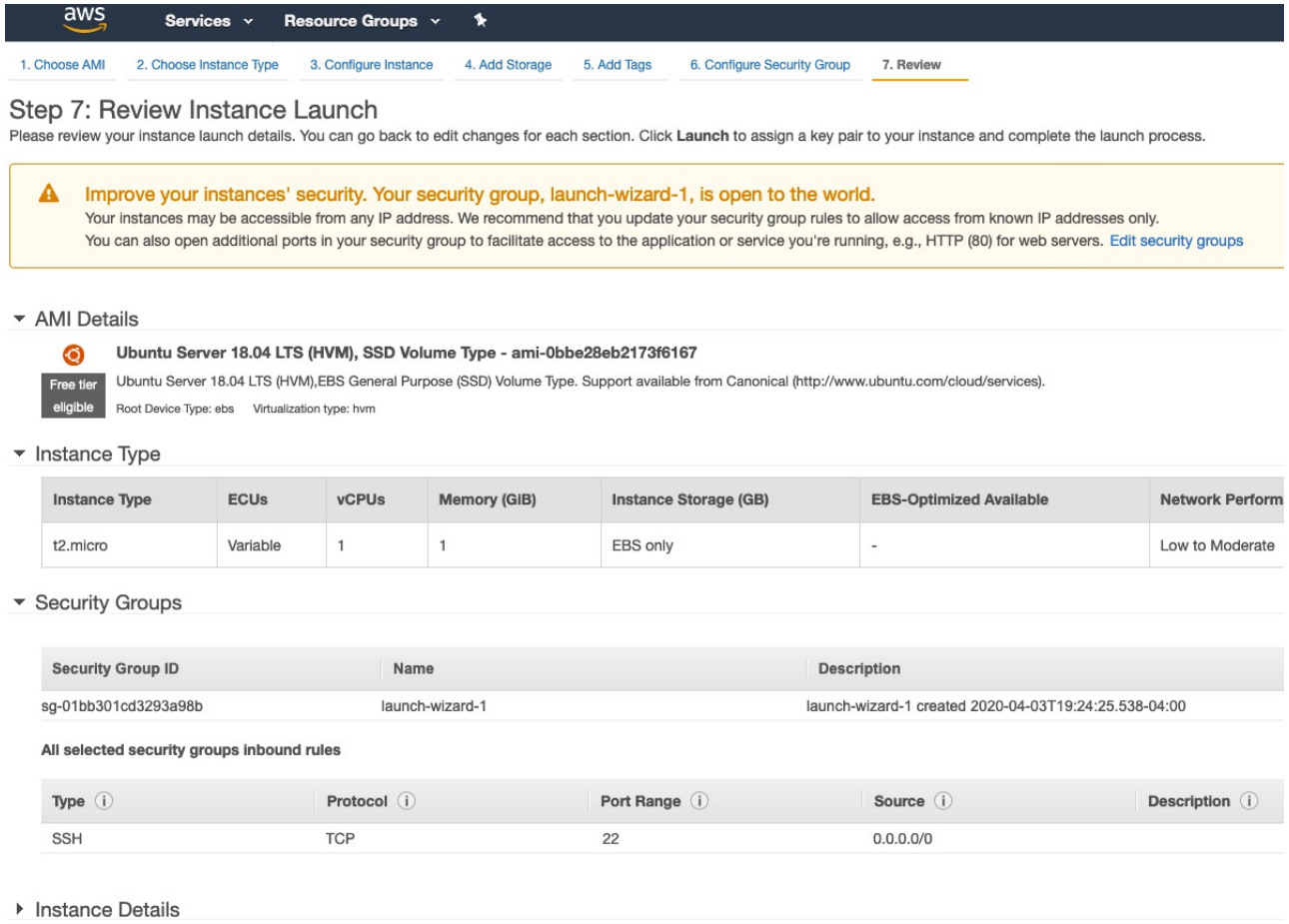
Launching an instance — Step 6

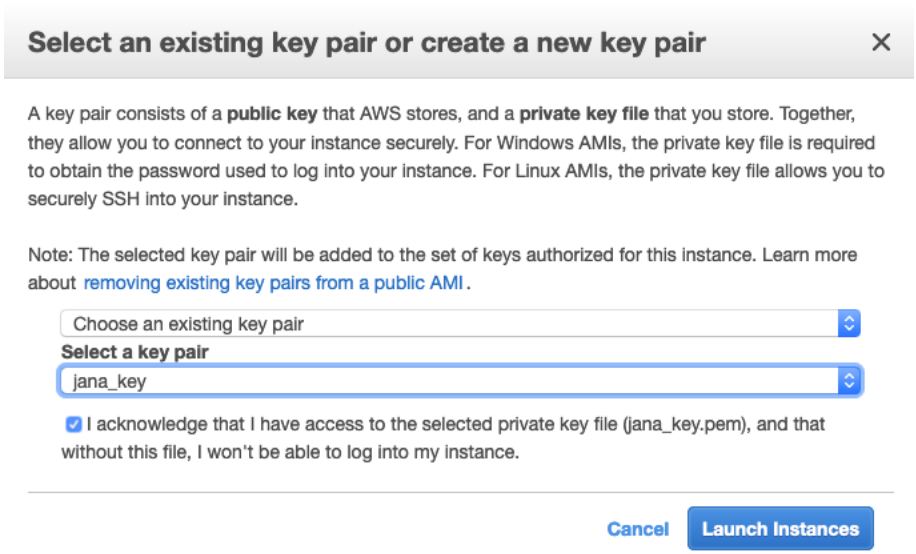




- Create a new security group if you don't have one
- Allow traffic only from your IP address
- Don't create a new security group every time you launch a new instance
- Don't allow all traffic
- Name your security group starting with your name, e.g. 'jana_security_group'

Launching an instance — Step 7

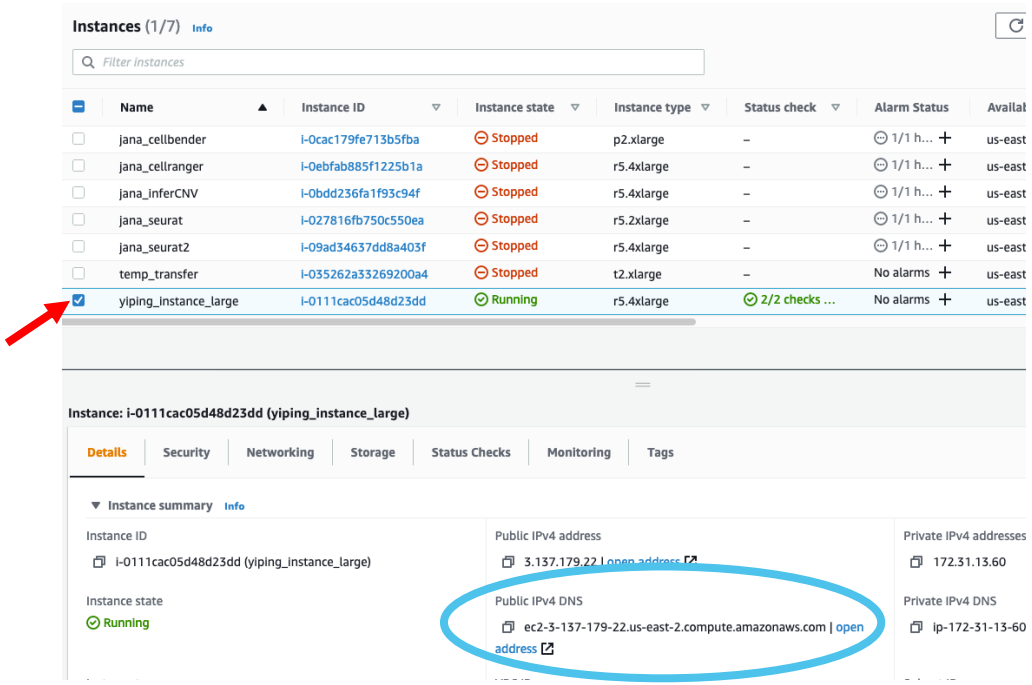




- Review your settings and launch your instance using your AWS key

CELL RANGER

SSH into your instance



- ssh (Secure Shell) is used to securely connect to a remote server/system (it transfers the data in encrypted form between the host and the client)
- Select your instance and copy the IP address (Public IPv4 DNS)
- Mount the remote folder to your local folder by specifying the directory of your AWS key and the IP address of the instance

```
# Create local folder as mounting point
mkdir AWS

# Mount your instance's home directory to your local AWS folder
sshfs -o IdentityFile=XXX.pem ubuntu@ec2-3-137-186-247.us-east-2.compute.amazonaws.com:/home/ubuntu/ ~/AWS

# SSH into your instance by specifying the directory of your AWS key and the IP address of the instance
ssh -Y -i xxx.pem ubuntu@ec2-3-137-186-247.us-east-2.compute.amazonaws.com
```

Update instance and install AWS CLI

- Update instance with:

```
sudo apt-get update; sudo apt-get upgrade -y
```

- Connect to your AWS account:

```
# install aws command line
curl "https://awscli.amazonaws.com/awscli-exe-linux-x86_64.zip" -o "awscliv2.zip"
sudo apt install unzip
unzip awscliv2.zip
sudo ./aws/install

# set up aws  s3 access
mkdir ~/.aws
echo "[default]" >> .aws/credentials
echo "aws_access_key_id = XXX"  >> .aws/credentials
echo "aws_secret_access_key = XXX" >> .aws/credentials

# list all buckets
aws s3 ls
```

Download and install Cell Ranger

- Copy download link for latest Cell Ranger version from: https://support.10xgenomics.com/single-cell-gene-expression/software/downloads/latest

```
curl -o cellranger-4.0.0.tar.gz ...
tar -zxvf cellranger-4.0.0.tar.gz
export PATH=/home/ubuntu/cellranger-4.0.0:$PATH
```

- Check if Cell Ranger works

```
cellranger
```

- Copy download link for latest 'Human reference (GRCh38) dataset required for Cell Ranger'

```
curl ...
mkdir reference
tar -zxvf refdata-gex-GRCh38-2020-A.tar.gz -C reference/
rm *.tar.gz
```

Create a "pre-mRNA" reference for snRNA-seq

UPDATE:
In the latest versions you don't need to generate a custom reference anymore for snRNA-seq (-> skip this)

1. Create a "pre-mRNA" GTF

Extract GTF annotation rows for transcripts based on the feature type transcript (column 3) of the original tab-delimited GTF and replace the feature type from transcript to exon.

```
# Examine GTF file structure
head reference/refdata-gex-GRCh38-2020-A/genes/genes.gtf

# Check gene transcript locus labels in original file
awk '{print $3}' reference/refdata-gex-GRCh38-2020-A/genes/genes.gtf | sort | uniq -c

# List each gene transcript locus as an exon
awk 'BEGIN{FS="\t"; OFS="\t"} $3 == "transcript"{ $3="exon"; print}' reference/refdata-gex-GRCh38-2020-A/genes/genes.gtf > references/GRCh38-2020-A_premrna.gtf

# Check gene transcript locus labels in new file
awk '{print $3}' reference/GRCh38-2020-A_premrna.gtf | sort | uniq -c
```

Create a "pre-mRNA" reference for snRNA-seq

UPDATE:
In the latest versions you don't need to generate a custom reference anymore for snRNA-seq (-> skip this)

2. Run cellranger mkref

Use the unmodified genome.fa file and the new GTF file as inputs to cellranger mkref.

```
cd reference
nohup cellranger mkref --genome=GRCh38-2020-A_premrna \
                --fasta=refdata-gex-GRCh38-2020-A/fasta/genome.fa \
                --genes=GRCh38-2020-A_premrna.gtf \
                --nthreads=16 > ref.out 2> ref.err &

# Check if mkref is running (leave with control + c)
cd
htop

# Press F9 to kill a job in htop
```

Transfer reference from S3 to instance

UPDATE:
In the latest versions you don't need to generate a custom reference anymore for snRNA-seq (-> skip this)
Use regular cellranger ref instead with introns flag

```
# transfer between instance and S3
cd ~
aws s3 sync s3://workshop-aws-data/reference/ reference/ --exclude '*' --include 'premrna' --dryrun
```

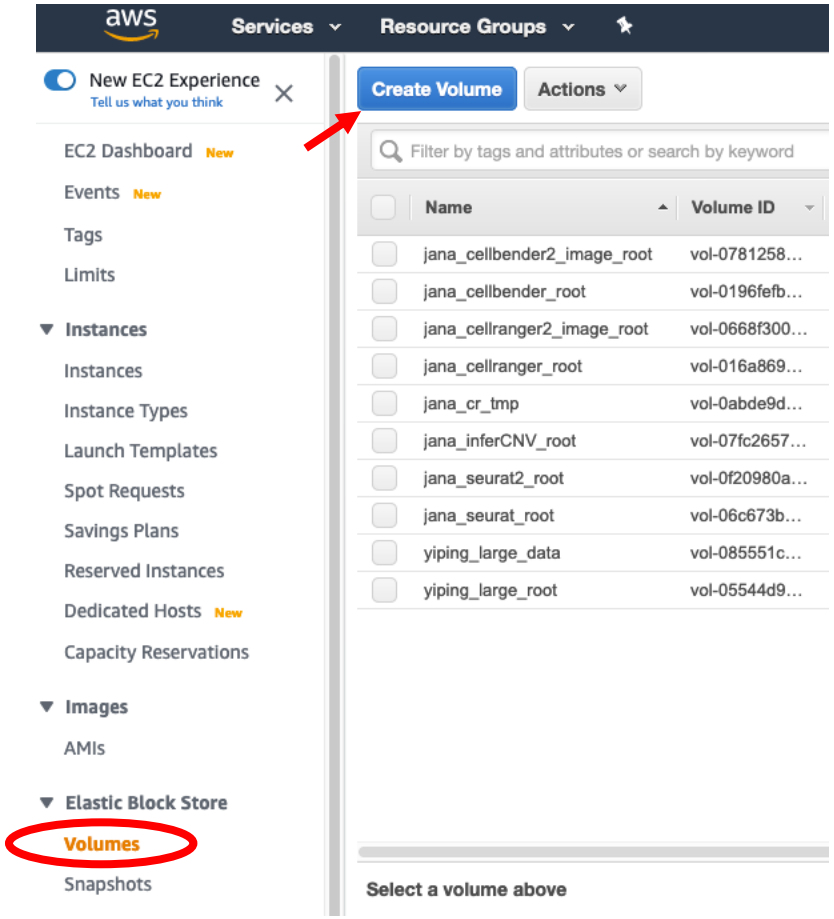
Source: S3 storage

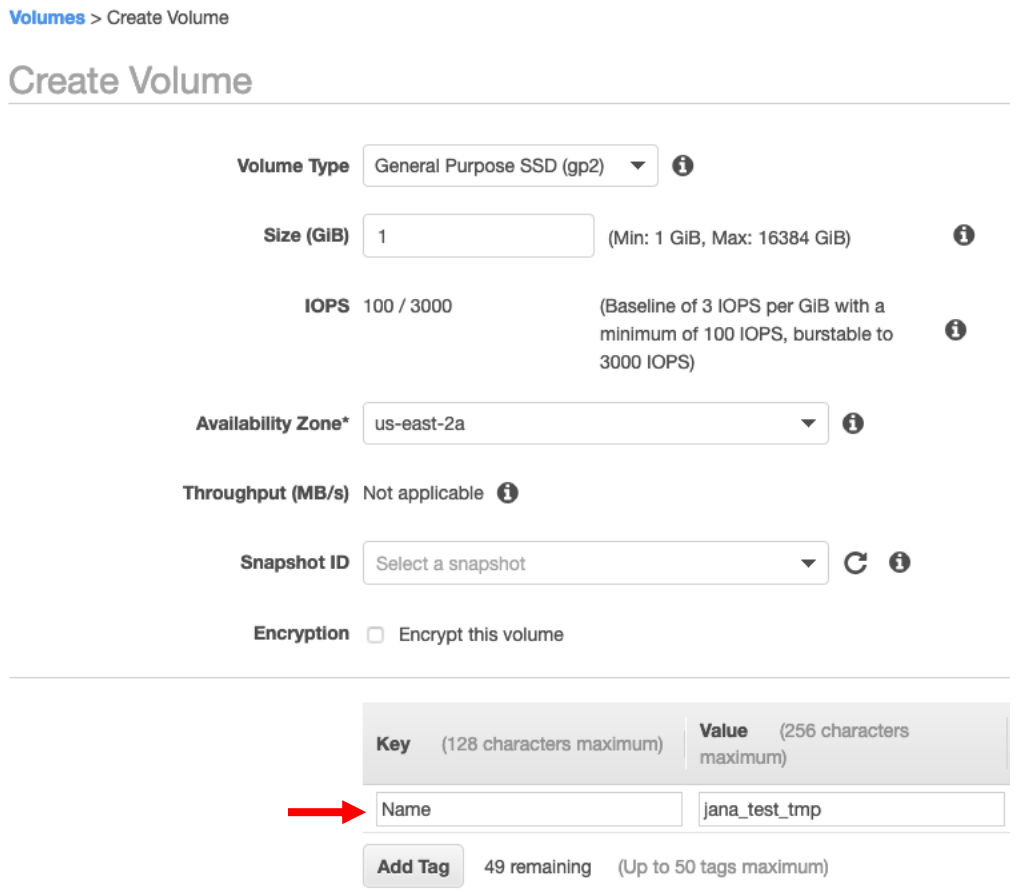
Target: your instance

Exclude everything apart from files with 'premrna'

- Directories have to end with /
- Use --dryrun first to make sure you selected the right directories
- Rerun without --dryrun

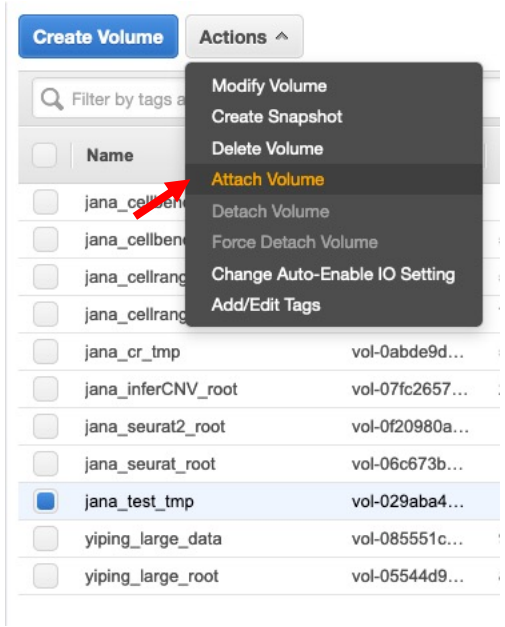
Create a volume





- Name your instances and volumes starting with your name
- E.g. ‘yiping_cellranger_tmp’
- Make sure volume is in same AZ as instance
- Today you’ll need 200 GiB

Attach and mount the volume to your instance




```
# Find the name of the volume (can be identified by size; it's probably nvme1n1)
lsblk
mkdir vol

# Format the volume to ext4 filesystem
sudo mkfs -t ext4 /dev/nvme1n1

# Mount volume
sudo mount /dev/nvme1n1 vol

# Change owner
sudo chown ubuntu:ubuntu vol
```

Downloading raw data to instance

Today we'll transfer the raw data of patient 5 (melanoma brain metastasis) from S3

```
aws s3 sync s3://workshop-aws-data/raw_data/BI5/ vol/
sudo chown ubuntu:ubuntu vol/*
```

Transfer the Cell Ranger scripts to your instance

```
aws s3 sync s3://workshop-aws-data/code/ ./ --exclude '*' --include 'cellranger*'
```

Downloading data from Genewiz to instance

Usually you'll have to download the raw data from Genewiz

```
# connect to Genewiz remote server (have password ready)
sftp cumc_izar_orders_gmail@sftp.genewiz.com

# go to local destination folder (here vol)
lcd ~/vol

# navigate to the remote folder with your data using ls and cd

# download all files in remote directory to local directory
mget *

# usually you'll receive three fastq files: R1, R2, I1
```

Run Cell Ranger: The main script

```
################## cellranger.sh ##################
#!/bin/sh -x

# provide 'BI5'
PAT=$1

cd vol
date -u > time_${PAT}.log

# execute
cellranger count --id=${PAT} --localcores=16 --localmem=128 \
                 --transcriptome=/home/ubuntu/reference/GRCh38-2020-A_premrna \
                 --fastqs=/home/ubuntu/vol/ --sample=${PAT} --chemistry SC3Pv3

date -u >> time_${PAT}.log
cd ..

# sync out
aws s3 sync /home/ubuntu/vol/${PAT}/ s3://workshop-aws-data/your_name/cellranger/${PAT}/ --exclude "*.fastq.gz" --quiet
#####################################################
```

Shebang: program loader is instructed to run the program /bin/sh, passing path/to/script as the first argument → tells computer how to read the script

Patient name will be passed as first variable, see next slide

Logging the time that Cell Ranger takes

Path to the snRNA-seq reference

threeprime for Single Cell 3′,
fiveprime for Single Cell 5′,
SC3Pv3 for Single Cell 3′ v3,
SC5P-PE for Single Cell 5′ paired-end (both R1 and R2 are used for alignment),
SC5P-R2 for Single Cell 5′ R2-only (where only R2 is used for alignment)

Path to downloaded fastqs

Sync files out to S3 when done

Run Cell Ranger: The wrapper script

- The wrapper script calls the main script in a loop for each sample (makes more sense with multiple samples: `for i in BI1 BI2 BI3 BI4 BI5`)
- `nohup` and `&` make sure the script keeps running when you exit the instance
- The output and error files for each sample can be found in individual files in the home directory

```
################## cellranger_wrapper.sh ##################
#!/bin/sh

cd ~

for i in BI5; do
  nohup cellranger.sh $i > cr_$i.out 2> cr_$i.err &
done
###########################################################
```

Run Cell Ranger

- Make the scripts executable using chmod

```
chmod +x cellranger.sh
chmod +x cellranger_wrapper.sh
```

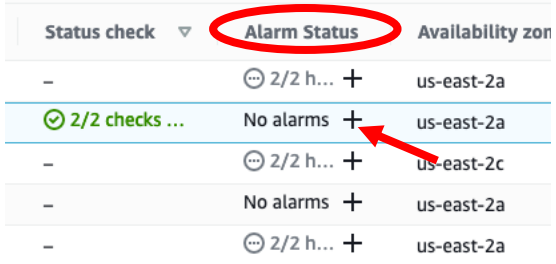
- Execute

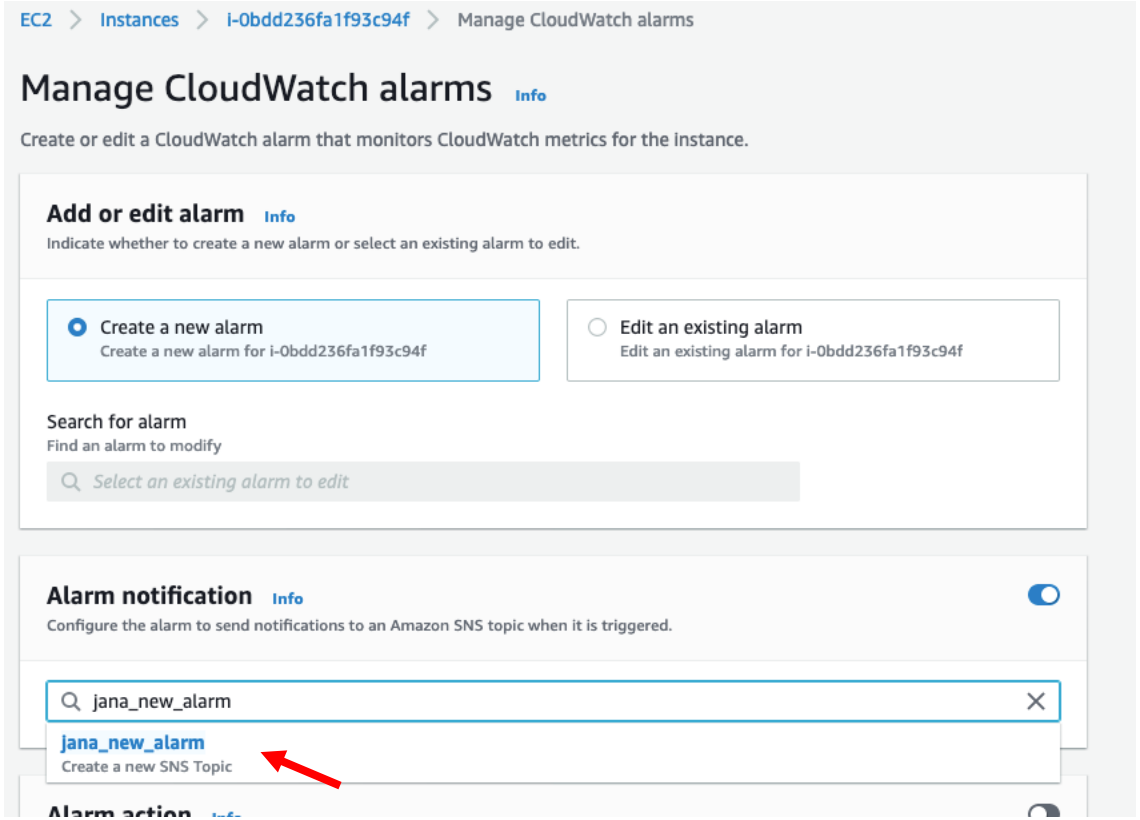
```
./cellranger_wrapper.sh
```

- Check if it's working

```
htop
```

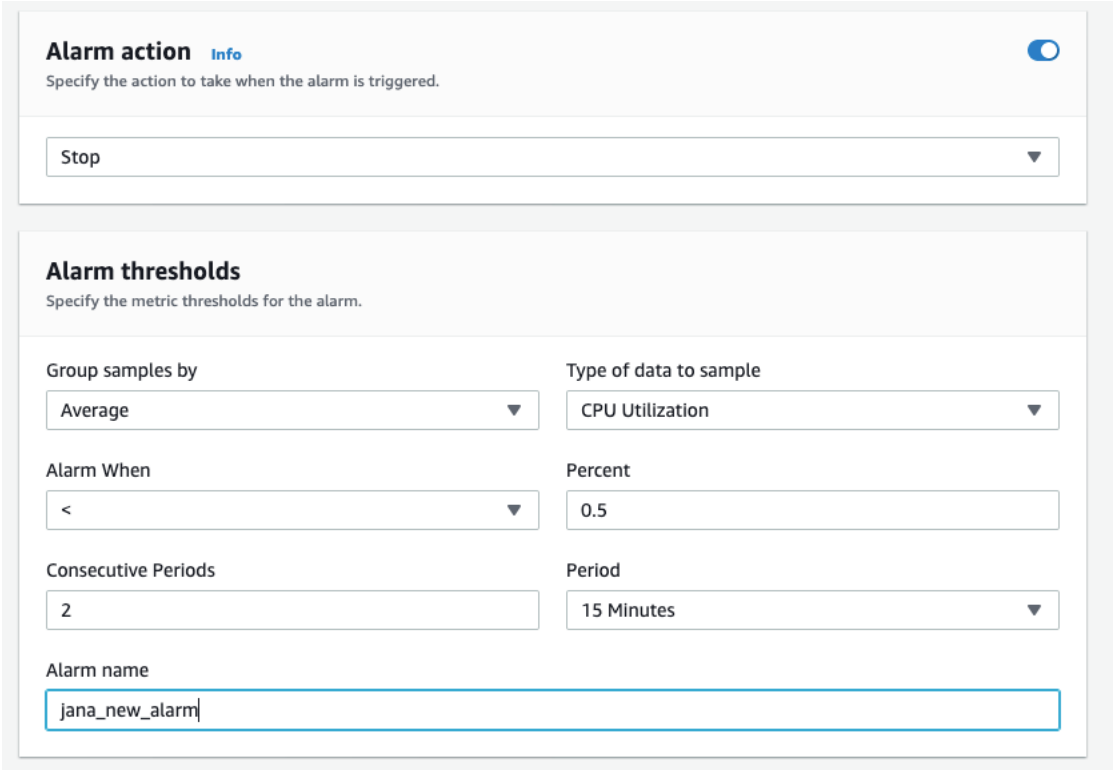
Set an alarm for your instance — Create alarm and notifications



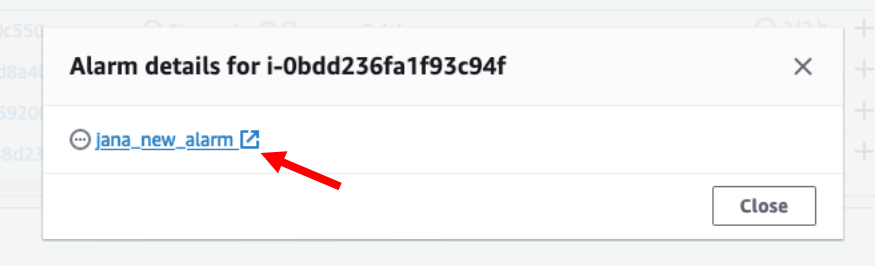


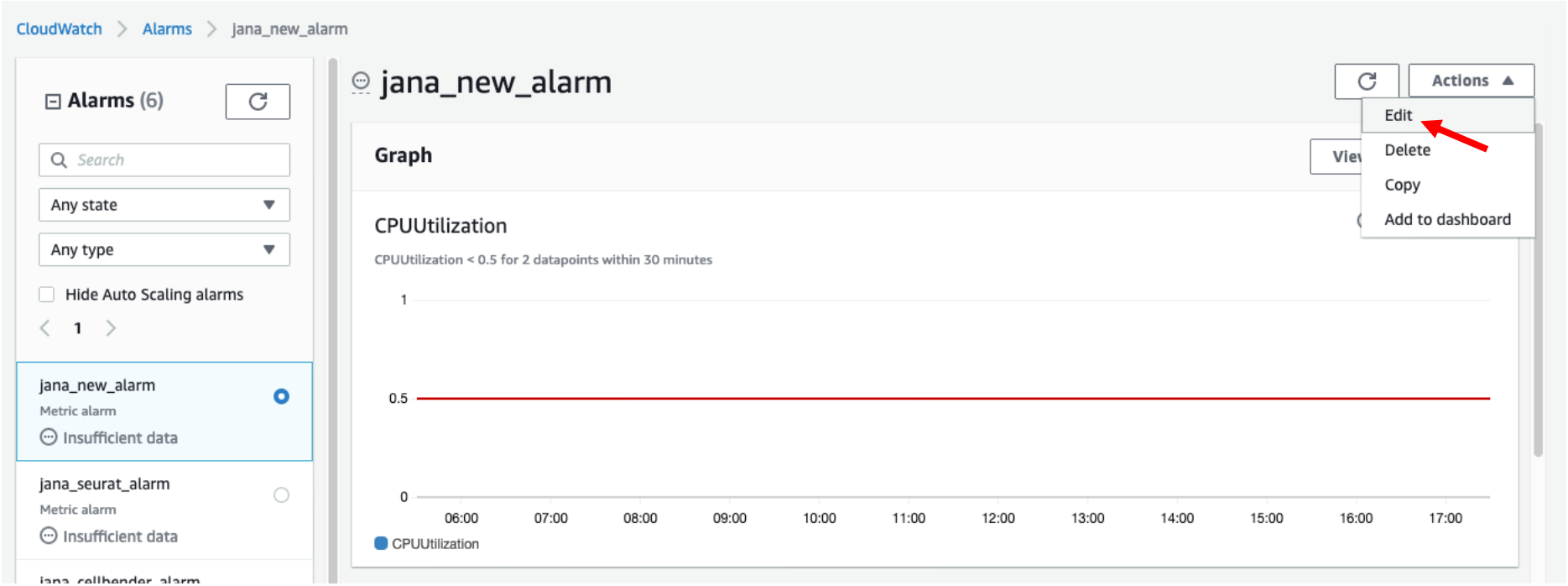
- Always set alarms for your instances to shut down automatically when they are not used anymore
- Forgetting to shut down an instance can become expensive
- You can also create alarms for your volumes

Set an alarm for your instance — Alarm action and threshold

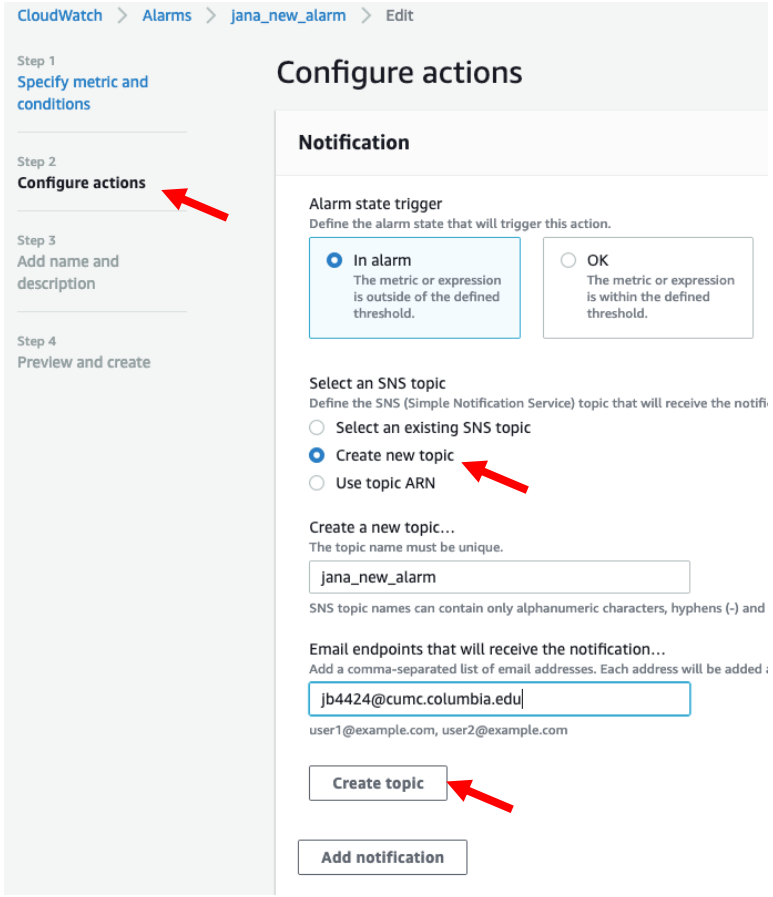


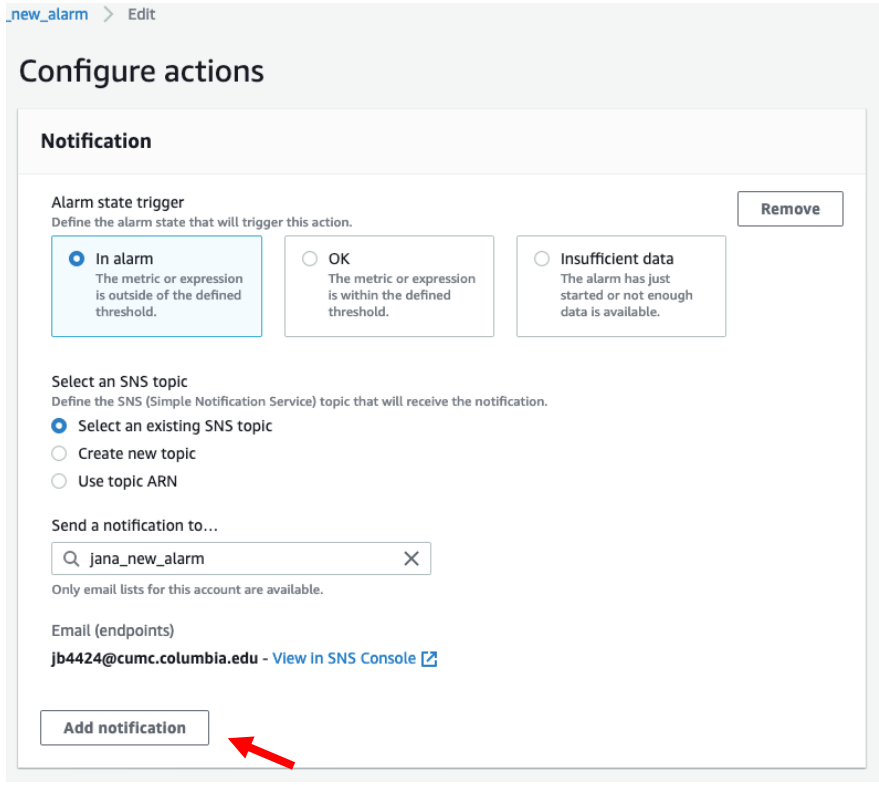
Set an alarm for your instance — Define details



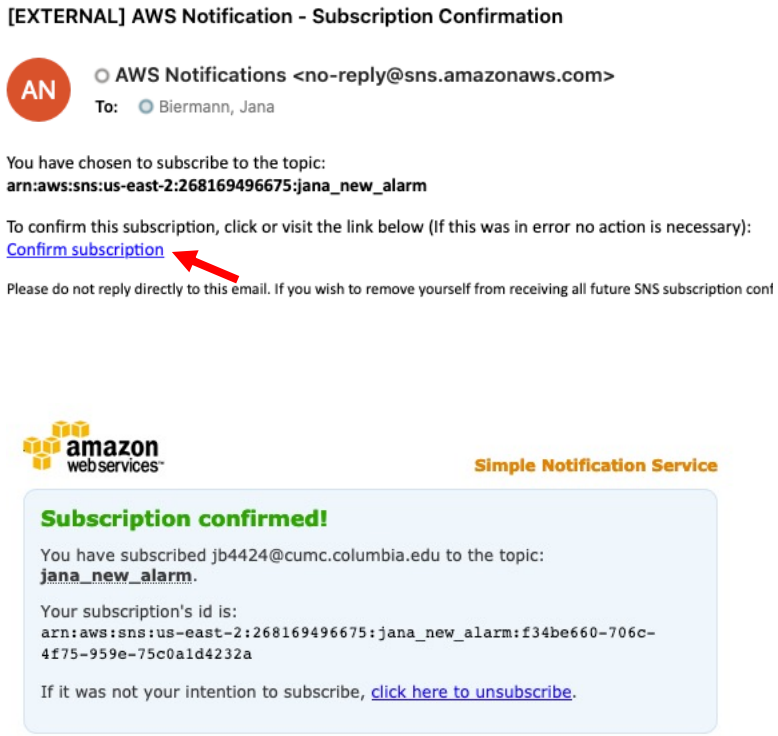


Set an alarm for your instance — Create SNS topic

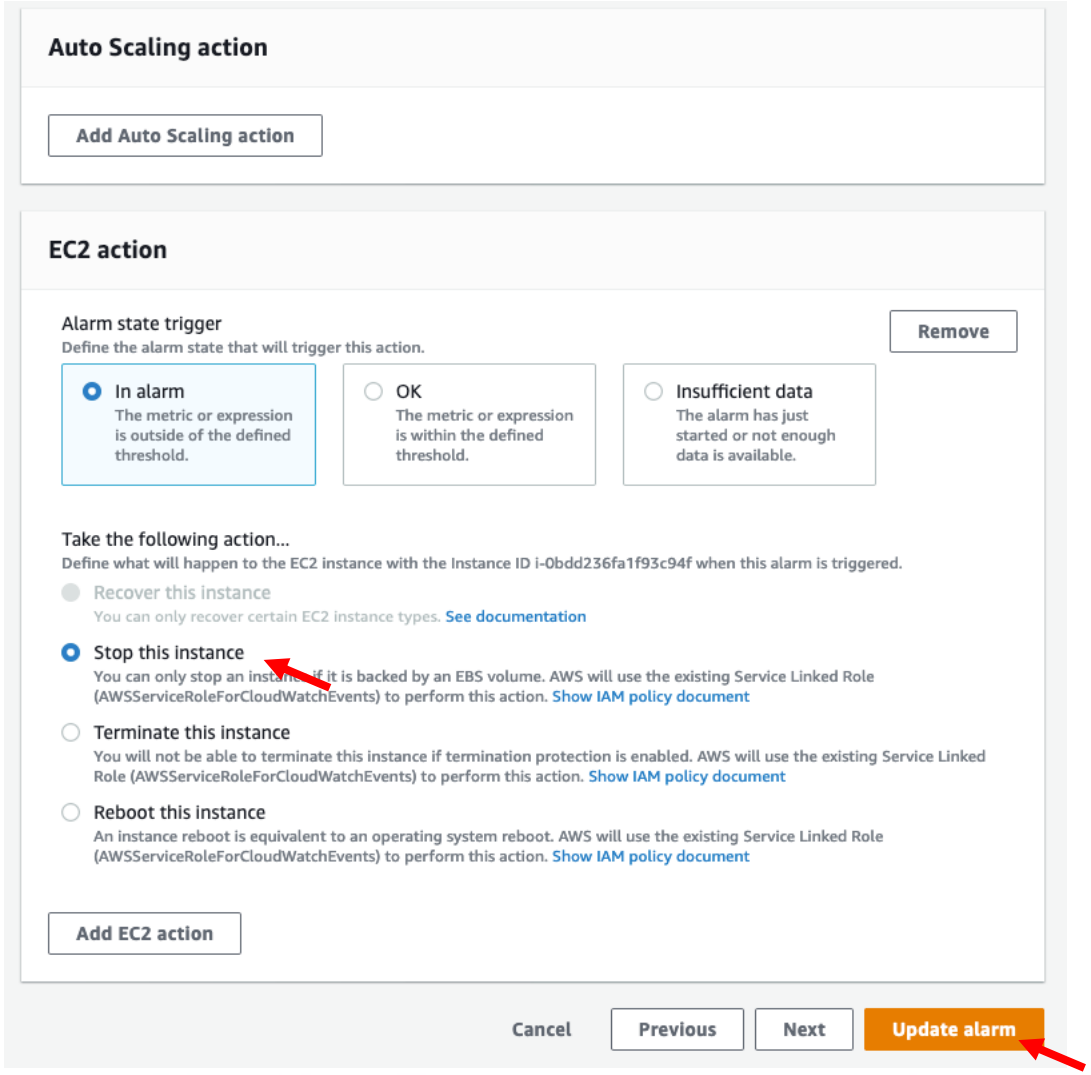




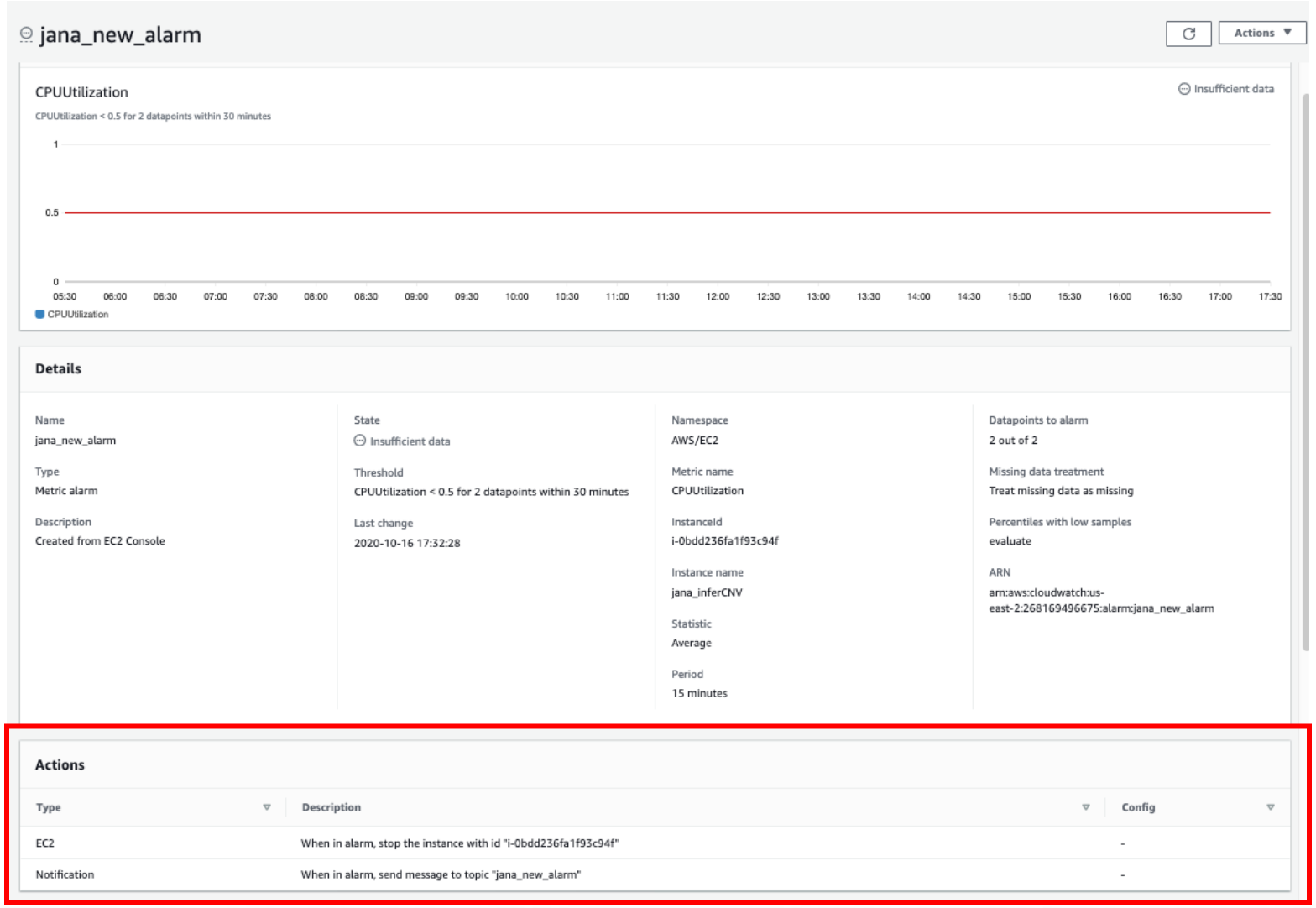
Email:



Set an alarm for your instance — Define EC2 action

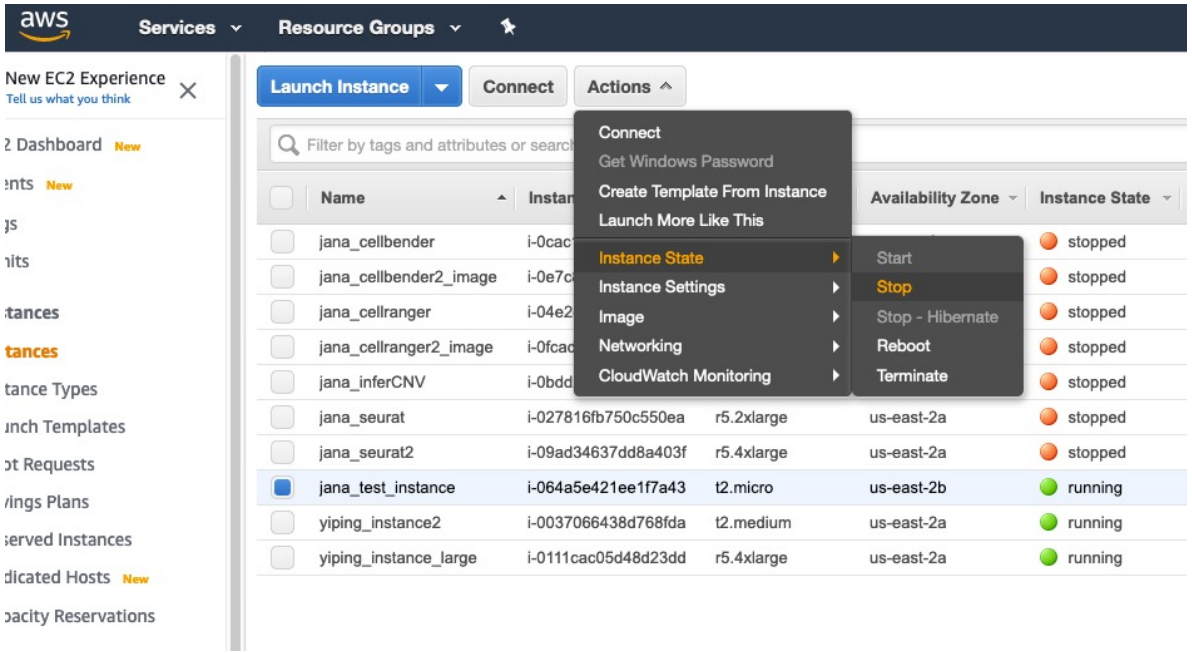


Set an alarm for your instance — Check notifications and actions



Tidying up

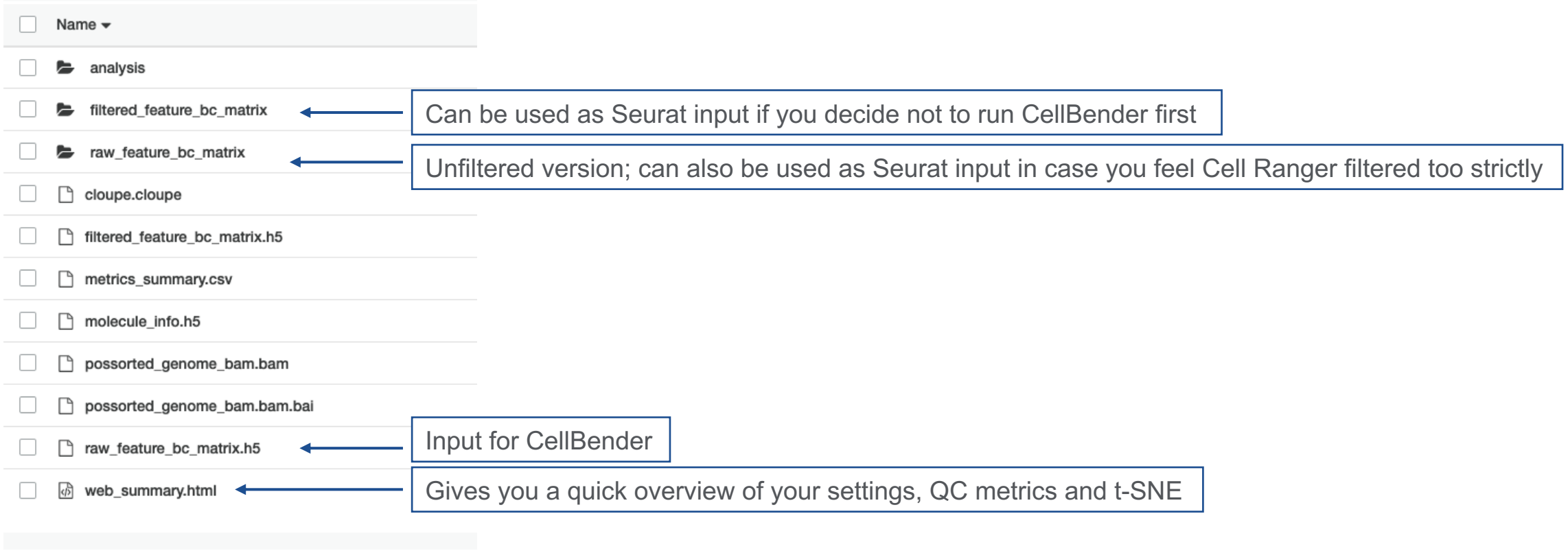
- Stop Cell Ranger on your instance using `htop` and F9
- Terminate instance
- Detach volume
- Delete volume



- Make sure only your instance is selected
- ‘Stop’ will shut down the instance until you start it again
- ‘Terminate’ will delete the instance (and the associated root volume unless you changed the settings)
- When instances are stopped, we only pay for the storage of their volume

Cell Ranger output

s3://workshop-aws-data/example/cellranger/BI5/outs



CELLBENDER

CellBender

- GitHub: https://github.com/broadinstitute/CellBender
- Preprint: https://www.biorxiv.org/content/10.1101/791699v1
- Model learns background RNA profile
- Distinguishes cell-containing droplets from empty droplets
- Generates background-free gene expression profiles

- CellBender requires access to Graphics Processing Units (GPUs)
  - GPU instance is recommended for most deep learning purposes
  - Training new models will be faster on a GPU instance than a CPU instance
  - Enable more parallelism for higher throughput on compute-intensive workloads

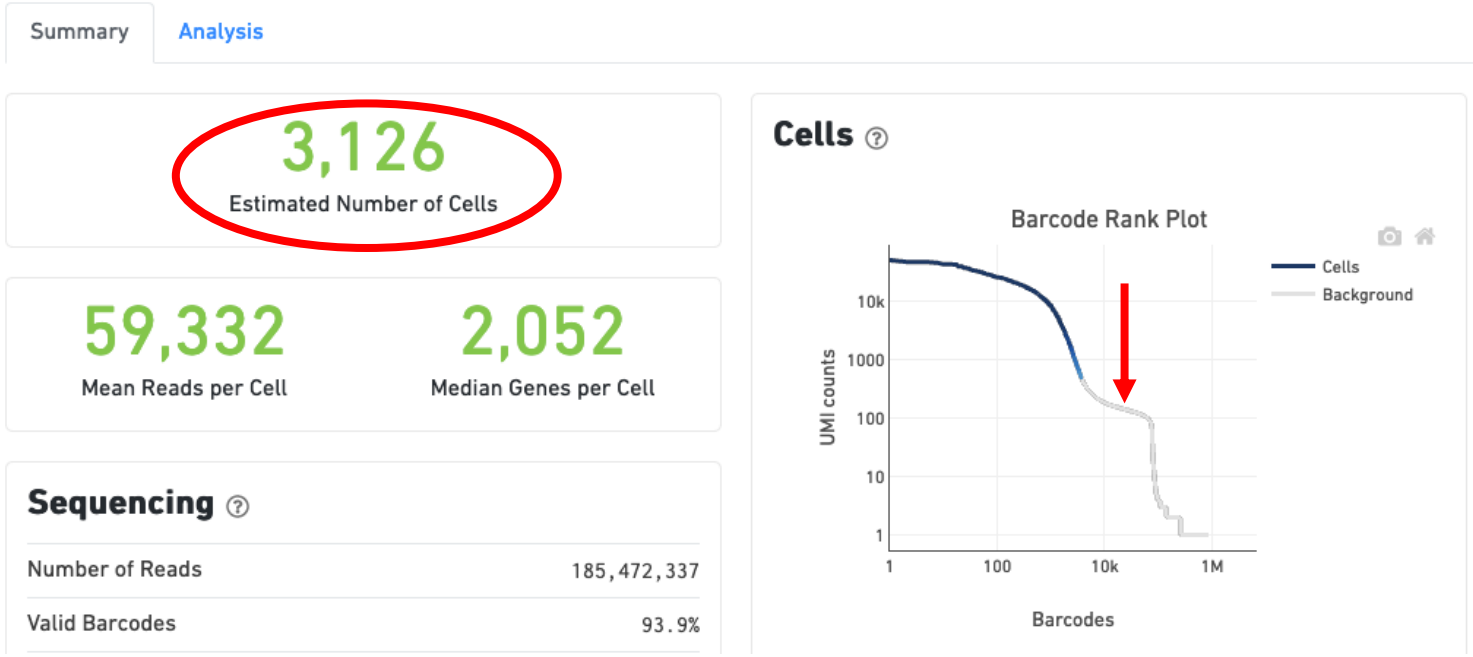
- CellBender uses CUDA (Compute Unified Device Architecture)
  - Parallel computing platform and application programming interface (API) model created by Nvidia
  - Allows to use a CUDA-enabled GPU for general purpose processing
  - CUDA platform is a software layer that gives direct access to the GPU's virtual instruction set and parallel computational elements

Getting the parameters for CellBender

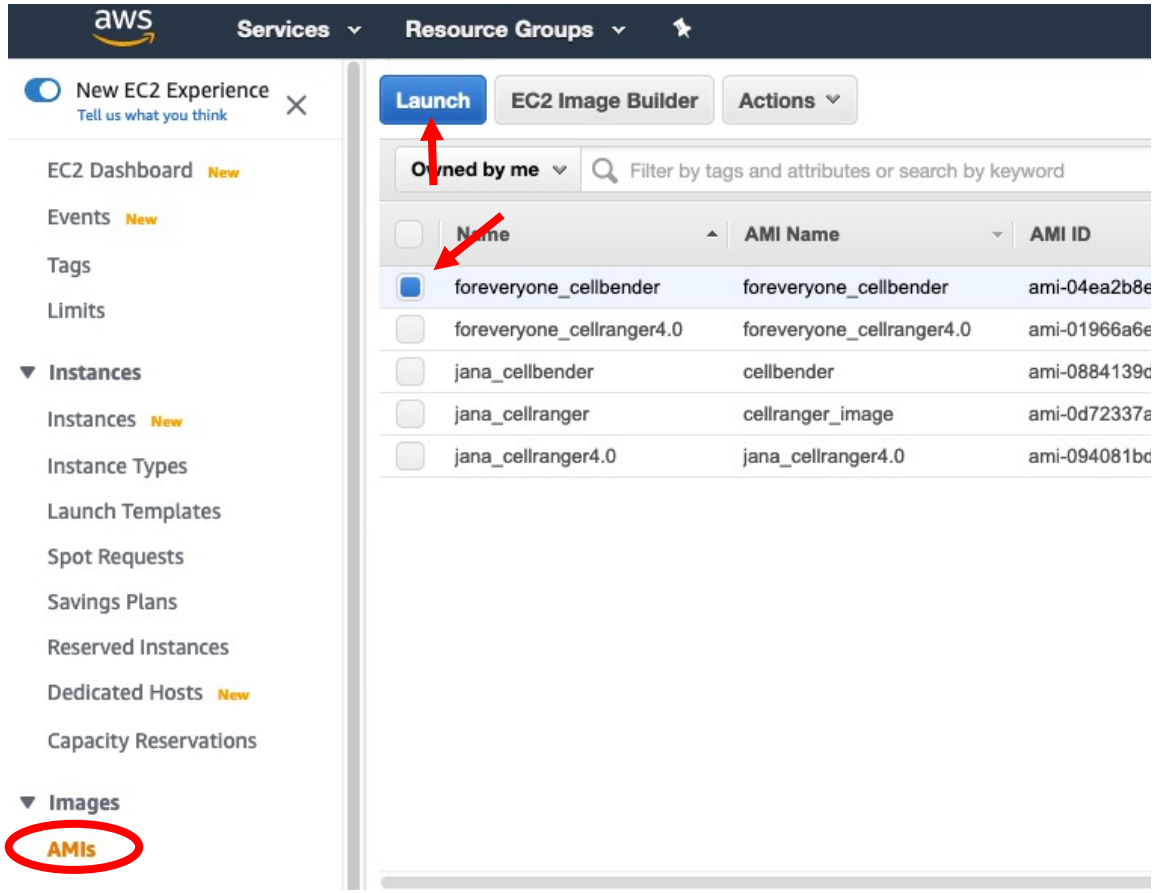
- Open web summary in S3:

workshop-aws-data/example/cellranger/BI5/outs/web_summary.html

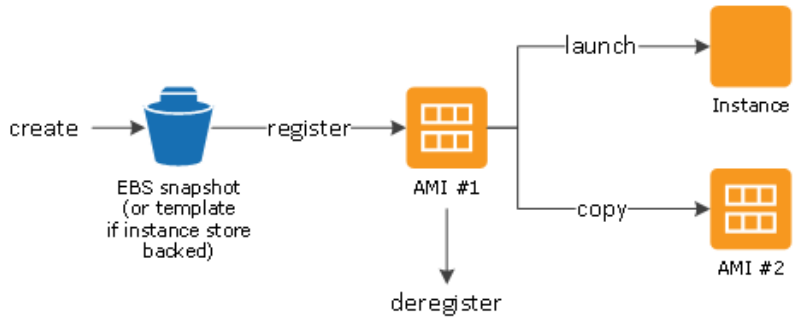
- You will need to look up two parameters for each sample individually:
  - Estimated Number of Cells: ~3,100
  - Total droplets included: 12,000



Starting an AMI CellBender instance



- Amazon Machine Image (AMI):
  - Provides the information required to launch an instance
  - EBS snapshots serve as a template for the root volume of the instance (for example, an operating system, an application server, and applications)



- Choose instance p2.xlarge

Setting up the CellBender instance

1. Update your instance
2. Add your AWS credentials
3. Verify that your instance has a GPU

```
lspci | grep -i nvidia
```

4. Activate the Conda environment

```
conda activate CellBender
```

5. Transfer the CellBender scripts to your instance

```
aws s3 sync s3://workshop-aws-data/code/ ./ --exclude '*' --include 'cellbender*'
```

Run CellBender: The main script

```
################## cellbender.sh ##################
#!/bin/sh

pat=$1
cells=$2
drop=$3

aws s3 sync s3://workshop-aws-data/example/cellranger/${pat}/outs/ data/${pat}/ --exclude '*' --include 'raw_feature_bc_matrix.h5'
sleep 15

cellbender remove-background \
                --input data/${pat}/raw_feature_bc_matrix.h5 \
                --output data/${pat}/${pat}.h5 \
                --expected-cells ${cells} \
                --total-droplets-included ${drop} \
                --cuda \
                --epochs 300

aws s3 sync data/${pat}/ s3://workshop-aws-data/your_name/cellbender/${pat}/ --exclude "*.out" --exclude '*.err' --exclude 'raw_feature*' --exclude
'.*' --quiet
#####################################################
```

Take the sample name, number of cells and droplet cut-off from the provided arguments (see next slide)

Transfer the raw matrix from S3

Obtain the number of cells and droplet cut-off from the provided arguments

Transfer the results to your S3 folder

Run CellBender: The wrapper script

```
################## cellbender_wrapper.sh ##################
#!/bin/sh

pat=$1
cells=$2
drop=$3

nohup ./cellbender.sh $pat $cells $drop > cb_$pat.out 2> cb_$pat.err &
###########################################################
```

- Execute

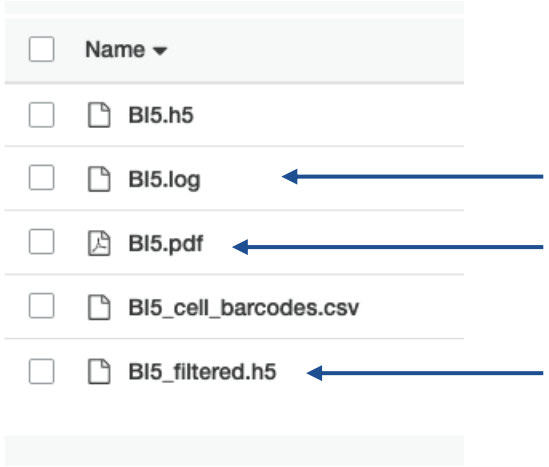
Provided arguments with the script:
$1 -> patient name
$2 -> expected-cells
$3 -> total-droplets-included

```
./cellbender_wrapper.sh BI5 3100 12000
```

Execute this script located in the current directory

CellBender output

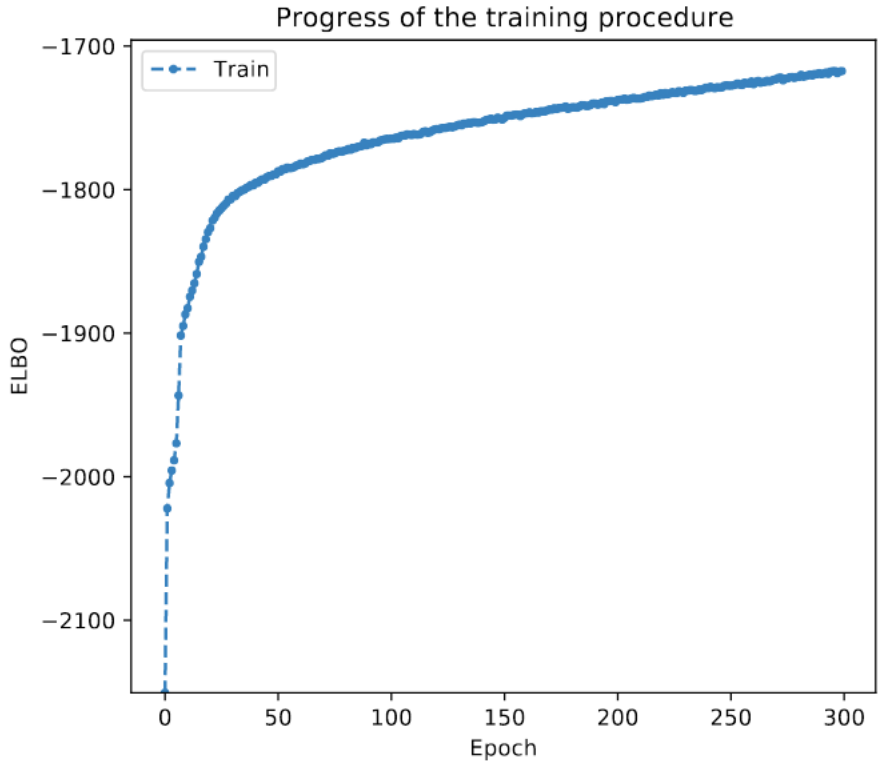
s3://workshop-aws-data/example/cellbender/BI5/



Check the log file to see if there were any problems during the run

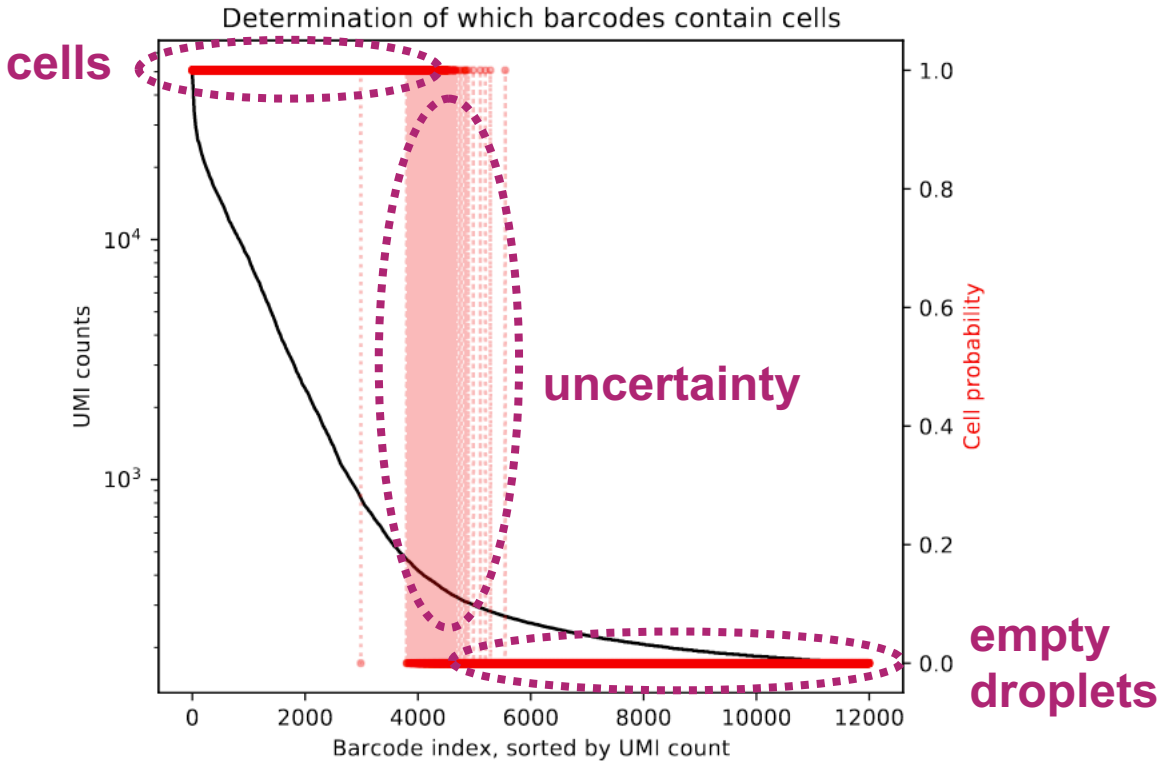
QC of modeling results

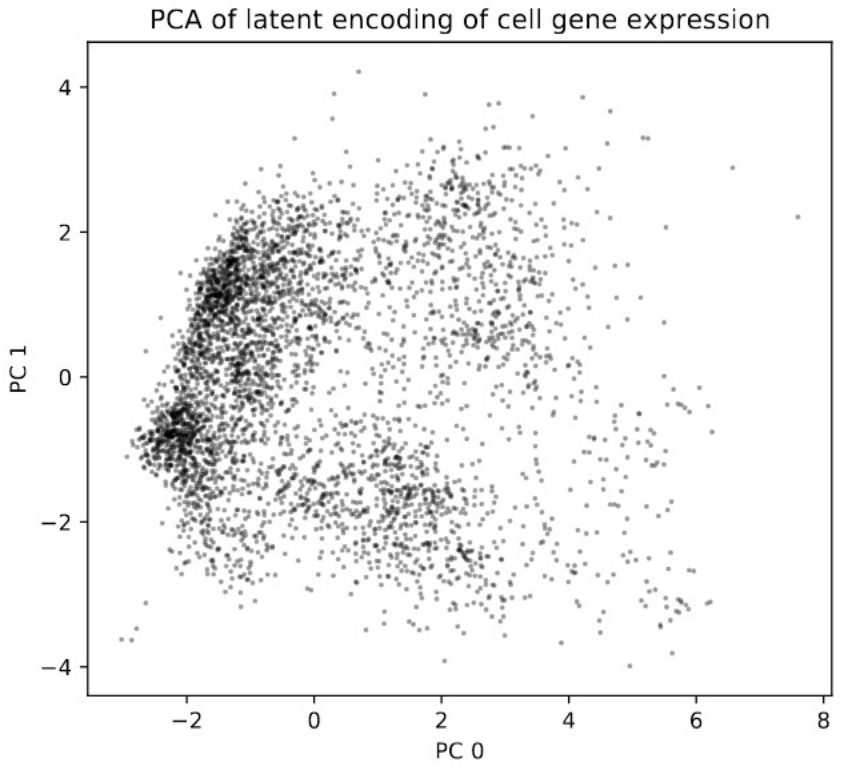
Input for Seurat



- The first graph should be approaching a saturation threshold
- Otherwise increase number of epochs (will take longer)

CellBender output





- The second plot shows the distribution of cells and empty droplets and the certainty of classification as either

- The third plot ideally shows several separate clusters (depends on what you sequenced)

SEURAT

Setting up Seurat

- We'll analyze the sample locally in Rstudio today
- Download the file BI5_filtered.h5 from the CellBender output and save it locally in ~/AWS_workshop/data/BI5
- Please name the folders exactly as suggested above
- Move the files seuratworkshop.R, scrublet_code.py, and dummy_signatures.csv into the folder ~/AWS_workshop/
- Open seuratworkshop.R using Rstudio

Loading single-cell data into Seurat

```
library(Seurat)
library(ggplot2)
library(Matrix)
library(SingleR)
library(SingleCellExperiment)
library(pheatmap)

#samplename is prefix of h5 file, for example BI5 in BI5_filtered.h5
#directoryname is name of folder where scripts, in this case ~/AWS_workshop
#h5 data file is then expected to be in the directory ~/AWS_workshop/data/BI5
#doubs is expected doublet rate, for later scrubbing of doublets
samplename <- “BI5”
directoryname <- “~/AWS_workshop”
doubs <- as.numeric(commandArgs()[3]) #.04 for this workshop

# Load dataset with Read10x_h5
#first argument is file name
#use.names = TRUE makes rows of the data be labeled with gene names, not gene ids
#unique.features = TRUE changes gene names in data, if necessary, to make them unique
seu.data <- Read10X_h5(paste0(directoryname,"/data/",samplename,”/”,samplename,"_filtered.h5"), use.names = TRUE, unique.features = TRUE)

# Initialize the Seurat object with the raw (non-normalized data)
#project argument sets project name
#min.cells = 1, include genes that occur in at least one cell
#min.features = 1, include cells that have at least one gene
seu_raw <- CreateSeuratObject(counts = seu.data, project = samplename, min.cells = 1, min.features = 1)

#include patient name metadata
seu_raw[["patient"]]<-samplename
```

Quality control filtering

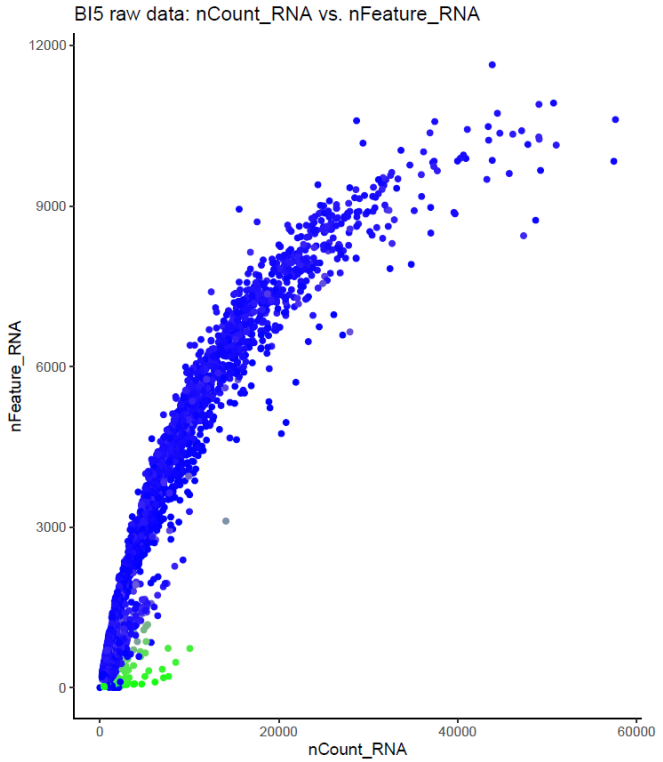
```
#determine mitochondrial percentage
#mitochondrial gene names begin with MT-
#PercentageFeatureSet finds percentage of all genes that start with MT-
#store in new variable percent.mt
seu_raw[["percent.mt"]] <- PercentageFeatureSet(seu_raw, pattern = "^MT-")

# identify doublets using scrublet
#expected_doublet_rate of .04 is based on experimental data, accurate value helps to guide search for true doublets
#use writeMM to write out raw count data to a file
#use a python script to read in the count file, run scrublet to remove doublets, and write out the result to another file
#read in results from that file back into R
matrixfilename = paste0(directoryname,"/",samplename,"_MM.txt")
scrubletoutputfile = paste0(directoryname,"/",samplename,"_doublets.txt")
writeMM(seu_raw@assays$RNA@counts,file=matrixfilename)
system(paste0("python3 scrublet_code.py ",matrixfilename," ",doubs," ",scrubletoutputfile))
scrubletoutput = read.table(scrubletoutputfile,header=T,quote=NULL)
#store doublet status, and score for strength of doublet prediction, in two new variables in seu_raw
seu_raw[['ScrubDoublet']]<-scrubletoutput$predicted_doublets
seu_raw[['ScrubDoublet_score']]<-scrubletoutput$doublet_scores

#use subset function to filter data based on min and max number of features (genes),
# min and max number of reads (counts), maximum mitochondrial percentage, and removing all doublets
minFeature<-200
maxFeature<- 7500
minCount<- 100
maxCount<- 40000
maxMT<-10
seu <- subset(seu_raw, subset = nFeature_RNA > minFeature & nFeature_RNA < maxFeature & nCount_RNA > minCount
        & nCount_RNA < maxCount & percent.mt < maxMT & ScrubDoublet ==F)
```

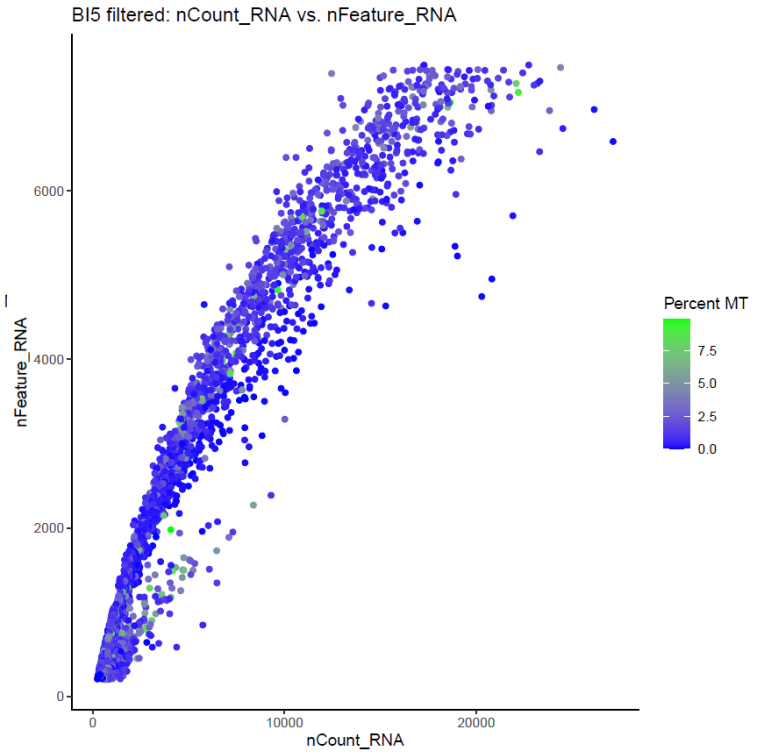
Scrublet filtering with Python

```
#!/usr/bin/env python3

import sys
import scrublet as scr
import scipy.io
import sys

#sys.arg is a list of words that are given as arguments for Python script
#in this case, three inputs are the name of the input matrix file written by R,
#the expected doublet rate, and the name of the output file to write to
inputfile=sys.argv[1]
doublet_rate=float(sys.argv[2])
outputfile=sys.argv[3]

#read in matrix inputfile with immread function, then format it further with .T and tocsc()
counts_matrix = scipy.io.mmread(inputfile).T.tocsc()
#initialize Scrublet object, using matrix and expected doublet rate
scrub = scr.Scrublet(counts_matrix, expected_doublet_rate=doublet_rate)
#analyze genes with min RNA count number>=1 in >=1 cells
#analyze genes whose variability is at or above the 85th percentile, and take 20 components of the PCA of these variable genes
doublet_scores, predicted_doublets = scrub.scrub_doublets(min_counts=1, min_cells=1, min_gene_variability_pctl=50, n_prin_comps=20)

#write out doublet results to output file
outFI = open(outputfile,'w')
outFI.write('predicted_doublets\tdoublet_scores\n')
for i in range(len(predicted_doublets)):
    outFI.write(str(predicted_doublets[i]).upper()+'\t'+str(doublet_scores[i])+'\n')

outFI.close()
```

Plot nCount vs. nFeature to check for quality control thresholds

```
pdf("workshop_QC_scatter_plot.pdf")

#print scatter plot for seu_raw using qplot function, with x axis being nCount, y nFeature, and color being percent.mt
#xlab, ylab, and main set x axis, y axis, and overall figure title
#scale_colour_gradient controls percent.mt colors
#labs function controls legend label for color
#theme_classic() changes overall graphic appearance
print(qplot(x=seu_raw@meta.data$nCount_RNA,y = seu_raw@meta.data$nFeature_RNA,
col=seu_raw@meta.data$percent.mt, xlab = "nCount_RNA",ylab = "nFeature_RNA", main =paste0(seu_raw@meta.data$orig.ident[1], " raw data: nCount_RNA vs. nFeature_RNA")) +
scale_colour_gradient(low="blue", high="green") + labs(color = "Percent MT") + theme_classic())

#same plot for quality filtered object seu
print(qplot(x=seu@meta.data$nCount_RNA,y = seu@meta.data$nFeature_RNA, col=seu@meta.data$percent.mt,
xlab = "nCount_RNA",
ylab = "nFeature_RNA", main =paste0(seu@meta.data$orig.ident[1], " filtered: nCount_RNA vs. nFeature_RNA")) +
scale_colour_gradient(low="blue", high="green") + labs(color = 'Percent MT') + theme_classic())

dev.off()

#filtering low feature/count cells removes technical noise
#filtering high feature/count cells remove likely doublets
```



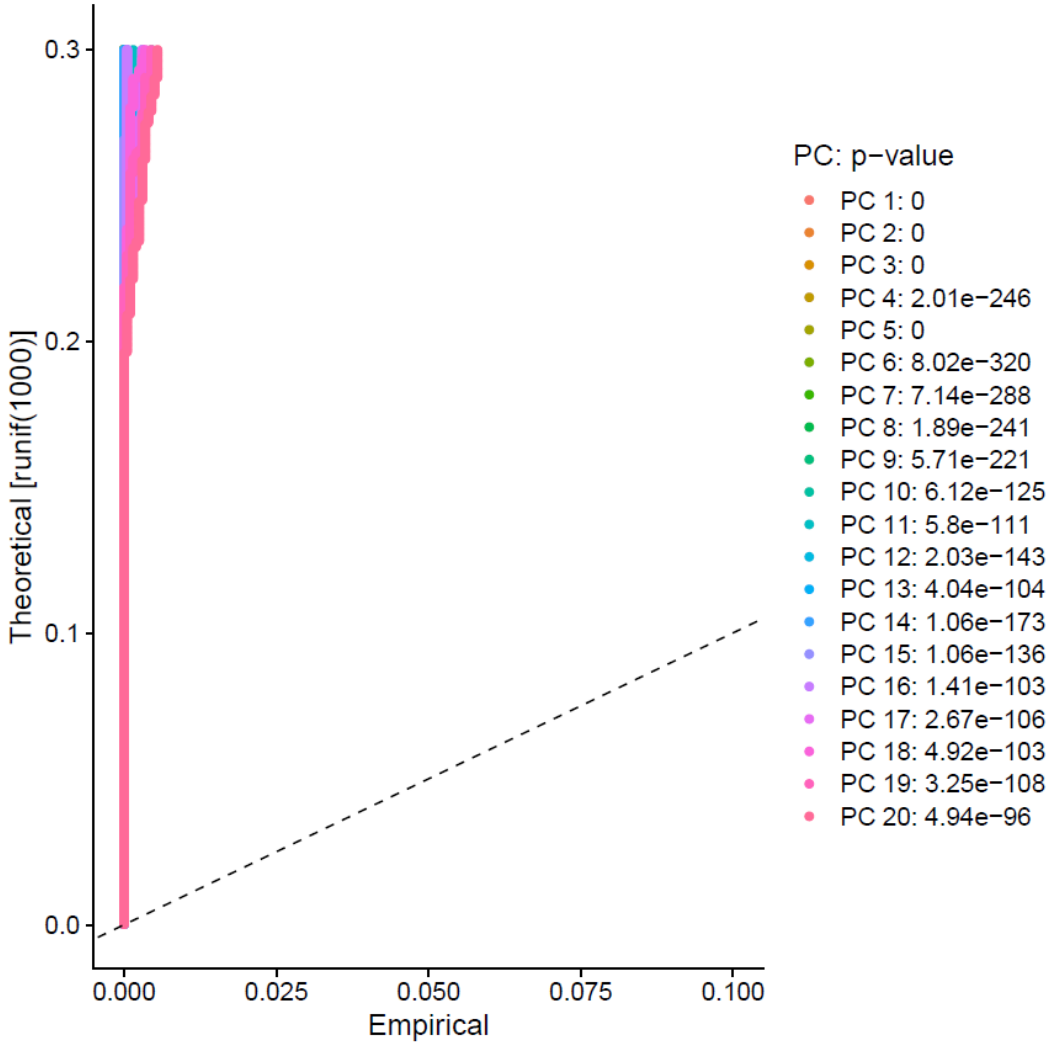


Canonical workflow to normalize data, and run PCA and UMAP

```
# normal workflow
#Log-normalizes data
seu <- NormalizeData(seu)

#find 2000 most variable genes in data
seu = FindVariableFeatures(seu, selection.method = "vst", nfeatures = 2000)

#shifts mean expression across cells 0, variance across cells to 1
seu <- ScaleData(object = seu)

#run PCA, to map cells to 2D PCA coordinates
seu <- RunPCA(object = seu, npcs = 100)

#Using dimensions 1-15 of the pca reduction, find closest neighboring cells to each single cell in dataset
seu <- FindNeighbors(seu, reduction = "pca", dims = 1:15)

#determine number of clusters within dataset, based on neighbors found above
seu <- FindClusters(seu)

#run UMAP, to map cells to 2D UMAP coordinates, using dimensions 1-20 of the pca reduction
seu <- RunUMAP(object = seu, dims = 1:20)
```

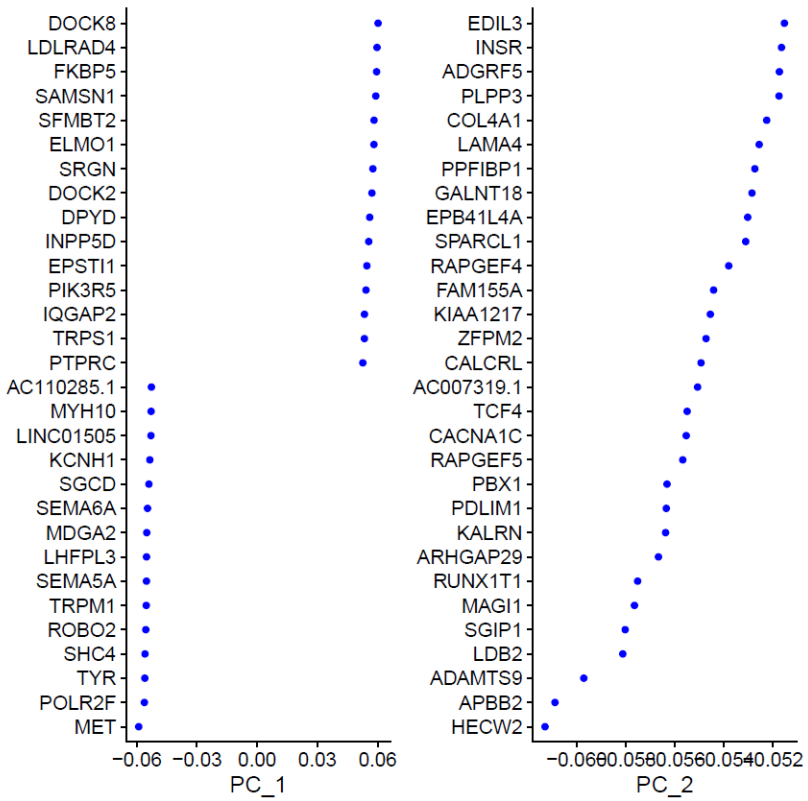
Determining number of significant PCs with Jackstraw

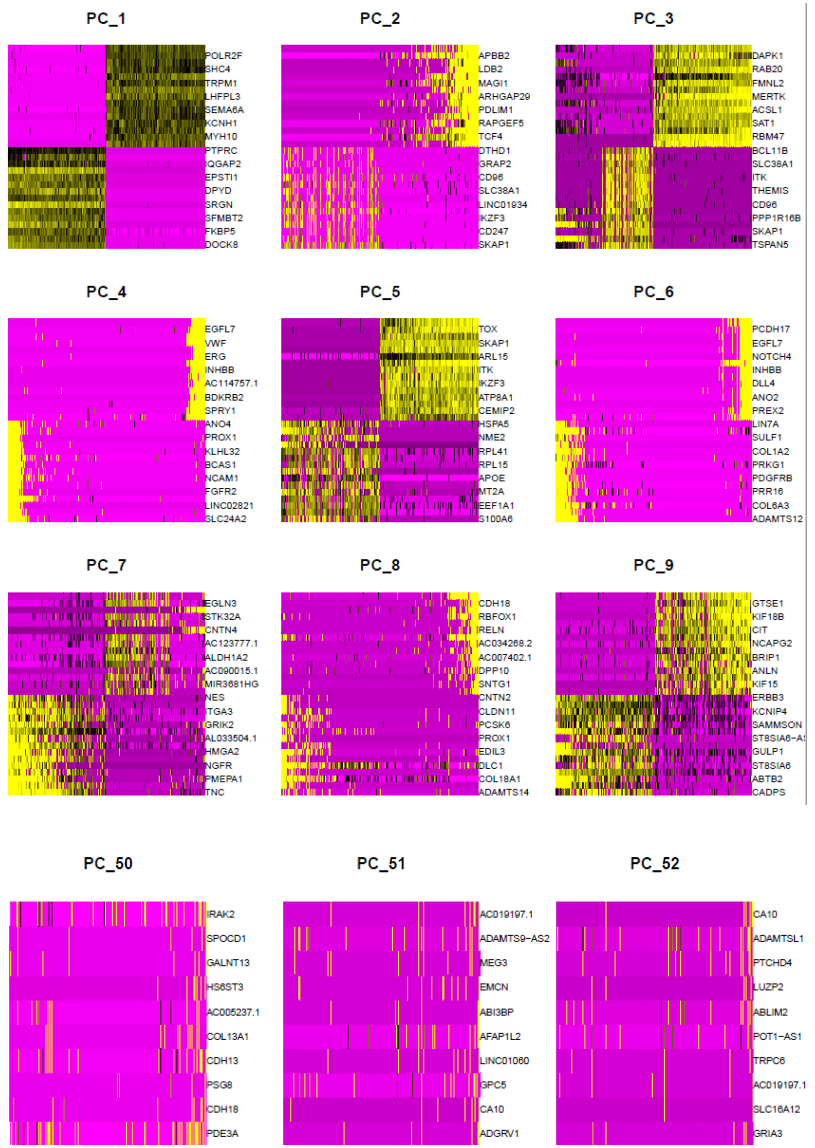
```
#Jackstraw procedure permutes expression of a subset of genes (default 1%)
#permute 100 times in num.replicate argument below
#rerun PCA, and add jackstraw scores for top 20 dimensions
seu <- JackStraw(seu, num.replicate = 100)
seu <- ScoreJackStraw(seu, reduction = "pca",dims = 1:20)

#For each PC, can then determine percentage of genes are associated with the
PC in permuted data, plotted on X axis
#On Y axis, plot percentage of genes associated with the PC in actual data
#Significant PCs are enriched in significantly associated genes, placed towards
left edge of graph
pdf("workshop_jackstraw_plot.pdf")
print(JackStrawPlot(seu, dims = 1:20))
dev.off()
```



Print heatmap of genes on each end of each PCA component

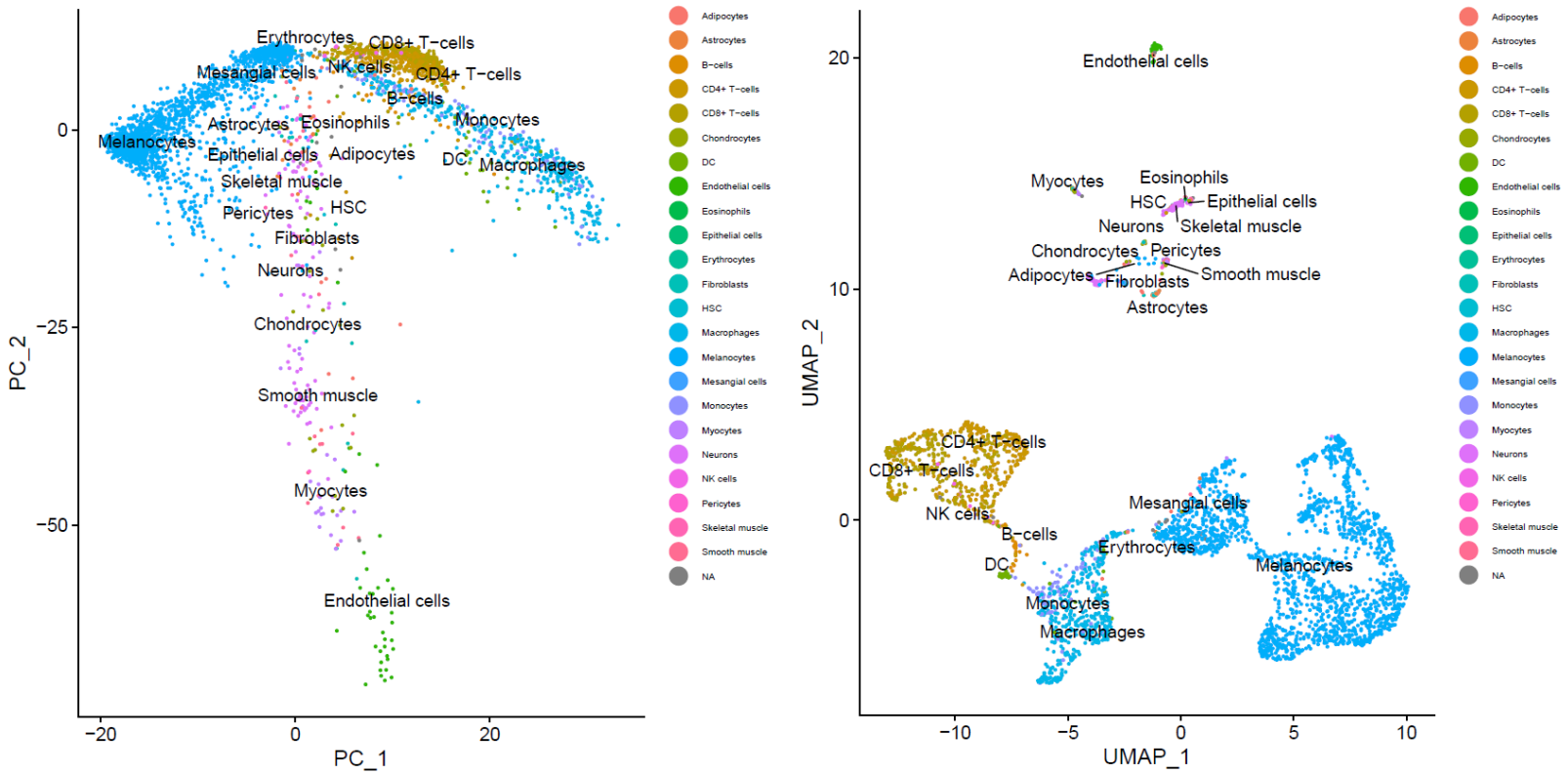
```
#print PCA loadings for components 1 and 2
pdf("workshop_pca_analysis.pdf")
print(VizDimLoadings(seu, dims = 1:2,
reduction = "pca"))

#cells are ordered along PCA component
#print heatmap of gene expression for 500
cells of seu, using balanced=TRUE, which
selects half from the top of a PCA
component, half from the bottom
#use PCA dimensions 1-9 for good PCs with
structure, or 50-52 for bad ones without
structure
print(DimHeatmap(seu, dims = 1:9, cells =
500, balanced = TRUE))
print(DimHeatmap(seu, dims =50:52, cells =
500, balanced = TRUE))
dev.off()
```





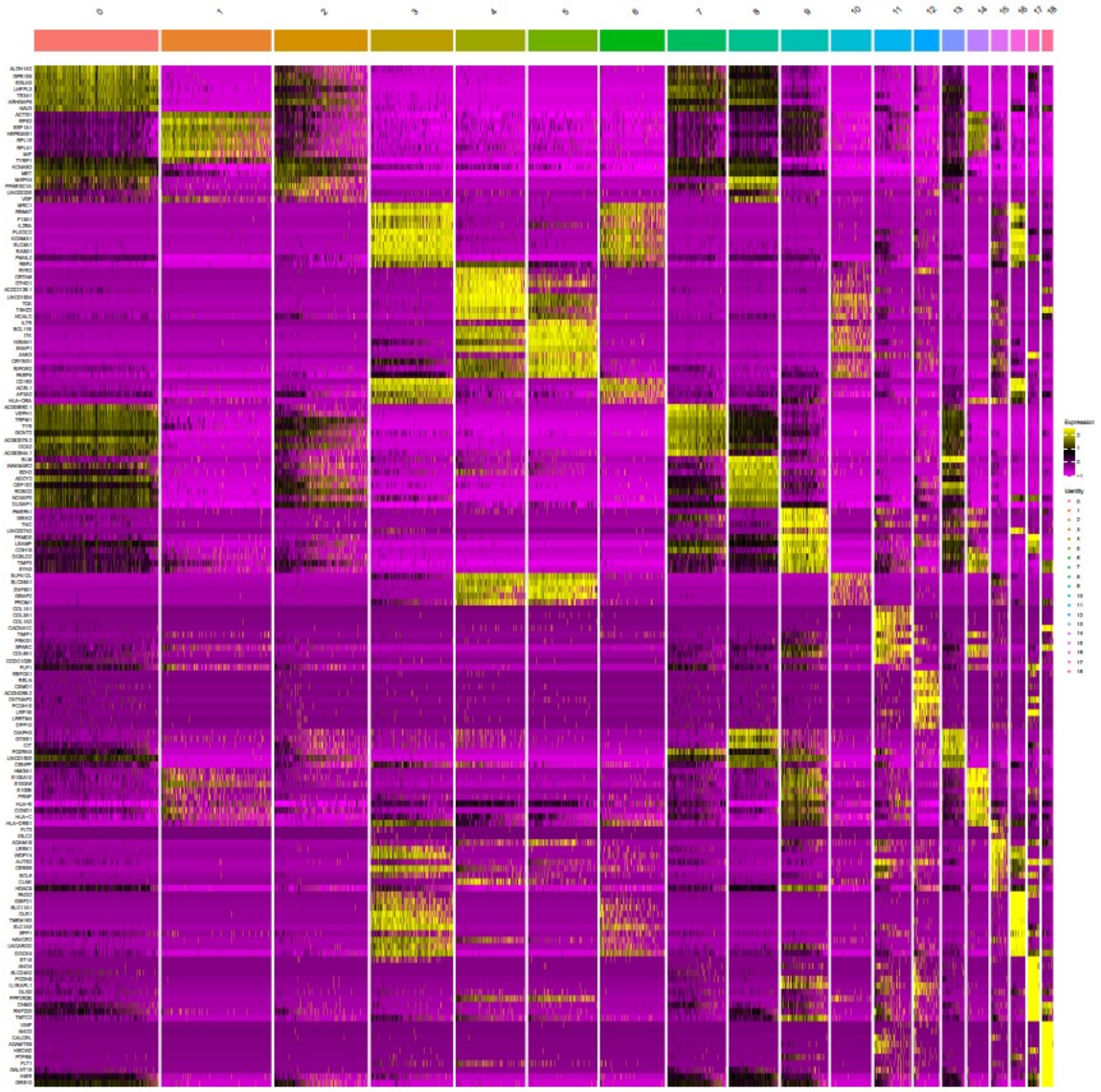
Print PCA and UMAP visualizations of single cell data

```
#plot umaps and pcas in single file
#pdf function creates a new file called seu_integrated.pdf
pdf(file = "workshop_umap_and_pca.pdf")

#print function plots a new figure on each page of the pdf
#DimPlot plots a 2D PCA or UMAP reduction of seu
#points in PCA or UMAP are colored by subset given in group.by argument
#label argument controls whether the groups are labeled in the figure
#guides and theme function are to format the legend, so it is a single column of labels, and takes less space in the figure
print(DimPlot(seu, reduction = "pca",label = T, group.by = 'celltype_bped_main') + guides(col = guide_legend(nrow = 30,override.aes = list(size=5))) +
theme(legend.text=element_text(size=6)))

print(DimPlot(seu, reduction = "umap",label = T,group.by = 'celltype_bped_main') + guides(col = guide_legend(nrow = 30,override.aes = list(size=5))) +
theme(legend.text=element_text(size=6)))

dev.off()
```



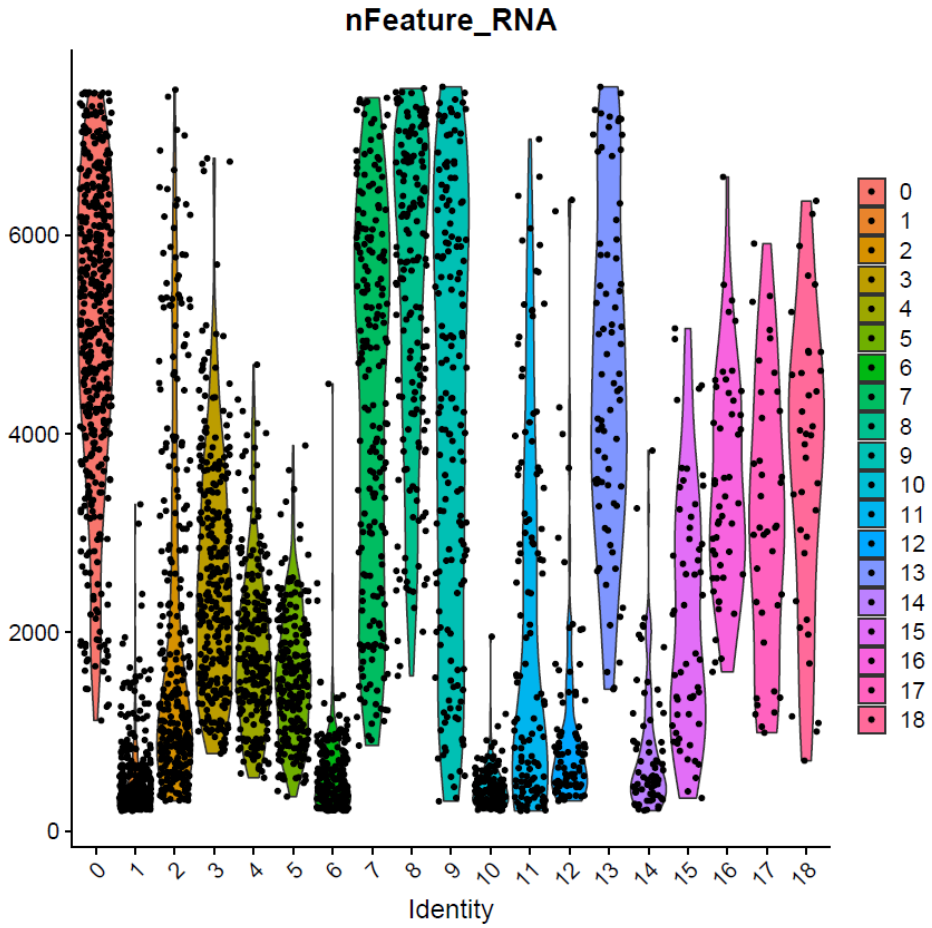
Find markers of differential expression

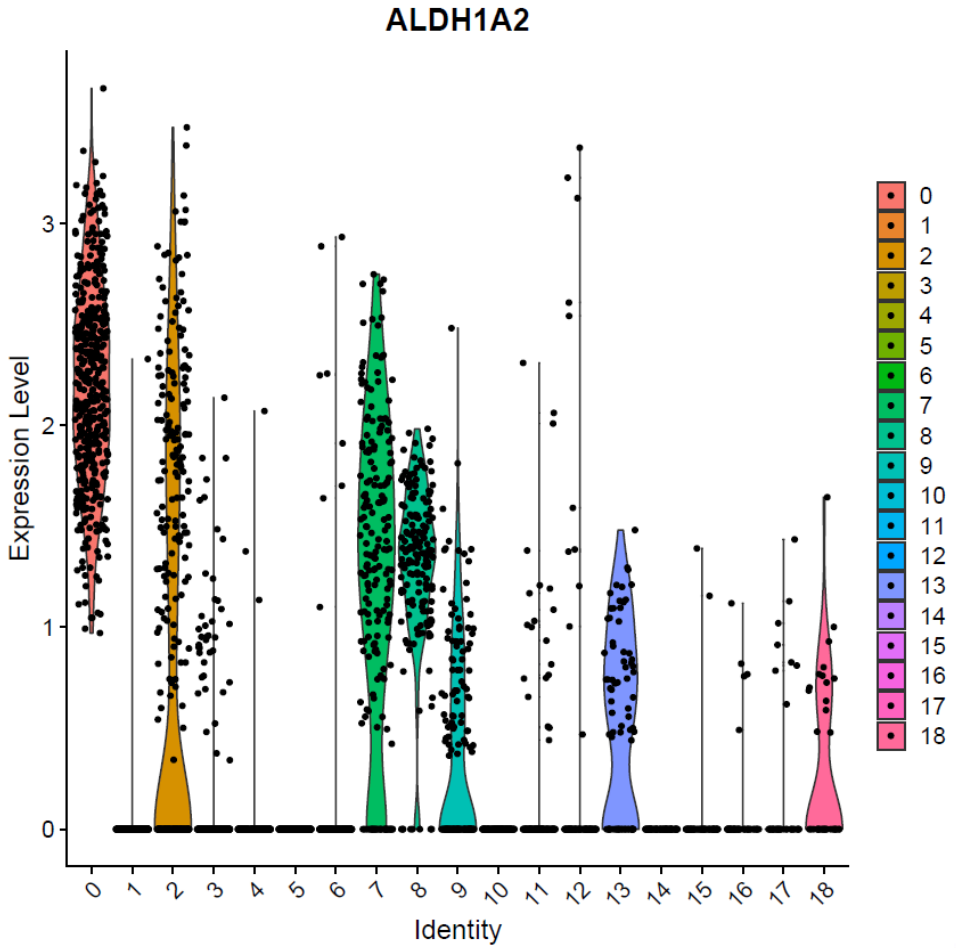
```
#find markers of differential expression between cluster 1 and all other cells
#only.pos controls whether only positive DE markers are found, or negative as well
#min.pct controls what percentage of cells must have non-zero expression of the marker
#logfc.threshold controls minimum log-fc threshold
cluster1.markers <- FindMarkers(seu, ident.1 = 1, min.pct = 0.25)

#find markers of differential expression between cluster 1 and cluster 2
cluster1vs2.markers <- FindMarkers(seu, ident.1 = 1, ident.2 = 2, min.pct = 0.25)

#find markers of differential expression for all clusters relative to each other
seu.markers <- FindAllMarkers(seu, only.pos = F, min.pct = 0.25, logfc.threshold = 0.25)

#determine top 10 markers in each cluster, ordered by avg_logFC
top10 = seu.markers %>% group_by(cluster) %>% top_n(n = 10, wt = avg_logFC)

#write top 100 markers to file
write.csv(top10,
paste0(directoryname,"/",samplename,"_allmarkers.csv"),row.names = F)

#print heatmap of top 10 cluster gene expression
#print figure 4x as large (default width and height is 7) to make labels display properly
pdf("workshop_top10_heatmap.pdf",width=28,height=28)
print(DoHeatmap(seu, features = top10$gene) + NoLegend())
dev.off()
```



Print violin plots of quality metrics and gene expression

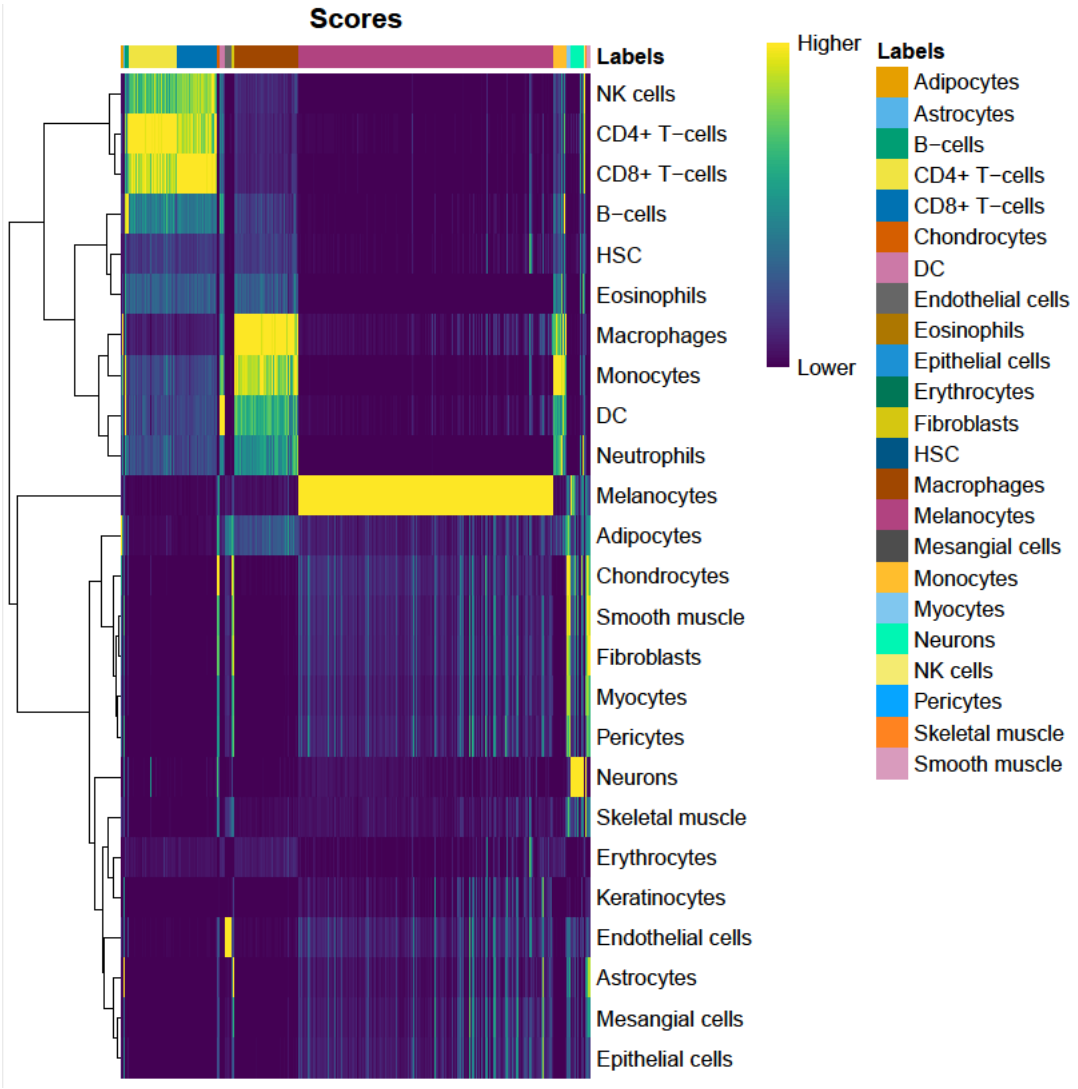
```
pdf("workshop_vlnplots.pdf")
print(VlnPlot(seu, features = c("nFeature_RNA")))
print(VlnPlot(seu, features = c("ALDH1A2")))
dev.off()
```





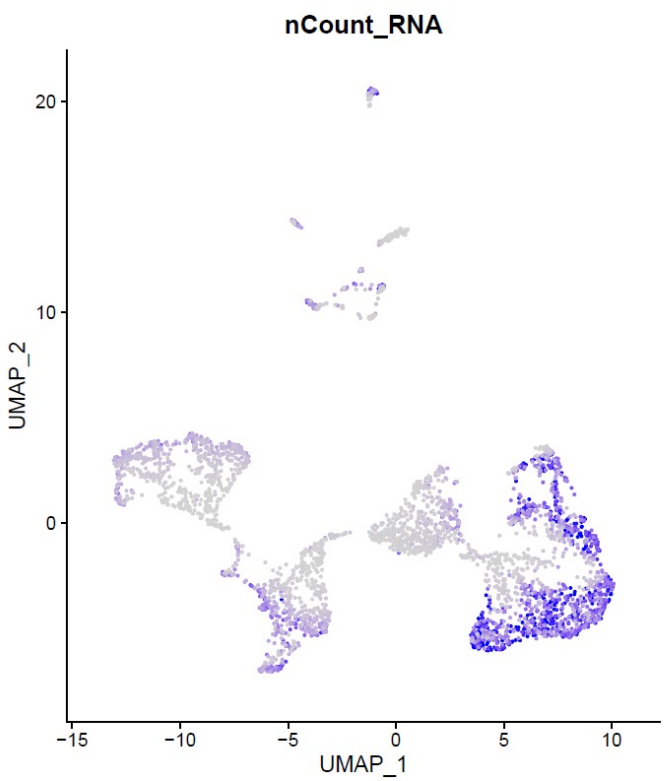
Annotate single-cell data with cell type classifications, using SingleR reference dataset

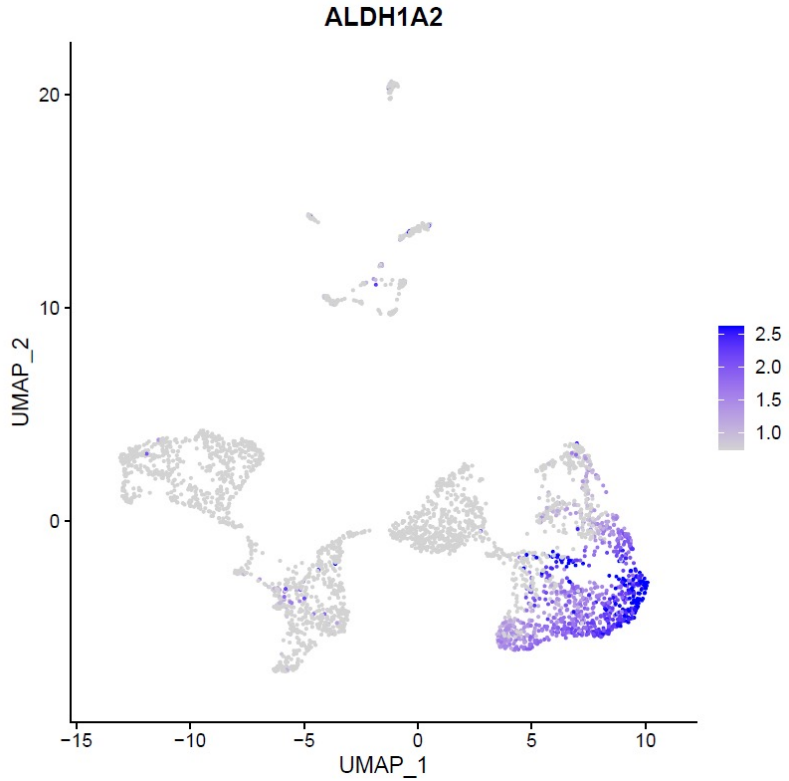
```
#load a reference celltype gene expression dataset using BlueprintEncodeData, store it in bped
bped <- BlueprintEncodeData()

#convert seu to a SingleCellExperiment variable seu_sce, for celltype assignment
seu_sce <- as.SingleCellExperiment(seu)

#use SingleR to assign predicted celltypes to seu_sce, using bped as a reference
#label the cells with label.main of bped, which represents
#the broadest major celltypes in the reference, such as "Epithelial", "Immune", etc.
pred_bped_main <- SingleR(test = seu_sce, ref = bped, labels = bped$label.main)

#filter predictions in pred_bped_main, then add the predictions to the original seu object as
celltype_bped_main
pruneScores(pred_bped_main)
seu[['celltype_bped_main']] <- pred_bped_main$pruned.labels

#can do the same thing with the label.fine in bped, which represents more fine-grained
celltypes, and store in celltype_bped_fine
pred_bped_fine <- SingleR(test = seu_sce, ref = bped, labels = bped$label.fine)
pruneScores(pred_bped_fine)
seu[['celltype_bped_fine']]<-pred_bped_fine$pruned.labels

#print heatmap of singleR scores for each cell type
pdf("workshop_singler_heatmap.pdf")
plotScoreHeatmap(pred_bped_main)
dev.off()
```



Print quality metric and gene expression strength across single-cell data

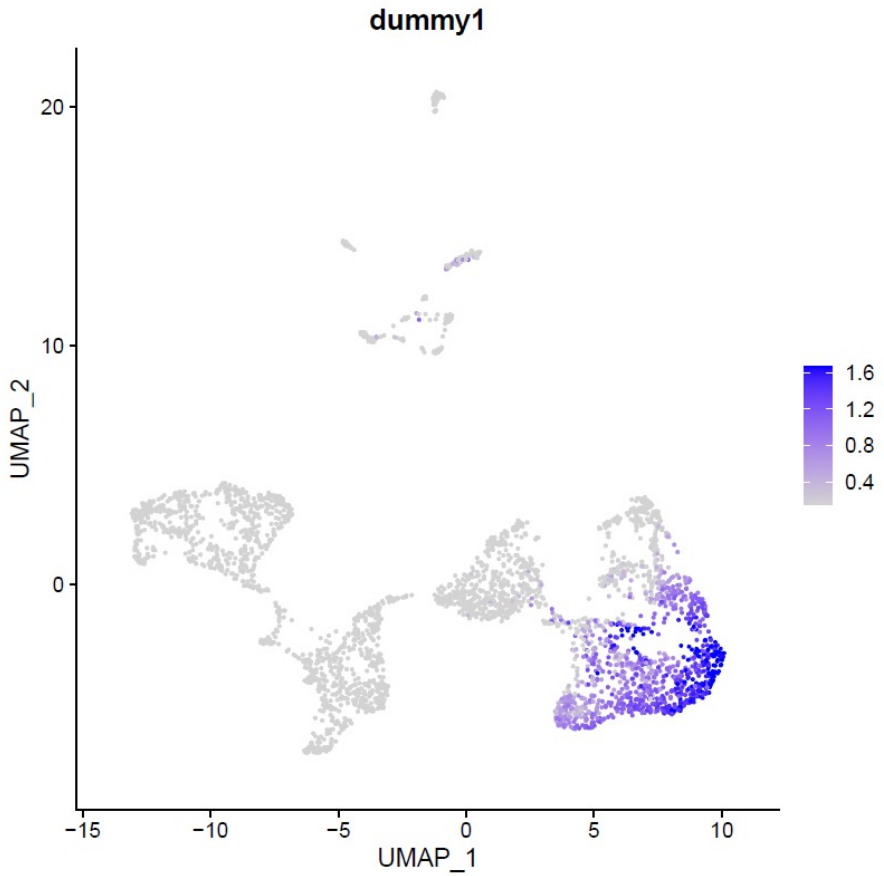
```
#FeaturePlot will plot the strength of each feature in blue across a UMAP
#stronger features corresponding to more blue
#features argument takes a list of features to plot
#features can be a list of quality metrics like percent.mt and nCount_RNA
#min.cutoff and max.cutoff determine thresholds below and above which not to plot.
#q9 and q90 indicate 9th and 90th percentiles as cutoffs
pdf("workshop_featureplot.pdf")
print(FeaturePlot(seu, features = c("nCount_RNA"), min.cutoff = "q9",max.cutoff = "q90"))

#features can also be a list of genes whose expression you want to see on a UMAP
#some genes may not be in the seu object. To check if a gene is present
#use "genename" %in% rownames(seu), which returns T if present, and F otherwise
#then use the following if expression, followed by FeaturePlot, to only plot a gene if present in in seu
if("ALDH1A2" %in% rownames(seu@assays$RNA@data)) {
print(FeaturePlot(seu, features = c("ALDH1A2"), min.cutoff = "q9",max.cutoff = "q90"))
}
dev.off()
```





Score cells based on strength of a gene expression signature

```
#read dummy_signatures.csv in with read.csv
#dummy is a set of DE genes in cluster 1 of the data
#if some signatures are longer than others, pad shorter signatures with NA values, using na.strings = ""
sigs <-
read.csv(paste0(directoryname,"/dummy_signatures.csv"),na.strings = '')
#run AddModuleScore for each signature, which will test for the signature given in the features argument
#na.omit(sigs[,c]) strips out NA values from each signature
#the name argument labels the module score in seu with the name of the signature
#however, by default, Seurat will add 1 to the name argument, thus name = “Signature” will result in a field called “Signature1” in the seu object
#assay = "RNA", so the module score is calculated on RNA values, and search=T, so if gene names in signature do not match exactly, Seurat will search for close matches in the data
for(c in 1:ncol(sigs)){
  seu<-AddModuleScore(object = seu,features =
list(na.omit(sigs[,c])),name = colnames(sigs)[c],assay = 'RNA',search=T)
}

#FeaturePlot
pdf("workshop_signature_featureplot.pdf")
print(FeaturePlot(seu, features = c("dummy1"), min.cutoff =
"q9",max.cutoff = "q90"))
dev.off()
```




```
#at any time in session, can save work in file using
saveRDS(seu, “seuratworkshopsaved.rds”)
#can load work back in with
seu = loadRDS(“seuratworkshopsaved.rds”)
```