Fred Hutch
Cancer Center

Introduction to Sequencing Data Analysis

Lecture 15

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Overview

I. Introduction to sequence data and resources

II. Tools for analyzing and visualizing sequencing data

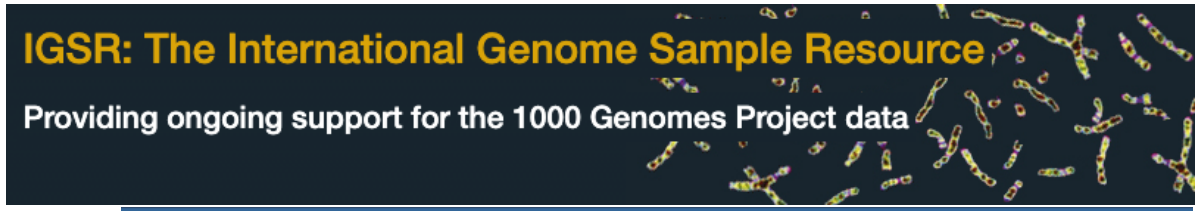
Overview: Learning Objectives

1. Sequence data
   - Databases and online resources for sequence data
   - Learn the common sequence data file formats
2. Tools for sequencing data
   - Tools to query, inspect, visualize an aligned sequence file
   - Learn the contents of sequence data files
   - Learn to generate sequencing metrics and to process sequence data
   - Learn about Python and R libraries/packages to read sequence data
3. Genome variant analysis (Background; Next Lecture)
   - Types of genomic variation
   - Tools to predict genomic variations
   - Learn the common file formats for variation data
   - Databases and online resources for human variation data

Sequence Data: International Consortia and Projects

1000 Genomes Project (https://www.internationalgenome.org/)

UK10K (https://www.uk10k.org/)



The 100,000 Genomes Project (https://www.genomicsengland.co.uk/)

- Rare disease, cancer, infectious disease

Genome 10K Project (https://genome10k.soe.ucsc.edu/)

- Genomic "zoo" of 16,000 vertebrate species

Exome Aggregation Consortium (ExAC) (http://exac.broadinstitute.org/)

Genome Aggregation Database (gnomAD) (https://gnomad.broadinstitute.org/)

The Cancer Genome Atlas (TCGA) (https://portal.gdc.cancer.gov/)

International Cancer Genome Consortium (ICGC) (https://icgc.org/)

Sequence Data: Databases and Online Resources

Common Repositories/Databases for human sequence data

1. NCBI Sequence Read Archive (SRA)

- Publicly available data submitted from studies (e.g. Gene Expression Omnibus [GEO])
- https://www.ncbi.nlm.nih.gov/gds/
- Controlled access (e.g. dbGaP)

2. European Genome Phenome Archive (EGA)

- https://www.ebi.ac.uk/ega/home

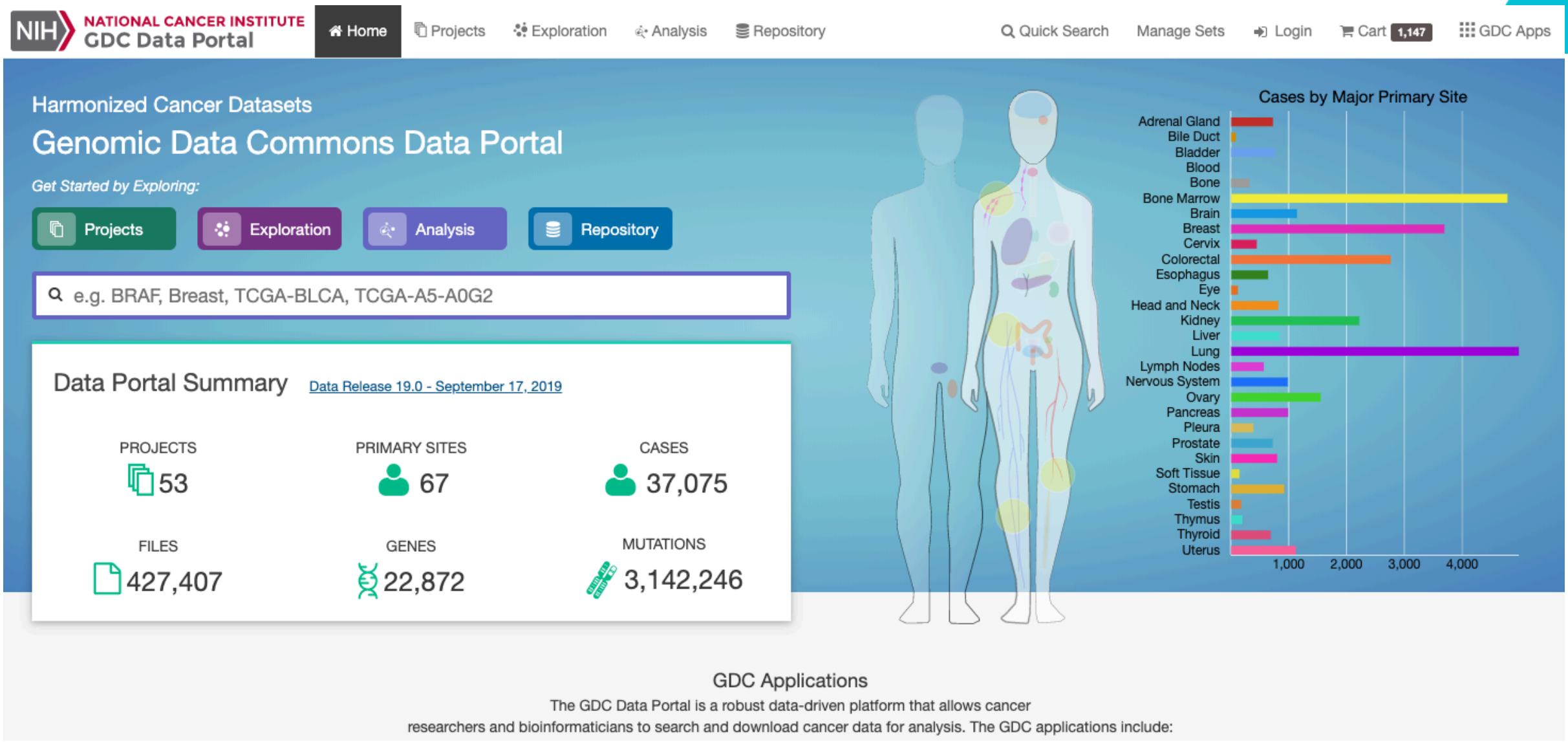
3. NIH NCI Genomic Data Commons (GDC) Data Portal

- https://portal.gdc.cancer.gov/
- Harmonized Cancer Datasets

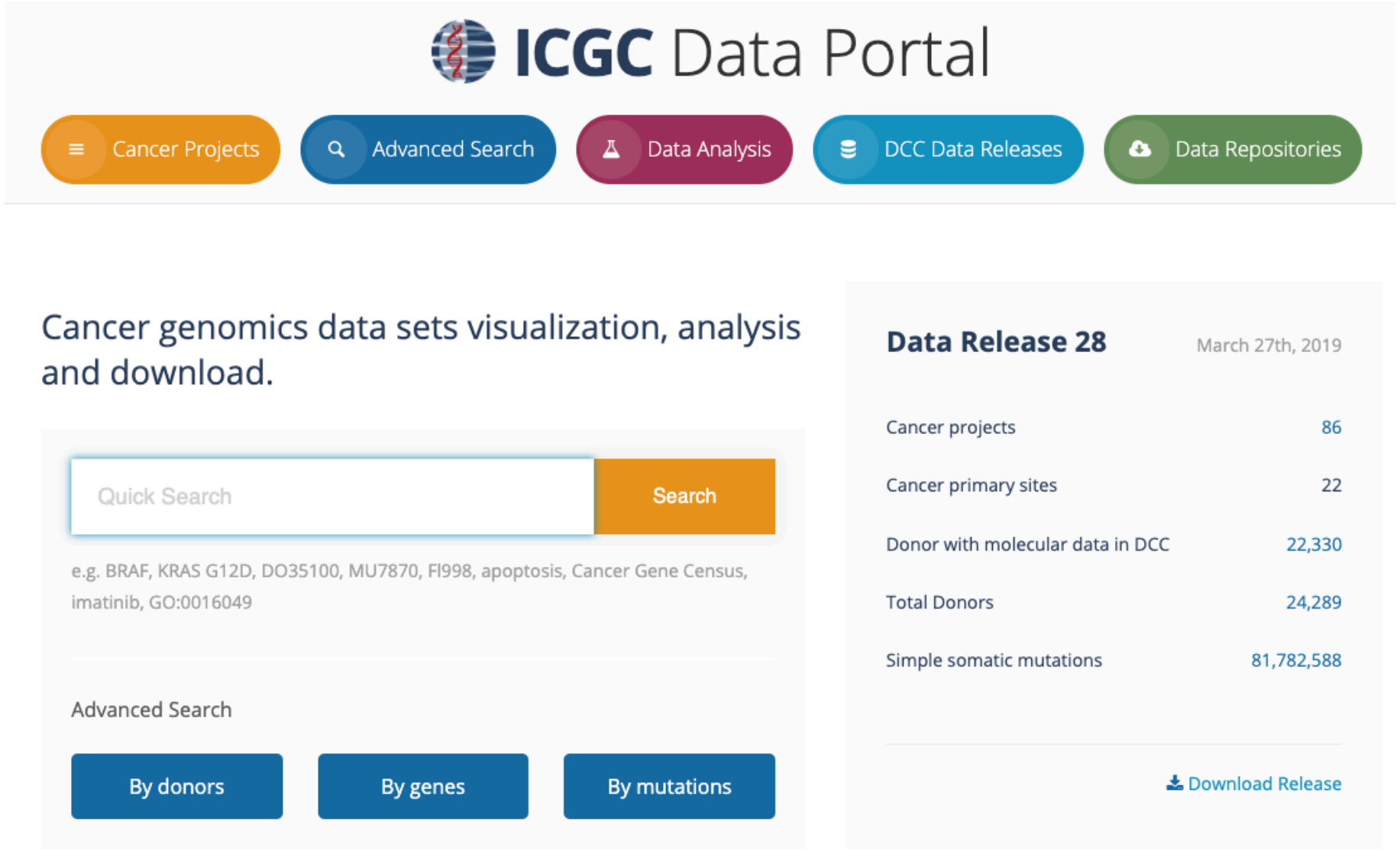
4. ICGC Data Portal

- https://dcc.icgc.org/

Sequence Data: Databases and Online Resources

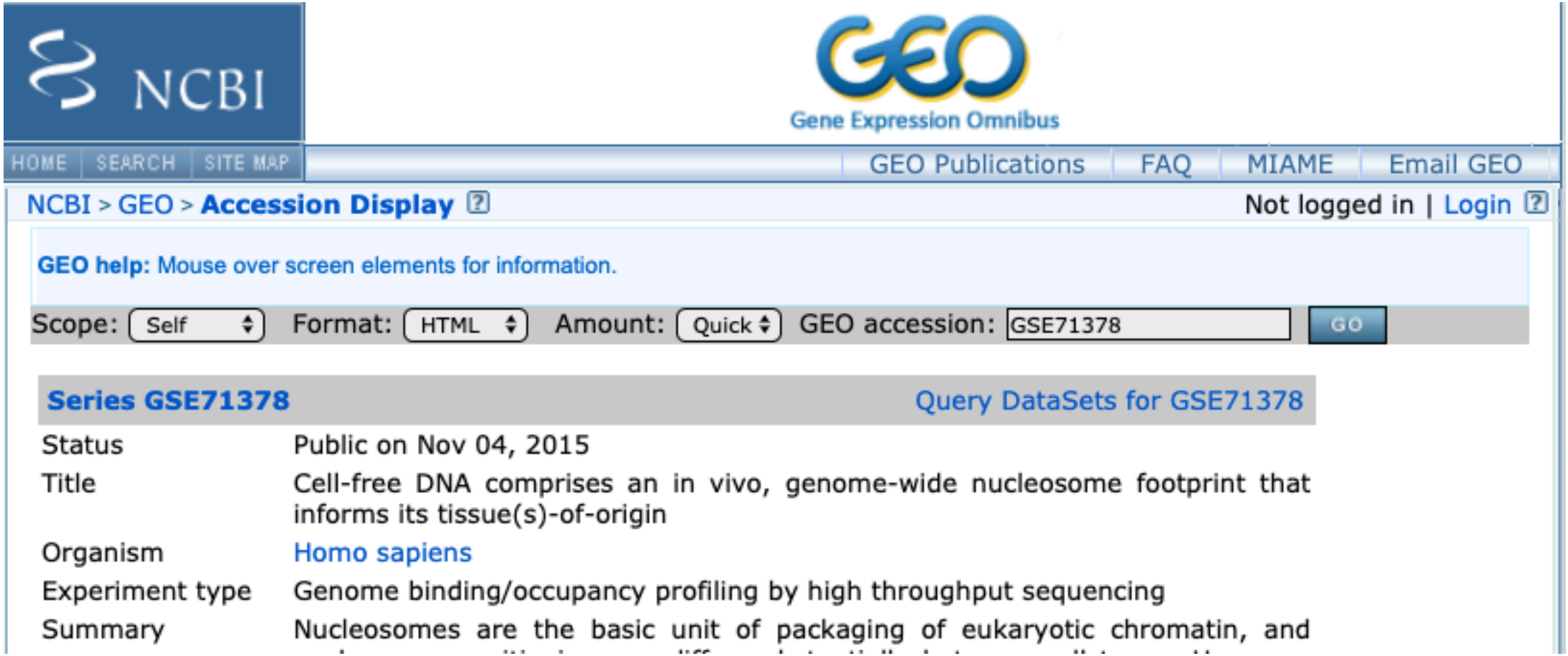


Sequence Data: Databases and Online Resources



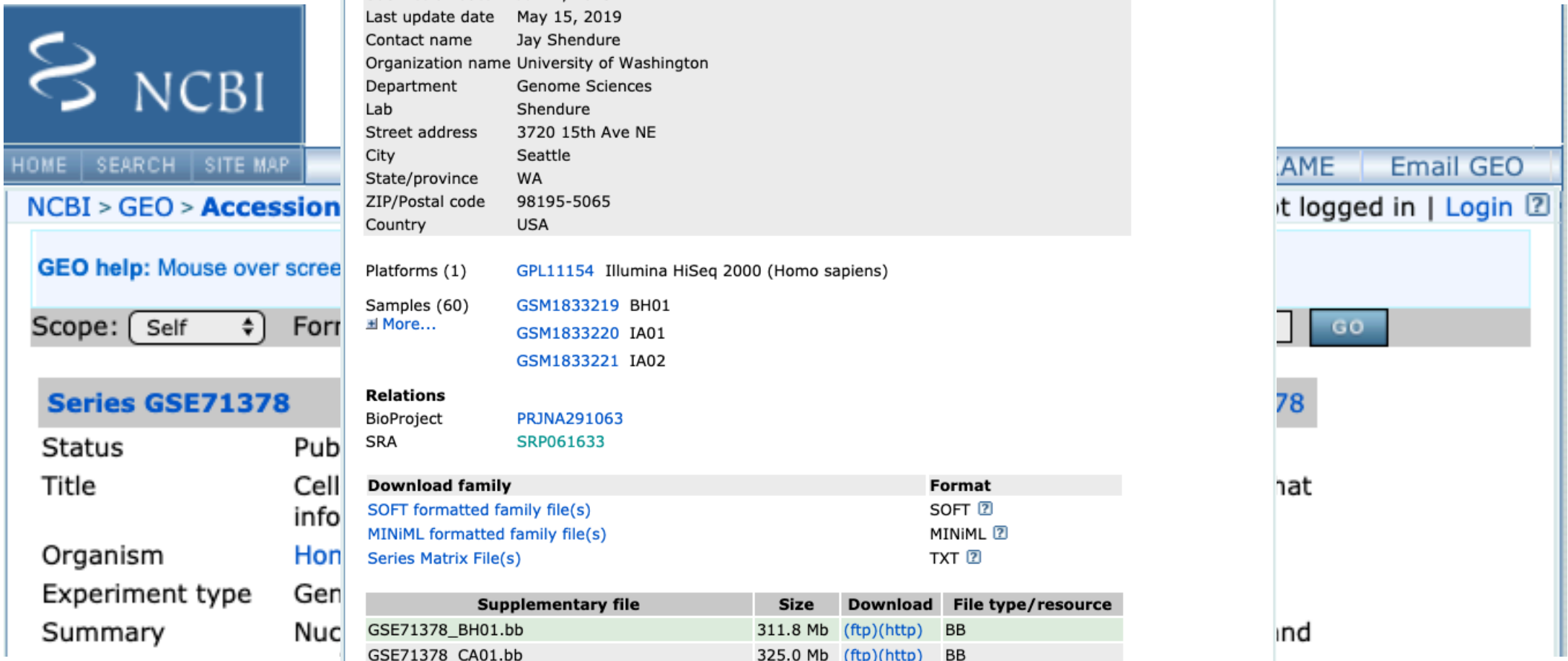
Sequence Data: Databases and Online Resources

Sequence Read Archive (SRA) & GEO example (GSE71378)



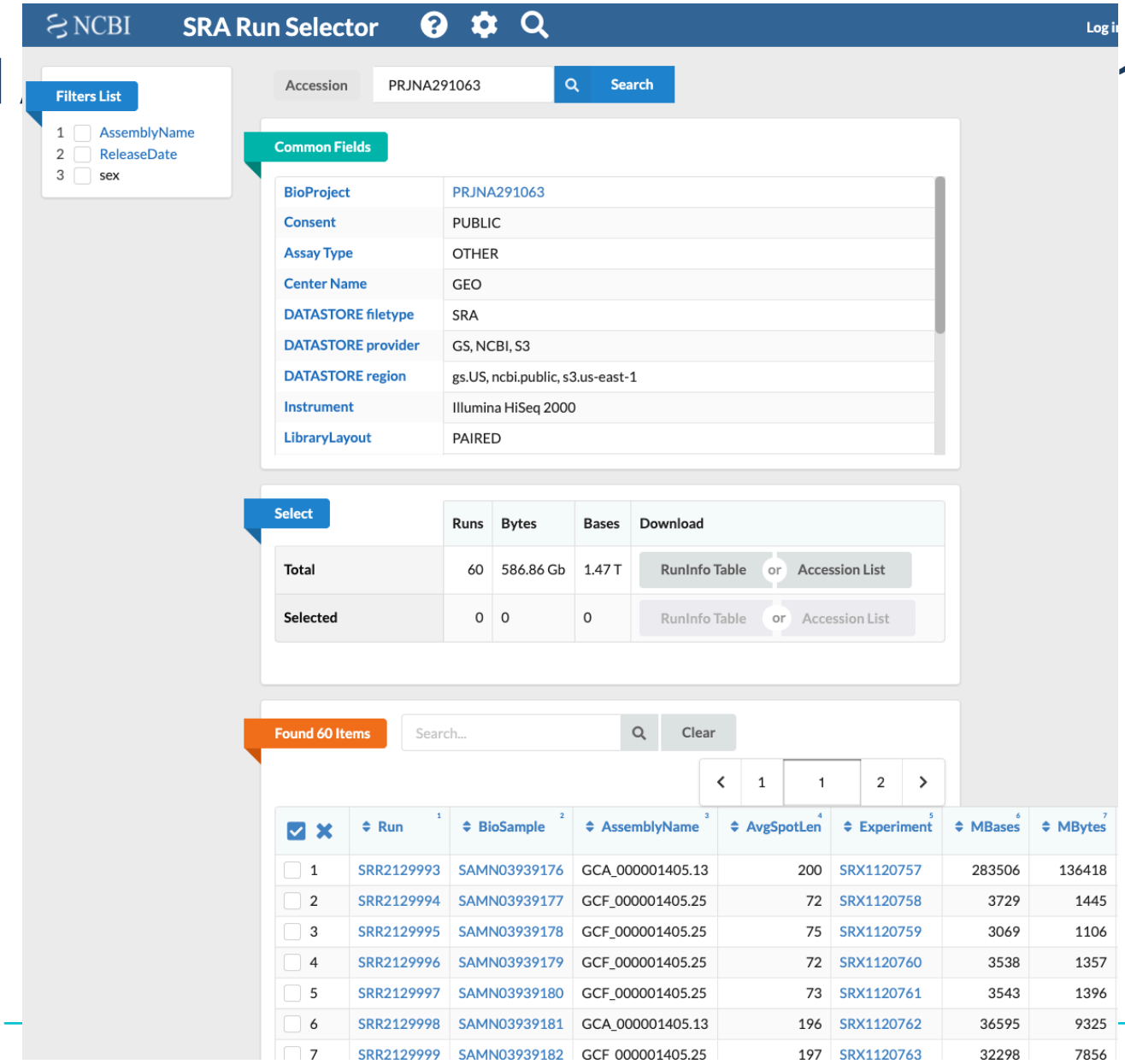
Sequence Data: Databases and Online Resources

Sequence Read … 1378)



Sequence Data: Databases and Online Resources

Sequence Read A 1378)



Sequence Data: File formats

Sequences

- Genome sequences - **FASTA** (.fasta or .fa)
- Sequenced reads - **FASTQ** (.fastq or .fq)

Sequence Alignment/Map Format

- https://samtools.github.io/hts-specs/SAMv1.pdf
- Sequence Alignment - **SAM** (.sam)
- Binary Alignment - **BAM** (.bam) or **CRAM** (.cram)

Sequence Data: Databases and Online Resources

Sequence Read Archive (SRA) & GEO example (GSE71378)

SRA Toolkit required to download and extract **`.sra`** files

- Download .sra file

```
prefetch SRR2130004
```

- Convert `.sra` file to fastq

```
fastq-dump SRR2130004 # use accession
fastq-dump SRR2130004.sra # use file if already downloaded
```

- Convert `.sra` file to SAM/BAM file

```
# will write data to a SAM file
sam-dump --header SRR2130004.sra > SAMN03160688.sam
# will write data to a BAM file
sam-dump --header SRR2130004.sra | samtools view -bS - > BRCA_IDC_cfDNA.bam
```

For your reference.

Sequence Data: Sequence alignment

Burrows-Wheeler Aligner, bwa (http://bio-bwa.sourceforge.net/)

- aln - for 35bp to 100bp reads
- mem - for reads with length 70bp to 1Mb (Recommended for most)

```
# If two fastq files, one for each mate of paired-end reads
bwa mem -M reference.fa BRCA_IDC_cfDNA_R1.fq BRCA_IDC_cfDNA_R2.fq > BRCA_IDC_cfDNA.bam


# If single fastq file with paired-end reads interleaved
bwa mem -M -p reference.fa BRCA_IDC_cfDNA.fq > BRCA_IDC_cfDNA.bam
```

Li H. and Durbin R. (2009) Fast and accurate short read alignment with Burrows-Wheeler Transform. Bioinformatics, 25:1754-60. [PMID: 19451168]

For your reference.

Tools for Sequencing Data: Overview

1. **Inspecting and Reading SAM/BAM files**
   - SAMtools
2. **Interactive Visualization**
   - Integrative Genomics Viewer (https://software.broadinstitute.org/software/igv)
3. **Sequencing metrics and Processing**
   - SAMtools
   - Genomic Analysis Toolkit (GATK) and Picard Tools

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1. Inspecting and Reading BAM Files

SAMtools (http://www.htslib.org/)

Demo & Exercise

Sequence Data: Inspecting and Reading BAM Files

SAMtools (http://www.htslib.org/)

- Indexing

```
samtools index BRCA_IDC_cfDNA.bam #required for all BAM files
```

- File operations

```
samtools sort BRCA_IDC_cfDNA.bam #sort by coordinate
```

- Statistics

```
samtools flagstat BRCA_IDC_cfDNA.bam #get general alignment metrics
```

- Viewing

```
# view header information
samtools view -H BRCA_IDC_cfDNA.bam

# view aligned reads at chr17:37844393
samtools view BRCA_IDC_cfDNA.bam 17:37844393
```

Sequence Data: SAM Format

https://samtools.github.io/hts-specs/SAMv1.pdf

A. Header information

```
samtools view -H BRCA_IDC_cfDNA.bam

@HD     VN:1.2  SO:coordinate
@SQ     SN:1    LN:249250621
@SQ     SN:2    LN:243199373
@SQ     SN:3    LN:198022430
@SQ     SN:4    LN:191154276
@SQ     SN:5    LN:180915260
@SQ     SN:6    LN:171115067
@SQ     SN:7    LN:159138663
@SQ     SN:8    LN:146364022
@SQ     SN:9    LN:141213431
...
```

Sequence Data: SAM Format

https://samtools.github.io/hts-specs/SAMv1.pdf

A. Header information

- `@HD`: Header line
  - `SO`: Sorting order of alignments (`unknown, unsorted, coordinate, queryname`)
- `@SD`: Reference sequence dictionary
  - `SN`: Reference sequence name - typically, one row for each chromosome
  - `LN`: Length of reference sequence
- `@RG`: Read group
  - `ID`: Read group identifier (must be unique)
  - `PL`: Platform or technology used (e.g. ILLUMINA)
  - `SM`: Sample ID and/or pool being sequenced
- `@PG`: Program/tool information
  - `ID`: Unique name, `PN`: Program name; `CL`: Command line

Sequence Data: SAM Format

https://samtools.github.io/hts-specs/SAMv1.pdf

B. Alignment information

```
samtools view BRCA_IDC_cfDNA.bam 17:37844393-37844393

...

41976152        163     17      37844359        60      39M     =       37844477
157
ACTCTCCGCTGAAGTCCACACAGTTTAAATTAAAGTTCC .AAAAFFFFFFFFFFF)FAFFFFFFFFFFFFFFFFFF
RG:Z:P12.17.7_Breast NH:i:1  NM:i:0
```

Sequence Data: SAM Format

https://samtools.github.io/hts-specs/SAMv1.pdf

B. Alignment information

```
samtools view BRCA_IDC_cfDNA.bam 17:37844393-37844393
```

Query (Read) ··· Name | Read Reference and Position | Mate's Reference and Position

```
41976152        163     17      37844359        60      39M     =       37844477
157
ACTCTCCGCTGAAGTCCACACAGTTTAAATTAAAGTTCC .AAAAFFFFFFFFFFF)FAFFFFFFFFFFFFFFFFF
RG:Z:P12.17.7_Breast NH:i:1  NM:i:0
```

Read Sequence

Sequence Data: SAM Format

https://samtools.github.io/hts-specs/SAMv1.pdf

B. Alignment information

```
samtools view BRCA_IDC_cfDNA.bam 17:37844393-37844393
--
41976152        163     17      37844359        60      39M     =       37844477
157
ACTCTCCGCTGAAGTCCACACAGTTTAAATTAAAGTTCC .AAAAFFFFFFFFFFF)FAFFFFFFFFFFFFFFFFF
RG:Z:P12.17.7_Breast NH:i:1  NM:i:0
```

Template Length (Insert Size or Fragment Size)

Flag

Mapping Quality

CIGAR string

Sequence Data: SAM Format

https://samtools.github.io/hts-specs/SAMv1.pdf

B. Alignment Format

1. QNAME: query (read) template name
2. FLAG: bitwise value describing the alignment
   - e.g. 4 - read is unmapped; 2 - proper pair; 1024 - PCR duplicate
   - https://www.samformat.info/sam-format-flag
3. RNAME: reference sequence name (i.e. chr1 or 1)
4. POS: position of aligned read (leftmost; 1-based)
5. MAPQ: Mapping quality
6. CIGAR: Code string to describe read alignment sequence match to reference
7. RNEXT: reference sequence name of mate read
8. PNEXT: position of mate read
9. TLEN: template (read) length; 0 if mates on different chromosomes
10. SEQ: sequence of mapped reads on forward genomic strand
11. QUAL: base qualities (Phred-scale)

Exercise: SAMtools

```
# While in dev container
conda activate samtools
# Go to directory where class data has been downloaded
cd myDataDirectory
```

1. Run samtools view header command on `BRCA_IDC_cfDNA.bam`
    a. What is the read group (@RG) ID?

2. Run samtools view at `17:7579472-7579472`
    a. What is the insert size?

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2. Interactive Visualization

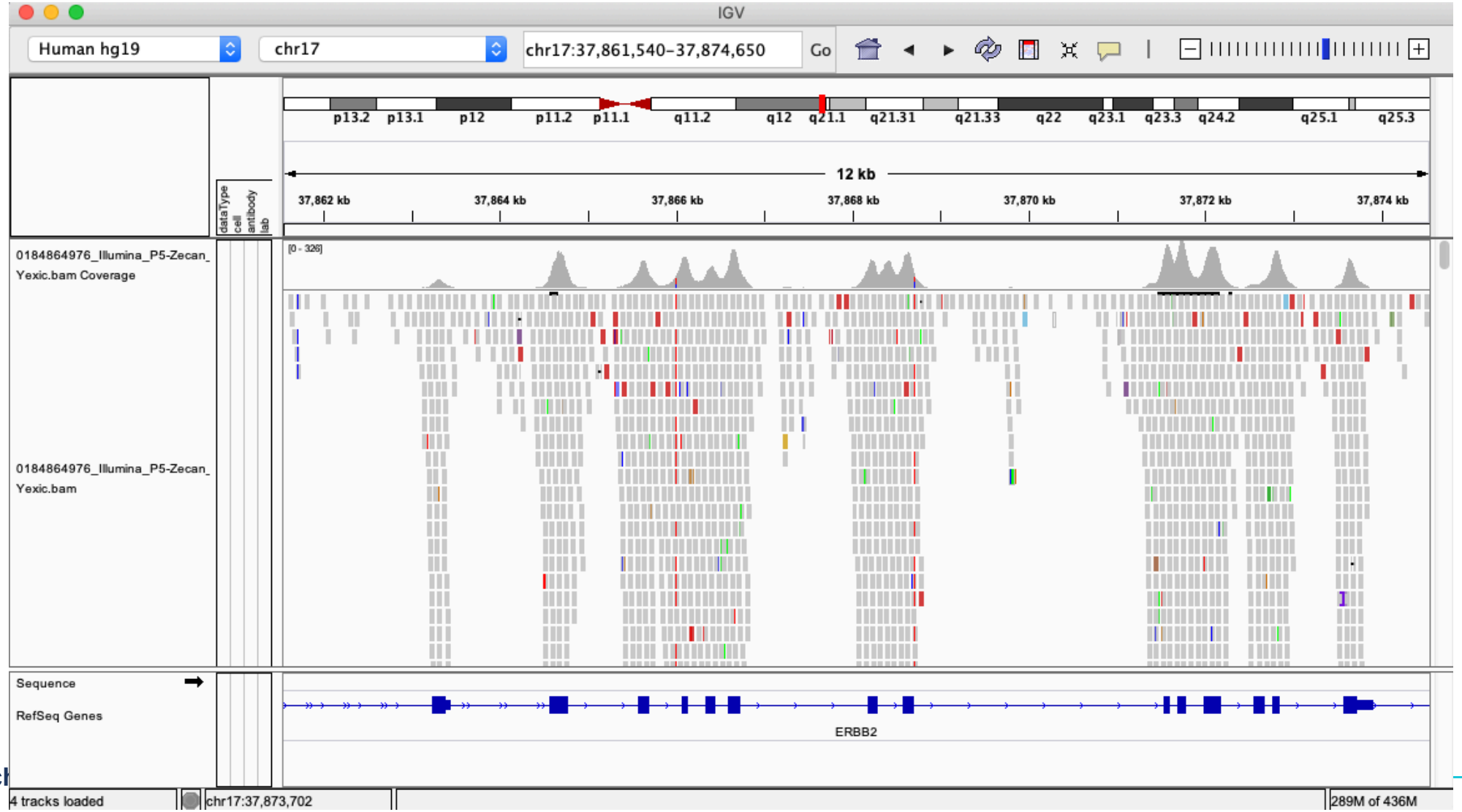
Integrative Genomics Viewer

(https://software.broadinstitute.org/software/igv)

Demo + Exercise

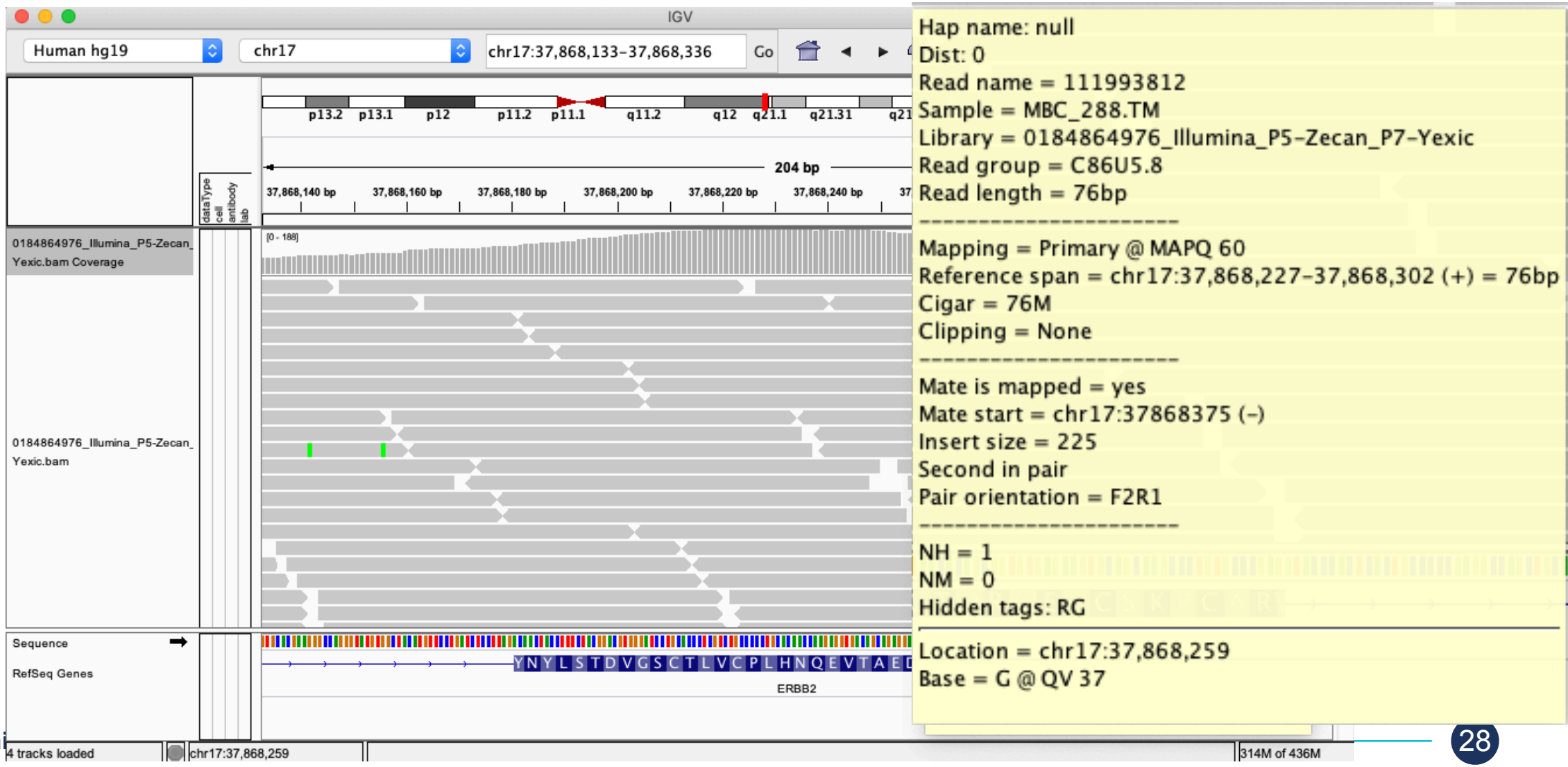
Tools for Sequencing Data: Interactive Visualization

Integrative Genomics Viewer (https://software.broadinstitute.org/software/igv)



Tools for Sequencing Data: Interactive Visualization

Integrative Genomics Viewer (https://software.broadinstitute.org/software/igv)



Exercise: IGV

Instructions:

Launch IGV-Web (https://igv.org/app/).

Tracks > Local File > select both BRCA_IDC_cfDNA.bam and BRCA_IDC_cfDNA.bam.bai

Questions:

1. Go to location chr17:7,579,517
    a. Which gene and exon # is at this location?
    b. How many reads match the reference? How many don't? What are the nucleotides bases?
2. Go to location chr13:32,912,062
    a. Which gene and exon # is at this location?
    b. What is the "Read length", "Insert size", and "CIGAR" for the read found here?
    c. File > Load from Server > Annotations > Variation and Repeats > *check* dbSNP
        i. What is the "Name" (rs ID) and "Class" of the SNP located at this position?

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3. Tools for Sequence Data Processing

PICARD and GATK

https://broadinstitute.github.io/picard/

https://software.broadinstitute.org/gatk/best-practices/

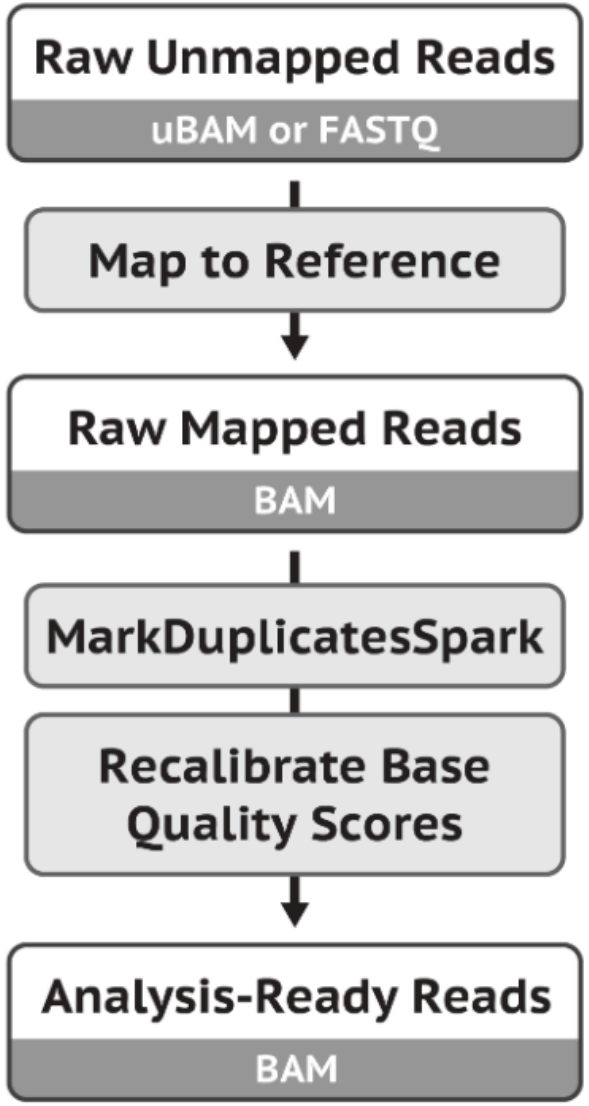
Demo + Exercise

Tools for Sequencing Data: Processing

Picard Tools & GATK4: Best practices

1. Mark Duplicates
    1. MarkDuplicates + SortSam (Picard)
2. Base Quality Score Recalibration (BQSR)
    1. BaseRecalibrator (GATK4)
    2. ApplyBQSR (GATK4)

```
picard MarkDuplicates \
INPUT=BRCA_IDC_cfDNA.bam \
REMOVE_DUPLICATES=false \
OUTPUT=BRCA_IDC_cfDNA.marked_duplicates.bam \
METRIC_FILE=BRCA_IDC_cfDNA.markDupMetrics.txt
```



https://software.broadinstitute.org/gatk/best-practices/

Tools for Sequencing Data: Sequencing Metrics

Picard Tools & GATK4: Best practices

3. Generate alignment metrics
    a. `CollectMultipleMetrics`
        - `CollectAlignmentSummaryMetrics`
        - `CollectInsertSizeMetrics`
    b. Collect assay-specific metrics
        - `CollectWgsMetrics` - Whole genome sequencing
        - `CollectHsMetrics` - Hybrid Selection (i.e. whole exome)
        - `CollectRnaSeqMetrics` - RNA-seq
        - `CollectTargetedPcrMetrics` - Targeted PCR amplicon sequencing
    c. `EstimateLibraryComplexity`
        a. Estimates the number of unique molecules in the library

https://broadinstitute.github.io/picard/command-line-overview.html
http://broadinstitute.github.io/picard/picard-metric-definitions.html

Tools for Sequencing Data: Sequencing Metrics

Picard Tools & GATK4: Best practices

3. Generate alignment metrics: (a) `CollectWgsMetrics`

```
picard CollectWgsMetrics \
INPUT=BRCA_IDC_cfDNA.bam \
OUTPUT=BRCA_IDC_cfDNA.alignMetrics.txt \
REFERENCE_SEQUENCE=hs37d5.fa \
VALIDATION_STRINGENCY=LENIENT
```

GENOME_TERRITORY	MEAN_COVERAGE	SD_COVERAGE	MEDIAN_COVERAGE	PCT_EXC_MAPQ	PCT_EXC_DUPE	PCT_1X	PCT_5X
2900340137	1.053882	1.383867	1	0.137741	0	0.578236	0.015963

https://broadinstitute.github.io/picard/command-line-overview.html
https://broadinstitute.github.io/picard/picard-metric-definitions.html#CollectWgsMetrics.WgsMetrics

Exercise: PICARD

Run `CollectAlignmentSummaryMetrics` for `BRCA_IDC_cfDNA.bam`

```
#While in Dev container
conda activate Picard
# Go to directory where class data has been downloaded
cd myDataDirectory
# Run Picard command
picard CollectAlignmentSummaryMetrics \
. . .
```

How many `PF_READS_ALIGNED` for `PAIR` Category?

https://broadinstitute.github.io/picard/command-line-overview.html

Tools for Sequencing Data: Accessing BAM files in R & Python

Python

- PySam

  https://pysam.readthedocs.io/en/latest/api.html

R and Bioconductor

- **Rsamtools**
  - Import BAM files into R
  - View the header information
  - Accessing read sequences, aligned positions, CIGAR, read names, etc
  - Large BAM files can be read in chunks to optimize memory
  - Create new BAM files using “Views” of a subset of reads

https://bioconductor.org/packages/release/bioc/vignettes/Rsamtools/inst/doc/Rsamtools-Overview.pdf

For your reference.

Lecture 15

R Bioconductor packages:

- VariantAnnotation
- GenomicRanges
- plyranges

Download data:

https://drive.google.com/drive/folders/13jM29nhzELyThKQXI27MrRXxbziVunQr?usp=sharing