NIBB ゲノムインフォマティックス・トレーニングコース2024
RNA-seq入門
2024.2.28-2024.2.29

RNA-Seqパイプライン ゲノムベースの解析法

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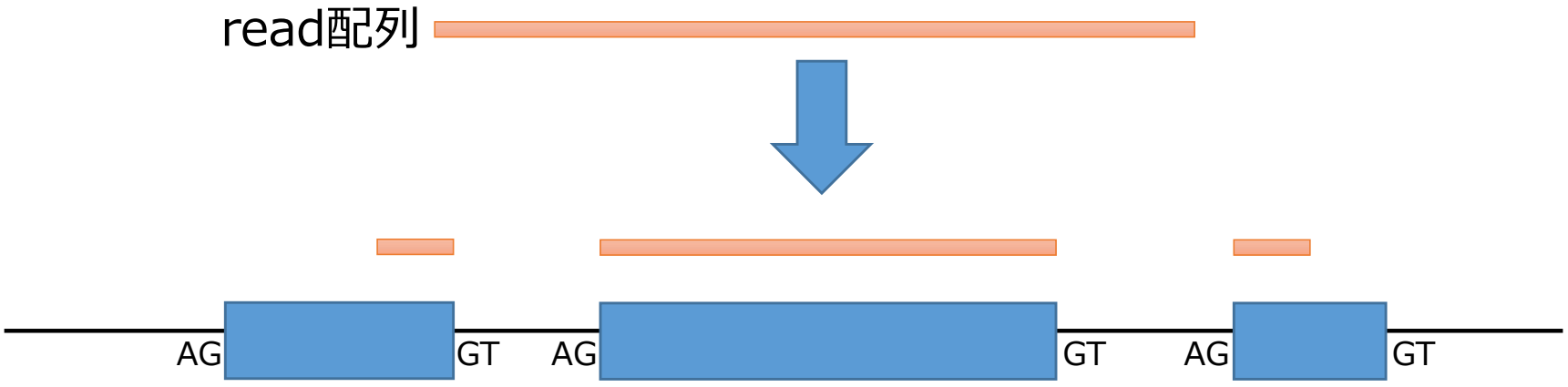
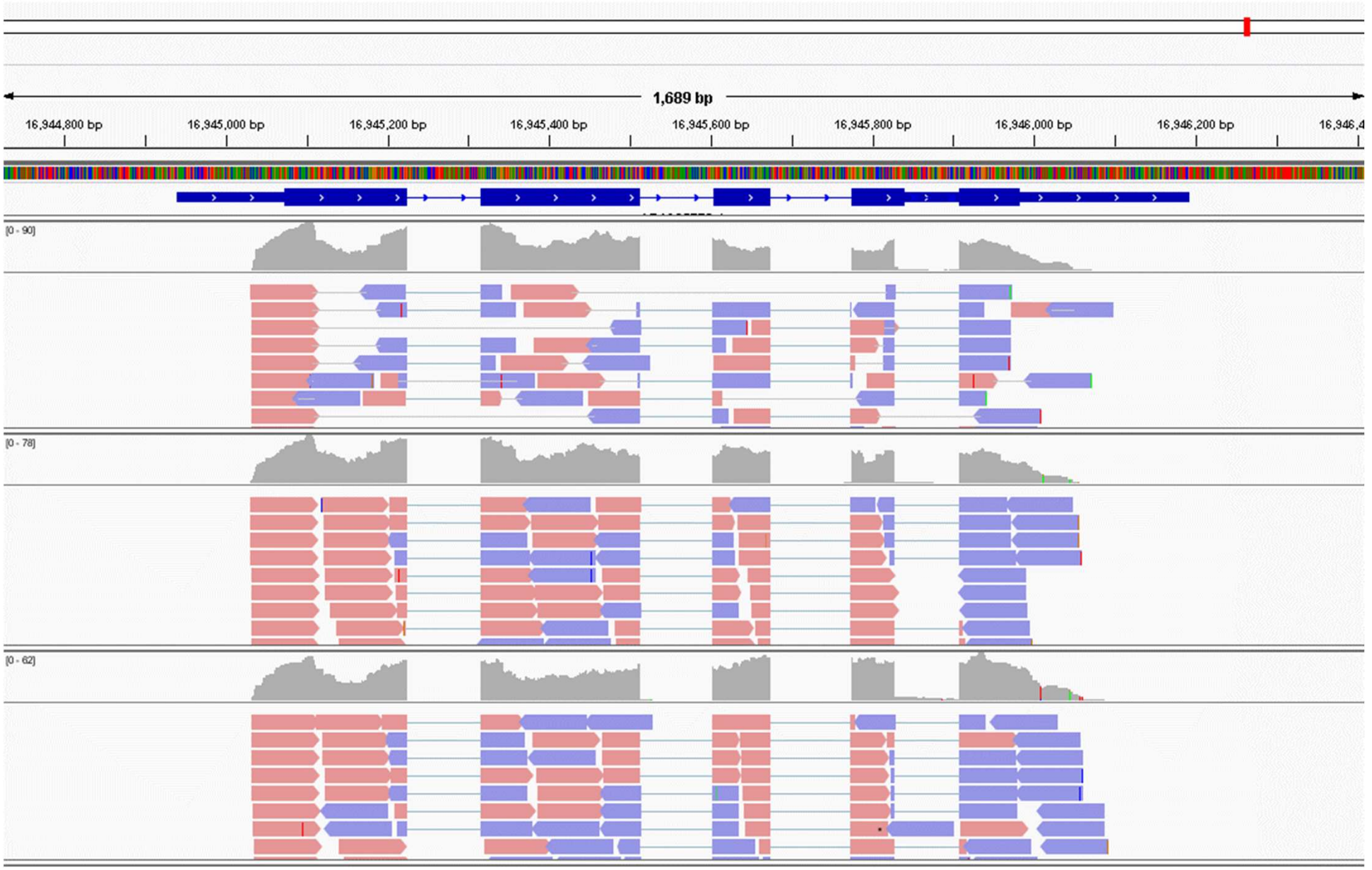
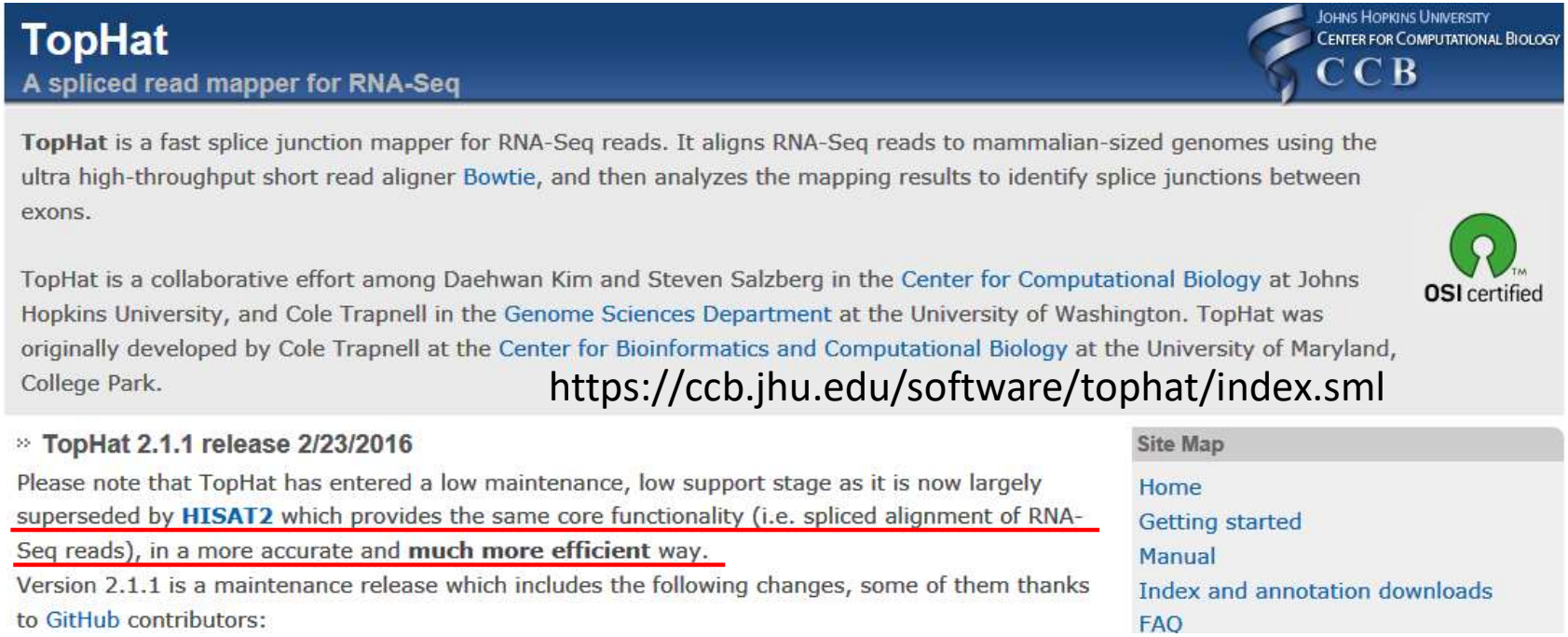
genomeをレファレンスとする場合

Reference配列がゲノム配列の場合、
イントロン配列のスプライシングを考慮した
アライメントを行う必要がある。
今回はHISAT2を用いる
他　STAR,Tophat, Blat(Plat), MapSplice2



実際こんな感じにアラインされる





Traditional 'Tuxedo' package

New 'Tuxedo' package

TopHat

↓

Cufflinks

→

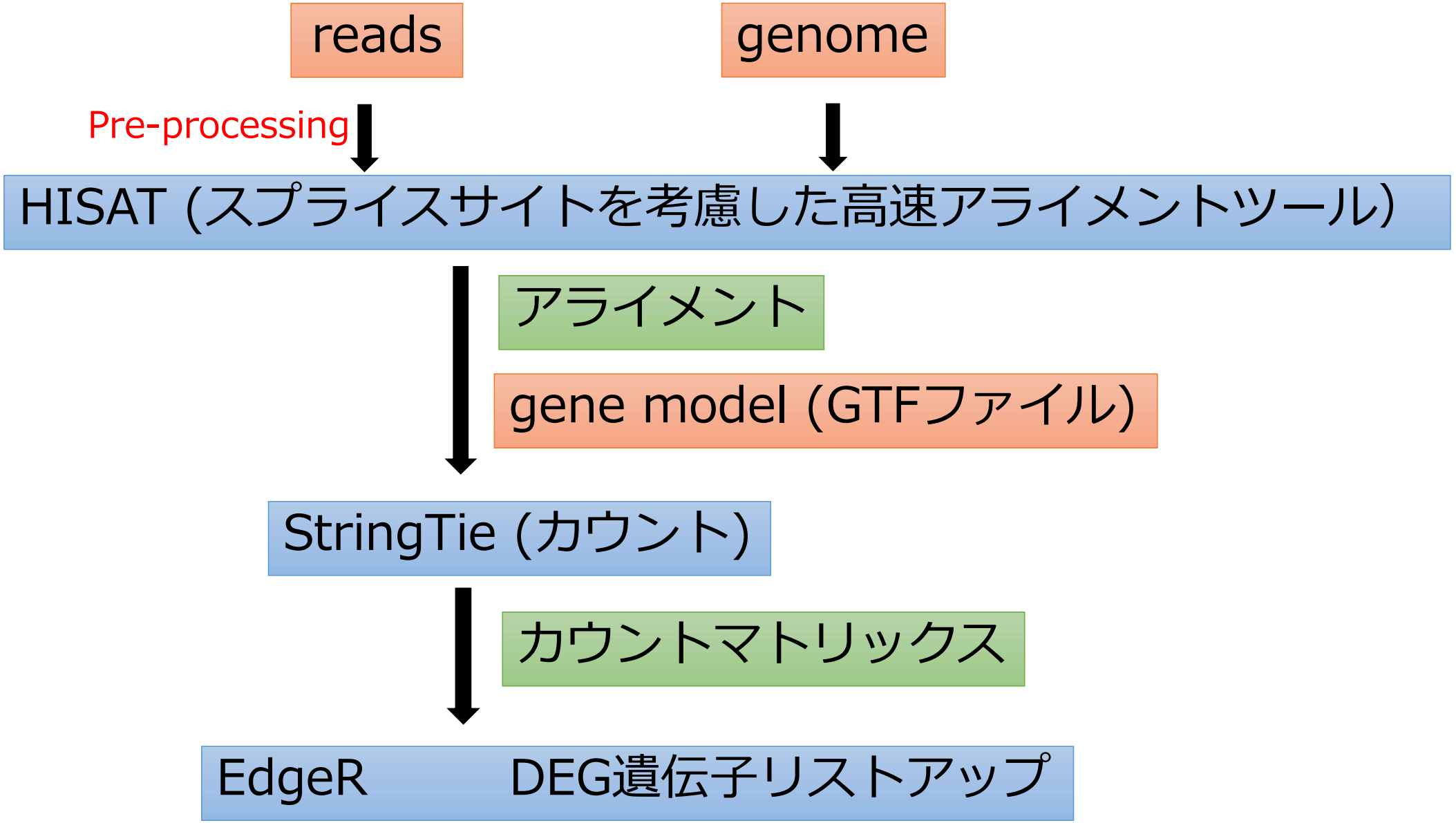
HISAT

↓

StringTie
Ballgown

劇的に解析速度が速くなった

本トレーニングコースでの流れ



HISAT2

HISAT2 is a fast and sensitive alignment program for mapping next-generation sequencing reads (both DNA and RNA) to a population of human genomes as well as to a single reference genome. Based on an extension of BWT for graphs (Sirén et al. 2014), we designed and implemented a graph FM index (GFM), an original approach and its first implementation. In addition to using one global GFM index that represents a population of human genomes, **HISAT2** uses a large set of small GFM indexes that collectively cover the whole genome. These small indexes (called local indexes), combined with several alignment strategies, enable rapid and accurate alignment of sequencing reads. This new indexing scheme is called a Hierarchical Graph FM index (HGFM).

TopHat2と比較して速い

HISAT-3N beta release 12/14/2020

HISAT-3N is a software system for analyzing nucleotide conversion sequencing reads. See the HISAT-3N for more details.

Index files are moved to the AWS Public Dataset Program. 9/3/2020

We have moved HISAT2 index files to the AWS Public Dataset Program. See the link for more details.

HISAT 2.2.1 release 7/24/2020

This patch version includes the following changes.

- Python3 support
- Remove the HISAT-genotype related scripts. HISAT-genotype moved to http://daehwankimlab.github.io/hisat-genotype/
- Fixed bugs related to `--read-lengths` option

Search

Funding

This work was supported in part by the National Human Genome Research Institute under grants R01-HG006102 and R01-HG006677, and NIH grants R01-LM06845 and R01-GM083873 and NSF grant CCF-0347992 to Steven L. Salzberg and by the Cancer Prevention Research Institute of Texas under grant RR170068 and NIH grant R01-GM135341 to Daehwan Kim

http://daehwankimlab.github.io/hisat2

Manual

Introduction

パラメータの意味など
詳しく知るためには、
必ずManualを見る

What is HISAT2?

HISAT2 is a fast and sensitive alignment program for mapping next-generation sequencing reads (whole-genome, transcriptome, and exome sequencing data) against the general human population (as well as against a single reference genome). Based on GCSA (an extension of BWT for a graph), we designed and implemented a graph FM index (GFM), an original approach and its first implementation to the best of our knowledge. In addition to using one global GFM index that represents general population, HISAT2 uses a large set of small GFM indexes that collectively cover the whole genome (each index representing a genomic region of 56 Kbp, with 55,000 indexes needed to cover human population). These small indexes (called local indexes) combined with several alignment strategies enable effective alignment of sequencing reads. This new indexing scheme is called Hierarchical Graph FM index (HGFM). We have developed HISAT 2 based on the HISAT and Bowtie2 implementations. HISAT2 outputs alignments in SAM format, enabling interoperation with a large number of other tools (e.g. SAMtools, GATK) that use SAM. HISAT2 is distributed under the GPLv3 license, and it runs on the command line under Linux, Mac OS X and Windows.

PROTOCOL

Transcript-level expression analysis of RNA-seq experiments with HISAT, StringTie and Ballgown

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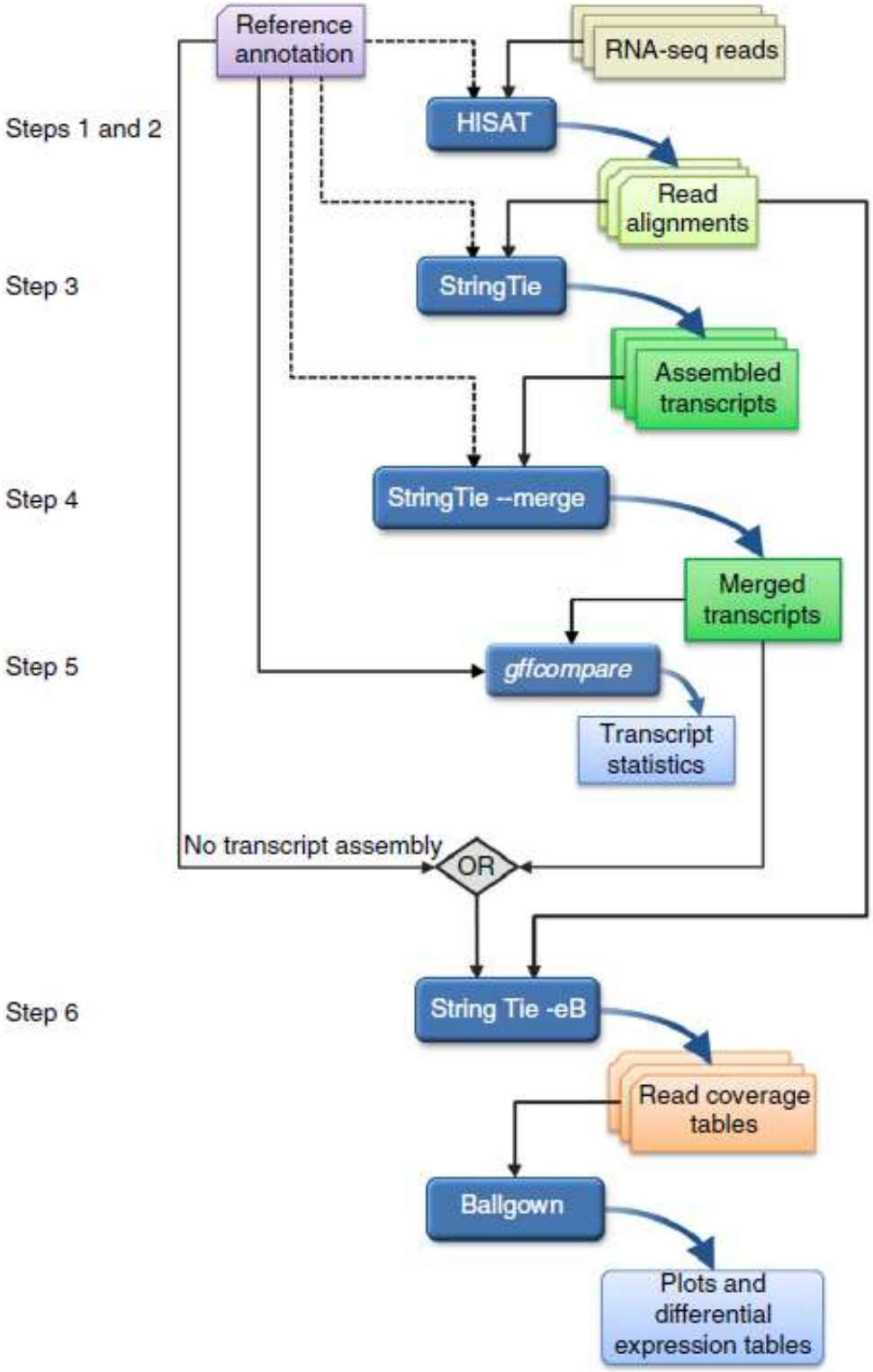
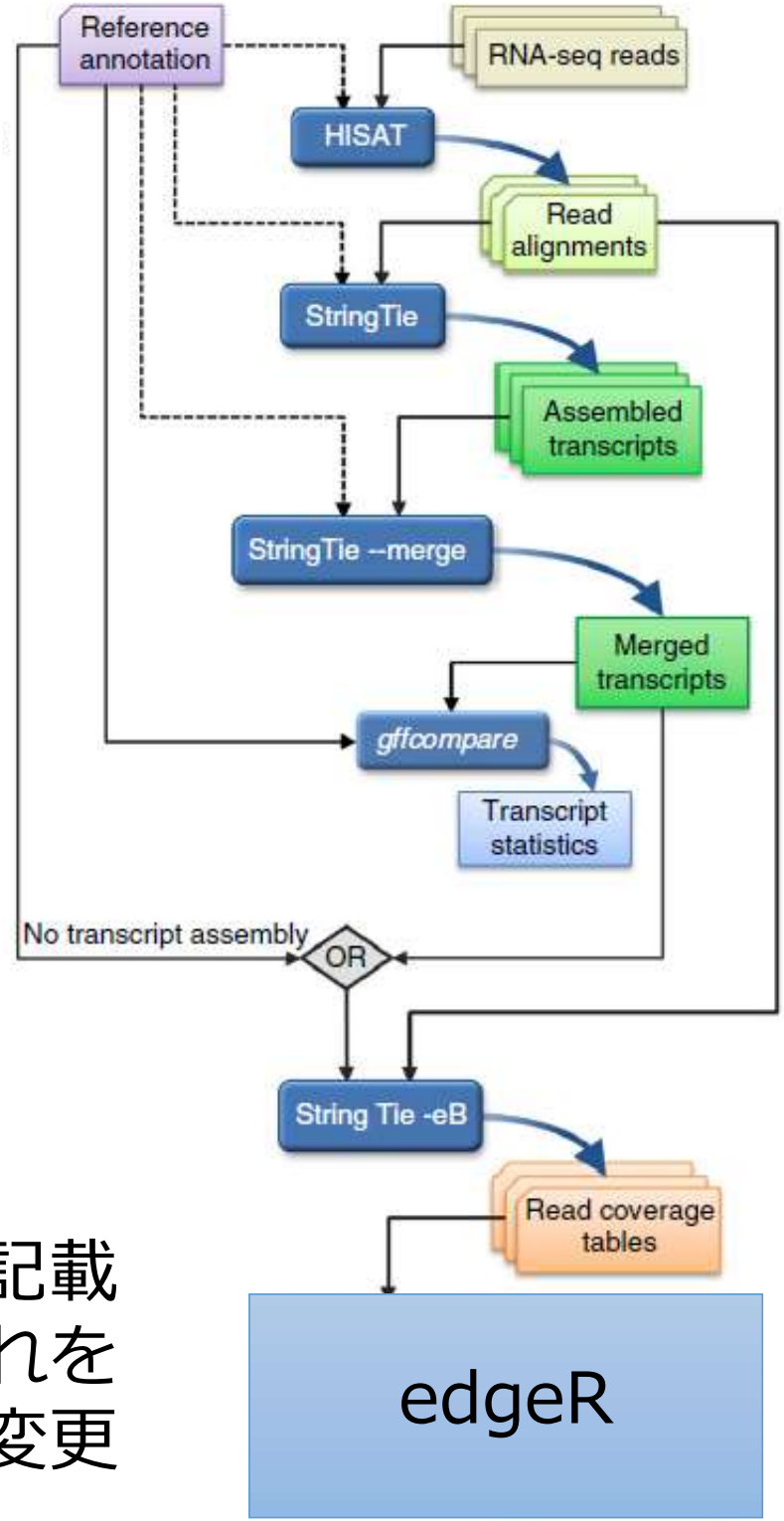
Published online 11 August 2016; doi:10.1038/nprot.2016.095



論文記載の流れを少し変更



hisat2-buildでリファレンスのインデックスを作る

```
$ hisat2-build -h
HISAT2 version 2.2.1 by Daehwan Kim (infphilo@gmail.com, http://www.ccb.jhu.edu/people/infphilo)
Usage: hisat2-build [options]* <reference_in> <ht2_index_base>
    reference_in            comma-separated list of files with ref sequences
    hisat2_index_base       write ht2 data to files with this dir/basename
Options:
    -c                      reference sequences given on cmd line (as
                            <reference_in>)
    --large-index           force generated index to be 'large', even if ref
                            has fewer than 4 billion nucleotides
    -a/--noauto             disable automatic -p/--bmax/--dcv memory-fitting
    -p <int>                number of threads
    --bmax <int>            max bucket sz for blockwise suffix-array builder
    --bmaxdivn <int>        max bucket sz as divisor of ref len (default: 4)
    --dcv <int>             diff-cover period for blockwise (default: 1024)
    --nodc                  disable diff-cover (algorithm becomes quadratic)
    -r/--noref              don't build .3/.4.ht2 (packed reference) portion
    -3/--justref            just build .3/.4.ht2 (packed reference) portion
    -o/--offrate <int>      SA is sampled every 2^offRate BWT chars (default: 5)
    -t/--ftabchars <int>    # of chars consumed in initial lookup (default: 10)
    --localoffrate <int>    SA (local) is sampled every 2^offRate BWT chars (default: 3)
    --localftabchars <int>  # of chars consumed in initial lookup in a local index (default: 6)
    --snp <path>            SNP file name
    --haplotype <path>      haplotype file name
    --ss <path>             Splice site file name
    --exon <path>           Exon file name
    --repeat-ref <path>     Repeat reference file name
    --repeat-info <path>    Repeat information file name
    --repeat-snp <path>     Repeat snp file name
    --repeat-haplotype <path>   Repeat haplotype file name
    --seed <int>            seed for random number generator
    -q/--quiet              disable verbose output (for debugging)
    -h/--help               print detailed description of tool and its options
    --usage                 print this usage message
    --version               print version information and quit
```

一部のモデル生物種以外は、リファレンス配列のインデックスを作る必要がある

実習1　hisat2-build

genome.faはArabidopsis thaliana (シロイヌナズナ）のレファレンスゲノム配列である。

中身を閲覧、query名およびreads数を確認せよ。

```
$ less genome.fa
$ grep '>' genome.fa
$ grep ‘>’ genome.fa|wc -l
```

indexを作製せよ。

```
$ hisat2-build genome.fa genome
```

新たに作製されたファイルを確認せよ。

```
$ ls –ltr      #新しいタイムスタンプのファイルが下になってリスト表示される
```

HISAT2基本コマンド

```
$ hisat2 -h
HISAT2 version 2.2.1 by Daehwan Kim (infphilo@gmail.com,
www.ccb.jhu.edu/people/infphilo)
Usage:
  hisat2 [options]* -x <ht2-idx> {-1 <m1> -2 <m2> | -U <r>} [-S <sam>]

  <ht2-idx>  Index filename prefix (minus trailing .X.ht2).
  <m1>       Files with #1 mates, paired with files in <m2>.
             Could be gzip'ed (extension: .gz) or bzip2'ed (extension: .bz2).
  <m2>       Files with #2 mates, paired with files in <m1>.
             Could be gzip'ed (extension: .gz) or bzip2'ed (extension: .bz2).
  <r>        Files with unpaired reads.
             Could be gzip'ed (extension: .gz) or bzip2'ed (extension: .bz2).
  <sam>      File for SAM output (default: stdout)

  <m1>, <m2>, <r> can be comma-separated lists (no whitespace) and can be
  specified many times.  E.g. '-U file1.fq,file2.fq -U file3.fq'.
```

結果はsamファイルで出力される

実習2　hisat2

read結果
2D2L_rep1_R1.fastq
2D2L_rep1_R2.fastq
を先にindexを作製したリファレンスにmapさせよ。

```
$ hisat2 -p 4 --dta ¥
-x genome ¥
-1 2D2L_rep1_R1.fastq ¥
-2 2D2L_rep1_R2.fastq ¥
-S 2D2L_rep1.sam
```

¥は表記上、分かりやすくするための改行を入れているが、入力の際は改行せずに入力するという意味

samファイルの内容を確認しよう

```
$ less 2D2L_rep1.sam
```

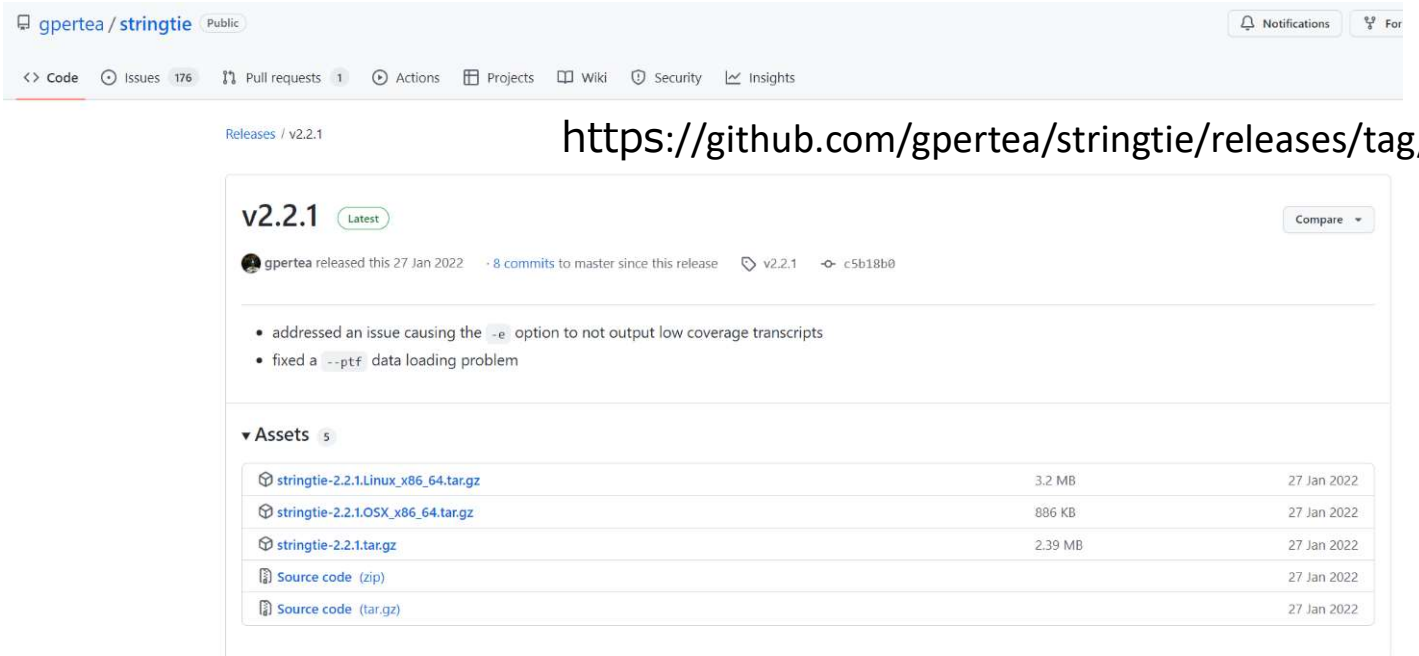
StringTie

Transcript assembly and quantification for RNA-Seq

JOHNS HOPKINS UNIVERSITY
CENTER FOR COMPUTATIONAL BIOLOGY
CCB

Overview

StringTie is a fast and highly efficient assembler of RNA-Seq alignments into potential transcripts. It uses a novel network flow algorithm as well as an optional *de novo* assembly step to assemble and quantitate full-length transcripts representing multiple splice variants for each gene locus. Its input can include not only alignments of short reads that can also be used by other transcript assemblers, but also alignments of longer sequences that have been assembled from those reads. In order to identify differentially expressed genes between experiments, StringTie's output can be processed by specialized software like Ballgown, Cuffdiff or other programs (DESeq2, edgeR, etc.).



https://github.com/gpertea/stringtie/releases/tag/v2.2.1

StringTieを用いてアラインされたreadを数える

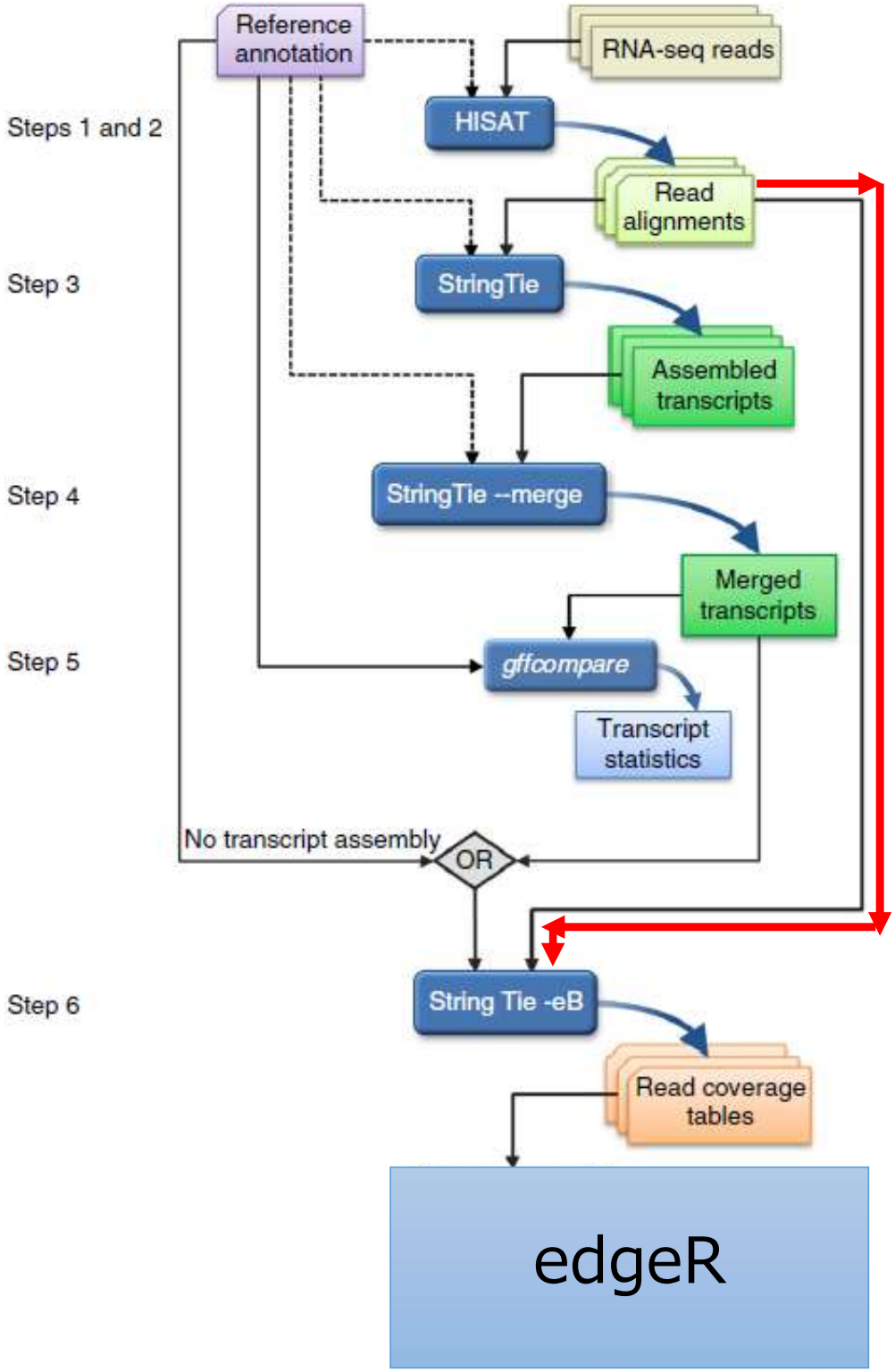
StringTieの解析の方向性として大きく2つある

・GTFファイルに記載された遺伝子モデルのみを数える

・新規な遺伝子モデルを見出し、それも含めて数える
新規な遺伝子モデルはサンプルによって異なりうるので、
得られた個々のモデルをStringTieのmerge modeでmerge
することで、全サンプルの新規遺伝子モデルが作製できる

StringTie基本コマンド

```
$ stringtie
StringTie v2.2.1 usage:

stringtie <in.bam ..> [-G <guide_gff>] [-l <prefix>] [-o <out.gtf>] [-p <cpus>]
 [-v] [-a <min_anchor_len>] [-m <min_len>] [-j <min_anchor_cov>] [-f <min_iso>]
 [-c <min_bundle_cov>] [-g <bdist>] [-u] [-L] [-e] [--viral] [-E <err_margin>]
 [--ptf <f_tab>] [-x <seqid,..>] [-A <gene_abund.out>] [-h] {-B|-b <dir_path>}
 [--mix] [--conservative] [--rf] [--fr]
Assemble RNA-Seq alignments into potential transcripts.
Options:
 --version : print just the version at stdout and exit
 --conservative : conservative transcript assembly, same as -t -c 1.5 -f 0.05
 --mix : both short and long read data alignments are provided
        (long read alignments must be the 2nd BAM/CRAM input file)
 --rf : assume stranded library fr-firststrand
 --fr : assume stranded library fr-secondstrand
 -G reference annotation to use for guiding the assembly process (GTF/GFF)
 --ptf : load point-features from a given 4 column feature file <f_tab>
 -o output path/file name for the assembled transcripts GTF (default: stdout)
         :
         :
         :
```



Case A

GTFファイルに記載された
遺伝子モデルのみを数える場合

StringTie
Transcript assembly and quantification for RNA-Seq
Johns Hopkins University Center for Computational Biology CCB
CCB » Software » StringTie
Home Manual FAQ

Running StringTie

The generic command line for the default usage has this format::

```
stringtie [-o <output.gtf>] [other_options] <read_alignments.bam>
```

The main input of the program (*<read_alignments.bam>*) must be a SAM, BAM or CRAM file with RNA-Seq read alignments sorted by their genomic location (for example the `accepted_hits.bam` file produced by TopHat or the output of HISAT2 after sorting and converting it using samtools as explained below).

The main output is a GTF file containing the structural definitions of the transcripts assembled by StringTie from the read alignment data. The name of the output file should be specified with the `-o` option. If this option is not used the output GTF records with the assembled transcripts will be printed to the standard output (and can be captured into a file using the `>` output redirect operator). **Note**:if the `--mix` option is used, StringTie expects *two alignment files* to be given as positional parameters, in a specific order: the short read alignments must be the *first file given* while the long read alignments must be the *second* input file. Both alignment files must be sorted by genomic location.

```
stringtie [-o <output.gtf>] --mix [other_options] <short_read_alns.bam> <long_read_alns.bam>
```

Note that the command line parser in StringTie allows arbitrary order and mixing of the positional parameters with the other options of the program, so the input alignment files can also precede or be given in between the other options -- the following command line is equivalent to the one above:

```
stringtie <short_read_alns.bam> <long_read_alns.bam> --mix [other_options] [-o <output.gtf>]
```

StringTie options

The following optional parameters can be specified when running `stringtie`:

`-h/--help`	Prints help message and exits.
`--version`	Prints version and exits.
`-o [<path/>]<out.gtf>`	Sets the name of the output GTF file where StringTie will write the assembled transcripts. This can be specified as a full path, in which case directories will be created as needed. By default StringTie writes the GTF at standard output.
`-p <int>`	Specify the number of processing threads (CPUs) to use for transcript assembly. The default is 1.
`-G <ref_ann.gff>`	Use a reference annotation file (in GTF or GFF3 format) to guide the assembly process. The output will include expressed reference transcripts as well as any novel transcripts that are assembled. This option is required by options -B, -b, -e, -C (see below).

inputはsortされたBAM

```
$ stringtie ¥
-e ¥
-p 4 ¥
-G genes.gtf ¥
-o count_genes.gtf ¥
hoge.sort.bam
```

-G reference annotation to use for guiding the assembly process (GTF/GFF3)
-e only estimate the abundance of given reference transcripts (requires -G)
-p number of threads (CPUs) to use (default: 1)
-o output path/file name for the assembled transcripts GTF (default: stdout)

個々のサンプルごとに行う

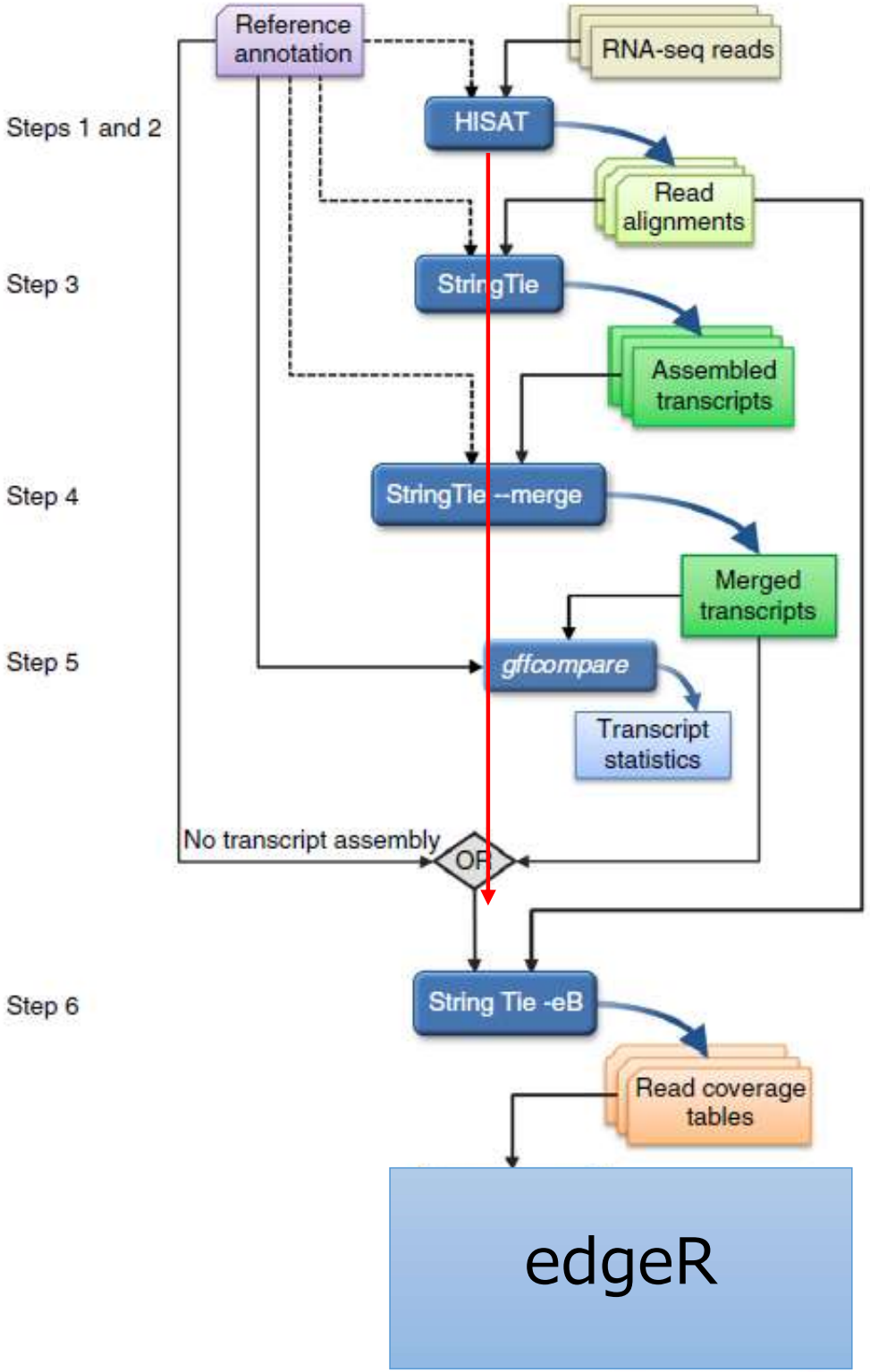
実習3　stringtie

GTFファイルに記載された遺伝子モデルのみを対象とするケース

HISATで作製したsamをsort.bamにし、StringTieにかける
hisat結果　2D2L_rep1.sam

```
$ samtools sort ¥
-@ 4 ¥
-o 2D2L_rep1.sort.bam ¥
2D2L_rep1.sam

$ stringtie -e -p 4 ¥
-G genes.gtf ¥
-o count_2D2L_rep1.gtf ¥
2D2L_rep1.sort.bam
```

samtools v1.3以降、
samファイルのsort,bam化
は同時にできる



Case B

新規な遺伝子モデルを見出し、
それも数える場合

```
$ stringtie ¥
-p 4 ¥
-G genes.gtf ¥
-o count_genes.gtf ¥
hoge.sort.bam
```

-G reference annotation to use for guiding the assembly process (GTF/GFF3)
-p number of threads (CPUs) to use (default: 1)
-o output path/file name for the assembled transcripts GTF (default: stdout)

-e の指定はなし

個々のサンプルごとに
全サンプルで行う。

StringTieのmerge modeでmerged_gtfファイルを作製する

```
$ stringtie ¥
--merge ¥
-p 4 ¥
-G genes.gtf ¥
-o stringtie_merged.gtf ¥
sample.list   #個々のgtfファイルのスペース区切りでの羅列も可
```

Sample.list　　gtfファイルの場所を指定したファイルを用意

Ex)
count_2D_rep1.gtf
count_2D_rep2.gtf
count_2D_rep3.gtf
count_2D2L_rep1.gtf
count_2D2L_rep2.gtf
count_2D2L_rep3.gtf

mergeしたgtfファイルを-Gで指定して、先と同様-eを指定し、個々のbamからカウントデータを得る

```
$ stringtie ¥
-e ¥
-p 4 ¥
-G stringtie_merged.gtf ¥
-o count_genes.gtf ¥
hoge.sort.bam
```

-G reference annotation to use for guiding the assembly process (GTF/GFF3)
-e only estimate the abundance of given reference transcripts (requires -G)
-p number of threads (CPUs) to use (default: 1)
-o output path/file name for the assembled transcripts GTF (default: stdout)

個々のサンプルごとに
全サンプルで行う。

実習4　stringtie

新規な遺伝子モデルを見出し、それも対象とするケース

ここではアラビドプシス5本の染色体のうち、
chr5にあるすべてのgtf情報を削除したgtfファイル
（genes_except_chr5.gtf)をrefとする。

HISATで作製したsamをsort.bamにし、StringTieにかける
hisat結果　2D2L_rep1.sam

```
$ stringtie -p 4 ¥
-G genes_except_chr5.gtf ¥
-o count_2D2L_rep1.gtf ¥
2D2L_rep1.sort.bam
```

-eを抜く

GTFファイルを比較するツール

The gffcompare utility

The program gffcompare can be used to compare, merge, annotate and estimate accuracy of one or more GFF files (the "query" files), when compared with a reference annotation (also provided as GFF/GTF). A more detailed documentation for the program and its output files can be found here (gffcompare documentation page)

https://ccb.jhu.edu/software/stringtie/gff.shtml#gffcompare

```
gffcompare ¥
-r gene.gtf ¥
-o merged ¥
stringtie_merged.gtf
```

```
# gffcompare v0.10.4 | Command line was:
#gffcompare -r genes.gtf -o merged stringtie_merged.gtf
#

#= Summary for dataset: stringtie_merged.gtf
#     Query mRNAs :   42241 in   33367 loci  (30667 multi-exon
transcripts)
#            (6233 multi-transcript loci, ~1.3 transcripts per
locus)
# Reference mRNAs :   41607 in   33350 loci  (30127 multi-exon)
# Super-loci w/ reference transcripts:    33240
#-----------------| Sensitivity | Precision  |
        Base level:   100.0     |     99.8    |
        Exon level:   100.0     |     99.4    |
      Intron level:   100.0     |     99.8    |
Intron chain level:   100.0     |     98.2    |
  Transcript level:   100.0     |     98.5    |
       Locus level:   100.0     |     99.9    |

     Matching intron chains:   30127
       Matching transcripts:   41607
              Matching loci:   33350

         Missed exons:       0/169264   (  0.0%)
          Novel exons:     102/170581   (  0.1%)
       Missed introns:       0/127896   (  0.0%)
        Novel introns:      55/128111   (  0.0%)
          Missed loci:       0/33350    (  0.0%)
           Novel loci:      37/33367    (  0.1%)
```

gene.gtf ← 既知model
stringtie_merged.gtf ← 含新規
model
この両者を比較できる

Hisat2→StringTieでの新規gene探索テスト

TAIR10 gene annotationのgenes.gtfをrefにしたものと、
合わせてそこからchr5の情報のみ除去したものの2種をrefとして

アラビドプシス　leaf, flowerのRNA-Seqデータ (PE101)
cutadapt後　218.6M PE reads, 42.00GbaseのRNA-Seqデータ

Hisat2
Stringtieは
genes.gtfおよびgenes_except_chr5.gtfをrefとして、
-eを付けず新規モデルも探索するオプションで実行。
Stringtie mergeにかけ、merged.gtfを得る。

gffcompareで比較

genes.gtf	41,611 mRNA (33,350 loci)
↓	
merged.gtf	52,535 mRNA (32,997 loci)

genes_except_chr5	32,322 mRNA (25,885 loci)
↓	
merged_except_chr5	47,874 mRNA (30,275 loci)

元のchr5にあるmodel	9,268 mRNA (7,453 loci)
新規にfinding出来たもの	7,127 mRNA (4,664 loci)

mRNA 76.9%　loci 62.6%
が見つけ出せた

Differential expression analysis^

Differential expression analysis

Together with HISAT and Ballgown, StringTie can be used for estimating differential expression across multiple RNA-Seq samples and generating plots and differential expression tables as described in our protocol paper.

⋮

Using StringTie with DESeq2 and edgeR

DESeq2 and edgeR are two popular Bioconductor packages for analyzing differential expression, which take as input a matrix of read counts mapped to particular genomic features (e.g., genes). We provide a Python script (`prepDE.py`, or the Python 3 version: `prepDE.py3`) that can be used to extract this read count information directly from the files generated by StringTie (run with the `-e` parameter).

カウントマトリックス作製

```
$ python prepDE.py -h
Usage: prepDE.py [options]

Generates two CSV files containing the count matrices for genes and
transcripts, using the coverage values found in the output of `stringtie -e`

Options:
  -h, --help            show this help message and exit
  -i INPUT, --input=INPUT, --in=INPUT
                        the parent directory of the sample sub-directories or
                        a textfile listing the paths to GTF files [default:
                        ballgown]
  -g G                  where to output the gene count matrix [default:
                        gene_count_matrix.csv
  -t T                  where to output the transcript count matrix [default:
                        transcript_count_matrix.csv]
  -l LENGTH, --length=LENGTH
                        the average read length [default: 75]
  -p PATTERN, --pattern=PATTERN
                        a regular expression that selects the sample
                        subdirectories
  -c, --cluster         whether to cluster genes that overlap with different
                        gene IDs, ignoring ones with geneID pattern (see
                        below)
  -s STRING, --string=STRING
                        if a different prefix is used for geneIDs assigned by
                        StringTie [default: MSTRG]
  -k KEY, --key=KEY     if clustering, what prefix to use for geneIDs assigned
                        by this script [default: prepG]
  --legend=LEGEND       if clustering, where to output the legend file mapping
                        transcripts to assigned geneIDs [default: legend.csv]
```

```
$ python prepDE.py
```

gene_count_matrix.csv
transcript_count_matrix.csv

Case study 2: Genome-based RNA-Seq pipeline
を進め、確認してみよう。

```
gene_id,2D2L_rep1,2D2L_rep2,2D2L_rep3,2D2L_rep4,2D_rep1,2D_rep2,2D_rep3,4D_rep1,4
D_rep2,4D_rep3,4D_rep4
AT4G22890,295,204,203,154,20,22,17,35,26,17,22
AT1G38440,0,0,0,0,0,0,0,0,0,0,0
AT3G27910,0,0,0,0,0,0,0,0,0,0,0
AT1G06620,3,0,6,0,0,3,4,9,0,3,0
AT5G54067,0,0,0,0,0,0,0,0,0,0,0
AT2G34630,52,13,10,18,9,0,3,11,7,12,11
AT2G46660,0,0,0,3,4,0,0,16,23,3,6
AT2G25590,13,7,7,12,3,4,7,21,15,13,15
AT1G43171,0,0,0,0,0,0,0,0,0,0,0
AT5G25130,3,5,3,5,0,0,0,0,0,0,0
AT2G32280,6,0,7,0,5,0,15,0,5,6,0
AT3G15020,5,0,4,7,40,9,23,9,18,10,0
AT5G61100,0,0,0,0,0,0,0,0,0,0,0
AT5G01650,42,15,27,13,35,19,33,0,23,10,18
AT5G05570,6,8,4,4,3,5,3,0,11,9,3
AT3G09770,47,30,25,10,3,14,14,38,46,13,26
AT3G10210,9,0,5,12,0,7,12,20,9,9,3
AT5G06000,0,0,0,5,7,0,5,0,0,0,0
AT5G64620,40,31,20,31,64,35,41,21,37,41,36
AT1G75280,36,45,36,44,8,11,14,16,10,4,11
```

このカウントマトリックスファイルをedgeRへのinputとして、transcript base解析で扱った同一の方法で解析を進める。

edgeRでの解析

このケースでは　,が区切りのテキストとして得られているので、read.csvを用いる。

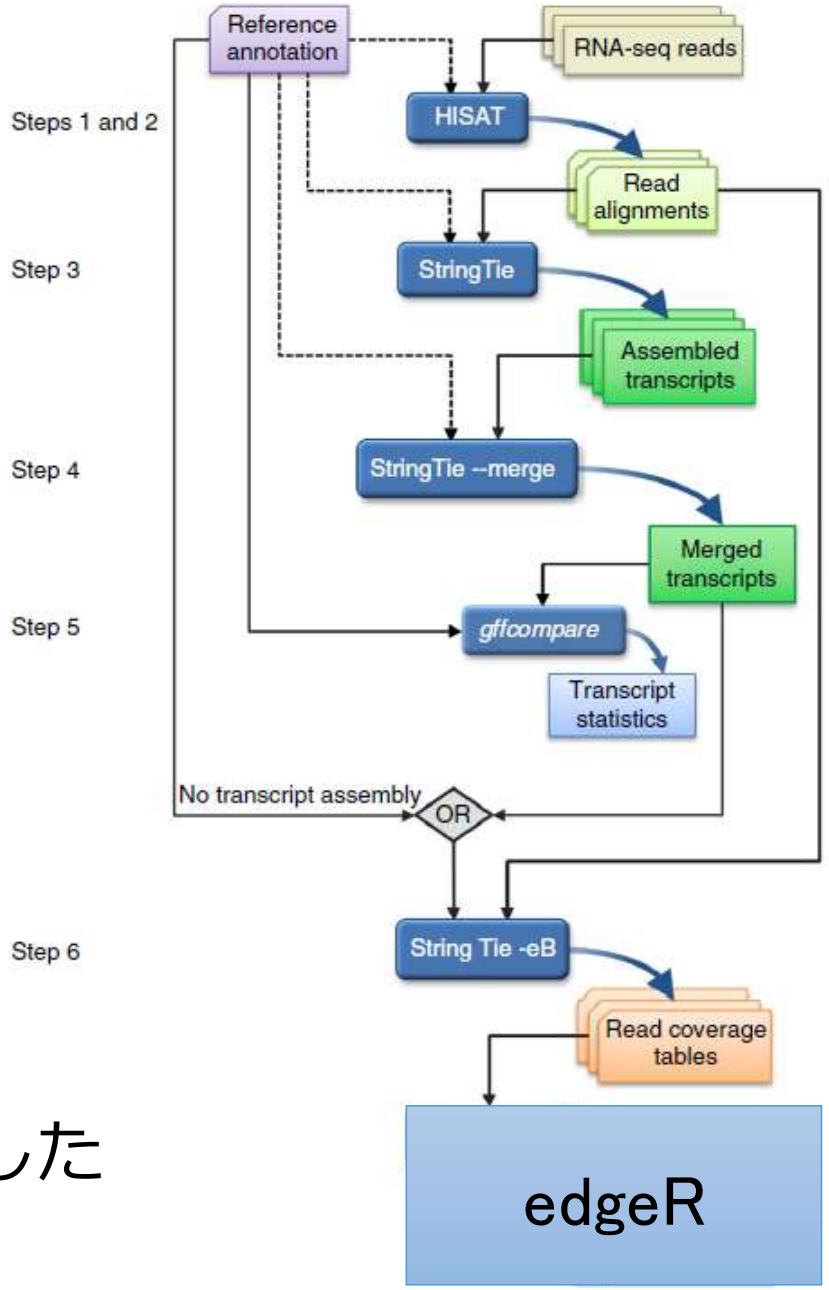
```
$ R
> library(edgeR)
> dat<-read.csv("gene_count_matrix.csv",row.names=1)
> group <- c(rep("2D",3),rep("2D2L",3))
> D<-DGEList(dat,group=group)
> D<-calcNormFactors(D)
> D<-estimateCommonDisp(D)
> D<-estimateTagwiseDisp(D)

2D vs 2D2Lの比較
> de_2D_2D2L <- exactTest(D,pair=c("2D","2D2L"))
> tmp <- topTags(de_2D_2D2L, n=nrow(de_2D_2D2L$table))
> write.table(tmp$table, "de.tagwise2.txt", sep="¥t", quote=F)
```

まとめ

HISAT

StringTie

edgeR



上記の流れを基盤にした、
genome baseのDEG解析を紹介した