基生研ゲノムインフォマティックス・トレーニングコース　2018春
RNA-seq入門 – NGSの基礎からde novo解析まで–
実践編 ： RNA-seq解析パイプライン
2018.03.08–2018.03.09

RNA-Seqパイプライン
ゲノムベースの解析法

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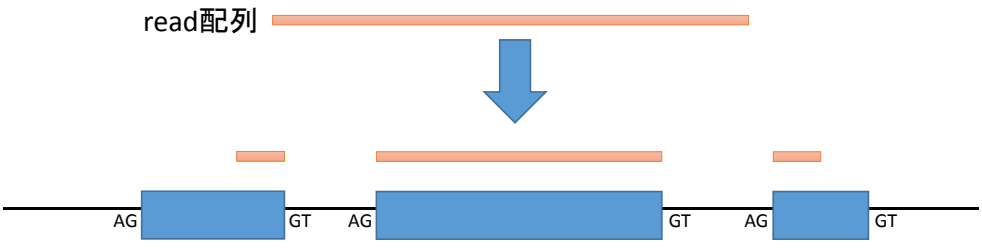
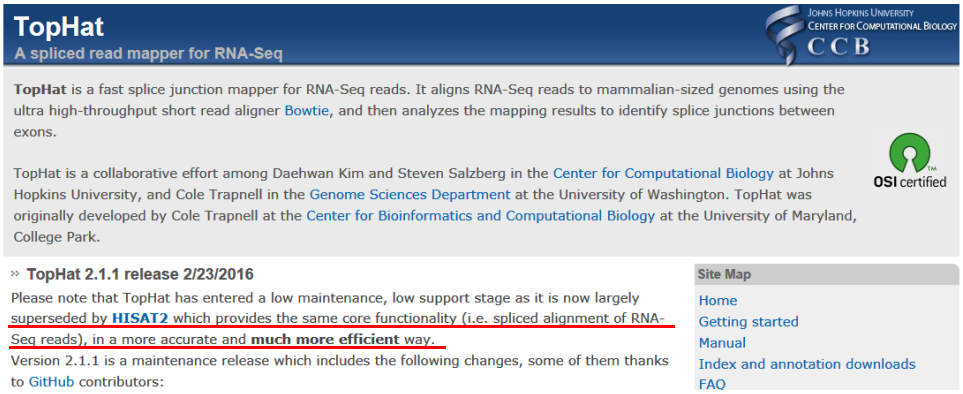
生物機能解析センター
山口勝司

genomeをレファレンスとする場合

レファレンス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HISATを用いる
他　Tophat, Blat, SpliceMap, MapSplice, GSMAP, QPALMA



実際こんな感じにアラインされる



Traditional 'Tuxedo' package

New 'Tuxedo' package

TopHat2

↓

Cufflinks

→

HISAT

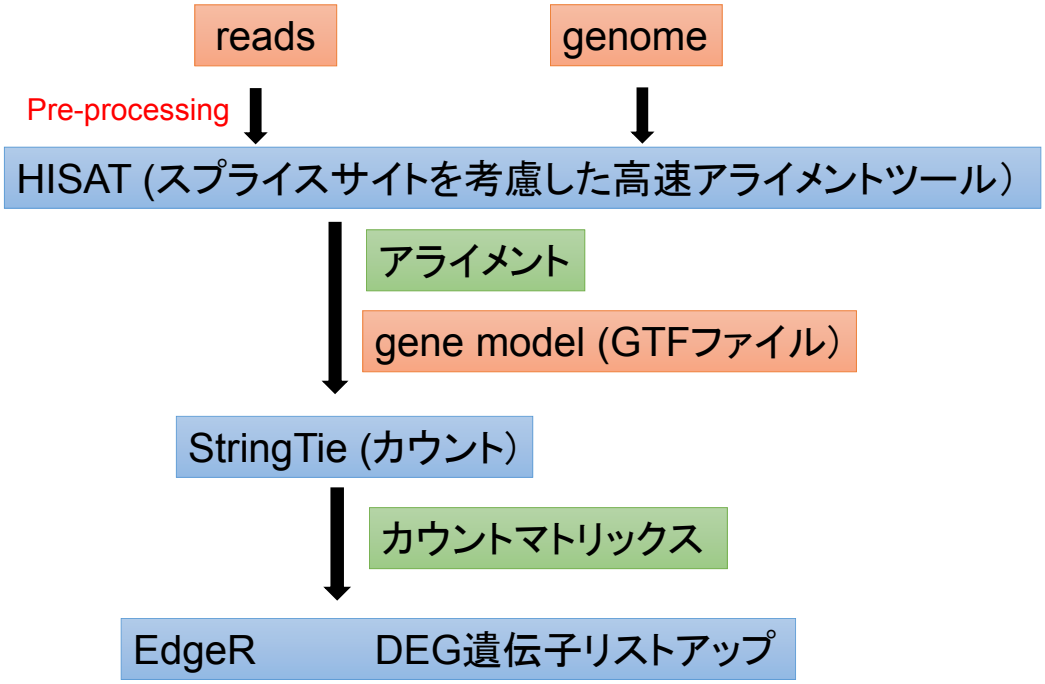
↓

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



HISAT

HISAT2

graph-based alignment of next generation sequencing reads to a population of genomes

Johns Hopkins University Center for Computational Biology CCB

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 to the best of our knowledge. 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 (each index representing a genomic region of 56 Kbp, with 55,000 indexes needed to cover the human population). 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).

OSI certified

TopHat2と比較して
とにかく速い

HISAT2 2.1.0 release 6/8/2017

- This major version includes the first release of HISAT-genotype, which currently performs HLA typing, DNA fingerprinting analysis, and CYP typing on whole genome sequencing (WGS) reads. We plan to extend the system so that it can analyze not just a few genes, but a whole human genome. Please refer to the HISAT-genotype website for more details.
- HISAT2 can be directly compiled and executed on Windows system using Visual Studio, thanks to Nigel Dyer.
- Implemented --new-summary option to output a new style of alignment summary, which is easier to parse for programming purposes.
- Implemented --summary-file option to output alignment summary to a file in addition to the terminal (e.g. stderr).
- Fixed discrepancy in HISAT2's alignment summary.
- Implemented --no-templatelen-adjustment option to disable automatic template length adjustment for RNA-seq reads.

Site Map

Home
Manual
FAQ

News and Updates

New releases and related tools will be announced through the Bowtie **mailing list**.

Getting Help

Please use hisat2.genomics@gmail.com for private communications only. Please do not email technical questions to HISAT2 contributors directly.

HISAT2

graph-based alignment of next generation sequencing reads to a population of genomes

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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

メジャーなモデル生物なら
indexが用意されている

News and Updates

New releases and related tools will be announced through the Bowtie **mailing list**.

Getting Help

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Releases

version 2.1.0 6/8/2017

- Source code
- Linux x86_64 binary
- Mac OS X x86_64 binary
- Windowns binary

Please cite:
Kim D, Langmead B and Salzberg SL. **HISAT: a fast spliced aligner with low memory requirements**. *Nature Methods* 2015

Indexes (see **note**)

H. sapiens, GRCh38

genome	3.9 GB
genome_snp	4.6 GB
genome_tran	4.1 GB
genome_snp_tran	4.6 GB

Introduction

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.

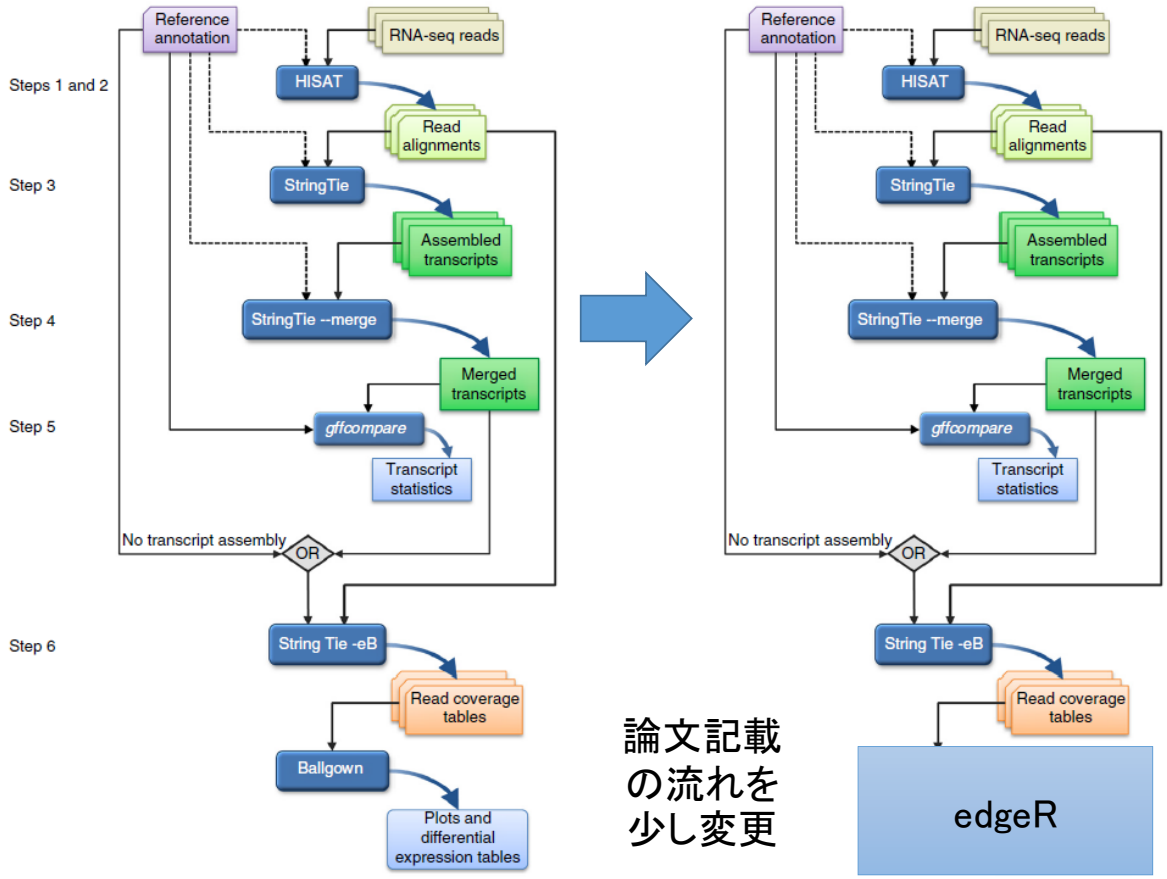
PROTOCOL

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

Mihaela Pertea[1,2], Daehwan Kim[1], Geo M Pertea[1], Jeffrey T Leek[3] & Steven L Salzberg[1–4]

[1]Center for Computational Biology, McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins School of Medicine, Baltimore, Maryland, USA. [2]Department of Computer Science, Whiting School of Engineering, Johns Hopkins University, Baltimore, Maryland, USA. [3]Department of Biostatistics, Bloomberg School of Public Health, Johns Hopkins University, Baltimore, Maryland, USA. [4]Department of Biomedical Engineering, Johns Hopkins University, Baltimore, Maryland, USA. Correspondence should be addressed to S.L.S. (salzberg@jhu.edu).

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



hisat-buildでリファレンスのインデックスを作る

```
$ hisat2-build -h
HISAT2 version 2.0.5 by Daehwan Kim (infphilo@gmail.com, http://www.ccb.jhu.edu/people/infphilo)
Usage: hisat2-build [options]* <reference_in> <bt2_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                      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.bt2 (packed reference) portion
    -3/--justref            just build .3/.4.bt2 (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
    --seed <int>            seed for random number generator
    -q/--quiet              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
```

ヒト・マウス等一部を除き、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
```

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
```

HISAT基本コマンド

```
$ hisat2 -h
HISAT2 version 2.0.5 by Daehwan Kim (infphilo@gmail.com,
www.ccb.jhu.edu/people/infphilo)
Usage:
  hisat2 [options]* -x <ht2-idx> {-1 <m1> -2 <m2> | -U <r> | --sra-acc <SRA accession
number>} [-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).
  <SRA accession number>        Comma-separated list of SRA accession numbers, e.g. --
sra-acc SRR353653,SRR353654.
  <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

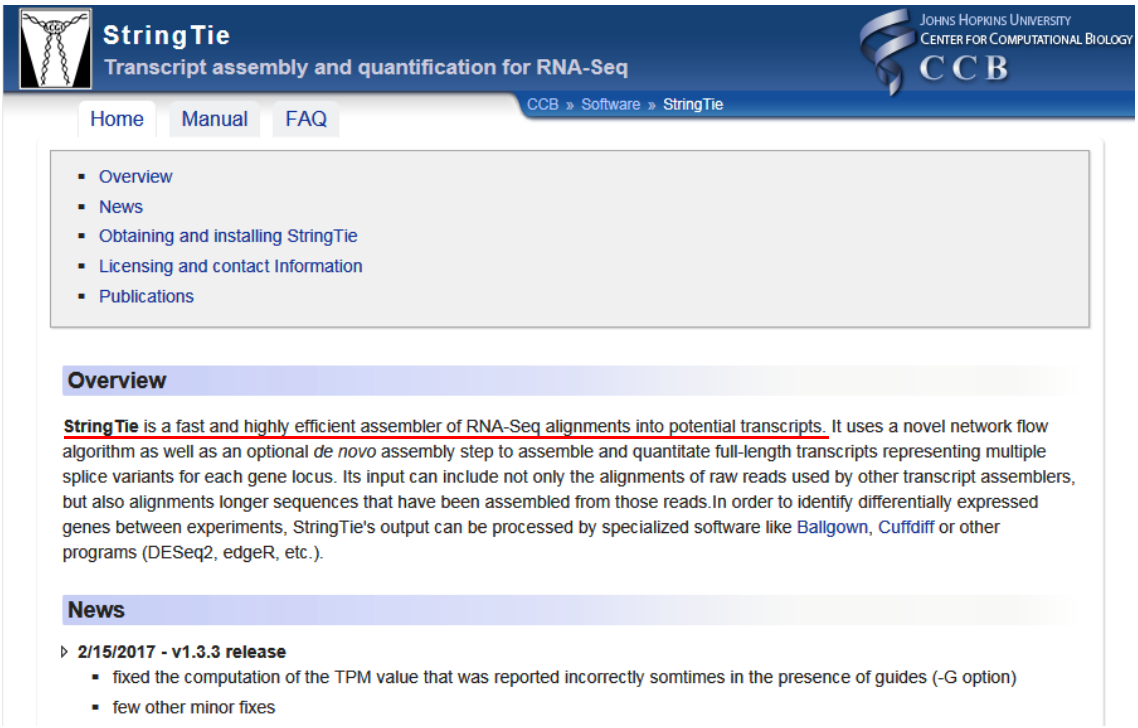
実習2　hisat2

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
```

samファイルの内容を確認しよう

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



StringTieを用いてアラインされたreadを数える

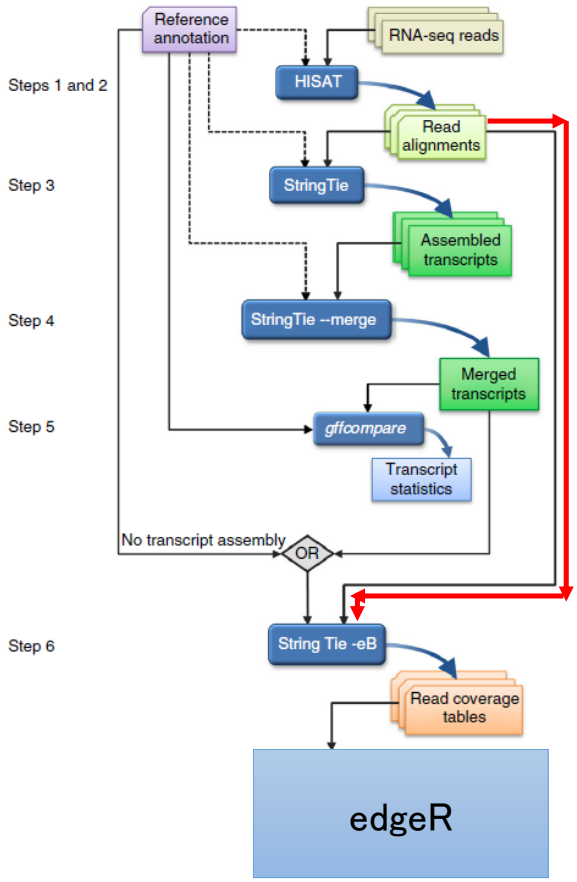
StringTieの解析の方向性として大きく2つある

・GTFファイルに記載された遺伝子モデルのみを数える

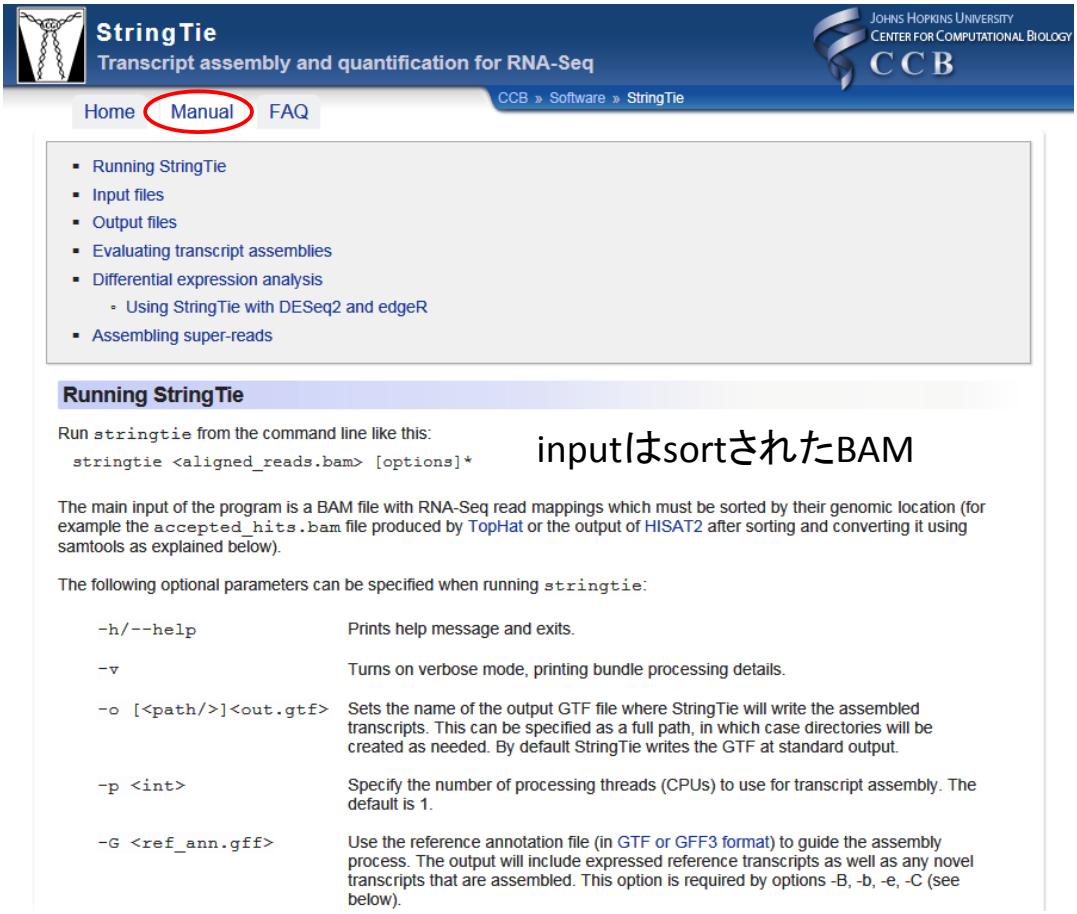
・新規な遺伝子モデルを見出し、それも数える
新規な遺伝子モデルはサンプルによって異なりうるので、
個々のモデルをStringTieのmerge modeでmergeし、
それを含めた、新しい遺伝子モデルを作製できる

StringTie基本コマンド

```
$ stringtie -h
StringTie v1.3.3 usage:
 stringtie <input.bam ..> [-G <guide_gff>] [-l <label>] [-o <out_gtf>] [-p
<cpus>]
  [-v] [-a <min_anchor_len>] [-m <min_tlen>] [-j <min_anchor_cov>] [-f
<min_iso>]
  [-C <coverage_file_name>] [-c <min_bundle_cov>] [-g <bdist>] [-u]
  [-e] [-x <seqid,..>] [-A <gene_abund.out>] [-h] {-B | -b <dir_path>}
Assemble RNA-Seq alignments into potential transcripts.
        :
        :
        :
Transcript merge usage mode:
  stringtie --merge [Options] { gtf_list | strg1.gtf ...}
With this option StringTie will assemble transcripts from multiple
input files generating a unified non-redundant set of isoforms. In this mode
the following options are available:
  -G <guide_gff>   reference annotation to include in the merging (GTF/GFF3)
  -o <out_gtf>     output file name for the merged transcripts GTF
                    (default: stdout)
        :
        :
        :
```



GTFファイルに記載された
遺伝子モデルのみを数える方式



StringTie
Transcript assembly and quantification for RNA-Seq

Johns Hopkins University Center for Computational Biology CCB

CCB » Software » StringTie

Home Manual FAQ

- Running StringTie
- Input files
- Output files
- Evaluating transcript assemblies
- Differential expression analysis
  - Using StringTie with DESeq2 and edgeR
- Assembling super-reads

Running StringTie

Run `stringtie` from the command line like this:

```
stringtie <aligned_reads.bam> [options]*
```

inputはsortされたBAM

The main input of the program is a BAM file with RNA-Seq read mappings which must be 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 following optional parameters can be specified when running `stringtie`:

`-h/--help`	Prints help message and exits.
`-v`	Turns on verbose mode, printing bundle processing details.
`-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 the 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).

```
$ stringtie \
-e \
-B \
-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)
-B enable output of Ballgown table files which will be created in the
same directory as the output GTF (requires -G, -o recommended)
-p number of threads (CPUs) to use (default: 1)
-o output path/file name for the assembled transcripts GTF (default: stdout)

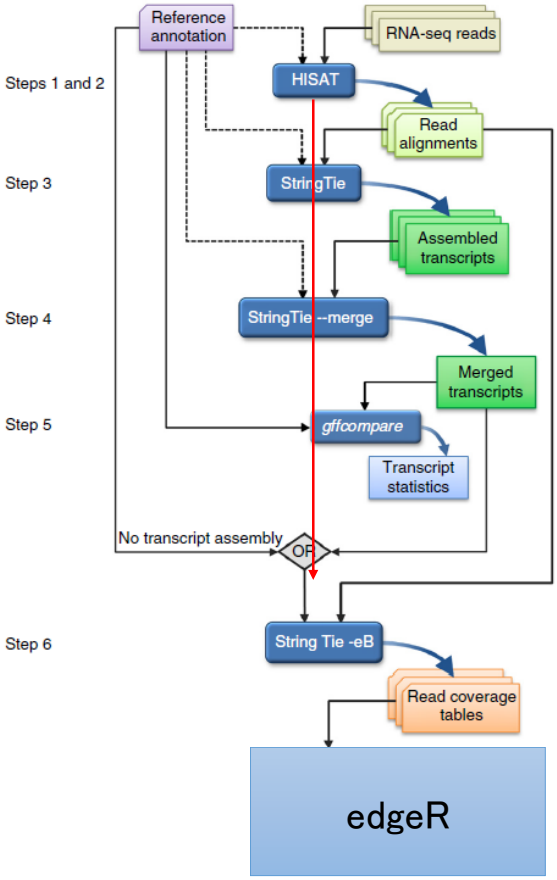
個々のサンプルごと行う

実習3　stringtie
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 -B -p 4 \
-G genes.gtf \
-o count_2D2L_rep1.gtf \
2D2L_rep1.sort.bam
```

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



新規な遺伝子モデルを見出し、
それも数える

```
$ 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 -Bの指定はなし

個々のサンプルごと行う

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
```

sample_lst.txt　　gtfファイルの場所を指定

```
./ballgown/2D_rep1/2D_rep1.gtf
./ballgown/2D_rep2/2D_rep2.gtf
./ballgown/2D_rep3/2D_rep3.gtf
./ballgown/2D_rep1/2D_rep1.gtf
./ballgown/2D_rep2/2D_rep2.gtf
./ballgown/2D_rep3/2D_rep3.gtf
./ballgown/2D_rep4/2D_rep4.gtf
./ballgown/4D_rep1/4D_rep1.gtf
./ballgown/4D_rep2/4D_rep2.gtf
./ballgown/4D_rep3/4D_rep3.gtf
./ballgown/4D_rep4/4D_rep4.gtf
```

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

```
$ stringtie \
-e \
-B \
-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)
-B enable output of Ballgown table files which will be created in the
same directory as the output GTF (requires -G, -o recommended)
-p number of threads (CPUs) to use (default: 1)
-o output path/file name for the assembled transcripts GTF (default: stdout)

個々のサンプルでおこなう

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 \
-G \
-o merged \
stringtie_merged.gtf
```

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

```
# gffcompare v0.10.4 | Command line was:
#gffcompare -r genes.gtf -G -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%)
```

Differential expression analysisへ

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`) 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
```

defaultではballgownフォルダー下にあるgtfファイルの
カウントマトリックスファイルが作成される。

gene_count_matrix.csv
transcript_count_matrix.csv

確認してみよう
less gene_count_matrix.csv

```
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("2D2L",4),rep("2D",3),rep("4D",4))
> 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

の流れを基盤にした、
genome baseのDEG解析を紹介した