

RNA-Seqパイプライン ゲノムベースの解析法

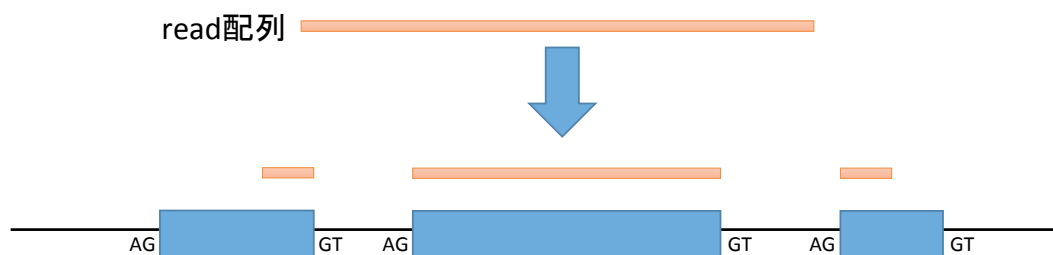
基礎生物学研究所
生物機能解析センター
山口勝司

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



TopHat

A spliced read mapper for RNA-Seq



TopHat is a fast splice junction mapper for RNA-Seq reads. It aligns RNA-Seq reads to mammalian-sized genomes using the ultra high-throughput short read aligner [Bowtie](#), and then analyzes the mapping results to identify splice junctions between exons.

TopHat is a collaborative effort among Daehwan Kim and Steven Salzberg in the [Center for Computational Biology](#) at Johns Hopkins University, and Cole Trapnell in the [Genome Sciences Department](#) at the University of Washington. TopHat was originally developed by Cole Trapnell at the [Center for Bioinformatics and Computational Biology](#) at the University of Maryland, College Park.



※ TopHat 2.1.1 release 2/23/2016

Please note that TopHat has entered a low maintenance, low support stage as it is now largely superseded by [HISAT2](#) which provides the same core functionality (i.e. spliced alignment of RNA-Seq reads), in a more accurate and **much more efficient** way.

Version 2.1.1 is a maintenance release which includes the following changes, some of them thanks to GitHub contributors:

Site Map

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- [Getting started](#)
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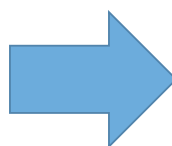
Traditional 'Tuxedo' package

New 'Tuxedo' package

TopHat2



Cufflinks



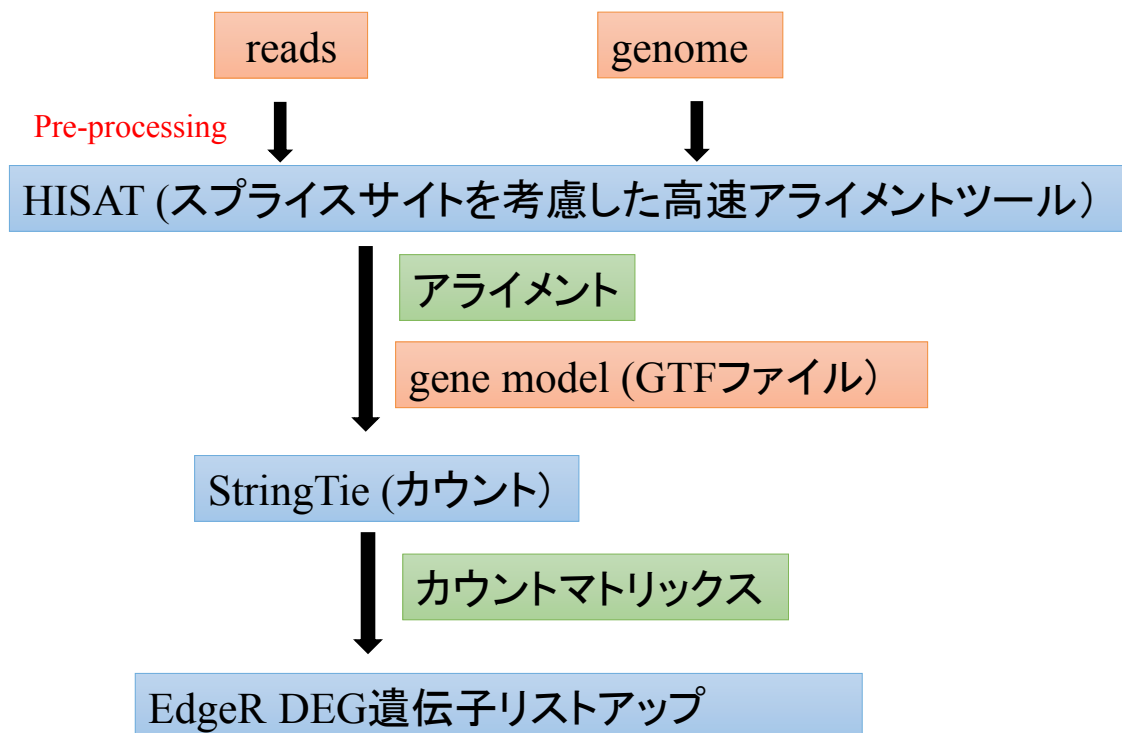
HISAT



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



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).



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-templaten-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.

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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

Site Map

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News and Updates

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Releases

version 2.1.0 6/8/2017
[Source code](#)
[Linux x86_64 binary](#)
[Mac OS X x86_64 binary](#)
[Windows 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](#))

<i>H. sapiens</i> , GRCh38	
<i>genome</i>	3.9 GB
<i>genome_snp</i>	4.6 GB
<i>genome_tran</i>	4.1 GB
<i>genome_snp_tran</i>	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 interoperability 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.

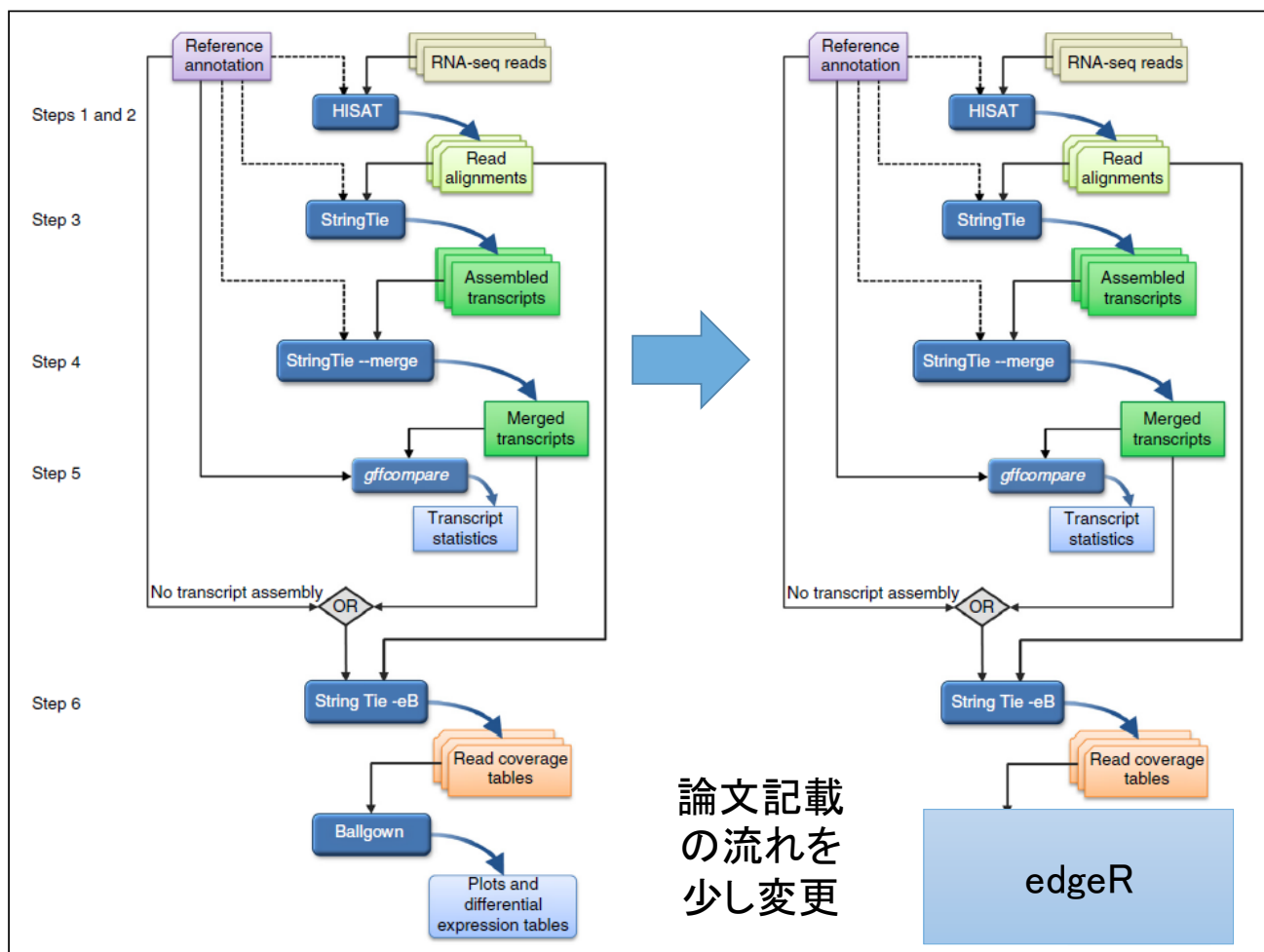
PROTOCOL

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



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)
  --nodd               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) のリ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
```



- Overview
- News
- Obtaining and installing StringTie
- Licensing and contact information
- Publications

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 the alignments of raw reads used by other transcript assemblers, but also alignments 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.).

News

- ▷ **11/2/2018 - v1.3.5 release** is a maintenance release providing a few minor changes and additions:
- spliced alignments produced by minimap2 (in SAM format) are now supported; there is no need to pre-process them in order to add the `XS` tag, the `"t3"` tag is now recognized as an alternative. **Note:** sorting of the SAM/BAM file is still required!
 - the default value for the `-M` option (maximum multi-mapping fraction) is now set to 1.0, such that transcripts assembled from only multi-mapped reads are no longer excluded (e.g. in case of multiple gene copies).
 - read alignments not having a transcription strand assigned (generally unspliced mappings) can be now automatically assigned the strand of the overlapping reference (guide) transcript, if any such overlap exists.

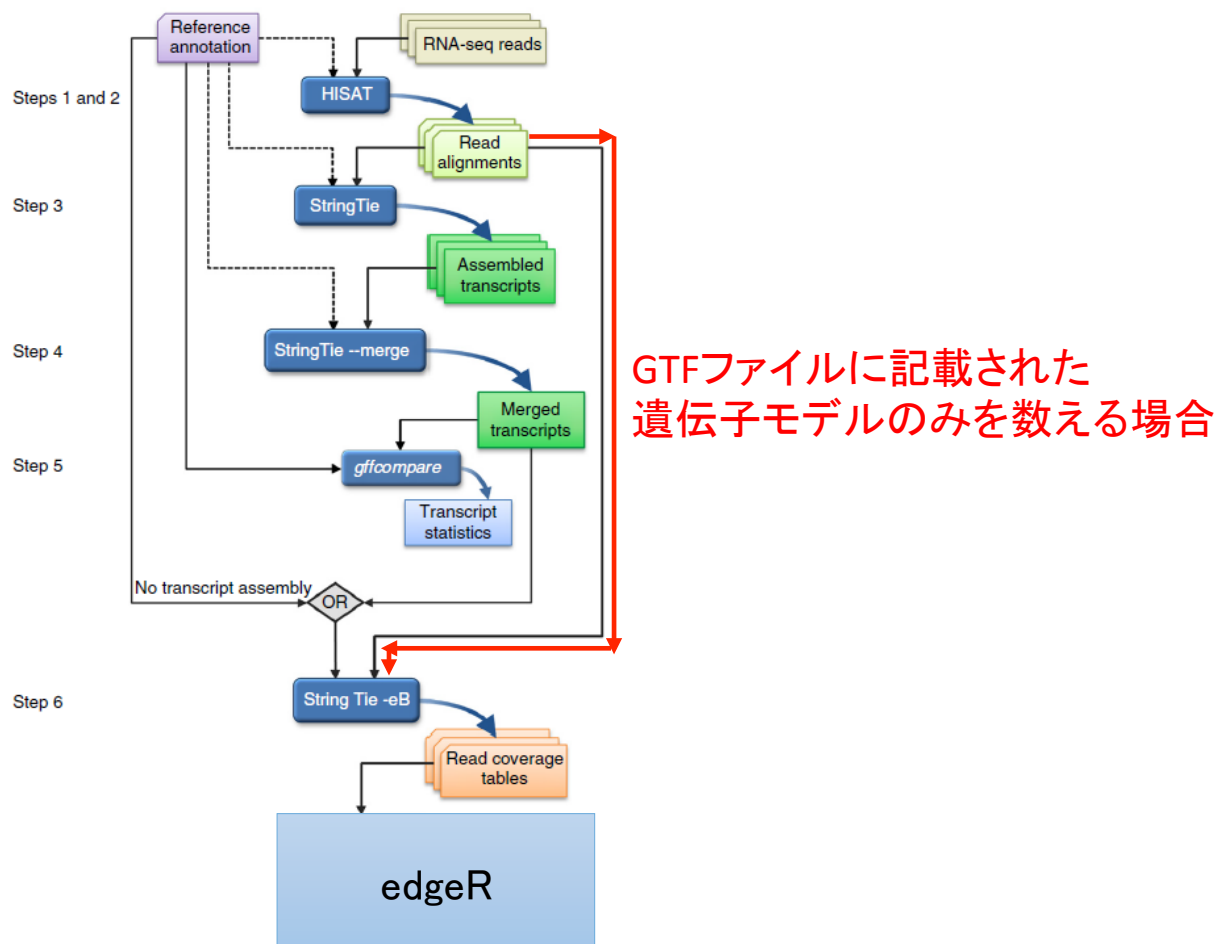
StringTieを用いてアラインされたreadを数える

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

L

```
$ ./stringtie -h
StringTie v1.3.4d 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 | strgl.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)
:
:
:
```





- 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`:

<code>-h/--help</code>	Prints help message and exits.
<code>-v</code>	Turns on verbose mode, printing bundle processing details.
<code>-o [<path/>]<out.gtf></code>	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.
<code>-p <int></code>	Specify the number of processing threads (CPUs) to use for transcript assembly. The default is 1.
<code>-G <ref_ann.gff></code>	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 <code>-B</code> , <code>-b</code> , <code>-e</code> , <code>-C</code> (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)

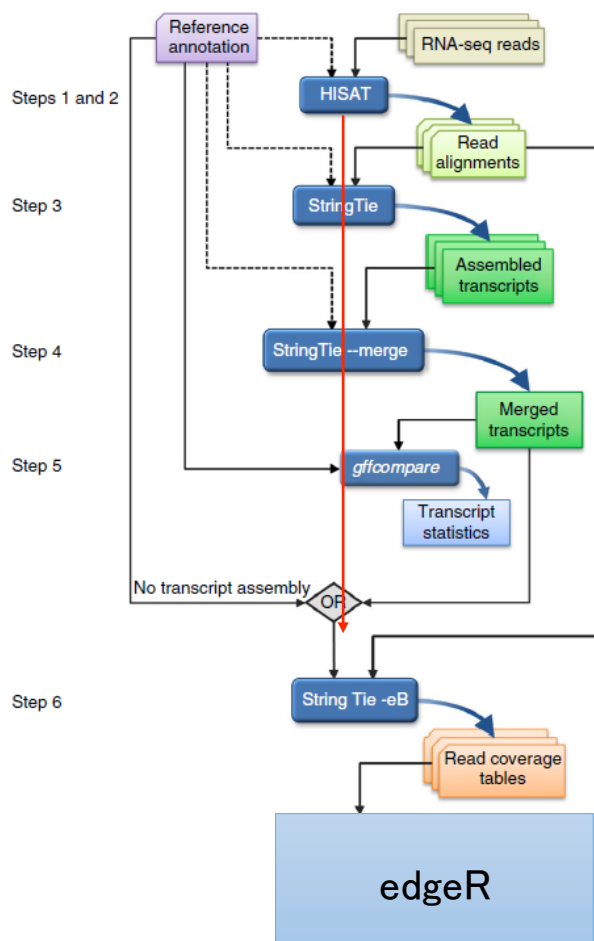
個々のサンプルごと行う

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 -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:

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

```
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_rep1,4D_rep2,4  
D_rep3,4D_rep4  
AT4G22890,295,204,203,154,20,22,17,35,26,17,22  
AT1G38440,0,0,0,0,0,0,0,0,0,0,0,0  
AT3G27910,0,0,0,0,0,0,0,0,0,0,0,0  
AT1G06620,3,0,6,0,0,3,4,9,0,3,0  
AT5G54067,0,0,0,0,0,0,0,0,0,0,0,0  
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  
AT1G43171,0,0,0,0,0,0,0,0,0,0,0,0  
AT5G25130,3,5,3,5,0,0,0,0,0,0,0,0  
AT2G32280,6,0,7,0,5,0,15,0,5,6,0  
AT3G15020,5,0,4,7,40,9,23,9,18,10,0  
AT5G61100,0,0,0,0,0,0,0,0,0,0,0,0  
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  
AT5G06000,0,0,0,5,7,0,5,0,0,0,0,0  
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、`read.csv`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

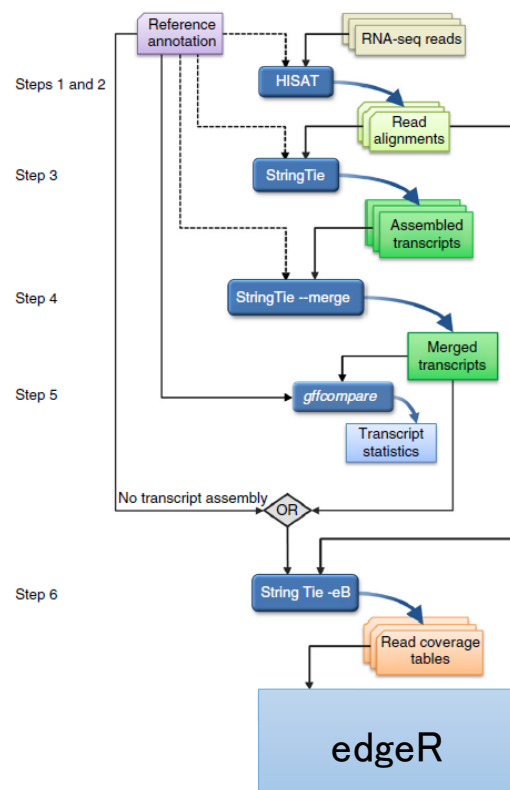
```
> de_2D_2D2L <- exactTest(D,pair=c("2D","2D2L"))
> tmp <- topTags(de_2D_2D2L, n=nrow(de_2D_2D2L$table))
> write.table(tmp$table, "de.tagwise2.txt", sep="\t", quote=F)
```

まとめ

HISAT

StringTie

edgeR



上記の流れを基盤にした、
genome baseのDEG解析を紹介した