INCOMPLETE DRAFT: Smashing single cells into k-mer sketches

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Abstract

Single-cell RNA-sequencing is a powerful technology for identifying novel and known cell types. However, the task of identifying new and novel cell types across species is nontrivial, especially when one or more of the species have poorly annotated genomes. Thus, there is an unmet need to quantitatively compare single-cell transcriptomes across species, without the need for a reference genome. To this end, we have developed a genome-agnostic method to compare molecular profiles using a lossy encoding on k-mers from putative protein-coding RNA-seq reads. We benchmark the annotation-agnostic methods on a bulk comparative transcriptomic dataset consisting of nine species and six tissues, showing that we can recapitulate the results as using only reads mapping to 1:1 orthologs of protein-coding genes, and we are able to resolve ... which can only be seen by using the reference-free k-mer method. We then show that k-mers can also be used for comparing transcriptomes built from long read sequencing, by comparing the cell-cell similarity nearest neighbor graphs built on k-mers from short reads and long reads from the same cells in a primate brain organoid system. We find that using k-mers on short reads has the advantage of resolving ... in comparison to using read counts from 1:1 gene orthologs, while long reads provide additional information in the form of ... Using k-mers, we were able to resolve cell types X in the primate brain organoid dataset, which was hidden using read counts alone. Thus, we have show the reference-free methods using the k-mers from single cells is a novel, annotation-agnostic method for comparing cells across species that is capable of identifying cell states unique to a particular organism, helping to build the cell type evolution tree of life.

Introduction

There are a predicted 8.7 million Eukaryotic species on earth [1], yet only 14% (1,233,500) have been catalogued and 0.000002% (200/8,700,000 = 2.3e-08) have genomes present in ENSEMBL Assemblies (as of ENSEMBL 98 – September 2019 release) [2]. And yet, the genome sequence is not enough. To truly understand the diversity of life on this planet, we need to determine not just the DNA blueprints of life, but understand the instantiation of the DNA, the cell types of the species. While sequencing DNA gives a quantitative measure of the nucleotide differences, it does not inform the functional strategies that change from DNA modifications due to speciation events . As new species can be defined by a new cell type. For example, the existence of a single cell type, the stinging cell called a "Cnidocyte" [3], a single-celled biological weapon, defines the phylum Cnidaria. Thus, entire clades, not only species, can be defined by the introduction of an additional cell type or state. However, it is unclear how many examples there are of this, and how it is possible to find cell types that are unique to one species. Thus, we aim to develop a computational method to compare cell types across species that is reference-agnostic and can find cell types that are novel and present in only one organism.

Organizations of existing cell states can also define novel organismal structures. For example, different physical organizations of similar cell types generate different genitalia in amniotes when comparing mammals to reptiles [4]

Determining common gene ancestry ("orthology") is a difficult problem.

Many approaches exist, reviewed by [5,6,7]. Generally, the approaches are structured in this way: (1) find orthologous groups of genes, (2) build gene trees, (3) build species trees, and (4) assign orthologs, as described in a recent approach (Orthofinder) [8]. In this approach, we are not interested in exactly reconstructing the species or gene trees, but rather inferring function based on cell type transcriptomes. Instead of exactly building the gene trees, we subset the protein-coding sequences into peptide words, and re-encode to lossy peptide encodings.

Determining common ancestry of cell types ("orthologous cell types") [9,10] is an additional difficult problem. Comparative transcriptomics begins with finding a common feature set for embedding molecular profiles across divergent species into a common space. Many researchers take the approach of using one-to-one orthologous genes [11,12,13,14,15,16], others use clusters of orthologous groups [19], others map reads onto a common genome derived from whole-genome alignment [20,21], or map onto native genomes [22] and re-annotate using a tool such as Comparative Annotation Toolkit [23].

Annotating one dataset's cell types from another can be performed using random forest models trained on the original dataset [24], using correlation between cell gene expression profiles as in Cell BLAST [25], locality-sensitive hashing of bit vectors of gene expression as in CellFishing.jl [26], or using a cell type hierarchy as in Garnett [27] and OnClass [28].

k-mers have been proposed for comparing single cells [29] as they are a fast, simple way to create cell-cell similarities. k-mers have also been used for orthologous gene detection [30] However, the work so far has focused on using annotated organisms and not cross-species analyses.

Reduced amino acid alphabets have been previously used to speed up database searches [31,32], protein fold prediction [33,34], and homology recognition [35]. We aim to find "orthologous reads" across species' transcriptomes. By representing each species' transcriptome as the set of k-mers, we can unbiasedly compare transcriptomes in an orthologous space without the need for knowing the orthologous genes ahead of time, or even the need for a reference genome. Additionally, we do not need to reduce the signal to only the genes with a 1:1 orthologous match.

Methods

Methods go here.

Experimental

Primate brain organoid protocols

We did things. One sentence per line. Prefer DOI for references, but for Biorxiv use the URL. DOI example: [5]. Biorxiv example: [8]. Multiple citations per line example: [5,8].

Single-cell capture of primate brain organoids

We did things. One sentence per line. Prefer DOI for references, but for Biorxiv use the URL. DOI example: [5]. Biorxiv example: [8]. Multiple citations per line example: [5,8].

Long read library prep

We did things. One sentence per line. Prefer DOI for references, but for Biorxiv use the URL. DOI example: [5]. Biorxiv example: [8]. Multiple citations per line example: [5,8].

Short read library prep

We did things. One sentence per line. Prefer DOI for references, but for Biorxiv use the URL. DOI example: [5]. Biorxiv example: [8]. Multiple citations per line example: [5,8].

Sequencing

We did things. One sentence per line. Prefer DOI for references, but for Biorxiv use the URL. DOI example: [5]. Biorxiv example: [8]. Multiple citations per line example: [5,8].

Computational

k-mer comparison of orthologous genes

We used ENSEMBL version 97. We did things. One sentence per line. Prefer DOI for references, but for Biorxiv use the URL. DOI example: [5]. Biorxiv example: [8]. Multiple citations per line example: [5,8].

Extraction of putative coding reads from RNA-seq

We did things. One sentence per line. Prefer DOI for references, but for Biorxiv use the URL. DOI example: [5]. Biorxiv example: [8]. Multiple citations per line example: [5,8].

$bam2fasta$ conversion

The `.bam` file generated by the Drop-seq [36] pipeline for the different primates in this study are in the order of 6-12 GB. The Drop-seq `.bam` files so obtained can attribute to few limitations as discussed below. Firstly, loading them in memory all at once would require a lot of RAM depending on how the program will allocate memory for different data typed tags in the `.bam` file. Secondly, if Drop-seq data is not accompanied by a barcodes file to filter the `.bam` file on, it would mean we would have to recursively go through the alignments in the bam file and deduce alignments with higher quality and combine sequences with already exisiting barcodes. This would need a look up

dictionary to be updated as it loops through the alignments in the `.bam` file and would search the look up dictionary as it updates the barcodes. In conclusion, this is a very memory intensive process that seemed to fail on even machines with 2TB RAM.

Hence we propose a method that could work on a computer with lesser RAM and not cause computer hangups. We released an open source pypi package for the same [37]. The package contains solution for the above discussed problem by sharding the `.bam` file into chunks of smaller `.bam` files and stores them in the machine's temporary folder, e.g. `/tmp`. The chunk size of the `.bam` file is a tunable parameter that can be accessed with `--line_count`; by default it is 1500 alignment lines. This process is done serially by iterating through the alignments in the `.bam` file, using `pysam`, a Python wrapper around samtools [38]. Now we employ a MapReduce [39] approach to the temporary `.bam` files to obtain all the reads per cell barcode in a `.fasta` file. In the "Map" step, we distribute the computation i.e parsing the barcode, determining the quality of the read, and if alignment is not duplicated, in parallel across multiple processes on the temporary shards of `.bam` files. These bam shards create temporary `.fasta` files that contain for each read: the cell barcode, unique molecular identifier (UMI), and the aligned sequence. There might be a cell barcode that would be present in different chunks of these sharded `.bam` files. As a result we would have multiple temporary `.fasta` files for the same barcodes. We implemented a method to find the unique barcodes based on these temporary `.fasta` file names and then assigning each of the unique barcodes all the temporary barcode `.fasta` files created by different `.bam` shards in a dictionary. In the "Reduce" step, we concatenate of strings of temporary `.fasta` file names, hence its memory consumption is less than it would be if appending to a list. These temporary `.fasta` files are then combined to one `.fasta` file per barcode by concatenating all the sequences obtained from different `.fasta` files. The concatenation of all sequences for each of the unique barcodes is also then parallelized to use multiple processes. For each of the cell barcodes, there is an option to obtain valid cell barcodes, based on the UMI count per cell barcode. For our datasets we have set the minimum number of UMIs per cell barcode to 1000, a common threshold. The minimum number of UMIs per cell barcode can be customized with the flag `--min-umi-per-barcode`. The computational resources and time taken for processing is as shown in Table [1].

Table 1: Human primate species bam file here is from a brain organoid for human

Primate	BAM file size(GB)	Time(hrs)	RAM(GB)	Processes
Human	12	7	16	32
Orangutan	9	4	16	32
Chimp	9	4	16	32

This method primarily gives us time performance improvement. It reduces time from days or just process running out of memory to hours. Depending on the size of `.bam` file and resources of the cluster/computer it can be further reduced.

Prefer DOI for references, but for Biorxiv use the URL. DOI example: [5]. Biorxiv example: [8]. Multiple citations per line example: [5,8].

Results

Figure 1 – k-mers are sufficient to detect orthologous genes

To determine whether short segments of sequences could detect gene orthologues, we k-merized orthologous genes derived from the ENSEMBL version 97 [40] COMPARA database [41] (Figure [1]). We compared human protein sequences to orthologous chimpanzee, mouse, (orangutan, bonobo,

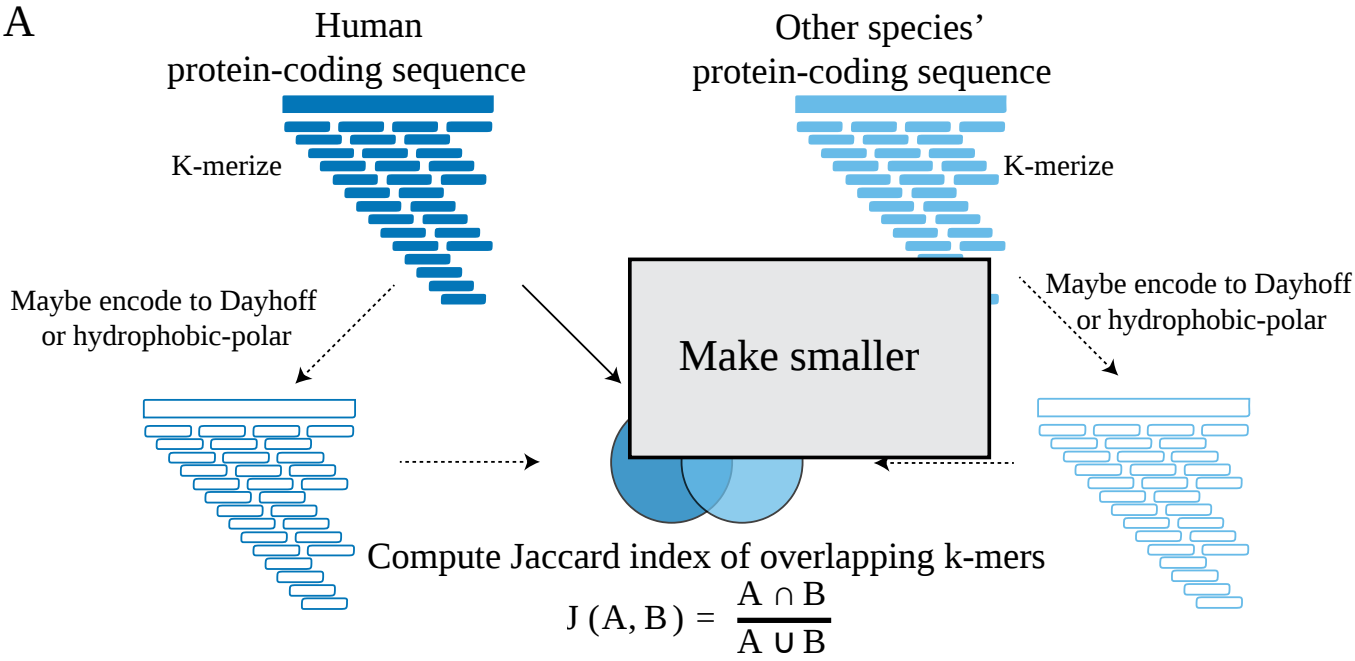
gorilla, macaque, opossum, platypus, chicken) protein sequences, as these are species used in [11]. As a background, we randomly chose 10 non-orthologous genes relative to the human gene. In addition to k-merizing the protein-coding sequence, we also re-encoded the protein-coding sequence into a six-letter Dayhoff alphabet [42], a nine-letter encoding [30], and a two-letter hydrophobic-polar encodings [43,44], show in Table [3].

Table 2: Dayhoff and hydrophobic-polar encodings are a reduced amino acid alphabet allowing for permissive cross-species sequence comparisons. For example, the amino acid sequence `SASHAFIERCE` would be Dayhoff-encoded to `bbbdbfecdac`, and HP-encoded to `phpphhhpppp`.

Amino acid	Property	Dayhoff	Hydrophobic-polar (HP)
C	Sulfur polymerization	a	p
A, G, P, S, T	Small	b	A, G, P: h
			S,T: p
D, E, N, Q	Acid and amide	c	p
H, K, R	Basic	d	p
I, L, M, V	Hydrophobic	e	h
F, W, Y	Aromatic	f	h

k-mer size may be tuned to find an “optimal” length of protein domains across the tree of life. Protein domain lengths follow a power law distribution where proteins with more domains, have shorter domains, whereas proteins with fewer domains, have fewer but longer domains [45,46].

A



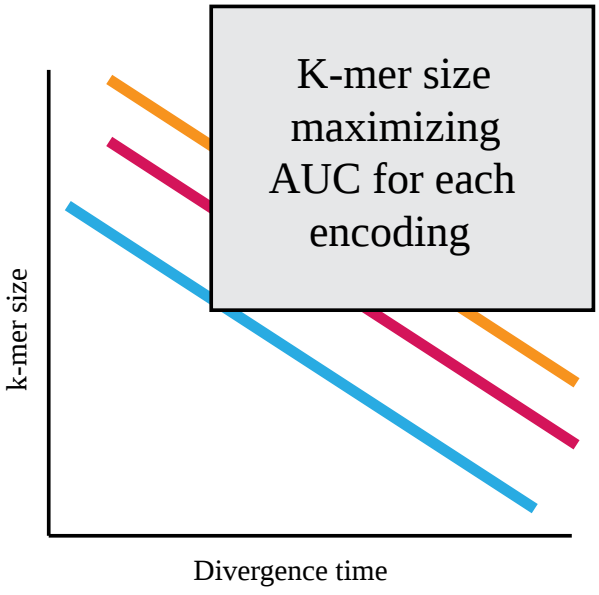
B

C

Update to Brawand2011 species, show diff between background

Update to Brawand2011 species

D



E

Placeholder

Figure 1: **A.** Overview of k-mer comparison of orthologous genes. The protein-coding sequence of each pair of known orthologs is k-merized, potentially encoded as Dayhoff or Hydrophobic polar, and then the Jaccard index (the intersection divided by the union) is computed on the k-mers. **B.** Jaccard similarity of orthologous genes in Dayhoff-encoded k-mer space relative to humans in eight species. x-axis, k-mer size; y-axis, Jaccard index. **C.**

We found that, consistent with previous knowledge, that 1:1 orthologues had higher k-mer similarities as determined by the Jaccard Index. This approach is similar to SwiftOrtho [30], a k-mer based orthology relationship finder.

Additionally, more recently diverged genes had higher k-mer similarity as well.

Outline

- Kmers can approximate orthologies
  - Jaccard similarity of orthologues is higher than non-orthologues
  - Benchmarking using https://orthology.benchmarkservice.org/cgi-bin/gateway.pl
  - Finding orthologues
    - Gold standard
      - ENSEMBL COMPARA
      - Quest for Orthologs consortium, Altenhoff, A. M., Boeckmann, B., Capella-Gutierrez, S., Dalquen, D. A., DeLuca, T., et al. (2016). Standardized benchmarking in the quest for orthologs. Nature Methods, 13(5), 425–430. http://doi.org/10.1038/nmeth.3830 [5]
    - Orthologous groups/Conserved Domain Database [47]

Figure 2 – k-mers from lossily-encoded putative protein-coding reads faithfully pull out reads from protein-coding genes within amniotes

A

B

C

Figure 2: Figure 2.

- Overview of kmermaid pipeline
  - Comparison of tissue across species
    - Partition reads to coding/noncoding bins
    - MinHash the Dayhoff-encoded coding sequences
    - Jaccard similarity on the MinHashes
- Which reads are found to have coding features but didn't map to the genome?
- Do these features map to novel genes or gene fusions?
- Kmers can find correct reading from of RNA-seq reads

- Human peptides → human, chimp, bonobo, orangutan, gorilla, macaque, mouse, opossum, playtpus, chicken RNAseq from Brawand2011 data
- Comparison to other methods: RNASamba [48]

Figure 3 – k-mers can pull out only reads from transcription factors and Amniotes can be compared on the MinHashes of their protein-coding sequences

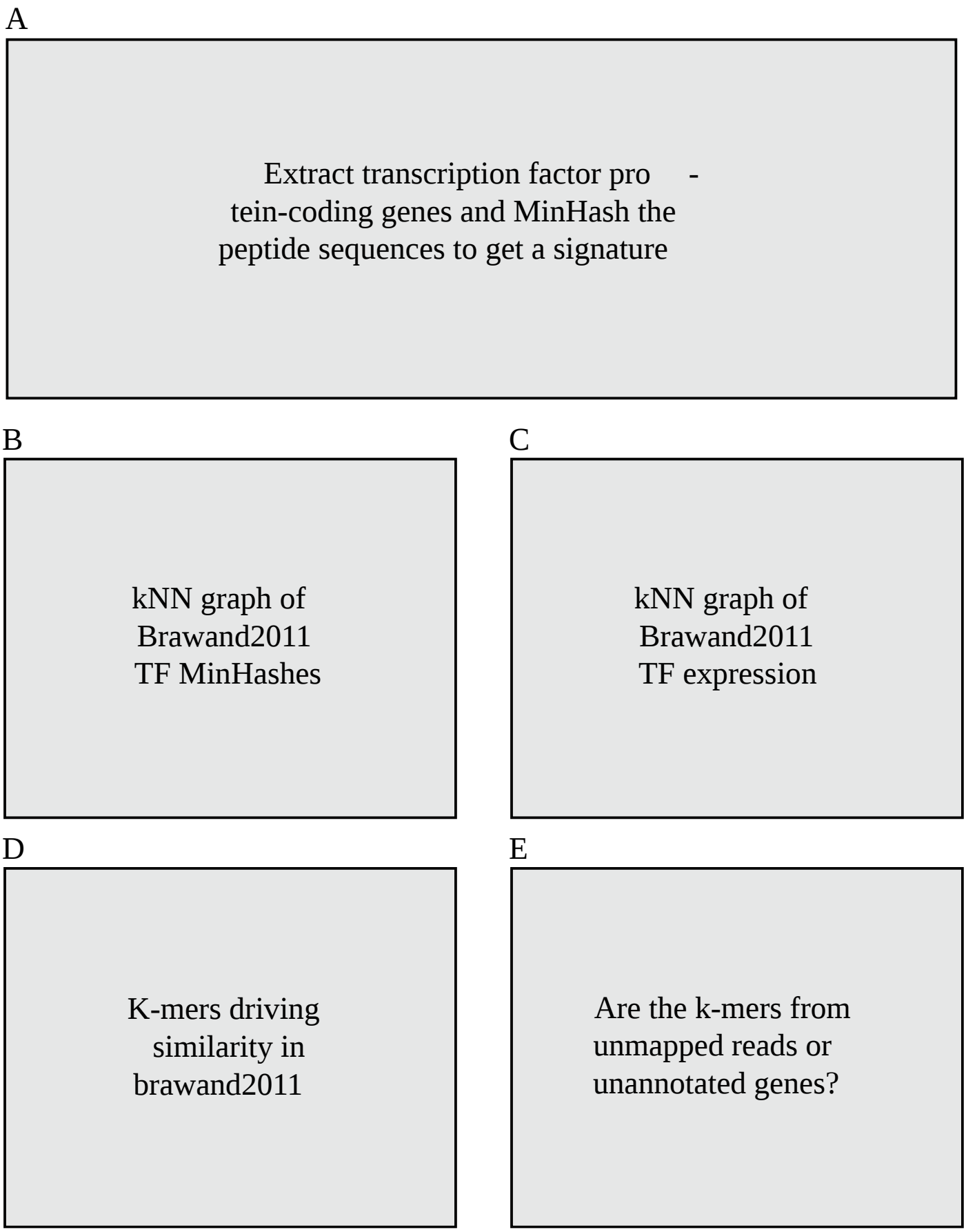


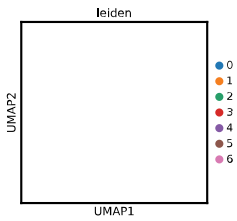
Figure 3: Figure 3.

- Kmers can find only transcription factor reads of TFs from RNA-seq reads
  - Human peptides → human, chimp, bonobo, orangutan, gorilla, macaque, mouse, opossum, playtpus, chicken RNAseq from Brawand2011 data

Figure 4 – k-mers can compare short and long read datasets in primate brain organoids

A

B



kNN/UMAP of
primate organoid
long reads
MinHashes

Figure 4: **A.** Overview of experimental system. Poly-A RNA molecules from single cells from primate brain organoids were captured using the Dolomite system, where molecule received a cell barcode and molecular barcode, was reverse transcribed and primed for full-length cDNA. Then the library was split for sequencing on the Illumina or PacBio platforms. For the Illumina platform, the library was first sheared to be compatible with the platform. **B**. UMAP of short read gene counts from Human (left), Chimp (middle), and Orangutan (right) organoids. **C.** UMAP of short read MinHashes with ksize=33, dayhoff encoding and a log2sketchsize=14. **D**. UMAP of long read MinHashes with ksize=33, dayhoff encoding, andl log2sketchsize=14. **E.** Examples of short reads with ambiguous coding sequence resolved by long reads. **F.** UMAP on short-read gene counts of 1:1 orthologous transcription factor genes across all species. **G.** UMAP on short-read MinHashes of all k-mers that match human transcription factor protein-coding peptide sequences. **H.** UMAP on long-read MinHashes of all k-mers that match human transcription factor protein-coding peptide sequences.

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Discussion

Conclusions and future directions go here.

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Supplemental Methods

Table 3: Dayhoff and hydrophobic-polar encodings are a reduced amino acid alphabet allowing for permissive cross-species sequence comparisons. For example, the amino acid sequence `SASHAFIERCE` would be Dayhoff-encoded to `bbbdbfecdac`, and HP-encoded to `phpphhhpppp`.

Amino acid	Property	Dayhoff	Hydrophobic-polar (HP)
C	Sulfur polymerization	a	p
A, G, P, S, T	Small	b	A, G, P: h
			S,T: p
D, E, N, Q	Acid and amide	c	p
H, K, R	Basic	d	p
I, L, M, V	Hydrophobic	e	h
F, W, Y	Aromatic	f	h

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