manuscript

July 12, 2021

1 Analysis of primary human hepatocytes LRTS

This notebook replicates the analysis from the paper

IsoTools: IsoTools: a python toolbox for long-read transcriptome sequencing (in preparation)

As it involves integration of short read data, the analysis is quite complex. For demonstration of the functionality of the isotools framework with respect to processing of LRTS data, the documentation provides more basic tutorials.

1.1 Preparation

1.1.1 Preparation of the working directory and download of reference and data files

```
cd /my/working/directory
# create some subdirectories
mkdir -p reference ccs refine flnc aligned rnaseq/fastq rnaseq/star/reference pickle cage 

# download the reference genome (806 MB)
genome_link='ftp://ftp.ebi.ac.uk/pub/databases/gencode/Gencode_human/release_36/GRCh38.p13
wget -P reference/ -O GRCh38.p13.genome.fa.gz ${genome_link}
gunzip reference/GRCh38.p13.genome.fa.gz

# download gencode reference annotation (46.2 MB)
gff='gencode.v36.chr_patch_hapl_scaff.annotation'
annotation_link= ftp://ftp.ebi.ac.uk/pub/databases/gencode/Gencode_human/release_36/${gff}
wget -P reference/ ${annotation_link}

# sort by chromosome and position
(zcat ${gff}.gff3.gz| grep ^"#" ; zcat reference/${gff}.gff3.gz|grep -v ^"#"| sort -k1,1 -
# create index
tabix -p gff reference/${gff}_sorted.gff3.gz

# download the isoseq circular consensus read (ccs) files (15.3 GB)
isoseq_link='wget ftp://ftp.sra.ebi.ac.uk/vol1/[accession: PRJEB46194 -  add link here whe
wget -P ccs -O VPA_ccs.bam ${isoseq_link}
isoseq_link='wget ftp://ftp.sra.ebi.ac.uk/vol1/[accession: PRJEB46194 -  add link here whe
wget -P ccs -O CTL_ccs.bam ${isoseq_link}
```

1.1.2 Processing of the CCS files

Install the pacbio isoseq3 workflow and apply the following steps to process the data:

```
#activate the isoseq3 environement (assuming it is called pacbio)
conda activate pacbioprimers=references/primers_NEB.fasta

# Primer removal(lima)
for file in ccs/*_ccs.bam; do
  fn=$(basename $file)
  sample=${fn%_ccs.bam}
  echo [$(date)] $sample
  lima -j 50 --isoseq --dump-clips $file $primers demux/${sample}_demux.bam
done

# Refine: filter reads and trim polyA tail
for file in demux/*_demux.primer_5p--primer_3p.bam; do
  fn=$(basename $file)
  sample=${fn%_demux.primer_5p--primer_3p.bam}
  echo [$(date)] $sample
  isoseq3 refine  --require-polya $file $primers flnc/${sample}_flnc.bam
done

# Alignment of the isoseq data.
# Use the pacbio version of minimap2 to align the flnc reads to the reference genome using
n_threads=60
ref='reference/GRCh38.p13.genome.fa'
for sample in VPA CTL
do pbmm2 align ${ref} flnc/${sample}_flnc.bam aligned/${sample}_aligned.sorted.bam --prese
done
```

1.1.3 Download and processing of RNA-seq data.

```
# download hepatocytes rna-seq data
for err in $(cut -f 35 illumina_samples.tsv); do
echo $err
fastq-dump --split-files $err
done
# download liver rna-seq data
for err in $(cat liver_samples_ERR.tsv); do
echo $err
fastq-dump --gzip --split-files $err
done

#merge rna-seq runs for each timepoint
illu_samples=illumina_samples.txt
for day in 1 2 3 6; do
  for tr in 'CTL' 'VPA'; do
    tr2='none'
    if [ $tr == 'VPA' ]; then tr2='valproic acid'; fi
    echo $day $tr $tr2
    bams=$(python -c "with open('${illu_samples}','r') as f: print(' '.join({l[34] for l in
    cat $(python -c "print(' '.join(['rnaseq/fastq/'+fn+'_1.fastq.gz' for fn in '$bams'.spl
    cat $(python -c "print(' '.join(['rnaseq/fastq/'+fn+'_2.fastq.gz' for fn in '$bams'.spl
  done
done

# alignment with star
star_ref=rnaseq/star/reference/
for fq1 in rnaseq/fastq/*_R1.fastq.gz
do
    bn=$(basename $fq1)
    sample=${bn%_R1.fastq.gz}
    fq2=rnaseq/fastq/${sample}_R2.fastq.gz
    out=rnaseq/star/${sample}_${set}_
    outbam=${out}Aligned.sortedByCoord.out.bam
    echo $sample
    $star --quantMode TranscriptomeSAM GeneCounts --runThreadN 16 --genomeDir $star_ref --
done

# rsem transcript and gene quantification
hg38fa=references/GRCh38.p13.genome.fa
anno=references/gencode.v36.chr_patch_hapl_scaff.annotation.gtf
rsem_ref=rnaseq/rsem/ref/$set
mkdir -p rnaseq/rsem/ref/
rsem-prepare-reference --gtf $anno -p 40 $hg38fa  $rsem_ref
#run rsem
for bam in rnaseq/star/*_${set}_Aligned.toTranscriptome.out.bam
do
    bn=$(basename $bam)
    sample=${bn%_Aligned.toTranscriptome.out.bam}
    out=rnaseq/rsem/${sample}
    if [ ! -s ${out}.isoforms.results ]; then
        echo $out
        rsem-calculate-expression -p 32 --bam --paired-end --no-bam-output --forward-prob
    fi
done

# rmats splice event quantification and differential splicing
# a) with reference derived events
rmats=/path/to/rmats/bin/rmats-turbo/rmats.py
rmats_dir=rnaseq/rMATS/isotools/
mkdir -p $rmats_dir
gtf=references/gencode/gencode.v36.chr_patch_hapl_scaff.annotation.gtf
find $dir/07-star/$set/ -name 'VPA*sortedByCoord.out.bam' -print0 | tr '\0' ,| sed 's/,*$/
find $dir/07-star/$set/ -name 'CTL*sortedByCoord.out.bam' -print0 | tr '\0' ,| sed 's/,*$/
rmats_dir=${rmats_dir}/events
```

```
python $rmats --b1 ${rmats_dir}/b1.txt --b2 ${rmats_dir}/b2.txt --gtf $gtf -t paired --rea
# b) with isotools derived events
rmats_dir=${rmats_dir}/events
gtf=references/gencode/gencode.v36.chr_patch_hapl_scaff.annotation.gtf
find rnaseq/star/ -name 'VPA*sortedByCoord.out.bam' -print0 | tr '\0' ,| sed 's/,*$//g'>${
find rnaseq/star/ -name 'CTL*sortedByCoord.out.bam' -print0 | tr '\0' ,| sed 's/,*$//g'>${
events_dir=${rmats_dir}/events
python $rmats --b1 ${rmats_dir}/b1.txt --b2 ${rmats_dir}/b2.txt --gtf $gtf -t paired --rea
```

1.1.4 Download of CAGE data from ENCODE

Use the ENCODE data browser website to download HepG2 CAGE peaks

1.2 Isotools analysis

[1]:
```
# import required modules
from  isotools.transcriptome import Transcriptome
from isotools.plots import plot_bar, plot_distr, plot_saturation
from isotools import __version__ as isotools_version
print (f'This is isotools version {isotools_version}')

import matplotlib.pyplot as plt
import numpy as np
import pandas as pd
import logging
logging.basicConfig(format='%(levelname)s:%(message)s', level=logging.INFO)
```

```
This is isotools version 0.2.0.dev3
```

[2]:
```
# set the file paths
path='./hepatocytes_isoseq'
project='manuscript'

isoseq_bam={sa:f'{path}/aligned/{sa}_S2_flnc_aligned.bam' for sa in ('CTL',␣
 ↪'VPA')}
genome_fn=f'{path}/references/gencode/GRCh38.p13.genome.fa'
ref_fn=f'{path}/references/gencode/gencode.v36.chr_patch_hapl_scaff.
 ↪annotation_sorted'
```

[3]:
```
#read basecall quality statistcs of first 100000 reads from the bam files as␣
 ↪initial qc
from isotools._utils import basequal_hist, error_rate
bcq={}
bcq['VPA']=basequal_hist(isoseq_bam["VPA"], n=100000, len_bins=np.
 ↪linspace(1000,10000,20))
bcq['CTL']=basequal_hist(isoseq_bam["CTL"], n=100000, len_bins=np.
 ↪linspace(1000,10000,20))
```

```
100%|      | 100000/100000 [03:06<00:00, 535.04 reads/s]
100%|      | 100000/100000 [02:03<00:00, 808.20 reads/s]
```

[4]:
```
for sa in ['VPA', 'CTL']:
    val=np.cumsum(bcq[sa].sum(1))/bcq[sa].sum().sum()
    print(f"for {sa}, {val['<1.00E+00 %']:.1%} reads have error rate <1%" )
```

```
INFO:Note: detected 128 virtual cores but NumExpr set to maximum of 64, check
"NUMEXPR_MAX_THREADS" environment variable.
INFO:Note: NumExpr detected 128 cores but "NUMEXPR_MAX_THREADS" not set, so
enforcing safe limit of 8.
INFO:NumExpr defaulting to 8 threads.
```

```
for VPA, 96.6% reads have error rate <1%
for CTL, 95.5% reads have error rate <1%
```

1.3 Import the data

If the dataset has been processed already, data is imported from pkl, otherwise from the gff/bam files.

[ ]:
```
out_fn=f'{path}/pickle'
try:
    isoseq=Transcriptome(out_fn+'_v36_isotools.pkl')
except FileNotFoundError:
    try:
        isoseq=Transcriptome.from_reference(ref_fn+'.isotools.pkl')
        isoseq.collapse_immune_genes()
    except FileNotFoundError:
        isoseq=Transcriptome.from_reference(ref_fn+'.gff3.gz')
        isoseq.save_reference(ref_fn+'.isotools.pkl')
        isoseq.collapse_immune_genes()
    #isoseq=Transcriptome('...')
    for sa,bam in  isoseq_bam.items():
        parts=os.path.basename(bam).split('_')
        group=parts[0]
        platform=parts[1]
        isoseq.add_sample_from_bam(bam, sample_name=sa, group=group,␣
 ↪platform=platform) #33+17 minutes

    isoseq.add_qc_metrics(genome_fn) #about 20 min
    isoseq.make_index()
    isoseq.save(out_fn+'_v36_isotools.pkl')
```

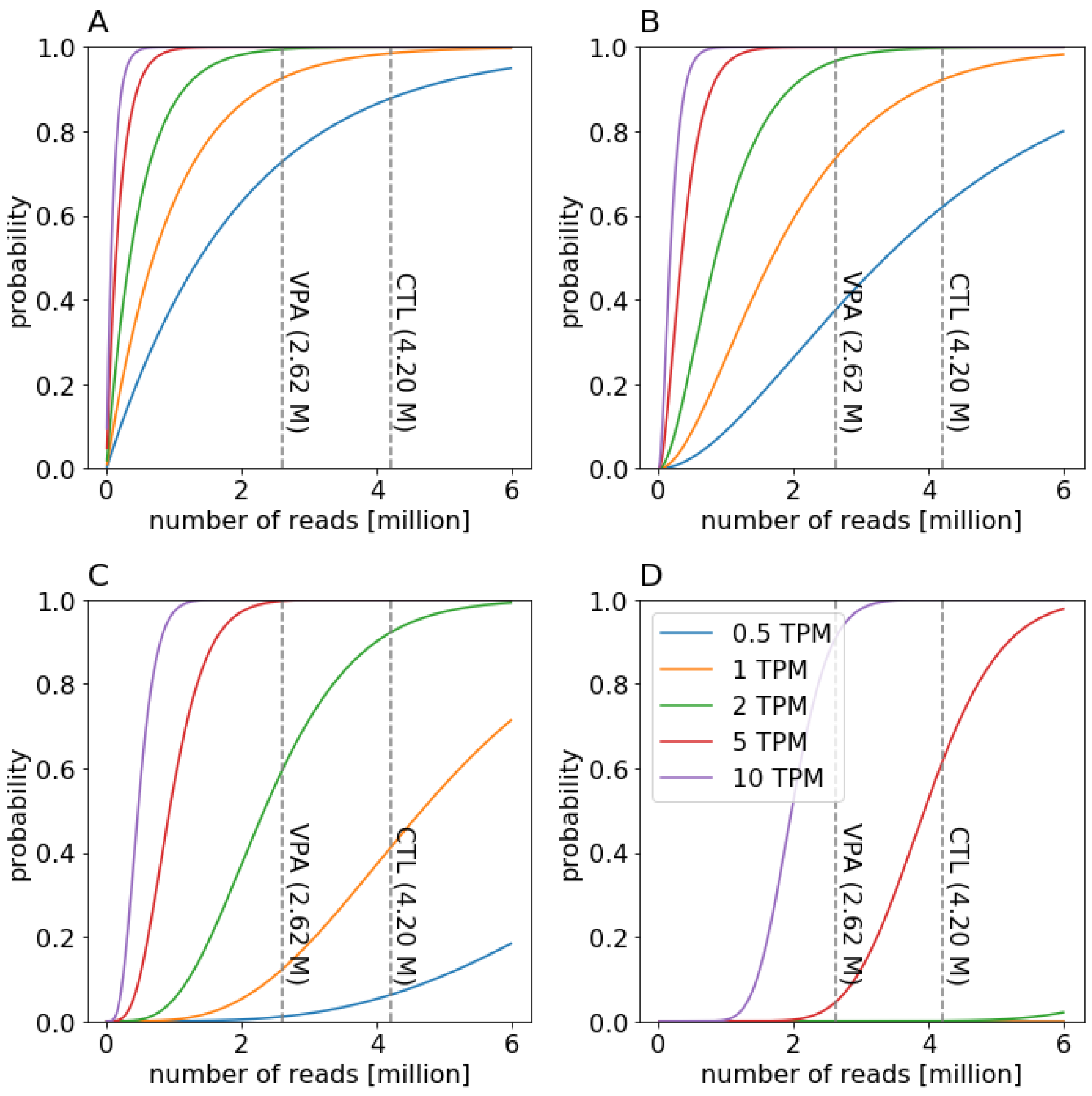
[ ]:
```
#import of illumina RNA seq
from glob import glob
illu_fn={}
```

```
for fn in sorted(glob(f'{path}/rnaseq/star/*_gencode_36_Aligned.sortedByCoord.
↪out.bam')):
    sn='_'.join(os.path.basename(fn).split('_')[:2])
    illu_fn[sn]=fn

isoseq.add_short_read_coverage(illu_fn)
```

1.4 Saturation analysis

This section performs the saturation analysis and replicates Figure 2 of the manuscript

[6]:
```
plt.rcParams["figure.figsize"] = (10,10)
plt.rcParams.update({'font.size': 16})

f,axs=plt.subplots(2,2)
for nr,(ax,cov_th) in enumerate(zip(axs.flatten(),[1,2,5,20])):
    plot_saturation(isoseq, ax=ax,cov_th=cov_th,␣
↪title='',x_range=(1e4,6e6,1e4), legend=False, xlabel='number of reads␣
↪[million]', ylabel='probability')
    ax.set_title('ABCD'[nr],{'fontsize':20}, loc='left', pad=10)
ax.legend(loc='upper left')
f.tight_layout()
f.savefig(out_fn+'_Fig2_saturation.png')
```



2 Artifacts and biases

This section computes the QC metrics and depicts them as in Figure 3 of the manuscript

```
[7]: #these are the default definitions for the filter tags (except HIGH_SUPPORT):

ref_transcript_filter={
              'UNSPLICED':'len(exons)==1',
              'MULTIEXON':'len(exons)>1',
              'HIGH_SUPPORT':'transcript_support_level=="1"',
              'INTERNAL_PRIMING':'len(exons)==1 and downstream_A_content>.5'}
```

```
isoseq.add_filter( ref_transcript_filter=ref_transcript_filter)
```

```
100%|      | 151670/151670 [00:59<00:00, 2563.64it/s]
```

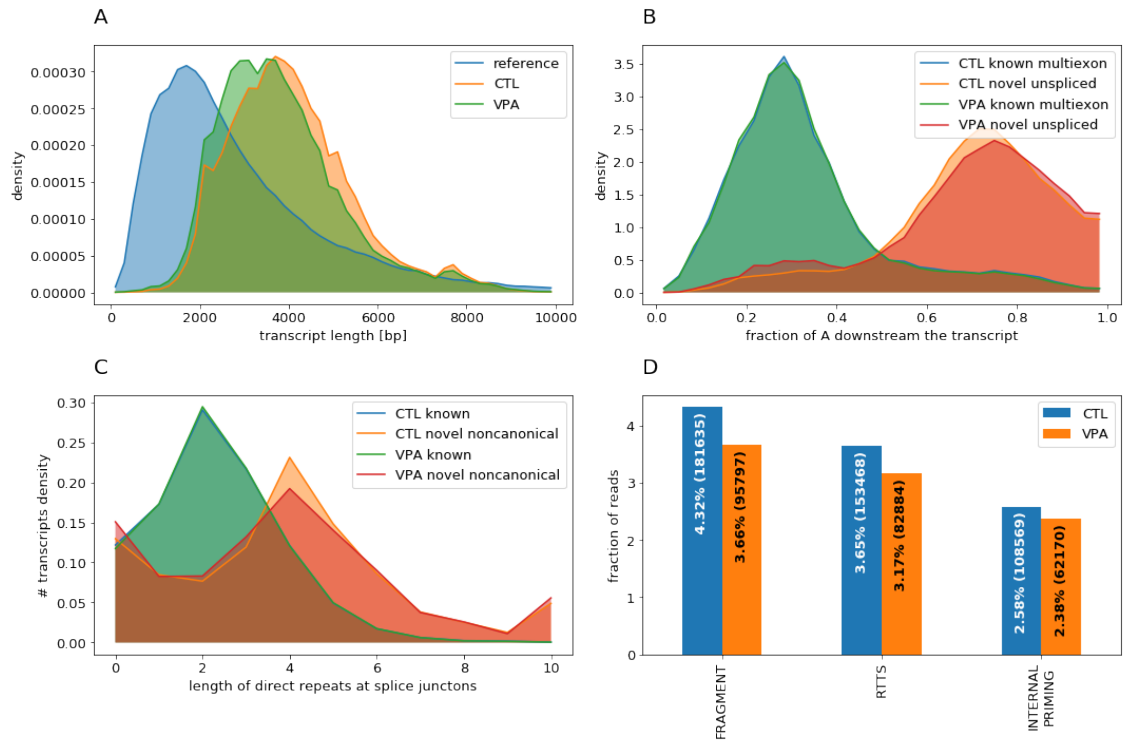
[8]:
```
#compute distributions of the QC metrics
tr_stats=[
    isoseq.transcript_length_hist(groups=isoseq.groups(), add_reference=True,␣
 ↪min_coverage=2,tr_filter=dict( remove=['NOVEL_GENE']),␣
 ↪ref_filter=dict(include=['HIGH_SUPPORT'])),
    isoseq.downstream_a_hist(groups=isoseq.groups(), tr_filter=dict(␣
 ↪remove=['NOVEL_GENE', 'UNSPLICED']), ref_filter=dict(remove=['UNSPLICED'])),
    isoseq.downstream_a_hist(groups=isoseq.groups(),␣
 ↪tr_filter=dict(include=['NOVEL_GENE', 'UNSPLICED'])),
    isoseq.direct_repeat_hist(groups=isoseq.groups(),bins=np.linspace(-.5,10.
 ↪5,12))]
for _,par in tr_stats:
    del par['title']
tr_stats.append((pd.concat([tr_stats[2][0].add_suffix(' novel␣
 ↪unspliced'),tr_stats[1][0].add_suffix(' known␣
 ↪multiexon')],axis=1),tr_stats[2][1]))
if 'liver' not in project:
    tr_stats[0]=(tr_stats[0][0][tr_stats[0][0].columns[[2,0,1]]],tr_stats[0][1])
    tr_stats[3]=(tr_stats[3][0][tr_stats[3][0].
 ↪columns[[2,0,3,1]]],tr_stats[3][1])
    tr_stats[4]=(tr_stats[4][0][tr_stats[4][0].
 ↪columns[[2,0,3,1]]],tr_stats[4][1])
```

[9]:
```
#compute histogram of filter stats
f_stats={th:isoseq.filter_stats( groups=isoseq.groups(),␣
 ↪weight_by_coverage=True,min_coverage=th) for th in [1]}
for i,th in enumerate(f_stats):
    f_stats[th][0].index=f_stats[th][0].index.str.replace('_','\n')
    del f_stats[th][1]['title']
f=sum(np.array([g.coverage[:,trid] for g,trid,tr in isoseq.
 ↪iter_transcripts(include=(['RTTS', 'INTERNAL_PRIMING', 'FRAGMENT']))]))
total=sum(np.array([g.coverage[:,trid] for g,trid,tr in isoseq.
 ↪iter_transcripts()]))
print(f'{f/total*100} percent filtered ({f} reads)')
```

```
[10.51445485  9.17255823] percent filtered ([441699 239879] reads)
```

[10]:
```
#Figure 3: QC plot
plt.rcParams["figure.figsize"] = (15,10)
plt.rcParams.update({'font.size': 13})

fig, axs = plt.subplots(2,2)
#A) transcript length
```

```
plot_distr(tr_stats[0][0],smooth=3,ax=axs[0,0],**tr_stats[0][1])
#B) internal priming
plot_distr(tr_stats[4][0],smooth=3,ax=axs[0,1],density=True,fill=True,␣
 ↪**tr_stats[4][1])
#C) RTTS
plot_distr(tr_stats[3][0],ax=axs[1,0],density=True,**tr_stats[3][1])
#D) frequency of artifacts
plot_bar(f_stats[1][0],ax=axs[1,1],drop_categories=['MULTIEXON','NOVEL\nTRANSCRIPT','NOVEL','U
 ↪],**f_stats[1][1])

for letter,ax in zip('ABCD',axs.flatten()):
    ax.set_title(letter,{'fontsize':20}, loc='left', pad=20)

plt.tight_layout()
plt.savefig(f"{out_fn}_Fig3_qc_metrics.png")
```



2.1 Integration of CAGE data

```
[11]: #Import cage files
from intervaltree import IntervalTree, Interval
from glob import glob
import os
peaks={}
```

```
encode_metadata=pd.read_csv(f'{path}/ENCODE_HepG2_CAGE/metadata.tsv', sep='\t',␣
↪index_col=0)

for file in glob(f'{path}/ENCODE_HepG2_CAGE/*.bed'):
    name=os.path.basename(file).replace('.bed','')
    cellline_name=encode_metadata.loc[name,'Biosample term name']
    assert encode_metadata.loc[name,'File assembly']=='GRCh38'
    print(f'{name}: {cellline_name}')

    with open(file) as fp:
        while True:
            line = fp.readline().rstrip().split('\t')
            if not line[0]:
                break
            peaks.setdefault(line[0]+line[5],[]).append(Interval(int(line[1]),␣
↪int(line[2]), name))

peaks={chrom:IntervalTree(intervals) for chrom, intervals in peaks.items() }
```

```
ENCFF089AFK: HepG2
ENCFF220OWX: HepG2
ENCFF241CGD: HepG2
ENCFF248QKX: HepG2
ENCFF373BNI: HepG2
ENCFF419FNU: HepG2
ENCFF875ILB: HepG2
ENCFF885VJU: HepG2
```

[12]:
```
# add cage data to isoseq transcripts
from tqdm import tqdm #2 minutes
pad=100 #consider the TSS +- 100 bases
for g,trid, tr in tqdm(isoseq.iter_transcripts(), total=isoseq.n_transcripts):
    tss=tr['exons'][0][0] if g.strand=='+' else tr['exons'][-1][1]
    tr['CAGE']=[i.data for i in peaks[g.chrom+g.strand].
↪overlap(tss-pad,tss+pad)] if g.chrom+g.strand in peaks else []
```

```
100%|      | 829444/829444 [03:56<00:00, 3509.60it/s]
```

[13]:
```
#18 seconds
for g,trid, tr in tqdm(isoseq.iter_ref_transcripts()):
    tss=tr['exons'][0][0] if g.strand=='+' else tr['exons'][-1][1]
    tr['CAGE']=[i.data for i in peaks[g.chrom+g.strand].
↪overlap(tss-pad,tss+pad)] if g.chrom+g.strand in peaks else []
```

```
253250it [00:46, 5482.06it/s]
```

[14]:
```
#check CAGE support of gencode transcripts
cage_ol_fragment=[]
for g,trid, tr in isoseq.iter_transcripts(include=['FRAGMENT']):
    #if sum(v for v in tr['coverage'].values())<2:
    #    continue
    if all(f[1] for f in tr['fragments']): #start exons missing:␣
 ↪tr[fragments][1]>0
        cage_ol_fragment.append(len(tr['CAGE']))
print(f'{sum(c>0 for c in cage_ol_fragment)/len(cage_ol_fragment)*100}% of␣
 ↪fragment tss have cage support')
cage_ol_gencode=[]
for g,trid, tr in isoseq.iter_transcripts(remove=['NOVEL_TRANSCRIPT']):
    if sum(v for v in tr['coverage'].values())<2:
        continue
    ref_tr=g.ref_transcripts[tr['annotation'][1]['FSM'][0]]
    if 'transcript_support_level' in ref_tr and␣
 ↪ref_tr['transcript_support_level']=='1':
        cage_ol_gencode.append(len(tr['CAGE']))
        #cage_ol_gencode.append(len(ref_tr['CAGE']))
print(f'{sum(c>0 for c in cage_ol_gencode)/len(cage_ol_gencode)*100}% of level␣
 ↪1 gencode FSM tss have cage support')
```

```
9.854125052962896% of fragment tss have cage support
76.87439457539554% of level 1 gencode FSM tss have cage support
```

[15]:
```
cage_ol_fragment={}
tss_cat=["5' fragment", "novel exonic TSS", "novel intronic TSS"]
bias=["RTTS", "FRAGMENT", "INTERNAL_PRIMING"]
for g,trid, tr in isoseq.iter_transcripts(include=tss_cat,min_coverage=2):#,␣
 ↪remove=bias):
    for cat in tss_cat:
        if cat in tr['annotation'][1]:
            cage_ol_fragment.setdefault(cat, []).append(len(tr['CAGE']))
for g,trid, tr in isoseq.iter_transcripts(remove=['NOVEL_TRANSCRIPT'],␣
 ↪min_coverage=2):
    ref_tr=g.ref_transcripts[tr['annotation'][1]['FSM'][0]]
    if 'transcript_support_level' in ref_tr and␣
 ↪ref_tr['transcript_support_level']=='1':
        cage_ol_fragment.setdefault('level 1 gencode FSM TSS', []).
 ↪append(len(tr['CAGE']))
for cat,ol in cage_ol_fragment.items():
    print(f'{sum(c>0 for c in ol)/len(ol):.3%} of {cat} have cage support')
```

```
31.121% of novel intronic TSS have cage support
10.125% of novel exonic TSS have cage support
11.695% of 5' fragment have cage support
76.874% of level 1 gencode FSM TSS have cage support
```

```
[16]: #adjust the requested number of cage
for cat,ol in cage_ol_fragment.items():
    print(f'{sum(c>0 for c in ol)/len(ol):.1%} of {cat} have cage support')
```

```
31.1% of novel intronic TSS have cage support
10.1% of novel exonic TSS have cage support
11.7% of 5' fragment have cage support
76.9% of level 1 gencode FSM TSS have cage support
```

2.2 Chimeric transcripts

In this section we analyze the chimeric alignments.

```
[17]: fusiontab=isoseq.chimeric_table().sort_values('total_cov', ascending=False)
fusion_cov=[ct[0] for ctl in isoseq.chimeric.values() for ct in ctl ]
cov_lic=[0,0]
for g,trid,tr in isoseq.iter_transcripts():
    if 'long_intron_chimeric' in tr:
        if 'VPA' in tr['coverage']:
            cov_lic[1]+=tr['coverage']['VPA']
        if 'CTL' in tr['coverage']:
            cov_lic[0]+=tr['coverage']['CTL']
print(f'total chimeric reads CTL: {cov_lic[0]+sum(c["CTL"] for c in fusion_cov␣
 ↪if "CTL" in c)}')
print(f'total chimeric reads VPA: {cov_lic[1]+sum(c["VPA"] for c in fusion_cov␣
 ↪if "VPA" in c)}')

malat1=fusiontab.loc[(fusiontab.gene1=='MALAT1') |(fusiontab.gene2=='MALAT1')]
other=fusiontab.loc[(fusiontab.gene1!='MALAT1') &(fusiontab.gene2!='MALAT1')]

print(f'malat1 CTL: {malat1.CTL_cov.sum()}')
print(f'malat1 VPA: {malat1.VPA_cov.sum()}')

print(f'{len(fusion_cov)} transcripts arise from chimeric reads, total␣
 ↪coverage={sum(sum(c.values()) for c in fusion_cov)} ({sum(c["CTL"] for c in␣
 ↪fusion_cov if "CTL" in c)} CTL +{sum(c["VPA"] for c in fusion_cov if "VPA"␣
 ↪in c)} VPA )')
print(f'accroding to table: total coverage={sum(fusiontab.total_cov)}␣
 ↪({sum(fusiontab.CTL_cov)} CTL + {sum(fusiontab.VPA_cov)} VPA <<- this counts␣
 ↪breakpoints not transcripts, hence ~30 more)')
# claim most are low coverage
sel=other.total_cov>=10
genes=set(other.gene1[sel]).union(set(other.gene2[sel]))
print(f'{(other.loc[sel].shape[0])} fusion candidates>10 reads, including␣
 ↪{len(genes)} genes')
fusiontab.to_csv(f'{out_fn}_tab1_chimeric_reads.csv', index=False)
malat1.to_csv(f'{out_fn}_tab1_malat1_chimeric_reads.csv', index=False)
```

```
malat1.head()
```

```
total chimeric reads CTL: 8489
total chimeric reads VPA: 5441
malat1 CTL: 2144
malat1 VPA: 1287
1327 transcripts arise from chimeric reads, total coverage=5523 (3737 CTL +1786
VPA )
accroding to table: total coverage=5585 (3791 CTL + 1794 VPA <<- this counts
breakpoints not transcripts, hence ~30 more)
21 fusion candidates>10 reads, including 30 genes
```

[17]:
```
                   name   chr1 strand1  breakpoint1           gene1   chr2  \
436       MALAT1_MALAT1  chr11       +     65500661          MALAT1  chr11
435       MALAT1_MALAT1  chr11       +     65502332          MALAT1  chr11
456       MALAT1_MALAT1  chr11       +     65500698          MALAT1  chr11
438  PB_novel_36558_MALAT1  chr11       +     65503695  PB_novel_36558  chr11
443  PB_novel_36558_MALAT1  chr11       +     65503804  PB_novel_36558  chr11

    strand2  breakpoint2   gene2  total_cov  CTL_cov  VPA_cov
436       +     65499092  MALAT1        593      332      261
435       +     65499375  MALAT1        507      299      208
456       +     65499091  MALAT1        202      106       96
438       +     65499071  MALAT1        173      113       60
443       +     65499089  MALAT1        135       89       46
```

[18]:
```
other[sel]
```

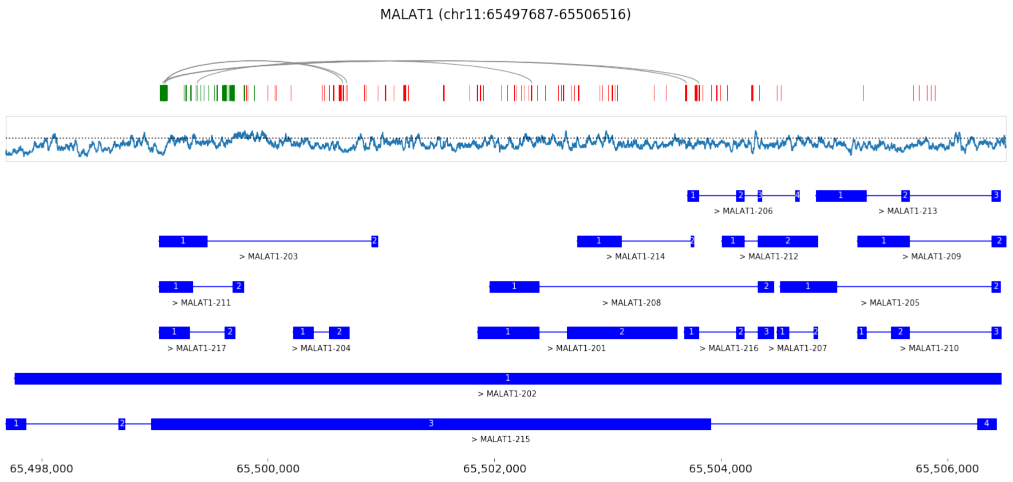
[18]:
```
                          name        chr1 strand1  breakpoint1  \
128          PB_novel_56121_MAGI1  KQ031385.1       -        14797
452  PB_novel_36558_PB_novel_36558       chr11       +     65502946
612                     GPC6_GPC6       chr13       +     93227617
647                 KANSL1_ARL17B       chr17       -     46094561
680                 KANSL1_ARL17B  KI270908.1       -       796667
645     PB_novel_48169_intergenic       chr17       -     43358962
415             NUTM2A-AS1_MINPP1       chr10       -     87326632
221          intergenic_AC270107.3        chr5       +     70401668
7                CROCC_intergenic        chr1       +     16954362
636                 BOLA2B_SMG1P5       chr16       -     30193361
628        OIP5-AS1_PB_novel_44179       chr15       +     41305438
421               intergenic_MOB2  KI270830.1       -        16636
348                 ALDH1B1_AKR1B10        chr9       +     38398350
33      PB_novel_03044_intergenic        chr1       +    149607547
499     PB_novel_36558_intergenic       chr11       +     65503696
223      PB_novel_18464_AC270107.2        chr5       +     70401668
46                      COPA_SOD2        chr1       -    160289755
263                  SERINC1_SOD2        chr6       -    122444162
```

```
312        SERPINE1_PB_novel_51071        chr7       +    101138798
607                      SCARB1_UBC       chr12      -    124863596
587      PB_novel_36766_intergenic       chr11      -     71563393

              gene1        chr2 strand2  breakpoint2           gene2  \
128  PB_novel_56121        chr3       -     65622090           MAGI1
452  PB_novel_36558       chr11       +     65499049  PB_novel_36558
612            GPC6       chr13       +     94286346            GPC6
647          KANSL1  KI270908.1       -      1090574          ARL17B
680          KANSL1  KI270908.1       -      1090574          ARL17B
645  PB_novel_48169       chr17       -     43365005      intergenic
415      NUTM2A-AS1       chr10       +     87449472          MINPP1
221      intergenic  KV575243.1       +        60936      AC270107.3
7             CROCC        chr1       -    148013326      intergenic
636          BOLA2B       chr16       -     30288789          SMG1P5
628        OIP5-AS1       chr15       +     41301213  PB_novel_44179
421      intergenic       chr11       -      1480886            MOB2
348         ALDH1B1        chr7       +    134527815         AKR1B10
33   PB_novel_03044        chr1       +    120556543      intergenic
499  PB_novel_36558       chr11       +     65499049      intergenic
223  PB_novel_18464  KV575243.1       +        40655      AC270107.2
46             COPA        chr6       -    159679795            SOD2
263         SERINC1        chr6       -    159679805            SOD2
312        SERPINE1       chr19       +     44662519  PB_novel_51071
607          SCARB1       chr12       -    124913776             UBC
587  PB_novel_36766       chr11       +     71563061      intergenic

     total_cov  CTL_cov  VPA_cov
128        272      151      121
452         47       35       12
612         31       26        5
647         30       23        7
680         29       23        6
645         27        5       22
415         24       13       11
221         22       16        6
7           20       12        8
636         19       16        3
628         18       10        8
421         17        5       12
348         16       16        0
33          15       13        2
499         13        8        5
223         13       13        0
46          13       11        2
263         12        8        4
312         10       10        0
```

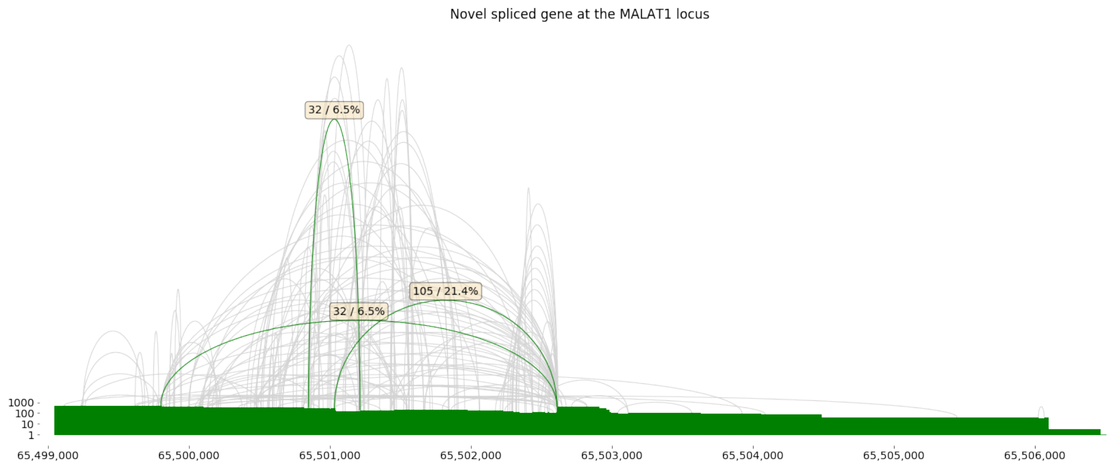
```
607          10         6        4
587          10         8        2
```

[19]:
```
# depict the MALAT1 locus (not shown in manuscript)
from pysam import AlignmentFile, FastaFile
# function to retrieve the genomic a content
def get_a_content(chrom, start, end, genome_file=genome_fn, wd=10):
    with FastaFile(genome_file) as genome_fh:
        seq=genome_fh.fetch(chrom, start-wd+1,end+wd)
    count={coi:[sum(c==coi for c in seq[:(2*wd)])] for coi in 'AT'}
    for i in range(2*wd,len(seq)):
        for coi in 'AT':
            last=count[coi][-1]
            count[coi].append(last+(seq[i]==coi)-(seq[i-2*wd]==coi))
    return [ca/(2*wd) if ca>count['T'][i] else count['T'][i]/(2*wd) for i,ca in␣
 ↪enumerate(count['A'])]
```

[20]:
```
from matplotlib import patches

plt.rcParams["figure.figsize"] = (25,10)
plt.rcParams.update({'font.size': 14})



fig, ax = plt.subplots(1)
ax.plot(malat1.breakpoint1, [7.5]*len(malat1),'r|',markersize= 20 )
ax.plot(malat1.breakpoint2, [7.5]*len(malat1),'g|',markersize= 20)
isoseq['MALAT1'].gene_track(ax=ax)
for _,row in malat1.head().iterrows():
    bp1,bp2=row.breakpoint1, row.breakpoint2
    bow=patches.Arc(((bp1+bp2)/2, 7.7), width=bp1-bp2, height=1,theta1=0,␣
 ↪theta2=180,linewidth=1,edgecolor='grey',zorder=1)
    ax.add_patch(bow)

g_start=min(tr['exons'][0][0] for tr in isoseq['MALAT1'].ref_transcripts)
g_end=max(tr['exons'][-1][1] for tr in isoseq['MALAT1'].ref_transcripts)
a_cont=get_a_content('chr11',g_start, g_end, wd=15)
ax.plot(range(g_start, g_end),[val+6 for val in a_cont])
ax.plot((g_start, g_end),(6.5,6.5), 'k:')
box=patches.Rectangle((g_start, 6), width=g_end-g_start,␣
 ↪height=1,linewidth=1,edgecolor='lightgrey',zorder=1, fill=False)
ax.add_patch(box)

ax.set_ylim(-.5,9)
ax.set_xlim(g_start-500,g_end+500)
#ax.axis('off')
ax.set(frame_on=False)
ax.get_yaxis().set_visible(False)
plt.savefig(f"{out_fn}_Fig_malat1.png")
```

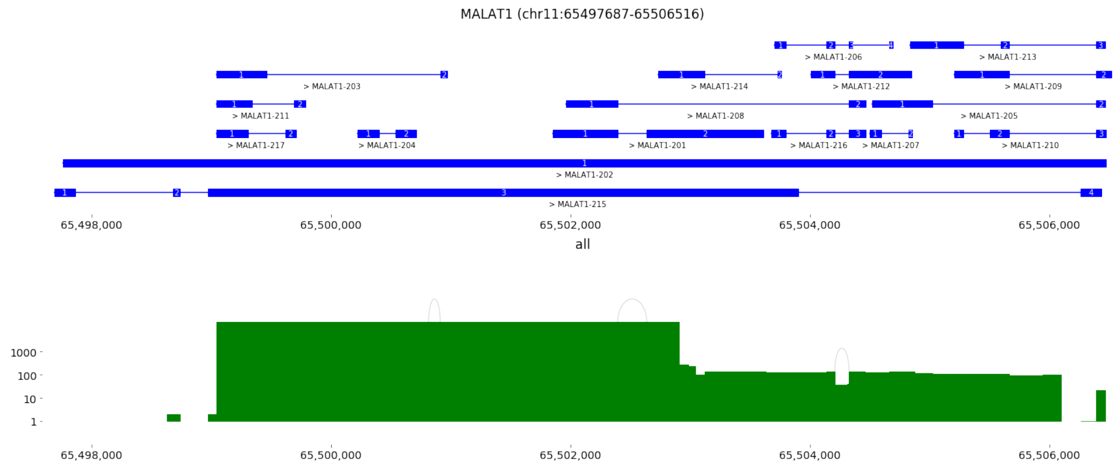


```
[21]: # some reads of that locus get assigned to a novel gene, since they are spliced␣
      ↪and do not share any junction with the annotation:
      plt.rcParams["figure.figsize"] = (25,10)
      g=isoseq["MALAT1"]
      malat1_locus=(g.chrom, g.start, g.end)
      for alt_g in sorted(list(isoseq.iter_genes(region=malat1_locus)), key=lambda x:␣
      ↪- x.coverage.sum()):
          if alt_g.name != 'MALAT1':
              f,axs=alt_g.sashimi_figure(draw_gene_track=False)
              axs[0].set_title("Novel spliced gene at the MALAT1 locus")
              break
```



[22]:
```
isoseq['MALAT1'].sashimi_figure()
```

[22]:
```
(<Figure size 1800x720 with 2 Axes>,
 array([<matplotlib.axes._subplots.AxesSubplot object at 0x7f67b8658bd0>,
        <matplotlib.axes._subplots.AxesSubplot object at 0x7f67b7523dd0>],
       dtype=object))
```



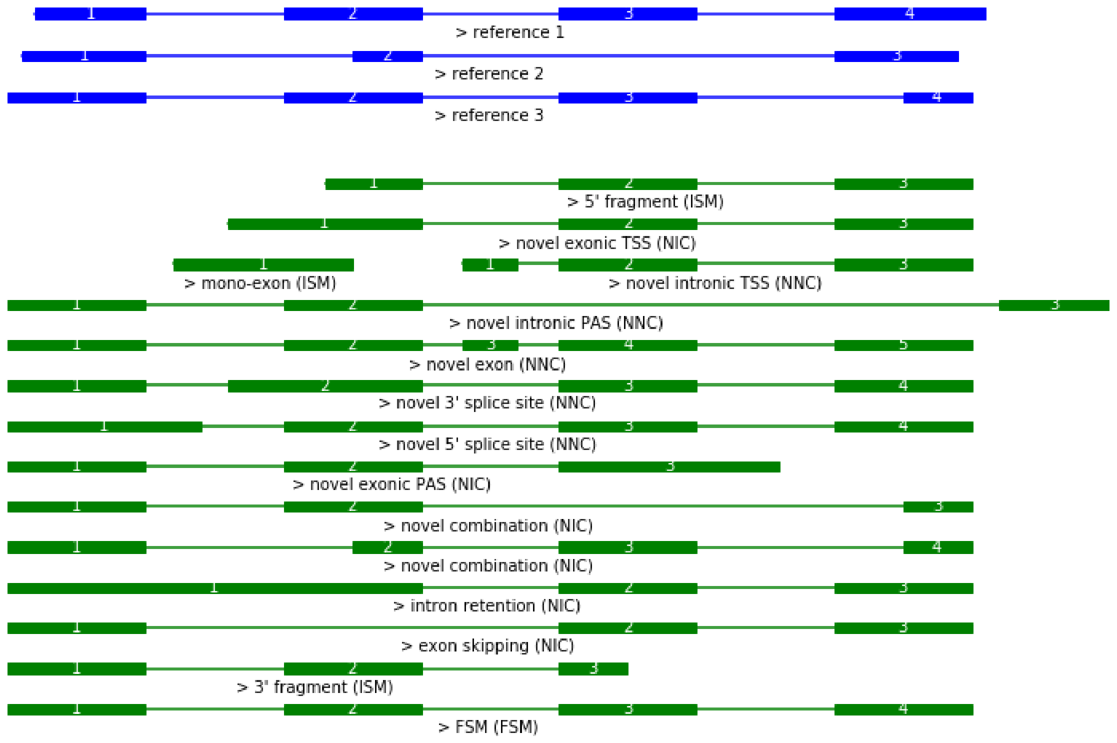
2.3 Novel isoform classification

In this section, we replicate the classification of novel transcripts, including Figure 4 of the manuscript

[23]:
```
import isotools
ref=[[[12,20],[30,40], [50,60],[70,81]],
     [[11,20],[35,40],          [70,79]],
     [[10,20],[30,40], [50,60],[75,80]]]
novel={'FSM':          [[10,20],[30,40], [50,60],[70,80]],
       "5' fragment": [[33,40], [50,60],[70,80]],
       "3' fragment": [[10,20],[30,40], [50,55]],
       "mono exon"  : [[22,35]],
       "exon skipping"     :  [[10,20], [50,60],[70,80]],
       "intron retention"  :  [[10,40], [50,60],[70,80]],
       "novel combination" :  [[10,20],[35,40], [50,60],[75,80]],
       "novel junction"   :   [[10,20],[30,40], [75,80]],
       "novel exonic TSS"  :  [[26,40], [50,60],[70,80]],
       "novel exonic PAS"  :  [[10,20],[30,40], [50,66]],
       "novel 5' splice site":[[10,24],[30,40], [50,60],[70,80]],
       "novel 3' splice site":[[10,20],[26,40], [50,60],[70,80]],
       "novel exon"  :        [[10,20],[30,40],[43,47], [50,60],[70,80]],
       "novel intronic TSS" : [[43,47],[50,60],[70,80]],
       "novel intronic PAS" : [[10,20],[30,40], [82,90]]}
```

```
ref={'transcripts':[{'exons':e, 'transcript_name':f'reference {i+1}'} for i,e␣
 ↪in enumerate(ref)]}
transcripts=[{'exons':e, 'transcript_name':n} for n,e in novel.items()]
example=isotools.Gene(10,80,{'strand':'+','ID':'example','reference':ref,␣
 ↪'transcripts':transcripts},None)
f,axs=plt.subplots(2,figsize=(10,7), gridspec_kw={'height_ratios': [1, 4]})
cat=['FSM','ISM','NIC','NNC','novel gene']
sg=example.ref_segment_graph
for novel in example.transcripts:
    alt_splice=sg.get_alternative_splicing(novel['exons'])
    print(f"{novel['transcript_name']}: {alt_splice[1]}")
    novel['transcript_name']=f"{','.join(alt_splice[1])} ({cat[alt_splice[0]]})␣
 ↪"

example.gene_track(ax=axs[0], x_range=[10,90], title='')
example.gene_track(reference=False,ax=axs[1], x_range=[10,90], title='',␣
 ↪color='green')
for ax in axs:
    ax.get_xaxis().set_visible(False)
f.tight_layout()
plt.savefig(f"{out_fn}_Fig4a_altsplice_example.png")
```

```
FSM: {'FSM': [0]}
5' fragment: {"5' fragment": [[12, 33]]}
3' fragment: {"3' fragment": [[55, 80]]}
mono exon: {'mono-exon': []}
exon skipping: {'exon skipping': [[30, 40]]}
intron retention: {'intron retention': [(20, 30)]}
novel combination: {'novel combination': []}
novel junction: {'novel combination': []}
novel exonic TSS: {'novel exonic TSS': [(26, 30)]}
novel exonic PAS: {'novel exonic PAS': [(60, 66)]}
novel 5' splice site: {"novel 5' splice site": [(24, -4)]}
novel 3' splice site: {"novel 3' splice site": [(26, 4)]}
novel exon: {'novel exon': [[43, 47]]}
novel intronic TSS: {'novel intronic TSS': [[43, 47]]}
novel intronic PAS: {'novel intronic PAS': [[82, 90]]}
```

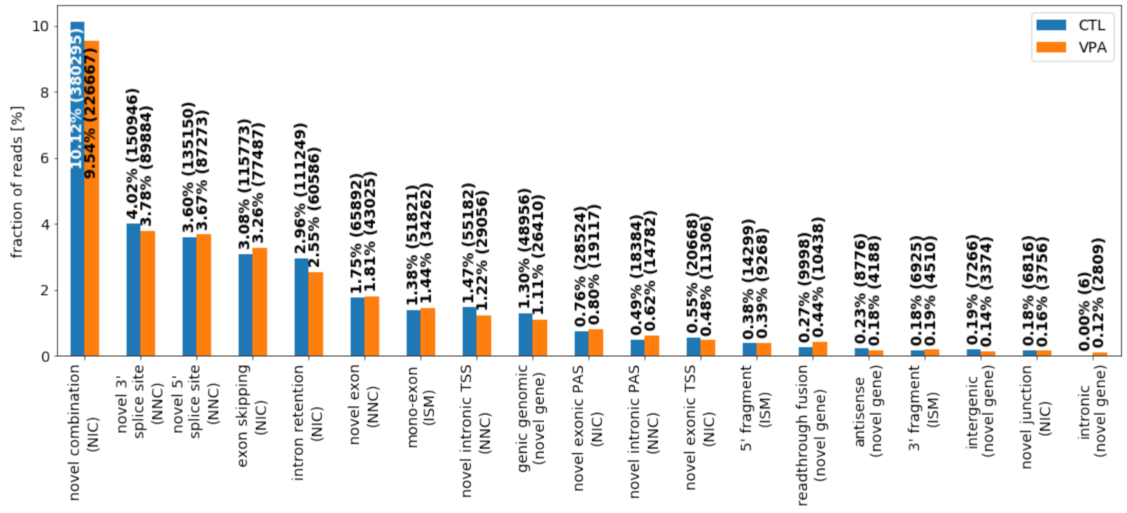


[24]:
```
# explain refined sqanti
# barplot (zu figure 3?)
cname=['FSM','ISM','NIC','NNC','novel gene']
cnr={}
for g, trid, tr in isoseq.iter_transcripts():
    for anno in tr['annotation'][1]:
        cnr[anno]=min(cnr.get(anno,5),tr['annotation'][0])
del cnr['FSM']
altsplice=[ isoseq.altsplice_stats(groups=isoseq.groups(),␣
↪weight_by_coverage=True, min_coverage=1, tr_filter=dict( remove=['RTTS',␣
↪'FRAGMENT', 'INTERNAL_PRIMING'])),
            isoseq.altsplice_stats(groups=isoseq.groups(),␣
↪weight_by_coverage=True, min_coverage=2, tr_filter=dict( remove=['RTTS',␣
↪'FRAGMENT', 'INTERNAL_PRIMING'])),
            isoseq.altsplice_stats(groups=isoseq.groups(),␣
↪weight_by_coverage=False, min_coverage=20, tr_filter=dict( remove=['RTTS',␣
↪'FRAGMENT', 'INTERNAL_PRIMING']))]
for i in range(3):
    altsplice[i][0].index=altsplice[i][0].index+[f'\n({cname[cnr[subcat]]})' if␣
↪subcat in cnr else '' for subcat in altsplice[i][0].index]
    altsplice[i][0].index=altsplice[i][0].index.str.replace('splice ','\nsplice␣
↪')
```

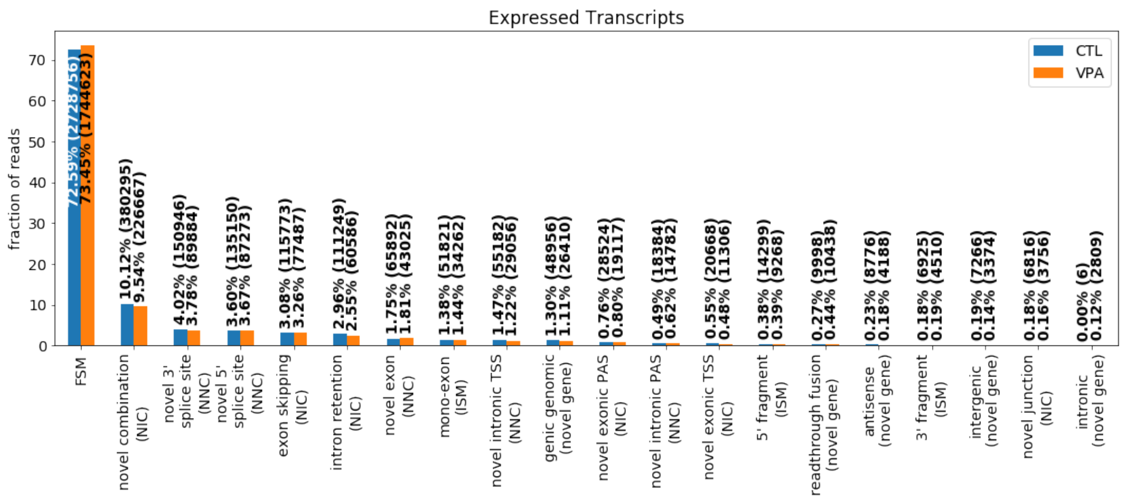
```
[25]: altsplice[0][0].loc["FSM"]/altsplice[0][0].loc["total"]
```

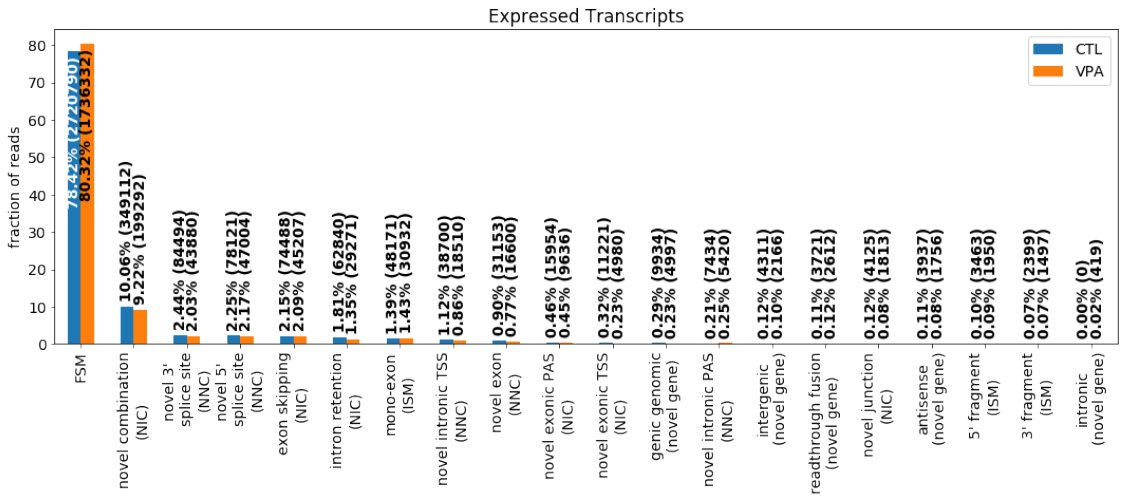
```
[25]: CTL    0.725892
      VPA    0.734485
      dtype: float64
```

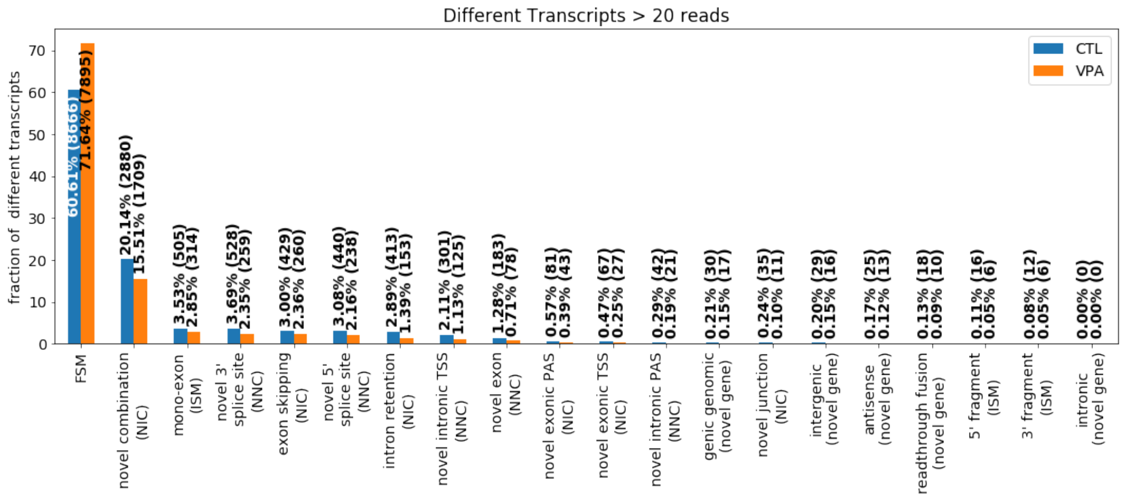
```
[26]: plt.rcParams["figure.figsize"] = (15,7)
      fig, ax = plt.subplots( 1)

      plot_bar(altsplice[0][0],ax=ax,ylabel='fraction of reads [%]',␣
       ↪drop_categories=['FSM'])
      #plot_bar(altsplice[1][0],ax=axs[1],**altsplice[1][1])
      fig.tight_layout()
      plt.savefig(f"{out_fn}_Fig4_altsplice.png")
```



```
[27]: #alternative plots for fig4
      plt.rcParams["figure.figsize"] = (15,20)
      fig, ax = plt.subplots( 3)

      for i in range(3):
          plot_bar(altsplice[i][0],ax=ax[i],**altsplice[i][1])
      plt.tight_layout()
```


Expressed Transcripts
fraction of reads
CTL
VPA
72.59% (2728756)
73.45% (1744623)
10.12% (380295)
9.54% (226667)
4.02% (150946)
3.78% (89884)
3.60% (135150)
3.67% (87273)
3.08% (115773)
3.26% (77487)
2.96% (111249)
2.55% (60586)
1.75% (65892)
1.81% (43025)
1.38% (51821)
1.44% (34262)
1.47% (55182)
1.22% (29056)
1.30% (48956)
1.11% (26410)
0.76% (28524)
0.80% (19117)
0.49% (18384)
0.62% (14782)
0.55% (20668)
0.48% (11306)
0.38% (14299)
0.39% (9268)
0.27% (9998)
0.44% (10438)
0.23% (8776)
0.18% (4188)
0.18% (6925)
0.19% (4510)
0.19% (7266)
0.14% (3374)
0.18% (6816)
0.16% (3756)
0.00% (6)
0.12% (2809)
FSM
novel combination (NIC)
novel 3' splice site (NNC)
novel 5' splice site (NNC)
exon skipping (NIC)
intron retention (NIC)
novel exon (NNC)
mono-exon (ISM)
novel intronic TSS (NNC)
genic genomic (novel gene)
novel exonic PAS (NIC)
novel intronic PAS (NNC)
novel exonic TSS (NIC)
5' fragment (ISM)
readthrough fusion (novel gene)
antisense (novel gene)
3' fragment (ISM)
intergenic (novel gene)
novel junction (NIC)
intronic (novel gene)


Expressed Transcripts
fraction of reads
CTL
VPA
78.42% (2720790)
80.32% (1736332)
10.06% (349112)
9.22% (199292)
2.44% (84494)
2.03% (43880)
2.25% (78121)
2.17% (47004)
2.15% (74488)
2.09% (45207)
1.81% (62840)
1.35% (29271)
1.39% (48171)
1.43% (30932)
1.12% (38700)
0.86% (18510)
0.90% (31153)
0.77% (16600)
0.46% (15954)
0.45% (9636)
0.32% (11221)
0.23% (4980)
0.29% (9934)
0.23% (4997)
0.21% (7434)
0.25% (5420)
0.12% (4311)
0.10% (2166)
0.11% (3721)
0.12% (2612)
0.12% (4125)
0.08% (1813)
0.11% (3937)
0.08% (1756)
0.10% (3463)
0.09% (1950)
0.07% (2399)
0.07% (1497)
0.00% (0)
0.02% (419)
FSM
novel combination (NIC)
novel 3' splice site (NNC)
novel 5' splice site (NNC)
exon skipping (NIC)
intron retention (NIC)
mono-exon (ISM)
novel intronic TSS (NNC)
novel exon (NNC)
novel exonic PAS (NIC)
novel exonic TSS (NIC)
genic genomic (novel gene)
novel intronic PAS (NNC)
intergenic (novel gene)
readthrough fusion (novel gene)
novel junction (NIC)
antisense (novel gene)
5' fragment (ISM)
3' fragment (ISM)
intronic (novel gene)


Different Transcripts > 20 reads
fraction of different transcripts
CTL
VPA
60.61% (8666)
71.64% (7895)
20.14% (2880)
15.51% (1709)
3.53% (505)
2.85% (314)
3.69% (528)
2.35% (259)
3.00% (429)
2.36% (260)
3.08% (440)
2.16% (238)
2.89% (413)
1.39% (153)
2.11% (301)
1.13% (125)
1.28% (183)
0.71% (78)
0.57% (81)
0.39% (43)
0.47% (67)
0.25% (27)
0.29% (42)
0.19% (21)
0.21% (30)
0.15% (17)
0.24% (35)
0.10% (11)
0.20% (29)
0.15% (16)
0.17% (25)
0.12% (13)
0.13% (18)
0.09% (10)
0.11% (16)
0.05% (6)
0.08% (12)
0.05% (6)
0.00% (0)
0.00% (0)
FSM
novel combination (NIC)
mono-exon (ISM)
novel 3' splice site (NNC)
exon skipping (NIC)
novel 5' splice site (NNC)
intron retention (NIC)
novel intronic TSS (NNC)
novel exon (NNC)
novel exonic PAS (NIC)
novel exonic TSS (NIC)
novel intronic PAS (NNC)
genic genomic (novel gene)
novel junction (NIC)
intergenic (novel gene)
antisense (novel gene)
readthrough fusion (novel gene)
5' fragment (ISM)
3' fragment (ISM)
intronic (novel gene)

2.4 Quantification

This section compares RNA-seq transcript quantification by rsem with isoseq read counts per transcript.

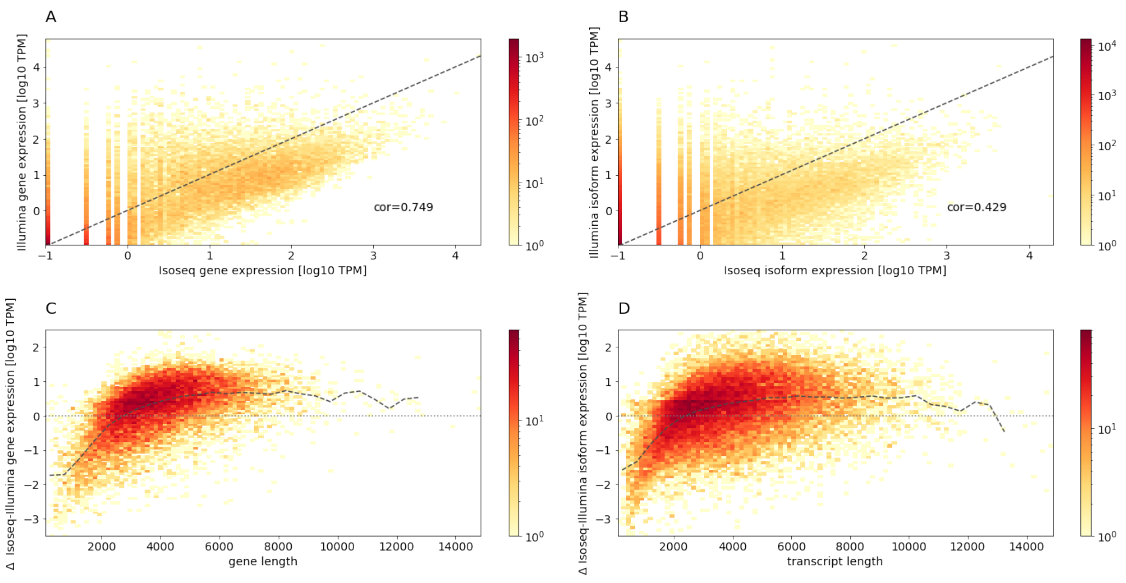
[28]:
```
illu_gene_tpm=pd.read_csv(f'{out_fn}_tab2_illumina_isoseq_gene_expression.csv').
 ↪set_index( "gene_id")
illu_transcript_tpm=pd.
 ↪read_csv(f'{out_fn}_tab3_illumina_isoseq_transcript_expression.csv').
 ↪set_index(["tr_id", "gene_id"])
illu_transcript_tpm.head()
```

[28]:
```
                                         gene_name         chr  start     end  \
tr_id             gene_id
ENST00000618686.1 ENSG00000278704.1  BX004987.1  GL000009.2  56139   58376
ENST00000613230.1 ENSG00000277400.1  AC145212.1  GL000194.1  53589  115018
ENST00000400754.4 ENSG00000274847.1       MAFIP  GL000194.1  53593  115055
ENST00000618679.1 ENSG00000277428.1       Y_RNA  GL000195.1  37433   37534
ENST00000612465.1 ENSG00000276256.1  AC011043.1  GL000195.1  42938   49164

                                    strand       gene_type  gene_level  \
tr_id             gene_id
ENST00000618686.1 ENSG00000278704.1      -  protein_coding           3
ENST00000613230.1 ENSG00000277400.1      -  protein_coding           3
ENST00000400754.4 ENSG00000274847.1      -  protein_coding           3
ENST00000618679.1 ENSG00000277428.1      -        misc_RNA           3
ENST00000612465.1 ENSG00000276256.1      -  protein_coding           3

                                    gene_tag transcript_type  \
tr_id             gene_id
ENST00000618686.1 ENSG00000278704.1     none  protein_coding
ENST00000613230.1 ENSG00000277400.1     none  protein_coding
ENST00000400754.4 ENSG00000274847.1     none  protein_coding
ENST00000618679.1 ENSG00000277428.1     none        misc_RNA
ENST00000612465.1 ENSG00000276256.1     none  protein_coding

                                    transcript_support_level              CDS  \
tr_id             gene_id
ENST00000618686.1 ENSG00000278704.1                      NaN   (58080, 58308)
ENST00000613230.1 ENSG00000277400.1                        1   (53646, 54021)
ENST00000400754.4 ENSG00000274847.1                        1  (54730, 112848)
ENST00000618679.1 ENSG00000277428.1                      NaN              NaN
ENST00000612465.1 ENSG00000276256.1                        2   (44723, 49117)

                                     rsem_genelength  isoseq_genelength  \
tr_id             gene_id
ENST00000618686.1 ENSG00000278704.1           2237.0                0.0
ENST00000613230.1 ENSG00000277400.1           2179.0             2203.0
```

```
ENST00000400754.4 ENSG00000274847.1          1599.0             1593.0
ENST00000618679.1 ENSG00000277428.1           101.0                0.0
ENST00000612465.1 ENSG00000276256.1          2195.0                0.0

                                     illumina_CTL_tpm  illumina_VPA_tpm  \
tr_id             gene_id
ENST00000618686.1 ENSG00000278704.1            0.0300            0.1100
ENST00000613230.1 ENSG00000277400.1            2.4825            5.9450
ENST00000400754.4 ENSG00000274847.1            5.9550           10.5675
ENST00000618679.1 ENSG00000277428.1            0.0000            0.0000
ENST00000612465.1 ENSG00000276256.1            3.4250            2.3475

                                     isoseq_CTL_tpm  isoseq_VPA_tpm
tr_id             gene_id
ENST00000618686.1 ENSG00000278704.1        0.000000        0.000000
ENST00000613230.1 ENSG00000277400.1        0.230754        3.736880
ENST00000400754.4 ENSG00000274847.1        0.000000        0.373688
ENST00000618679.1 ENSG00000277428.1        0.000000        0.000000
ENST00000612465.1 ENSG00000276256.1        0.000000        0.000000
```

[29]:
```
plt.rcParams["figure.figsize"] = (20,10)

from matplotlib.colors import LogNorm
from isotools._utils import pairwise

f=(illu_gene_tpm.illumina_CTL_tpm>0.01 ) #&(illu_gene_tpm.isoseq_CTL_tpm>0␣
↪)#&(illu_gene_tpm.rsem_genelength>4000 )
fig, axs = plt.subplots( 2,2)

x=np.log10(illu_gene_tpm.isoseq_CTL_tpm[f]+.1)
y=np.log10(illu_gene_tpm.illumina_CTL_tpm[f]+.1)
hist=axs[0,0].hist2d(x,y,100,norm=LogNorm(),cmap='YlOrRd')
fig.colorbar(hist[3], ax=axs[0,0])
axs[0,0].plot([-2, 5], [-2, 5], ls="--", c=".3")
axs[0,0].set_xlabel('Isoseq gene expression [log10 TPM]')
axs[0,0].set_ylabel('Illumina gene expression [log10 TPM]')
axs[0,0].text(3,0,f'cor={np.corrcoef(x,y)[1,0]:.3f}')


f=(illu_transcript_tpm.illumina_CTL_tpm>0.01 ) #&(illu_transcript_tpm.
↪isoseq_CTL_tpm>0 )
x=np.log10(illu_transcript_tpm.isoseq_CTL_tpm[f]+.1)
y=np.log10(illu_transcript_tpm.illumina_CTL_tpm[f]+.1)
hist=axs[0,1].hist2d(x,y,100,norm=LogNorm(),cmap='YlOrRd')
fig.colorbar(hist[3], ax=axs[0,1])
axs[0,1].plot([-2, 5], [-2, 5], ls="--", c=".3")
axs[0,1].set_xlabel('Isoseq isoform expression [log10 TPM]')
```

```
axs[0,1].set_ylabel('Illumina isoform expression [log10 TPM]')
axs[0,1].text(3,0,f'cor={np.corrcoef(x,y)[1,0]:.3f}')


bins=range(0,14000,500)
f=(illu_gene_tpm.illumina_CTL_tpm>0.01) & (illu_gene_tpm.isoseq_CTL_tpm>0 )&␣
 ↪(illu_gene_tpm.isoseq_genelength<15000)
y=np.log10(illu_gene_tpm.isoseq_CTL_tpm[f]+.1)-np.log10(illu_gene_tpm.
 ↪illumina_CTL_tpm[f]+.1)
#x=[gl for gl,sel in zip(glen,f) if sel]
#x=[gl for gl,sel in zip(illu_glen,f) if sel]
x=illu_gene_tpm.isoseq_genelength[f]
mean_y=[sum(ybin)/len(ybin) if ybin else np.nan for ybin in [[yi for xi,yi in␣
 ↪zip(x,y) if lb<xi<=ub ] for lb,ub in pairwise(bins)]]
hist=axs[1,0].hist2d(x,y, 100,norm=LogNorm(),cmap='YlOrRd')
fig.colorbar(hist[3], ax=axs[1,0])
axs[1,0].plot([(a+b)/2 for a,b in pairwise(bins)],mean_y , ls="--", c=".3")
axs[1,0].axhline( ls=":", c=".5")
axs[1,0].set_xlabel('gene length')
axs[1,0].set_ylabel(r'$\Delta$  Isoseq-Illumina gene expression [log10 TPM]')
axs[1,0].set_ylim(-3.5,2.5)

#axs[1,0].text(4000,-2,f'this is based on mean reference (gencode) transcript␣
 ↪length\nweighted by isoseq expression')

f=(illu_transcript_tpm.illumina_CTL_tpm>0.01) & (illu_transcript_tpm.
 ↪isoseq_CTL_tpm>0 ) & (illu_transcript_tpm.isoseq_genelength<15000)
y=np.log10(illu_transcript_tpm.isoseq_CTL_tpm[f]+.1)-np.
 ↪log10(illu_transcript_tpm.illumina_CTL_tpm[f]+.1)
x=illu_transcript_tpm.isoseq_genelength[f]
mean_y=[sum(ybin)/len(ybin) if ybin else np.nan for ybin in [[yi for xi,yi in␣
 ↪zip(x,y) if lb<xi<=ub ] for lb,ub in pairwise(bins)]]
hist=axs[1,1].hist2d(x,y, 100,norm=LogNorm(),cmap='YlOrRd')
fig.colorbar(hist[3], ax=axs[1,1])
axs[1,1].plot([(a+b)/2 for a,b in pairwise(bins)],mean_y , ls="--", c=".3")
axs[1,1].axhline( ls=":", c=".5")
axs[1,1].set_xlabel('transcript length')
axs[1,1].set_ylabel(r'$\Delta$ Isoseq-Illumina isoform expression [log10 TPM]')
axs[1,1].set_ylim(-3.5,2.5)

for letter,ax in zip('ABCD',axs.flatten()):
    ax.set_title(letter,{'fontsize':20}, loc='left', pad=20)

fig.tight_layout()
plt.savefig(f"{out_fn}_Fig5_Isoseq_vs_Illumina_expression.png")
```



2.5 Differential splicing

In this section we apply the statistical tests, to identify differential splicing events between CTL and VPA treated hepatocytes.

[30]:
```
#min_cov both paths must have that many reads (combined)
#min_n: each sample must have that many reads over both paths
res=isoseq.altsplice_test(groups=['CTL', 'VPA'],min_n=10).sort_values('pvalue')
res.to_csv(f'{out_fn}_tab4_differential_splicing.csv', index=True)
#res=pd.read_csv(f'{out_fn}_tab4_differential_splicing.csv')
res.head()
```

```
INFO:testing differential splicing for CTL (1) vs VPA (1) using proportions test
100%|          | 151670/151670 [14:32<00:00, 173.78it/s]
```

[30]:
```
           gene             gene_id  chrom strand     start       end  \
18570  SLC39A14  ENSG00000104635.15   chr8      +  22408496  22414779
1634      GNG12   ENSG00000172380.6   chr1      -  67717963  67718084
3658       PSAP  ENSG00000197746.14  chr10      -  71822007  71825836
10513    PPP1CB  ENSG00000213639.10   chr2      +  28751678  28751820
10514    PPP1CB  ENSG00000213639.10   chr2      +  28751803  28752176

      splice_type  novel          padj         pvalue   CTL_PSI  CTL_disp  \
18570          ME  False  0.000000e+00   0.000000e+00  0.506763         0
1634          TSS   True  4.654688e-267  4.627616e-271  0.049881         0
3658          5AS   True  5.911088e-259  8.815064e-263  0.089206         0
10513         TSS  False  2.159496e-186  4.293873e-190  0.099855         0
10514         TSS   True  4.173779e-174  1.037376e-177  0.858659         0
```

```
        VPA_PSI  VPA_disp  total_PSI  total_disp  CTL_CTL_in_cov  \
18570  0.210480         0   0.404409           0            7568
1634   0.290463         0   0.144611           0             335
3658   0.265184         0   0.188241           0             938
10513  0.525737         0   0.309033           0             207
10514  0.433783         0   0.649975           0            1780

       CTL_CTL_total_cov  VPA_VPA_in_cov  VPA_VPA_total_cov
18570              14934            1659               7882
1634                6716            1267               4362
3658               10515            3589              13534
10513               2073            1052               2001
10514               2073             868               2001
```

[31]:
```
sig=(res.padj<.01) & (res.splice_type.isin( ["ME", "ES","5AS","3AS","IR"]))
#res.loc[sig].head(20)
res.loc[res.gene=='MCL1']
```

[31]:
```
     gene             gene_id chrom strand      start        end splice_type  \
662  MCL1  ENSG00000143384.14  chr1      -  150577491  150578842         5AS

     novel      padj    pvalue   CTL_PSI  CTL_disp   VPA_PSI  VPA_disp  \
662  False  0.033524  0.004895  0.712121         0  0.481013         0

     total_PSI  total_disp  CTL_CTL_in_cov  CTL_CTL_total_cov  VPA_VPA_in_cov  \
662   0.586207           0              47                 66              38

     VPA_VPA_total_cov
662                 79
```

[32]:
```
sig=(res.padj<.01) & (res.splice_type.isin( ["ME", "ES","5AS","3AS","IR"]))
groups=['CTL', 'VPA']
print(f'{sum(sig)} differential splice sites in {len(res.loc[sig,"gene"].
 ↪unique())} genes for {" vs ".join(groups)}')
```

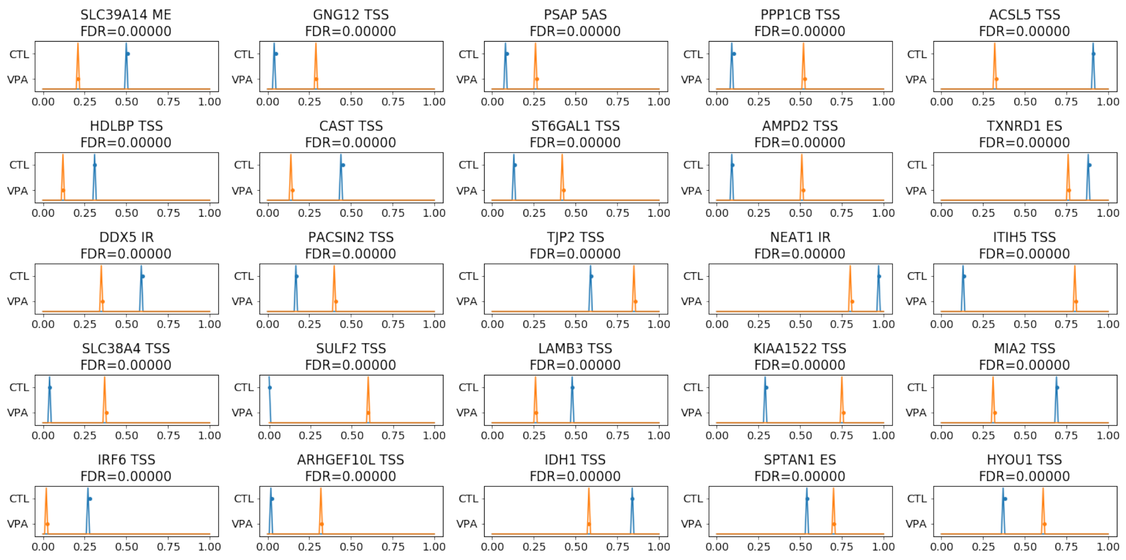
```
806 differential splice sites in 556 genes for CTL vs VPA
```

[33]:
```
for st in ["ME", "ES","5AS","3AS","IR","TSS","PAS"]:
    sel=(res.padj<.01) &(res.splice_type==st)
    print(f'{sum(sel)} differential {st} sites ({sum(sel)/sum(sig):.1%}) in␣
 ↪{len(res.loc[sel,"gene"].unique())} genes for {" vs ".join(groups)}')
```

```
26 differential ME sites (3.2%) in 20 genes for CTL vs VPA
259 differential ES sites (32.1%) in 239 genes for CTL vs VPA
105 differential 5AS sites (13.0%) in 98 genes for CTL vs VPA
62 differential 3AS sites (7.7%) in 61 genes for CTL vs VPA
354 differential IR sites (43.9%) in 233 genes for CTL vs VPA
```

```
974 differential TSS sites (120.8%) in 646 genes for CTL vs VPA
306 differential PAS sites (38.0%) in 239 genes for CTL vs VPA
```

[34]:
```
plotted=isotools.plots.plot_diff_results(res,min_support=1)
```

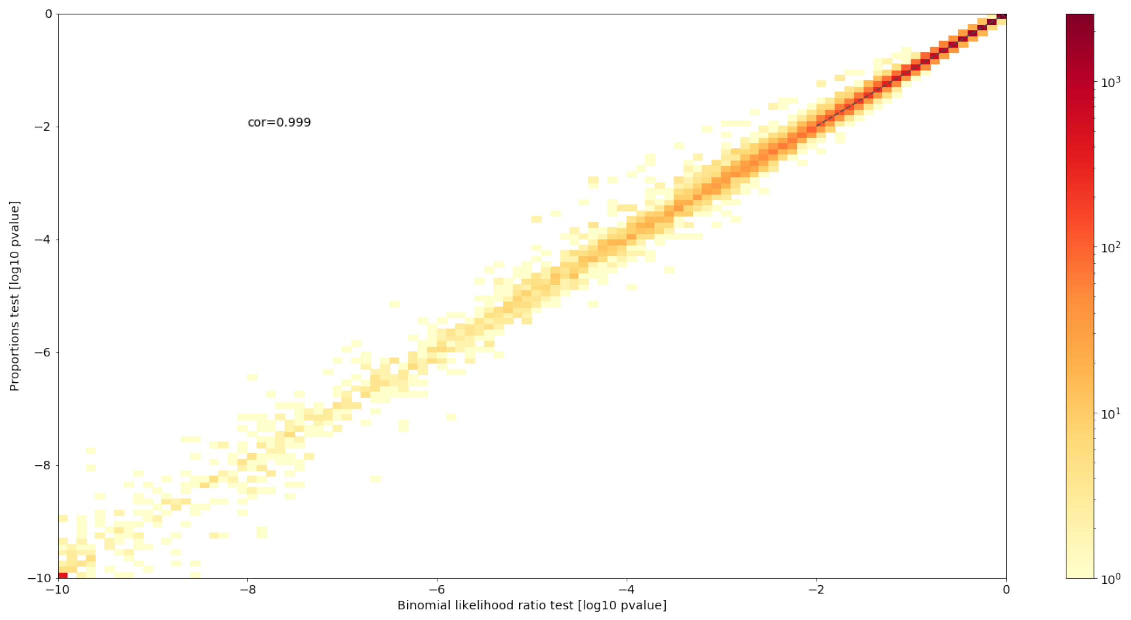


[35]:
```
#compare to alternative test
res_lr=isoseq.altsplice_test(groups=['CTL', 'VPA'],min_n=10, test='binom_lr').
 ↪sort_values('pvalue')
res_lr.to_csv(f'{out_fn}_tab4_lr_differential_splicing.csv', index=True)
#res=pd.read_csv(f'{out_fn}_tab4_differential_splicing.csv')
res_lr.head()
res['lr_pvalue']=res_lr['pvalue']
```

```
INFO:testing differential splicing for CTL (1) vs VPA (1) using binom_lr test
100%|      | 151670/151670 [11:56<00:00, 211.77it/s]
```

[36]:
```
from matplotlib.colors import LogNorm

fig, ax=plt.subplots()
x=np.log10(res['lr_pvalue']+1e-10)
y=np.log10(res['pvalue']+1e-10)
#x=res['lr_pvalue']
#y=res['pvalue']
hist=ax.hist2d(x,y,100,norm=LogNorm(),cmap='YlOrRd')
fig.colorbar(hist[3], ax=ax)
ax.plot([-2, 5], [-2, 5], ls="--", c=".3")
ax.set_xlabel('Binomial likelihood ratio test [log10 pvalue]')
ax.set_ylabel('Proportions test [log10 pvalue]')
ax.text(-8,-2,f'cor={np.corrcoef(x,y)[1,0]:.3f}')
```

```
fig.tight_layout()
plt.savefig(f"{out_fn}_SupplFig_proportions_vs_lr_test.png")
```



```
[ ]:
```