Supplementary Data 1. The codes used to analyze Two-colour FRET traces and Three-colour FRET traces.

<Read me>

*The code runs on Matlab versions 2017a or newer 2022b on Windows.

*Copy the code and run.

*Try testing the 'two-colour FRET traces' with 'two-colour FRET traces' code, a window with traces with will appear in about 1-2 minutes.

>code for Two-colour FRET trace analysis

```
function plotalextrace(path)
path='E:\Disc E\TIRFM analysis\Separation traces\save traces s\saved-traces';
close all;
close all hidden;
fclose('all');
judge=1;
fretline=0;
cd(path);
disp(path);
          timeunit=0.5*2;
savepath1=[path '/saved-traces-d'];
mkdir(savepath1);
savepath2=[path '/saved-traces-s'];
mkdir(savepath2);
savepath3=[path '/saved-traces-a'];
mkdir(savepath3);
savepath4=[path '/saved-traces-h'];
mkdir(savepath4);
savepath5=[path '/saved-traces-g'];
mkdir(savepath5);
 savepath= [path '/saved-traces'];
mkdir(savepath);
A=dir;
numfiles=size(A);


figure;
hdl3=gcf;
k=1;
ks=[];
ksn=1;
%for i=1:numfiles,
while (k < numfiles(1))
  if A(k).isdir ~=0
     k=k+1;
  end
  if A(k).isdir == 0
    sigfname=A(k).name;      %sigfname is signal file name
    k=k+1;
    if (sigfname(end-2:end) == 'csv')
   %     if (sigfname(20:23) == 'pair')
                  if (sigfname(16:19) == 'pair')
```

```
  %  if (sigfname(15:18) == 'pair')
     %if (sigfname(1:4) == 'pair')
%         state=dlmread([sigfname(1:end-3) 'txt']);
%        if state(1) == judge
         ks(ksn)=k;
         ksn=ksn+1;
         cd(path);
         sigfid=fopen(sigfname,'r');                %signal file id
         firstheaderline=textscan(sigfid, '%f:%f-%f, %f:%f-%f,');
         temp1 = textscan(sigfid, '%f, %f, ,', 'headerlines', 1);
%               firstheaderline=textscan(sigfid, '%f:%f-%f, %f-%f, FRET');
%              temp1 = textscan(sigfid, '%f, %f, %f, ,', 'headerlines', 1);
%              firstheaderline=textscan(sigfid, '%f:%f-%f, %f-%f, FRET, ');
%             temp1 = textscan(sigfid, '%f, %f, %f,', 'headerlines', 1);
         data1=cell2mat(temp1(2));
         data2=cell2mat(temp1(1));
         length=size(data1);


         time=(1:length/2)*timeunit;
         figure(hdl3);
         subplot(3,1,1);
         plot(time,data1(1:length/2),'g',time,data2(1:length/2),'b');%,time,stdev,'r');
         title(['Molecule ' num2str(k) ' of ' num2str(numfiles(1))]);
         grid on;
         zoom on;

%                    temp=axis;
%            temp(1)=0;
%            temp(2)=100;
%            axis(temp);
%            grid on;
%            zoom on;


         subplot(3,1,2);

plot(time,data1(length/2+1:length),'g',time,data2(length/2+1:length),'r');%,time,stdev,'r');
         grid on;
         zoom on;
        %            firstheaderline2=cell2mat(firstheaderline(1:4));
        %  title([sigfname  num2str(firstheaderline2)]);
%                    temp=axis;
%            temp(1)=0;
```

```
%           temp(2)=100;
%           axis(temp);
%           grid on;
%           zoom on;



        subplot(3,1,3);
        fret=zeros(length);
%           fret=(data2/0.72)./(data1/0.59+data2/0.72);
        fret=(data2)./(data1+data2);
        fret=fret.*double((data1+data2)>fretline);
        plot(time, fret(1:length/2), 'black');
        title(['FRET']);
        temp=axis;
        temp(3)=0;
        temp(4)=1;
        axis(temp);
        grid on;
        zoom on;

        reply=input('return to continue, f for fret, c for co-locol, b to go back ','s');
         if reply=='h'
           copyfile(sigfname,savepath4);
           copyfile(sigfname,savepath);
           k=k;
         end
         if reply=='g'
           copyfile(sigfname,savepath5);
           copyfile(sigfname,savepath);
           k=k;
        end
        if reply=='d'
           copyfile(sigfname,savepath1);
           copyfile(sigfname,savepath);
           k=k;
        end
        if reply=='s'
           copyfile(sigfname,savepath2);
           copyfile(sigfname,savepath);
           k=k;
        end
        if reply=='a'
           copyfile(sigfname,savepath3);
```

```
            copyfile(sigfname,savepath);
            k=k;
          end
         if reply=='b'
            ksn=ksn-2;
            k=ks(ksn)-1;
          else
            k=k;
          end
          fclose(sigfid);
 %       end
       end
     end
   end
end

fclose('all');
disp('done');
end
```

>code for Three-colour FRET trace analysis

```
function plotalextrace(path)
path='I:\SuiLab\Sudi\20190220\1102-000';
close all;
close all hidden;
fclose('all');
judge=1;
fretline=0;
cd(path);
disp(path);
          timeunit=0.5*2;
savepath1=[path '/saved-traces-d'];
mkdir(savepath1);
savepath2=[path '/saved-traces-s'];
mkdir(savepath2);
savepath3=[path '/saved-traces-a'];
mkdir(savepath3);
savepath4=[path '/saved-traces-h'];
mkdir(savepath4);
savepath5=[path '/saved-traces-g'];
mkdir(savepath5);
 savepath= [path '/saved-traces'];
mkdir(savepath);
A=dir;
numfiles=size(A);


figure;
hdl3=gcf;
k=1;
ks=[];
ksn=1;
%for i=1:numfiles,
while (k < numfiles(1))
  if A(k).isdir ~=0
     k=k+1;
  end
  if A(k).isdir == 0
    sigfname=A(k).name;      %sigfname is signal file name
    k=k+1;
    if (sigfname(end-2:end) == 'csv')
   %     if (sigfname(20:23) == 'pair')
                  if (sigfname(16:19) == 'pair')
```

```
  %  if (sigfname(15:18) == 'pair')
     %if (sigfname(1:4) == 'pair')
%        state=dlmread([sigfname(1:end-3) 'txt']);
%       if state(1) == judge
         ks(ksn)=k;
         ksn=ksn+1;
         cd(path);
         sigfid=fopen(sigfname,'r');                %signal file id
         firstheaderline=textscan(sigfid, '%f:%f-%f, %f:%f-%f, %f:%f-%f,');
         temp1 = textscan(sigfid, '%f, %f, %f, ,', 'headerlines', 1);
%              firstheaderline=textscan(sigfid, '%f:%f-%f, %f-%f, FRET');
%             temp1 = textscan(sigfid, '%f, %f, %f, ,', 'headerlines', 1);
%             firstheaderline=textscan(sigfid, '%f:%f-%f, %f-%f, FRET, ');
%           temp1 = textscan(sigfid, '%f, %f, %f,', 'headerlines', 1);
         data1=cell2mat(temp1(2));
         data2=cell2mat(temp1(1));
         data3=cell2mat(temp1(3));
         length=size(data1);


         time=(1:length/2)*timeunit;
         figure(hdl3);
         subplot(2,1,1);

plot(time,data1(1:length/2),'g',time,data2(1:length/2),'blue',time,data3(1:length/2),'black');%,ti
me,stdev,'r');
         title(['Molecule ' num2str(k) ' of ' num2str(numfiles(1))]);
         grid on;
         zoom on;

%                    temp=axis;
%           temp(1)=0;
%           temp(2)=100;
%           axis(temp);
%           grid on;
%           zoom on;


         subplot(2,1,2);

plot(time,data1(length/2+1:length),'g',time,data2(length/2+1:length),'blue',time,data3(length/
2+1:length),'black');%,time,stdev,'r');
         grid on;
         zoom on;
```

```
        %            firstheaderline2=cell2mat(firstheaderline(1:4));
        %  title([sigfname  num2str(firstheaderline2)]);
%                    temp=axis;
%           temp(1)=0;
%           temp(2)=100;
%           axis(temp);
%           grid on;
%           zoom on;



%           subplot(3,1,3);
%           fret=zeros(length);
% %           fret=(data2/0.72)./(data1/0.59+data2/0.72);
%           fret35=(data1(1:length))./(data1(1:length)+data2(1:length));
%           fret37=(data3(1:length))./(data3(1:length)+data2(1:length));
%           fret57=(data3(1:length))./(data1(1:length)+data3(1:length));
%           fret=fret.*double((data1(1:length)+data2(1:length))>fretline);
%           plot(time, fret35(1:length), 'b', time, fret37(1:length),
%           'red', time, fret57 (1:length),'black');, time, stdev, 'r';
%           title(['FRET']);
%           temp=axis;
%           temp(3)=0;
%           temp(4)=1;
%           axis(temp);
%           grid on;
%           zoom on;

          reply=input('return to continue, f for fret, c for co-locol, b to go back ','s');
           if reply=='h'
             copyfile(sigfname,savepath4);
             copyfile(sigfname,savepath);
             k=k;
           end
           if reply=='g'
             copyfile(sigfname,savepath5);
             copyfile(sigfname,savepath);
             k=k;
          end
          if reply=='d'
             copyfile(sigfname,savepath1);
             copyfile(sigfname,savepath);
             k=k;
          end
```

```
          if reply=='s'
             copyfile(sigfname,savepath2);
             copyfile(sigfname,savepath);
             k=k;
          end
          if reply=='a'
             copyfile(sigfname,savepath3);
             copyfile(sigfname,savepath);
             k=k;
          end
         if reply=='b'
             ksn=ksn-2;
             k=ks(ksn)-1;
          else
             k=k;
          end
          fclose(sigfid);
 %       end
       end
     end
   end
end

fclose('all');
disp('done');
end
```