```
clc
clear all
close all
addpath('D:\Program Files\MATLAB\R2013a\work\')

%%  File information
%-------------------------------------------------------------------------
----
basename='HBV'; %File name base
columnW=[5 12]; %Number of images in each set
FolderNames={'3colortestcontrol' '3colortest'}; %Folder name with each
set of images
%Assignment of channel numbers
%-----------------------------
dapc=1;
stainc=4;
cy5c=3;
rep=50;
%-----------------------------
outputfile='3color.xlsx'; %Excell output file
folder='HBVcc_Edu_hNTCP_BAC_NRG_21317';
%-------------------------------------------------------------------------
----
%% Segmentation parameters
cluster=3; %base k-means clustering size
medsiz=30; %median filter size

%%
%Folder loop
for uu=1:length(FolderNames)
    fileFolder = ['D:\Program Files\MATLAB\R2013a\work\' folder '\'
sprintf('%s',FolderNames{uu})];
    cd(fileFolder)
    %% Initalize vectors
    totalcells=zeros(columnW(uu),1);
    totalred= totalcells;
    redtotalcelloverlap= totalcells;
    totalredthick= totalcells;

    totalcy5= totalcells;
    cy5totalcelloverlap= totalcells;
    totalcy5thick= totalcells;
    totalboth= totalcells;
    totalboththick= totalcells;
    bothtotalcelloverlap= totalcells;
    %%
    %Position Loop
    for w=1:columnW(uu)
        %% Read files
        if  w<10
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```
            dapin=[basename '_t00'  num2str(w) '_c00' num2str(dapc)];
            redn=[basename '_t00'  num2str(w) '_c00'
num2str(stainc)];
            cy5n=[basename '_t00'  num2str(w) '_c00' num2str(cy5c)];
        else
            dapin=[basename '_t0'  num2str(w) '_c00' num2str(dapc)];
            redn=[basename '_t0'  num2str(w) '_c00' num2str(stainc)];
            cy5n=[basename '_t0'  num2str(w) '_c00' num2str(cy5c)];
        end
        dapi=imread([dapin '.tif']);
        red=imread([redn '.tif']);
        cy5=imread([cy5n '.tif']);
        [a b]=size(cy5);
        %% Perform image operations
        B = medfilt2(red, [medsiz medsiz]); %Filter out punctate
structures
        BC = medfilt2(cy5, [medsiz medsiz]);
        outlie=red-B; %Subtract filter image to segment punctate
structures
        outliecy5=cy5-BC;
        imageavg=mean(red(:)); %Calculate the mean of the image
        imageavgcy5=mean(cy5(:));
        imagestd=std(double(red(:))); %Calculate the standard devation
of the image
        imagestdcy5=std(double(cy5(:)));
        [redG, Gdir]=imgradient(red); %Calculate the image graident
        [cy5G, cy5Gdir]=imgradient(cy5);

        %Use kmeans clustering to segment images

%-----------------------------------------------------------------
        [v4 , c4, breakbin4] = kmeanssubbreakrep(red,
cluster,floor(numel(dapi)),rep); %Segment high pixels
        redhigh=c4>2;
        [v2 , c2, breakbin2] = kmeanssubbreakrep(redG,
cluster+1,floor(numel(dapi)),rep); %Segment high graident regions
        redGhigh=c2>3;
        [vv4 , cc4, breakbin4c] = kmeanssubbreakrep(cy5,
cluster,floor(numel(dapi)),rep); %Segment high pixels
        cy5high=cc4>1;
        [vv2 , cc2, breakbin2c] = kmeanssubbreakrep(cy5G,
cluster,floor(numel(dapi)),rep); %Segment high graident regions
        cy5Ghigh=cc2>1;

%-----------------------------------------------------------------
        %%
        nobackred=redhigh&redGhigh; %Overlap of segmented images
        nobackcy5=cy5high&cy5Ghigh; %Overlap of segmented images

        if nnz(nobackcy5)>(0.2*a*b)
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```
            nobackcy5=zeros(a,b);
        end
        if nnz(nobackred)>(0.2*a*b)
            nobackred=zeros(a,b);
        end
        figure(5)
        imshow(nobackred)
        figure(6)
        imshow(nobackcy5)
       % stop
        %Segment nuclei from DAPI image

%-----------------------------------------------------------------
        [v1 , c1] = kmeanssub(dapi, cluster,floor(numel(dapi)/10));
        noback=c1>2;
        figure(2)
        imshow(noback)

%-----------------------------------------------------------------
        % Clear structures too small or large to be nuclei
        noback=bwareaopen(noback,8);
        toobig=bwareaopen(noback,1000);
        noback=noback-toobig;

        nobackallcell=imdilate(noback,strel('disk',4)); %Enlarge
nuclei to get an assumed cell area
        largernoback=bwmorph(noback,'thicken',10); %Thickened nuclei
to get more cell area but more concervative than nobackallcell

        %Remove too small and too large stainings
        nobackred=bwareaopen(nobackred,8);
        nobackred=imclearborder(nobackred);
        toobigred=bwareaopen(nobackred,900);
        nobackred=nobackred-toobigred;


        nobackcy5c=bwareaopen(nobackcy5,30);
        nobackcy5c=imclose(nobackcy5c,strel('disk',1));
        nobackcy5c=imfill(nobackcy5c,'holes');
        nobackcy5c=imclearborder(nobackcy5c);
        nobackcy5=nobackcy5c;

        %Calculate overlaps - assumed cell area (dilate nuclei, allows
overlap)
        overlapredlargemas=nobackallcell&nobackred;
        overlapcy5largemas=nobackallcell&nobackcy5;
        redtotalcelloverlap(w)=max(max(bwlabel(overlapredlargemas)));
        cy5totalcelloverlap(w)=max(max(bwlabel(overlapcy5largemas)));

bothtotalcelloverlap(w)=nnz(intersect(unique(bwlabel(nobackallcell).*(
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overlapredlargemas)),
unique(bwlabel(nobackallcell).*(overlapcy5largemas))));

        %Label Nuclei
        ldapi=bwlabel(noback);
        ldapithick=bwlabel(largernoback);


        totalcells(w)=max(max(ldapi));

        %Count nuclear overlaps
        overlapim=ldapi.*double(ldapi&nobackred);
        overlapimcy5=ldapi.*double(ldapi&nobackcy5);
        idxon=nonzeros(unique(overlapim));
        idxoncy5=nonzeros(unique(overlapimcy5));

idxonboth=nonzeros(intersect(unique(overlapimcy5),unique(overlapim)));
        totalred(w)=numel(idxon);
        totalcy5(w)=numel(idxoncy5);
        totalboth(w)=numel(idxoncy5);

        %Count nuclear overlaps - thick nuclei (expand nuceli without
causeing overlap)
        overlapimthick=ldapithick.*double(ldapithick&nobackred);
        overlapimthickcy5=ldapithick.*double(ldapithick&nobackcy5);
        idxonthickcy5=nonzeros(unique(overlapimthickcy5));
        idxonthick=nonzeros(unique(overlapimthick));

idxonthickboth=nonzeros(intersect(unique(overlapimthickcy5),unique(ove
rlapimthick)));
        totalredthick(w)=numel(idxonthick);
        totalcy5thick(w)=numel(idxonthickcy5);
        totalboththick(w)=numel(idxonthickcy5);


        %Show segmented image
        figure(4)
        imshow(noback)
        hold on
       [yy xx]=find(nobackred);
        plot(xx,yy,'.r')
        [yyc xxc]=find(nobackcy5c);
        plot(xxc,yyc,'.y')
        [yyc xxc]=find(nobackcy5c&nobackred);
        plot(xxc,yyc,'.b')
        hold off
        pause(0.05)
        saveas(gcf,['segmented' num2str(w) '.png'])

    end
```

```
    %Save data to files between loops
    save([FolderNames{uu} num2str(uu)],'totalcells',
'totalred','totalredthick','redtotalcelloverlap','totalcy5','totalboth
','totalcy5thick','totalboththick','cy5totalcelloverlap')
end

%% Write data to excell file
columnW=[5 12];
for uu=1:length(FolderNames)
    fileFolder = ['D:\Program Files\MATLAB\R2013a\work\' folder '\'
sprintf('%s',FolderNames{uu})];
    cd(fileFolder)
    load([FolderNames{uu} num2str(uu)])

    cd(['D:\Program Files\MATLAB\R2013a\work\' folder '\'])


    if uu==1
        firstend=columnW(uu)+1;
        start=2;
        xlswrite(outputfile,{'nuclei','overlaping nuclei','overlaping
thick','overlapping assumed cell'},'Red','A1:D1')
        xlswrite(outputfile,{'nuclei','overlaping nuclei','overlaping
thick','overlapping assumed cell'},'Cy5','A1:D1')
        xlswrite(outputfile,{'nuclei','overlaping nuclei','overlaping
thick','overlapping assumed cell'},'both','A1:D1')
    else
        firstend=columnW(uu)+lastend;
        start=lastend+1;
    end
        xlswrite(outputfile,totalcells,'Red',['A' num2str(start) ':A'
num2str(firstend)])
        xlswrite(outputfile,totalred,'Red',['B' num2str(start) ':B'
num2str(firstend)])
        xlswrite(outputfile,totalredthick,'Red',['C' num2str(start)
':C' num2str(firstend)])
        xlswrite(outputfile,redtotalcelloverlap,'Red',['D'
num2str(start) ':D' num2str(firstend)])

        xlswrite(outputfile,totalcells,'Cy5',['A' num2str(start) ':A'
num2str(firstend)])
        xlswrite(outputfile,totalcy5,'Cy5',['B' num2str(start) ':B'
num2str(firstend)])
        xlswrite(outputfile,totalcy5thick,'Cy5',['C' num2str(start)
':C' num2str(firstend)])
        xlswrite(outputfile,cy5totalcelloverlap,'Cy5',['D'
num2str(start) ':D' num2str(firstend)])

        xlswrite(outputfile,totalcells,'Both',['A' num2str(start) ':A'
num2str(firstend)])
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```
        xlswrite(outputfile,totalboth,'Both',['B' num2str(start) ':B'
num2str(firstend)])
        xlswrite(outputfile,totalboththick,'Both',['C' num2str(start)
':C' num2str(firstend)])
        xlswrite(outputfile,bothtotalcelloverlap,'Both',['D'
num2str(start) ':D' num2str(firstend)])

        lastend=firstend+1;


end
%%
```