

Supporting Information

Zinc binding inhibits cellular uptake and antifungal activity of Histatin-5 in *Candida albicans*

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MATLAB script for image intensity analysis.

Figure S1. Synthesis of Hist-5*.

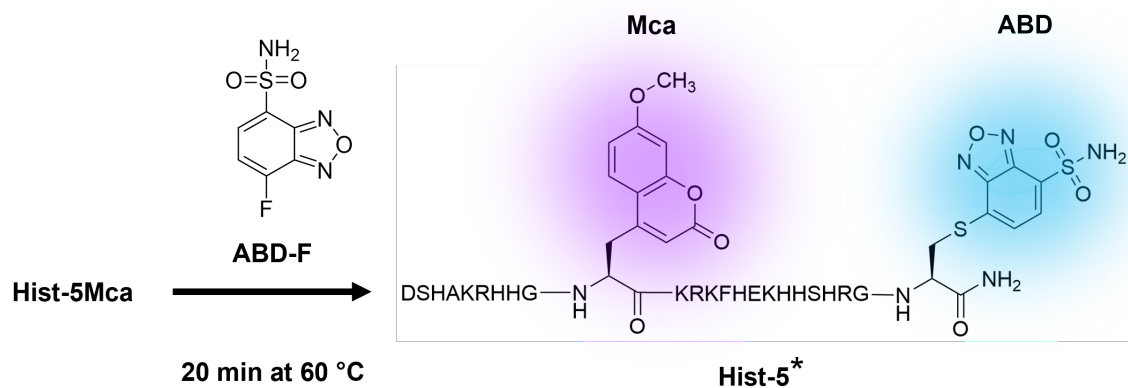


Figure S1. Synthesis of Hist-5*. Fmoc-protected methoxycoumarin (Mca) was incorporated into the solid phase peptide synthesis of Hist-5 yielding a singly labeled peptide, Hist-5Mca. Purified Hist-5Mca was reacted in a 1:1 molar ratio with 4-Fluoro-7-sulfamoylbenzofurazan (ABD-F) for 20 min in a water bath at 60 °C yielding the doubly labeled Hist-5 peptide, Hist-5*.

Figure S2. HPLC and MS Characterization of Hist-5.

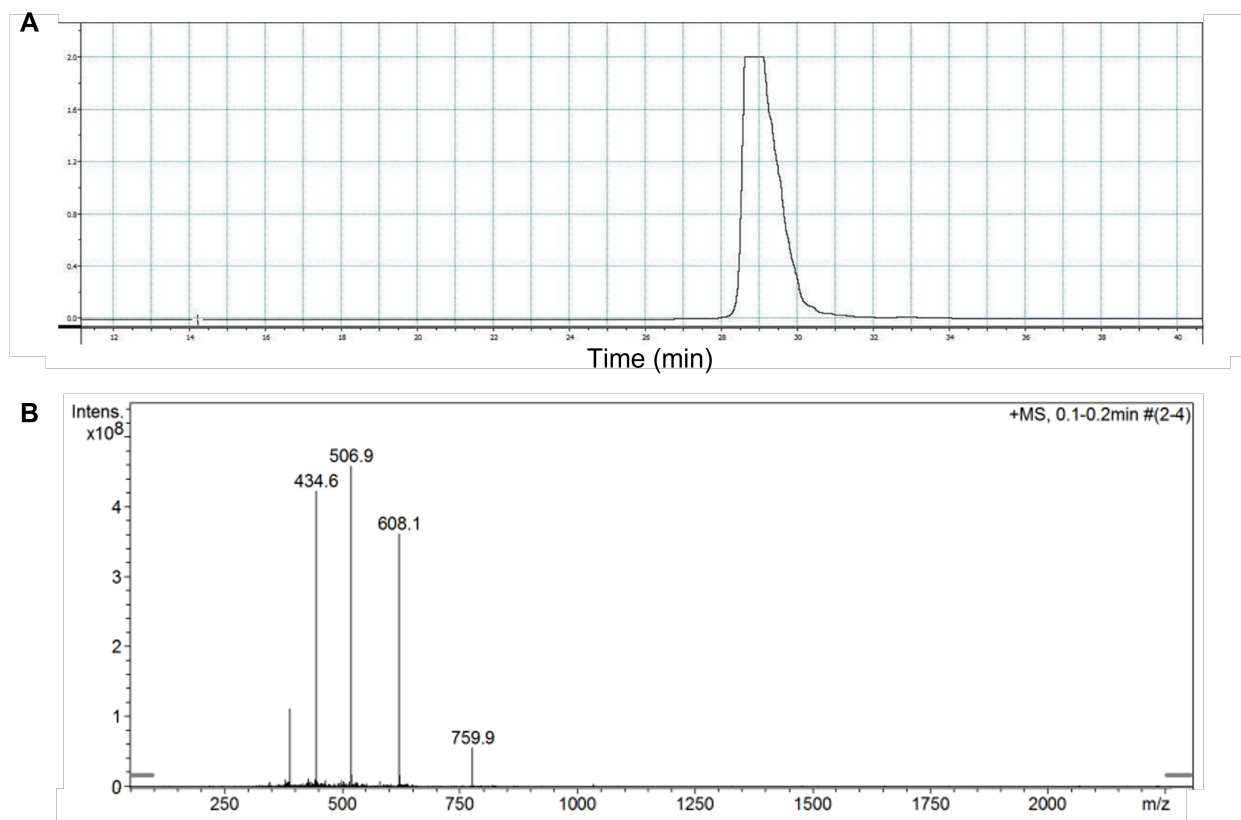


Figure S2. HPLC and MS Characterization of Hist-5. (A) Analytic HPLC trace of Hist-5 detection at 280 nm, retention time ~ 28.8 min. (B) Mass spectrum of Hist-5. Observed masses confirmed by electrospray ionization mass spectrometry: calculated mass for Hist-5 3034.5, found $(M + 4H^+)$ 759.9 m/z, $(M + 5H^+)$ 608.1 m/z, $(M + 6H^+)$ 506.9 m/z, $(M + 7H^+)$ 434.6 m/z.

Figure S3. HPLC and MS Characterization of Hist-5Mca.

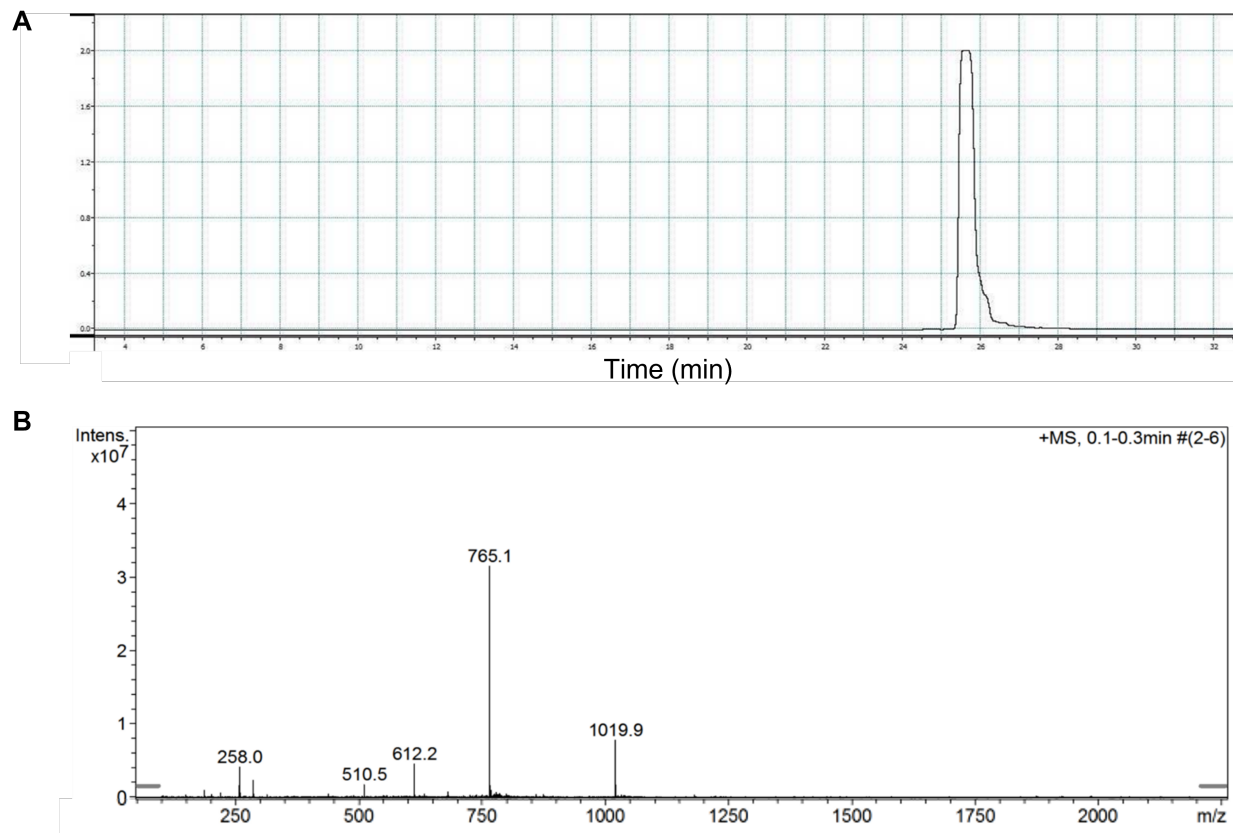


Figure S3. HPLC and MS Characterization of Hist-5Mca. (A) Analytic HPLC trace of Hist-5Mca detection at 325 nm, retention time ~25.7 min. (B) Mass spectrum of Hist-5Mca. Observed masses confirmed by electrospray ionization mass spectrometry: calculated mass for Hist-5-Mca 3056.5, found ($M + 3H^+$) 1019.9 m/z, ($M + 4H^+$) 765.1 m/z, ($M + 5H^+$) 612.2 m/z, ($M + 6H^+$) 510.5 m/z.

Figure S4. HPLC and MS Characterization of Hist-5*.

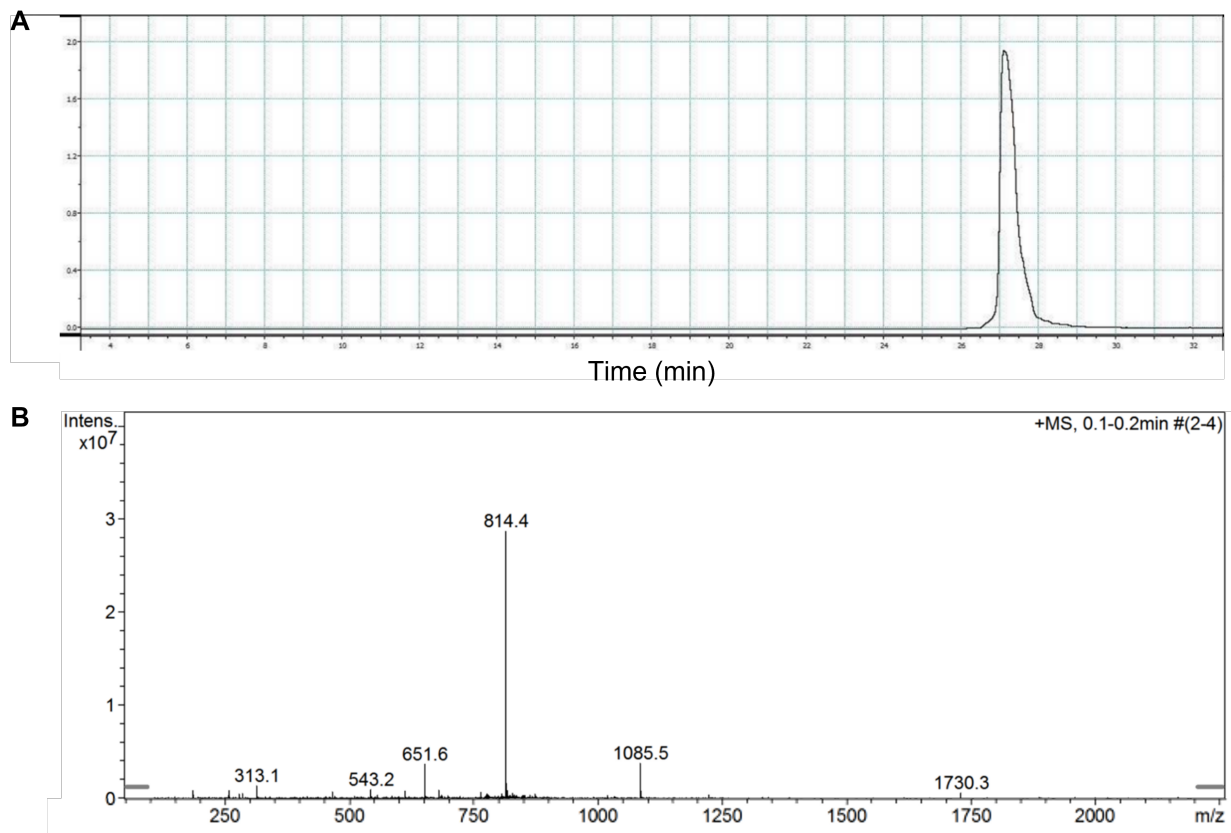


Figure S4. HPLC and MS Characterization of Hist-5*. (A) Analytic HPLC trace of Hist-5* detection at 325 nm, retention time \sim 27.1 min. (B) Mass spectrum of Hist-5*. Observed masses confirmed by electrospray ionization mass spectrometry: calculated mass for Hist-5* 3253.5, found (M + 3H⁺) 1085.5 m/z, (M + 4H⁺) 814.4 m/z, (M + 5H⁺) 651.6 m/z, (M + 6H⁺) 543.2 m/z.

Figure S5. Fluorescence signals from Mca and ABD fluorophores on Hist-5* colocalize.

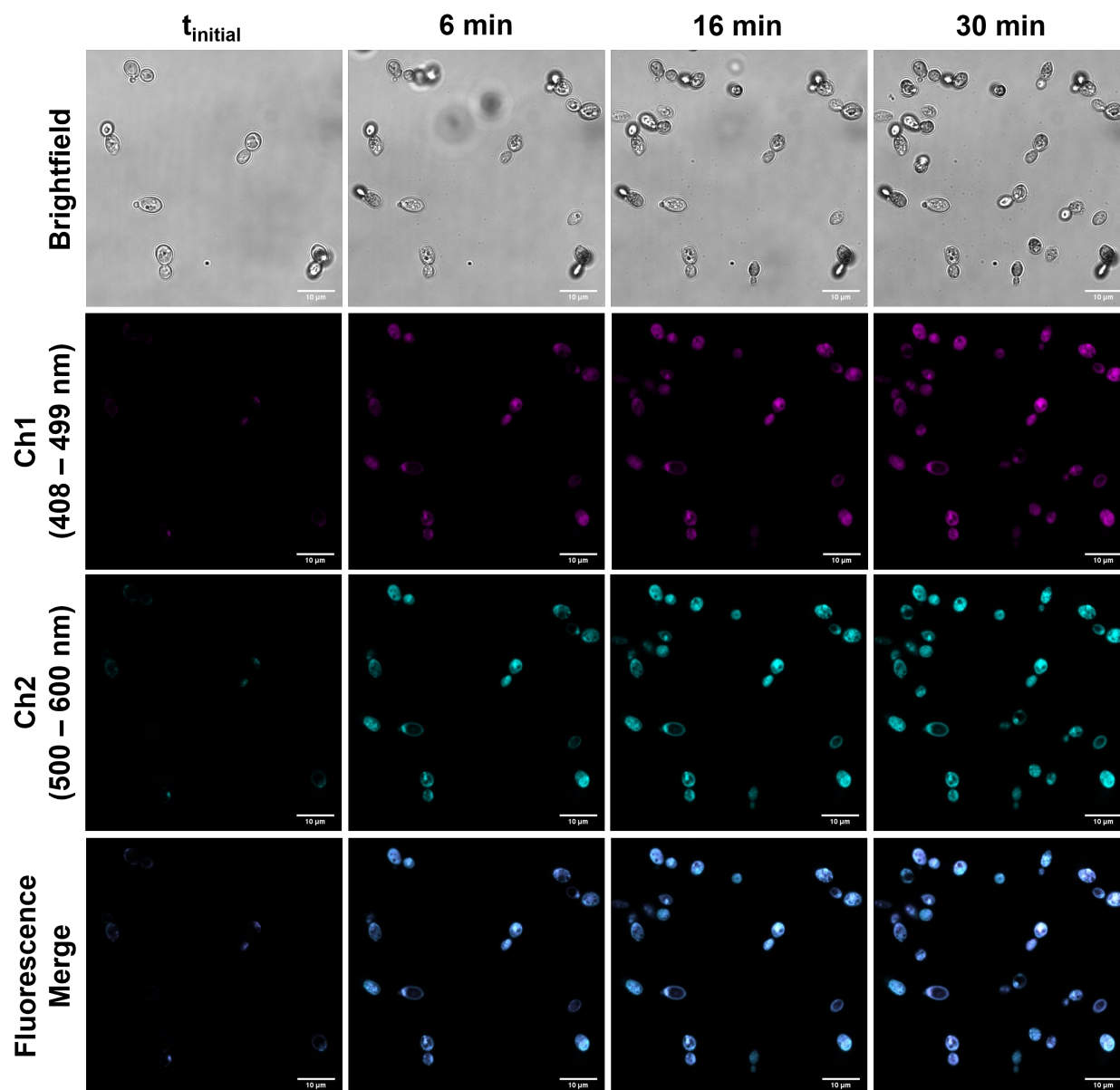


Figure S5. Fluorescence signals from Mca and ABD fluorophores on Hist-5* colocalize. Timelapse microscopy images of cells treated with 12.5 μM Hist-5* at room temperature (RT) over 30 min in 1 mM potassium phosphate buffer pH 7.4 (PPB). Scale bar = 10 μm .

Figure S6. Control cells exhibit no fluorescence signal.

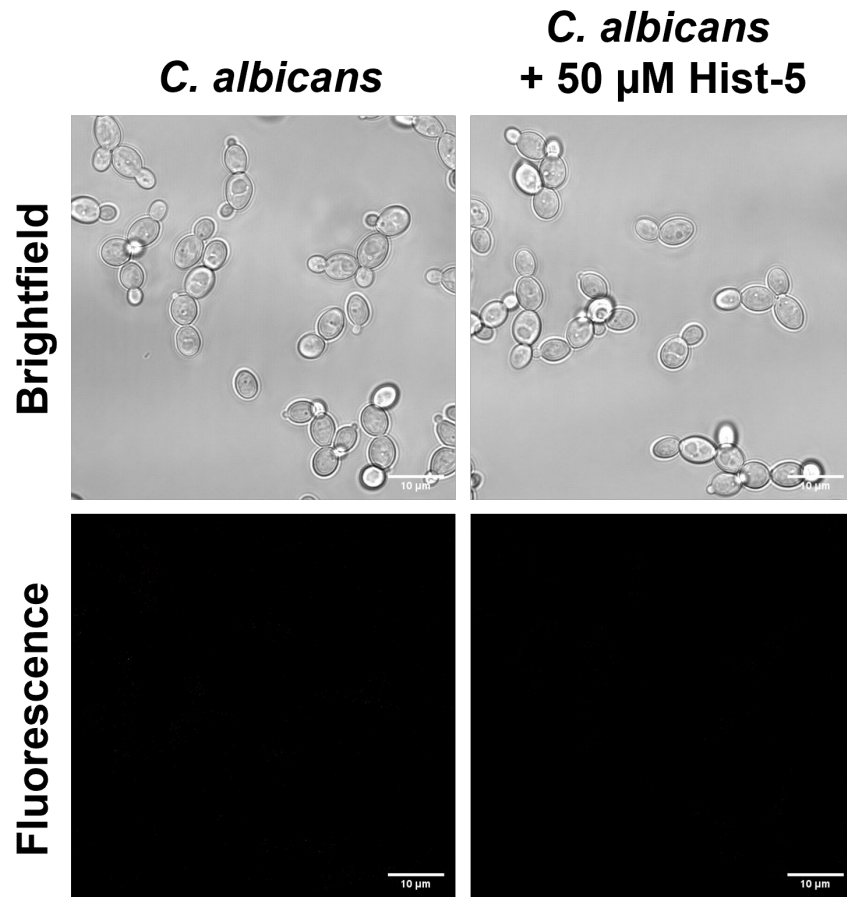


Figure S6. Control cells exhibit no fluorescence signal. Confocal fluorescence microscopy images of untreated *C. albicans* cells and cells treated with 50 μ M Hist-5 at RT for 5 min in PPB.

Figure S7. Fungicidal activity of Hist-5* is inhibited by increasing concentration of Zn²⁺.

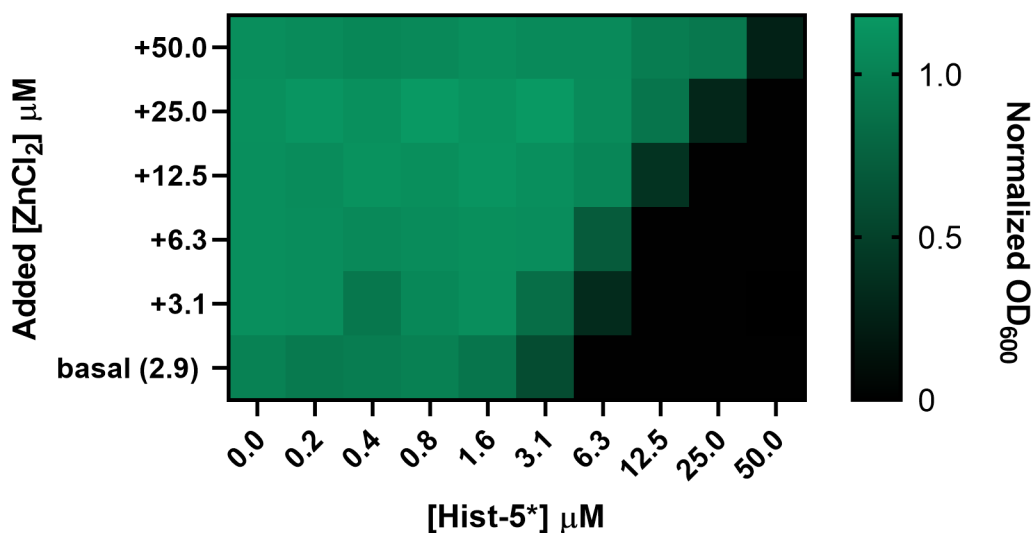


Figure S7. Fungicidal activity of Hist-5* is inhibited by increasing concentration of Zn²⁺. *C. albicans* cells were pre-incubated in PPB pH 7.4 for 1.5 h at 37 °C with increasing concentrations of Hist-5* and supplemental ZnCl₂; concentrations are indicated in the figure axes. Aliquots were resuspended in 50 mM Tris-buffered synthetic defined media (SD+), pH 7.4, and cell growth was measured by OD₆₀₀ after incubation for 48 h at 30 °C. Values represent the average from three separate biological replicates.

Figure S8. Modulation of cell growth by Zn^{2+} at sub-MIC levels of Hist-5, revealing that Zn^{2+} improves Hist-5 activity at a ratio of 1:2 Zn:peptide under these conditions.

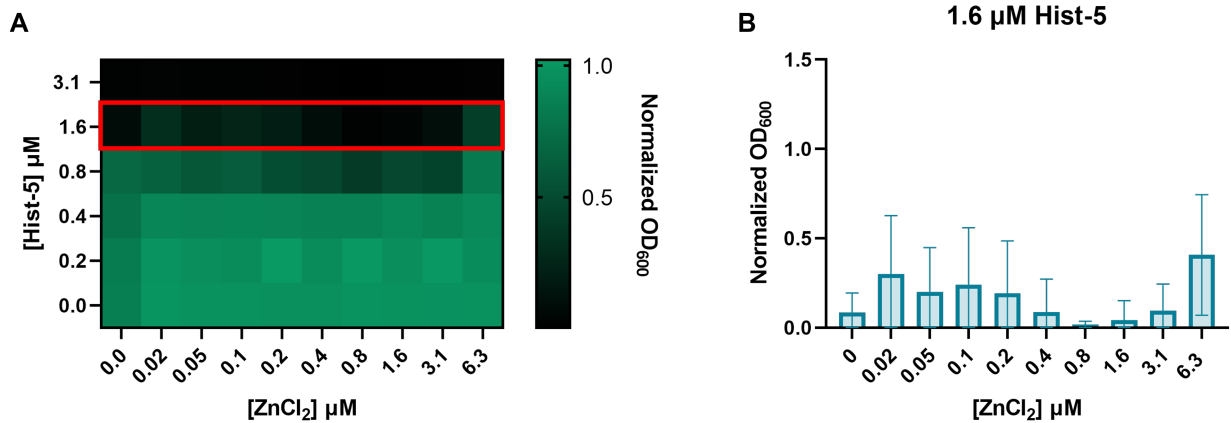


Figure S8. Modulation of cell growth by Zn^{2+} at sub-MIC levels of Hist-5, revealing that Zn^{2+} improves Hist-5 activity at a ratio of 1:2 Zn:peptide under these conditions. (A) *C. albicans* cells were pre-incubated in PPB pH 7.4 for 1.5 h at 37 °C with increasing concentrations of Hist-5 and supplemental ZnCl₂; concentrations are indicated in the figure axes. Aliquots were resuspended in 50 mM Tris-buffered synthetic defined Zn-free media (SD-Zn), pH 7.4, and cell growth was measured by OD₆₀₀ after incubation for 48 h at 30 °C. **(B)** Data from the 1.6 μM Hist-5 row (red box in A) replotted as a bar graph to zoom in the nuanced modulatory effect of Zn^{2+} under these conditions. Values and error bars represent the average and standard deviation from three separate biological replicates.

Figure S9. Zn^{2+} addition blocks peptide internalization.

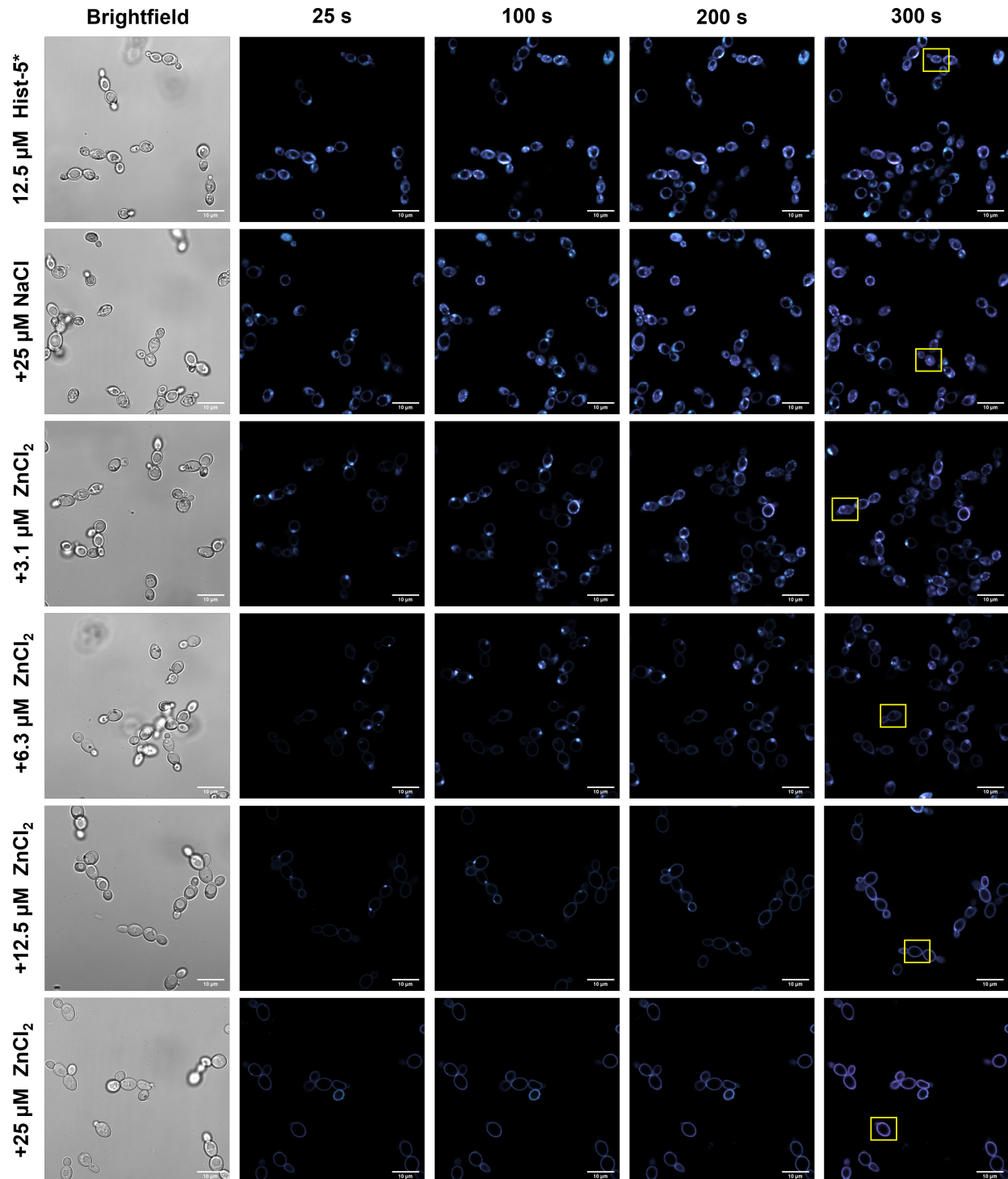


Figure S9. Zn^{2+} addition blocks peptide internalization. Timelapse microscopy images of *C. albicans* cells treated with 12.5 μM Hist-5* alone, peptide+2 eq. NaCl, or varying concentrations of $ZnCl_2$ (0.25, 0.5, 1, or 2 eq. Zn^{2+}) at RT over 5 min in PPB.

Figure S10. Zn-induced adherence of Hist-5* to the cell surface is long-lived.

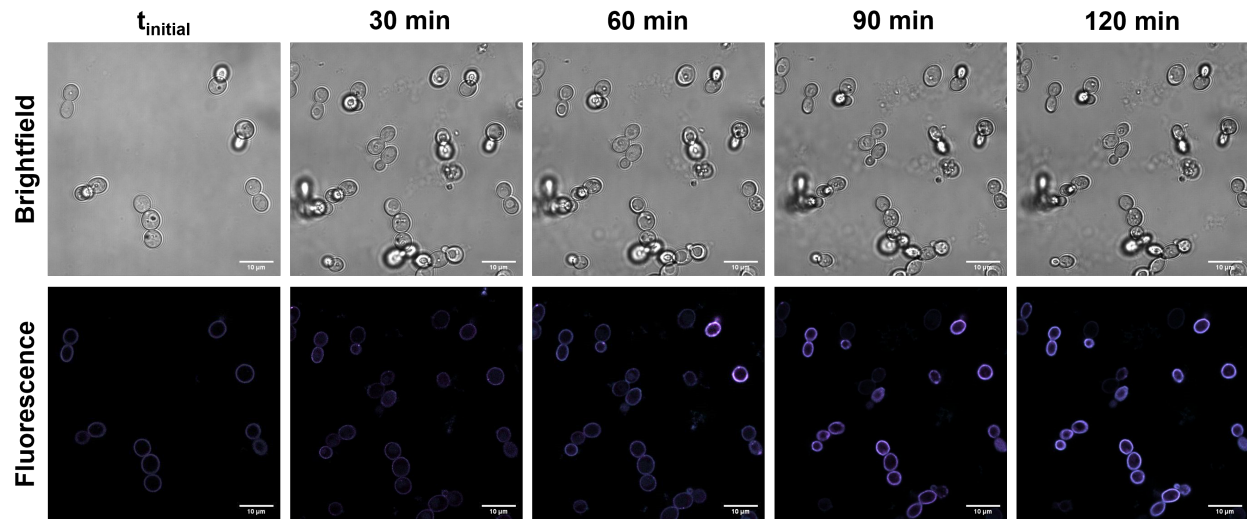


Figure S10. Zn-induced adherence of Hist-5* to the cell surface is long-lived. Timelapse microscopy images of *C. albicans* cells treated with 12.5 μM Hist-5* and 12.5 μM ZnCl_2 at RT over 2 h in PPB.

Figure S11. Surface-bound effect of Hist-5* is Zn-specific.

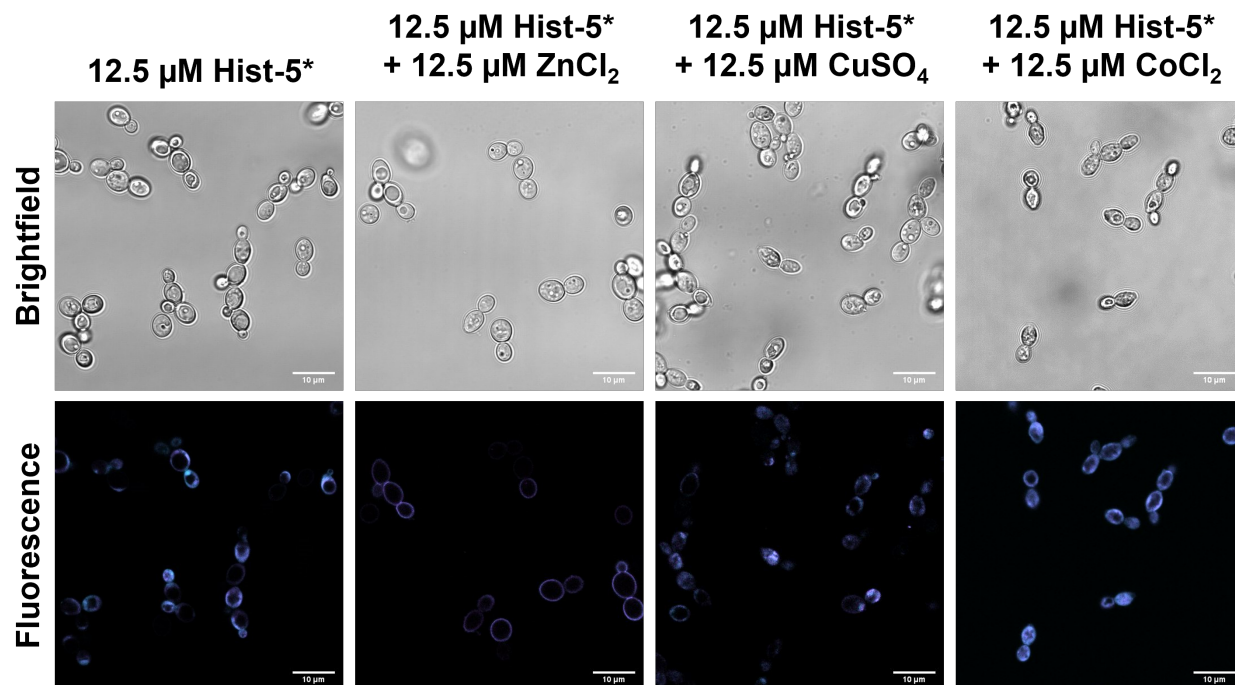


Figure S11. Surface-bound effect of Hist-5* is Zn-specific. Confocal microscopy images of *C. albicans* cells treated with 12.5 μM Hist-5* alone or 12.5 μM Hist-5*+12.5 μM ZnCl_2 , 12.5 μM CuSO_4 , or 12.5 μM CoCl_2 at RT for 5 min in PPB.

Figure S12. In vitro formation of a ternary complex between Hist-5, ZQ, and Zn^{2+} .

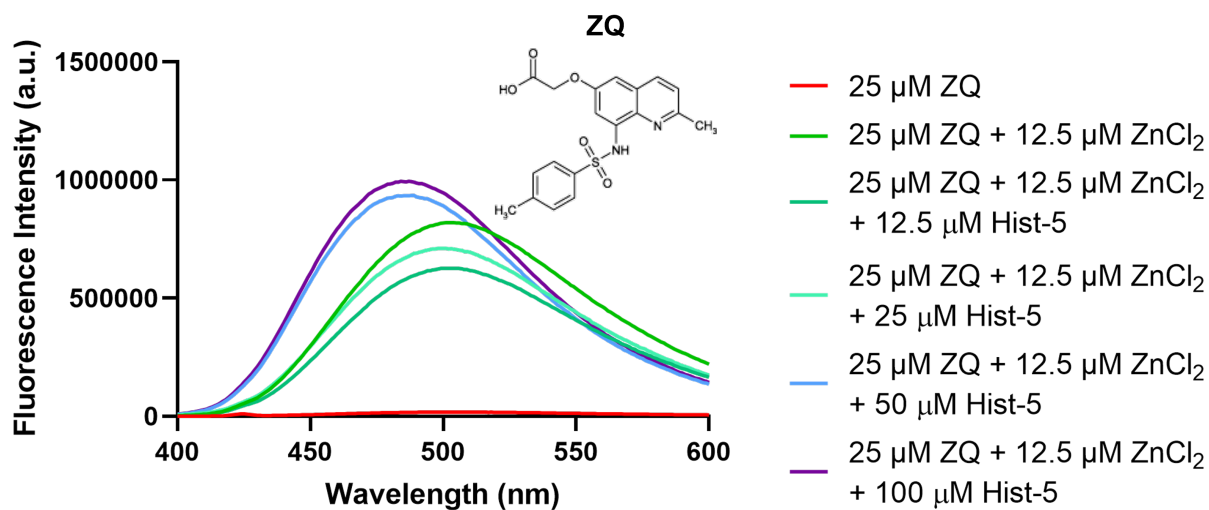


Figure S12. In vitro formation of a ternary complex between Hist-5, ZQ, and Zn^{2+} . Titration of Hist-5 into a solution containing 25 μM ZQ and 12.5 μM in ZnCl₂ PPB pH 7.4. Structure of ZQ free acid shown in the right-hand corner.

Figure S13. EDTA and S100A12 protein do not induce membrane permeability.

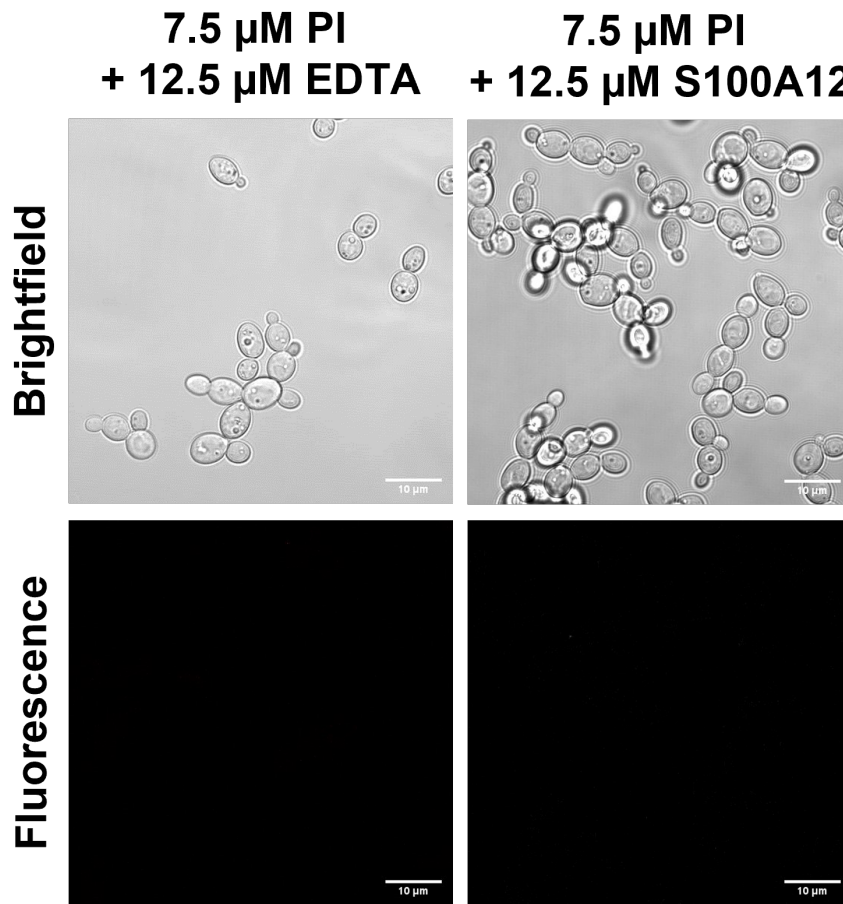


Figure S13. EDTA and S100A12 protein do not induce membrane permeability. Confocal microscopy images of *C. albicans* cells treated with 7.5 μ M PI and 12.5 μ M EDTA or 12.5 μ M S100A12 at RT for 5 min in PPB.

MATLAB script for image intensity analysis. This script calculates the corrected total cell fluorescence (CTCF) value for each slice of a z-stack across all times within a confocal timelapse microscopy image.

$$CTCF = \frac{(Area\ of\ selected\ cell) * (Average\ pixel\ intensity\ within\ the\ area)}{(Area\ of\ background) * (Average\ pixel\ intensity\ of\ background)}$$

```
%% This script reads *.czi files containing image stacks for
% quantifying cell intensities.
% It uses open source bio-formats v5.3 dependencies for MATLAB
% extract bformatlab.zip, downloadable from
% https://downloads.openmicroscopy.org/bio-formats/5.3.4/
% and copy file path below
% Written by Keerthi Anand
% 1/21/2020
% modified 3/22/20 to optimize run time
clc
close all
%%

% Paste full file path of extracted dependencies folder here
bfFolder='C:\Users\Joanna\Documents\MATLAB\bformatlab';
addpath(genpath(bfFolder))

% Open dialog Box to load .czi file
[file,path,indx] = uigetfile('*.czi');

% Change to directory
cd(path)

% Extract all stacks and metadata
volume=bfOpen3DVolume([path file]);

% Extract data, which is located in the first cell
data=volume{1,1}{1,1};

% Number of stacks by 3 channels
maxStacks=size(data,3)/3;

% This is header info for data written out for each ROI/Cell
outputSummaryData={'Filename' 'Time' 'Frame' 'CH1 Area' 'CH1 Mean' 'CH1 ID' ...
    'CH1 CTFC' 'CH1 Mean Bgd' 'CH2 Area' 'CH2 Mean' 'CH2 ID' 'CH2 CTFC' ...
    'CH2 Mean Bgd'};

% Scale for area covered by each pixel, custom for your imaging field
factor=67.48^2/512^2;

% Second cell contains metadata, use this to find number of time points
% (numTotalTimePoints) and number z stacks (numTotalZStacks)
metadata = volume{1, 2};
metadataKeys = metadata.keySet().iterator();
```

```

numTotalTimePoints=1;
numTotalZStacks=1;

% Search through metadata to find fields containing number timepoints and
% z stacks
for z=1:metadata.size()
    key = metadataKeys.nextElement();
    value = metadata.get(key);

    if contains(key, 'DimensionT')
        numTotalTimePoints=str2double(value);
    end
    if contains(key, 'SizeZ')
        numTotalZStacks=str2double(value);
    end
    allMetadata{z,1}=key;
    allMetadata{z,2}=value;
    % fprintf('%s = %s\n', key, value)
end

% Specify colormaps for vizualizing channels
colormapMagenta=zeros([256 3]); % colormap for magenta
colormapMagenta(:,[1 3])=repmat(linspace(0,1,256)',[1 2]);
colormapCyan=zeros([256 3]); % colormap for cyan
colormapCyan(:,[2 3])=repmat(linspace(0,1,256)',[1 2]);

% Set intensity thresholds for chan 1 and 2 to get rid of noise
noiseThresholdChan1=15; % 15 works well but play around
noiseThresholdChan2=15;

% First run, set flag to 0
reuseROI=0;

% numTotalTimePoints = number Timepoints
% numTotalZStacks = number z Stacks
% channels arranged tmpt1-z1-chans1,2,3;tmpt1-z2-chans1,2,3 ...
% Figure out how many images there are and iterate through them

% Allocate space for saving intensities through time
intensityThruTimeChan1=zeros(size(data,1),size(data,2),numTotalTimePoints);
intensityThruTimeChan2=zeros(size(data,1),size(data,2),numTotalTimePoints);

% idxDataEntry will be counter to add data to summary output file.
idxDataEntry=1;
for idxTime=1:numTotalTimePoints % Iterate through time

    %Allocate space
    intensityThruStacksChan1=zeros(size(data,1),size(data,2),numTotalZStacks);
    intensityThruStacksChan2=zeros(size(data,1),size(data,2),numTotalZStacks);

    % Iterate through z stacks
    for z=1:numTotalZStacks
        % Unique index to find data for particular
        % z-stack and time point
        imLoc=(idxTime-1)*numTotalZStacks*3+(z-1)*3;
    end
end

```

```

chan1=data(:,:,imLoc+1); % Mca fluorophore
chan2=data(:,:,imLoc+2); % ABD fluorophore
chan3=data(:,:,imLoc+3); % Bright field

%Save intensity through stacks
intensityThruStacksChan1(:,:,z)=chan1;
intensityThruStacksChan2(:,:,z)=chan2;

% Draw ROI for first time point and first z stack, we will
% assume cells don't move too much
if z==1 && idxTime==1 % if first z stack, draw ROIs
    figure(1)
    subplot(1,1,1)
    % Displays brightfield image
    imagesc(chan3)
    title(['File: ' file ' Bright Field'],'Interpreter', 'none')
    colormap(gca,'gray')
    axis image

    % First ROI is around cell of interest
    if reuseROI==0 || exist('main','var')==0
        roiCell = imfreehand();
        % Coordinates of ROI
        xyCell = roiCell.getPosition;
        hold on;
        % plot boundaries of ROI
        plot(xyCell(:, 1), xyCell(:, 2), 'b.', 'LineWidth', 2, 'MarkerSize',
10);

        mainCellMask = createMask(roiCell);
    end

    % Second ROI is around region in background
    if reuseROI==0 || exist('bgd1','var')==0
        roiBgd1 = imfreehand();
        % Coordinates of ROI
        xyBgd1 = roiBgd1.getPosition;
        hold on;
        % plot boundaries of ROI
        plot(xyBgd1(:, 1), xyBgd1(:, 2), 'r.', 'LineWidth', 2, 'MarkerSize',
10);

        bgdMask1 = createMask(roiBgd1);
    end

    % Third ROI is around region in background
    if reuseROI==0 || exist('bgd2','var')==0
        roiBgd2 = imfreehand();
        % Coordinates of ROI
        xyBgd2 = roiBgd2.getPosition;
        hold on;
        % plot boundaries of ROI
        plot(xyBgd2(:, 1), xyBgd2(:, 2), 'r.', 'LineWidth', 2, 'MarkerSize',
10);

        bgdMask2 = createMask(roiBgd2);

    end
end

```

```

10);
    % Fourth ROI is around region in background
    if reuseROI==0 || exist('bgd3','var')==0
        roiBgd3 = imfreehand();
        % Coordinates of ROI
        xyBgd3 = roiBgd3.getPosition;
        hold on;
        % plot boundaries of ROI
        plot(xyBgd3(:, 1), xyBgd3(:, 2), 'r.', 'LineWidth', 2, 'MarkerSize',
            10);
        bgdMask3 = createMask(roiBgd3);
    end

else % if later zstack or tmpt, just calc values using existing ROI
    figure(1),

    % Display the three different channels as subplots
    h=subplot(2,2,1);
    imagesc(chan1,[0 50]),
    title('Channel 1')
    colormap(gca,colormapMagenta)
    axis image
    h2=subplot(2,2,2);
    imagesc(chan2,[0 50]),
    title(['T: ' num2str(idxTime) ' Z: ' num2str(z) ],'Channel 2'})
    colormap(gca,colormapCyan)
    axis image
    h3=subplot(2,2,3);
    imagesc(chan3),
    title('Bright Field')
    colormap(gca,'gray')
    axis image

    % Plot Cell ROI
    subplot(2,2,3)
    hold on
    plot(xyCell(:, 1), xyCell(:, 2), 'b.', 'LineWidth', 2, 'MarkerSize', 10);
    % Plot Background ROI 1
    hold on
    plot(xyBgd1(:, 1), xyBgd1(:, 2), 'r.', 'LineWidth', 2, 'MarkerSize', 10);
    % Plot Background ROI 2
    hold on
    plot(xyBgd2(:, 1), xyBgd2(:, 2), 'r.', 'LineWidth', 2, 'MarkerSize', 10);
    % Plot Background ROI 3
    hold on
    plot(xyBgd3(:, 1), xyBgd3(:, 2), 'r.', 'LineWidth', 2, 'MarkerSize', 10);
end

% Calculate the mean values of three background ROIs in chan 1 (Mca)
meanNoiseIntensities(z,1,2)=mean(chan1(bgdMask1));
meanNoiseIntensities(z,1,3)=mean(chan1(bgdMask2));
meanNoiseIntensities(z,1,4)=mean(chan1(bgdMask3));
meanNoiseIntensityBgdChan1=mean(squeeze(meanNoiseIntensities(z,1,2:end)));

% Calculate the mean values of three background ROIs in chan 2 (ABD)

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meanNoiseIntensities(z,2,2)=mean(chan2(bgdMask1));
meanNoiseIntensities(z,2,3)=mean(chan2(bgdMask2));
meanNoiseIntensities(z,2,4)=mean(chan2(bgdMask3));
meanNoiseIntensityBgdChan2=mean(squeeze(meanNoiseIntensities(z,2,2:end)));

% Create a mask that calculates only the mean of data with
% intensity above the background, and display this mask
acceptMask=mainCellMask &
chan1>(meanNoiseIntensityBgdChan1+noiseThresholdChan1) &
chan2>(meanNoiseIntensityBgdChan2+noiseThresholdChan2);
subplot(2,2,4)
imagesc(acceptMask),
title('Thresholded Mask')
colormap(gca,'gray')
axis image

% Calculate average intensity of cell in chan 1
meanNoiseIntensities(z,1,1)=mean(chan1(acceptMask));
% Calculate average intensity of cell in chan 2
meanNoiseIntensities(z,2,1)=mean(chan2(acceptMask));

% Sum up area of cell
areas=sum(sum(double(acceptMask)));

% Compute Integrated Density chan 1
ID(z,1)=factor*areas*meanNoiseIntensities(z,1,1);
% Compute Integrated Density chan 2
ID(z,2)=factor*areas*meanNoiseIntensities(z,2,1);

% Compute Corrected Total Cell Fluorescence chan 1
CFTC(z,1)=factor*areas*(meanNoiseIntensities(z,1,1)-
meanNoiseIntensityBgdChan1);
% Compute Corrected Total Cell Fluorescence chan 2
CFTC(z,2)=factor*areas*(meanNoiseIntensities(z,2,1)-
meanNoiseIntensityBgdChan2);

% Uncomment for display of ID and CFTC values (may slow down script)
% figure(1)
% subplot(2,2,1)
% xlabel({'ID: ' num2str(ID(i,1))},['CFTC: ' num2str(CFTC(i,1))])
% subplot(2,2,2)
% xlabel({'ID: ' num2str(ID(i,2))},['CFTC: ' num2str(CFTC(i,2))])

% update figure
drawnow

% Save summary data to output file and increment counter
idxDataEntry=idxDataEntry+1;
outputSummaryData(idxDataEntry,:)={file idxTime z areas*factor
meanNoiseIntensities(z,1,1) ID(z,1) CFTC(z,1) meanNoiseIntensityBgdChan1 areas*factor
meanNoiseIntensities(z,2,1) ID(z,2) CFTC(z,2) meanNoiseIntensityBgdChan2};
end
% Save Intensity through stacks
intensityThruTimeChan1(:,:,idxTime)=sum(intensityThruStacksChan1,3);
intensityThruTimeChan2(:,:,idxTime)=sum(intensityThruStacksChan2,3);

```

```

end

%% Saves ROI and stat data under custom suffix
time=clock;
time=num2str(time(1:end-1));
time(time == ' ') = [];

% Prompt for user suffix
prompt = {'Enter ROI identifier eg Cell1 or C1'};
dlgTitle = 'Input';
dims = [1 40];
defInput = {[time '_C1'],'hsv'};
answer = inputdlg(prompt,dlgTitle,dims,defInput);

fileSuffix=[answer{1}];
saveFilename=[file(1:end-4) '_' fileSuffix '.mat'];

% Save data to .mat file, load this to reuse masks, change reuse flag to 1
save(saveFilename,'mainCellMask','bgdMask1','bgdMask2','bgdMask3','outputSummaryData'
,'intensityThruTimeChan1','intensityThruTimeChan2','noiseThresholdChan1','noiseThresh
oldChan2','acceptMask','xyCell','xyBgd1','xyBgd2','xyBgd3')

```