Title: Fabrication and characterization of a thick, viable bi-layered stem cell-derived surrogate for future myocardial tissue regeneration

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Supplemental Materials and Methods

Flow cytometry analysis

Flow cytometry analysis was performed as described previously [50-52]. Briefly, cells were dissociated using 0.25 % trypsin and resuspended as single cells, permeabilized in 0.1 % Triton X-100 at 4 °C for 10 min, then incubated with primary and secondary antibodies for 30 min at 4 °C with 3 x 5 min wash in between. Finally, cells were resuspended in 2% fetal bovine serum/phosphate-buffered saline (FBS/PBS) containing 5 µL of propidium iodide (10 µg/mL) and evaluated with a FACS Aria instrument (BD Biosciences, USA). Antibodies used, along with dilutions, are listed Supplemental Table 1.

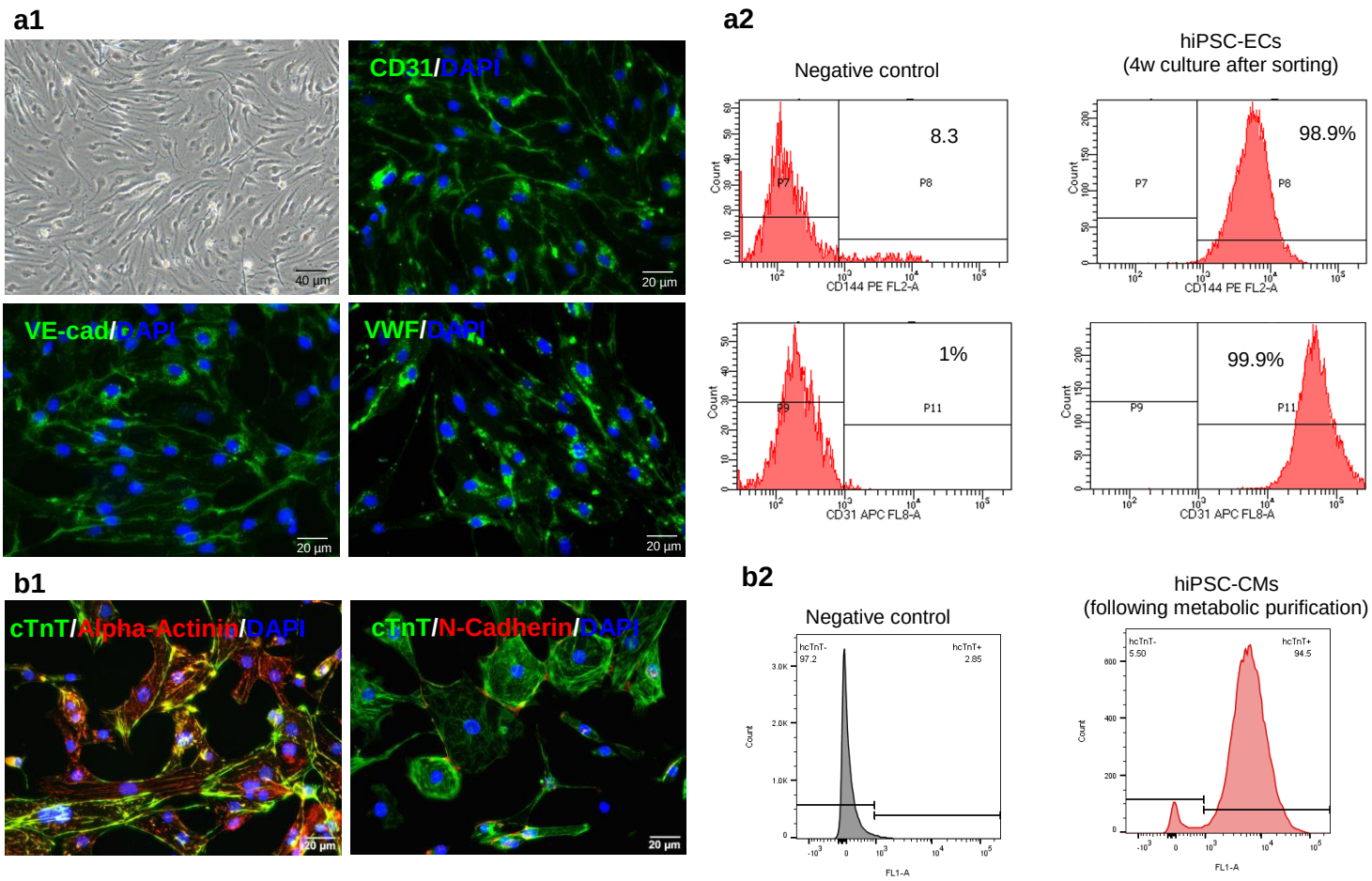
Cardiomyocyte proliferation assay

Cardiomyocyte proliferation was tested using a proliferation assay from CyQUANT Direct Cell Proliferation Assay Kit (Invitrogen, Cat# C35011). Briefly, CM proliferation was determined via green fluorescent nucleic acid staining of the nucleus, after which the fluorescence intensity of the readout was obtained 120 min after reagent addition via microplate reader. Based on the results, there was no significant difference between the proliferation of 28-day-old and 42-day-old hiPSC-CMs (see Supplemental Figure 2). Student t-test was performed, $p = 0.6565$ ($n = 6$).

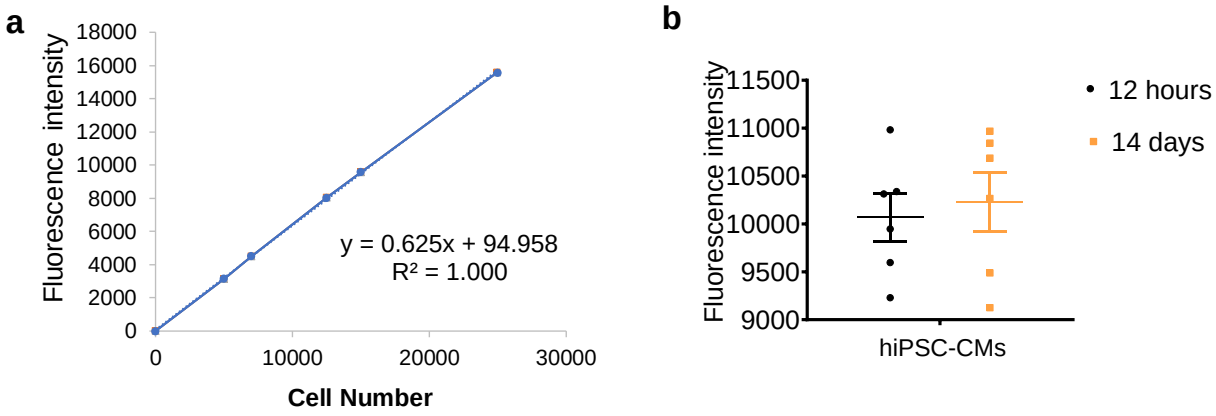
Supplemental Table 1: Antibodies used for flow cytometry, FACS, and immunofluorescent staining.

Antibody Name	Application	Company	Catalog Number
Alexa Fluor® 647 Mouse Anti-Human CD31	FACS	BD Biosciences	561654
Cardiac Troponin T Monoclonal Antibody (13-11)	Flow Analysis	Invitrogen	MA5-12960
Rabbit Anti-Cardiac Troponin T antibody [EPR3695]	Immunofluorescent Staining	Abcam	91605
Mouse Anti-Cardiac Troponin T antibody [1F11]	Immunofluorescent Staining	Abcam	10214
Goat anti-Mouse IgG Secondary Antibody, Alexa Fluor 555	Immunofluorescent Staining	Invitrogen	A32727
Donkey anti-Mouse IgG Secondary Antibody, Alexa Fluor 488	Immunofluorescent Staining	Invitrogen	A21202
Donkey anti-Rabbit IgG Secondary Antibody, Alexa Fluor 488	Immunofluorescent Staining	Invitrogen	A21206
Donkey anti-Rabbit IgG Secondary Antibody, Alexa Fluor 555	Immunofluorescent Staining	Invitrogen	A31572
Rabbit Anti-Collagen I antibody	Immunofluorescent Staining	Abcam	34710
Mouse Anti-CD31 antibody [JC/70A]	Immunofluorescent Staining	Abcam	9498
Rabbit Anti-Fibrinogen beta chain antibody	Immunofluorescent Staining	Abcam	137830
Rabbit Anti-Collagen III antibody	Immunofluorescent Staining	Abcam	7778
Rabbit Anti-Fibronectin antibody	Immunofluorescent Staining	Abcam	2413
Rabbit Anti-Collagen IV antibody	Immunofluorescent Staining	Abcam	6586
Rabbit Anti-Laminin antibody	Immunofluorescent Staining	Abcam	11575
Rabbit Anti-VE Cadherin	Immunofluorescent Staining	Abcam	33168
Mouse Anti-VWF Antibody (F8/86)	Immunofluorescent Staining	Santa Cruz Biotech	53466
Rabbit Anti-N-Cadherin	Immunofluorescent Staining	Abcam	18203
Anti-Alpha Actinin	Immunofluorescent Staining	Sigma	A7811
Anti-Connexin 43 / GJA1 antibody - Intercellular Junction Marker	Immunofluorescent Staining	Abcam	11370
Mouse Anti-Ryanodine Receptor antibody [C3-33]	Immunofluorescent Staining	Abcam	2827
JPH2 Polyclonal Antibody	Immunofluorescent Staining	Thermo Fisher	40-5300

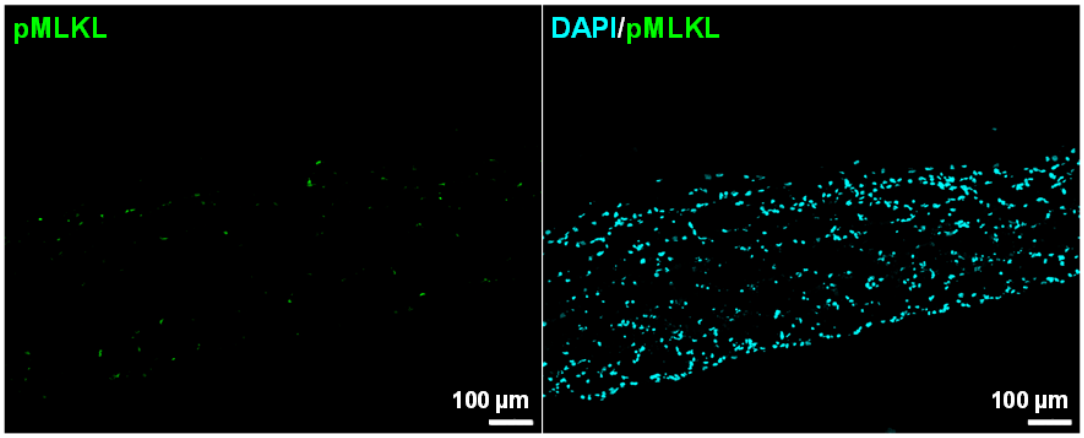
Supplemental Figures



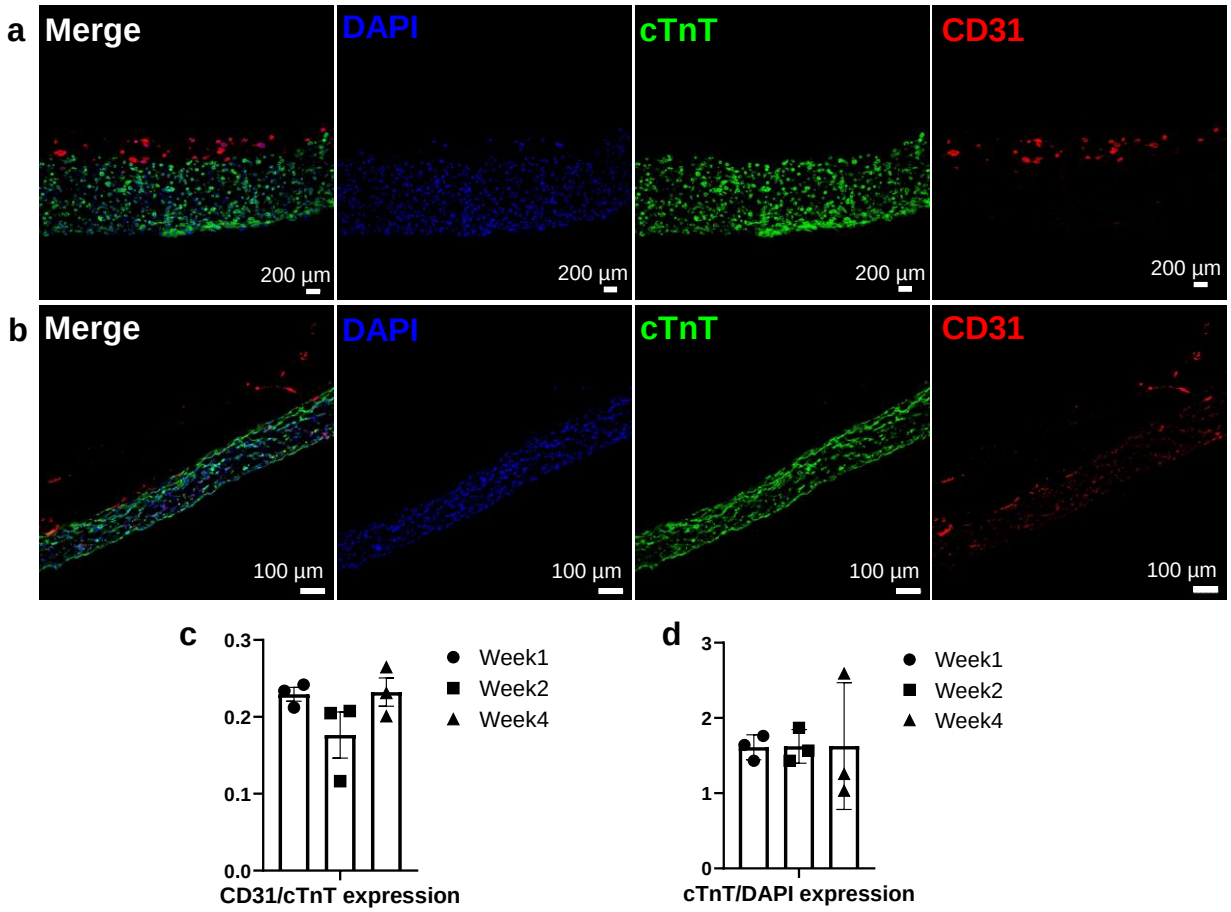
Supplemental Figure 1: Characterization data confirming differentiation and purification for iECs (a) and iCMs (b) using immunofluorescent staining (1) and flow cytometry (2) analysis.



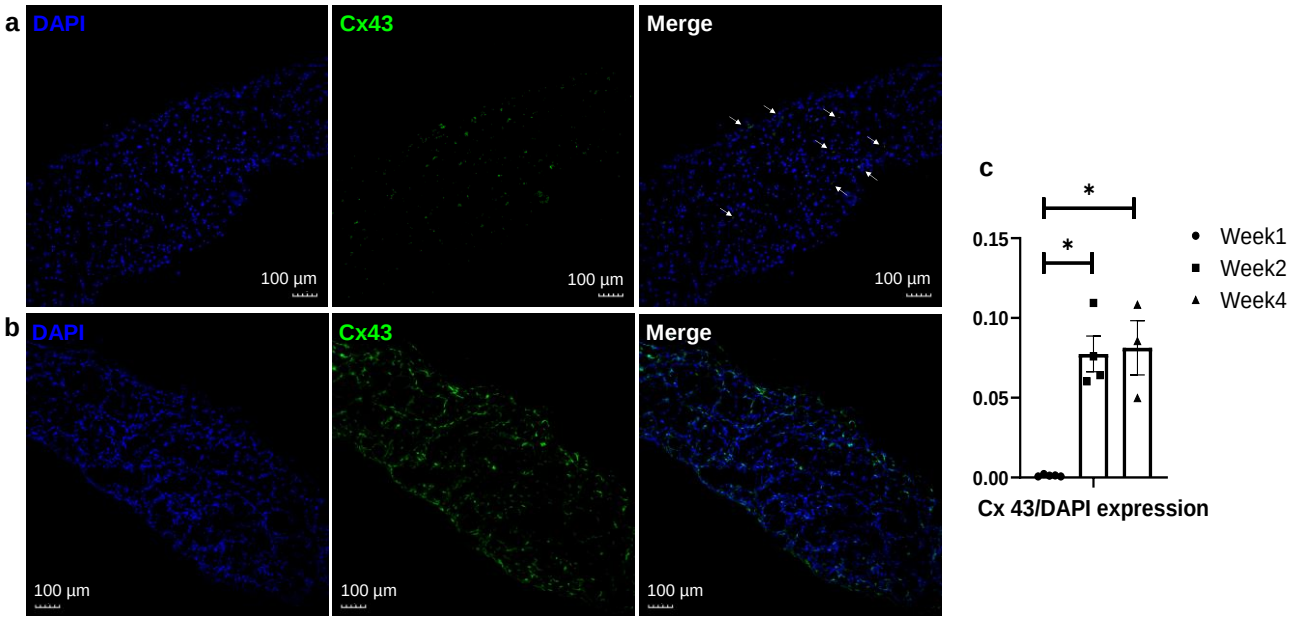
Supplemental Figure 2: Fluorescence-based cell proliferation assay showing the (a) standard curve and (b) the resulting measurements at Day 28 (12 hours) of hiPSC-CMs culture in 96-well plate (15,000 cells/well) as well as measurements at Day 42 (14 days). p = 0.6565 (n = 6)



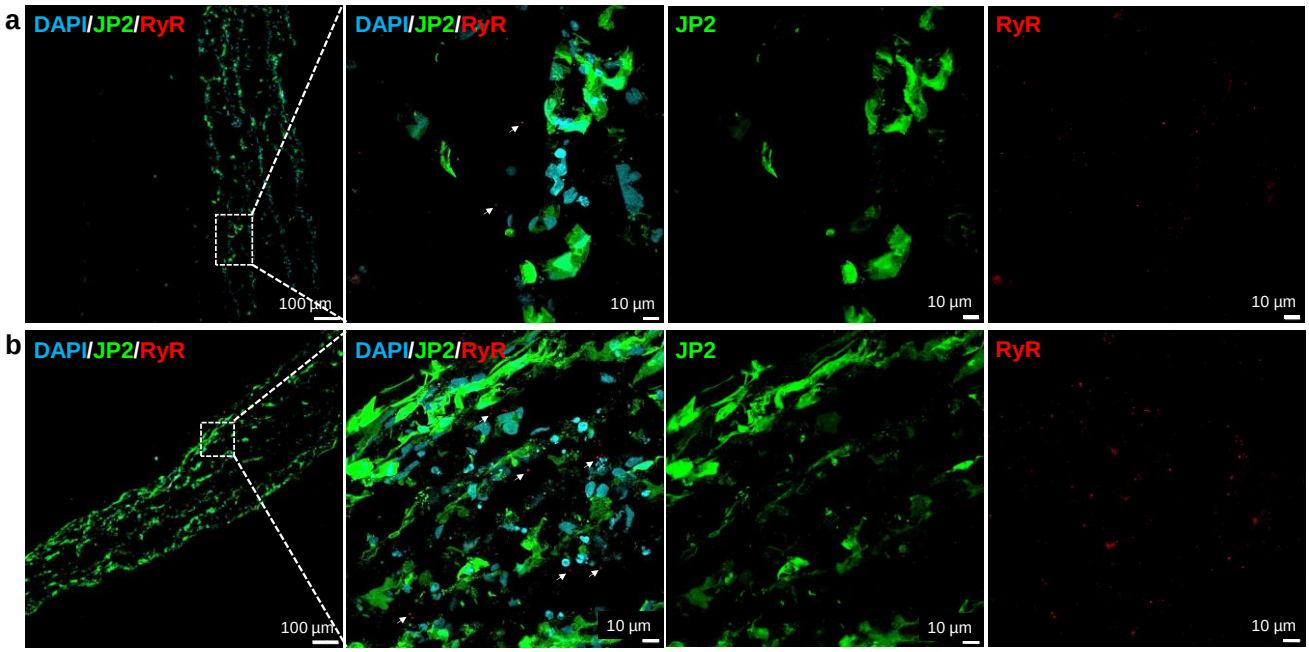
Supplemental Figure 3: Immunofluorescent staining showing presence of DAPI and phosphorylated MLKL (Ser358, pMLKL) after 4 weeks in culture.



Supplemental Figure 4: Immunofluorescent staining showing presence of DAPI, cTnT and CD31 after (a) 1 week in culture and (b) 4 weeks in culture. Quantification of expression intensity and subsequent expression ratios for all 4 weeks for (c) CD31/cTnT expression as well as (d) cTnT/DAPI. No statistically significant difference noted between groups for (c) or for (d), n = 3.



Supplemental Figure 5: Immunofluorescent staining showing connexin 43 (Cx43) gap junction presence after (a) 1 week in culture and (b) 2 weeks in culture, along with the (c) quantification of the ratio of Cx43/DAPI fluorescence expression. *p < 0.05, with n = 4



Supplemental Figure 6: Confocal micrograph of junctophilin (JP2) and ryanodine receptor (RyR) expression levels in cardiac tissue surrogates after (a) 7 days, and (b) 28 days in culture, respectively.

Supplemental Matlab code: Generalized Maxwell model of order four

```
%% Load datasets
% Raw data sheets used in this file/program should only consist of numbers
% Delete the graphs and the titles in all the columns or MatLab won't
% recognize the file
% convert the Excel file to a .csv if necessary
% Ensure that all the files are in the same folder
% Ensure the directory is set (left click the tab at the top to
% set directory)
%%

load('PDMS6edit.csv') % loads the Raw data file generated with Low Force Testbench into MatLab
Do = 2.830*10^-3;          % Sample thickness - sample-dependent (meters)
PlotOn = 1; % 1 = plot, 0 = don't plot
%FileName = 'DataSet02';     % creates a new destination to store results

%%
%   Section converts the load obtained from instrument to Stress measurements,
%   and identifies point were sample strain was introduced

Force = PDMS6edit(:,7); % Load value in gram-force

Force = Force/101.971621;   %Gram-force to Newton

Area = 4.165*10^-5; % Area of sample (in m2)

Stress = Force / Area; % Y-Values in Graph (Stress)

Time = PDMS6edit(:,2); % Elapsed Time (s) (temporarily uses entire Time column before selecting for the desired
range)

%plot (Time, Stress)

Ep = 0.1;    % 10 percent sample strain

[StressVal, StressPos] = max(Stress(:));   %find max Y-value

%%
%create new matrix - only consider data points relevant to 10 percent
%strain

NewMat = [Time, Stress];    %assign Time to column1 and Stress to column2 of NewMatrix
k = 1;

for i = StressPos:size(NewMat, 1)
    maxT(k) = Time(i) - Time(StressPos);
    maxS(k) = Stress(i);

    k = k+1;
end

max_mat = [maxT', maxS'];    %this is the new matrix containing only the experimental data relevant to the strain
experiment

%plot (maxT, maxS)

%%
opts = fitoptions( 'Method', 'NonlinearLeastSquares' );
ft = fittype( 'a + b0*exp(-c0*x) + b1*exp(-c1*x) + b2*exp(-c2*x) + b3*exp(-c3*x)', 'independent', 'x', 'dependent', 'y');
            %model fitting
opts.DiffMaxChange = 100000;     %how much the coefficients of each a, b0, c0 etc. changes with ongoing iterations
```

```
opts.DiffMinChange = 1e-10;
opts.Display = 'Off';
opts.Lower = [0 0 0 0 0 0 0 0 0];  %lower limits of exponents should be zero at minimum
opts.MaxFunEvals = 10000;
opts.MaxIter = 100000;        %number of iterations
opts.Robust = 'LAR';        %LAR refers to least absolute residuals - ensures more robust fit of model
opts.StartPoint = [36000 3500 4500 4500 1800 0.002 1e-04 1e-05 1e-06];
opts.Upper = [100000 10000 10000 10000 10000 10000 10000 10000 10000];     %ceiling of a, b0, c0 etc

[fitresult, gof] = fit(maxT', maxS', ft, opts);

% Plot fit with data.
Name = 'Result %d';

%figure( 'Name', sprintf(Name));
h = plot( fitresult,  maxT', maxS' );

set(h,'LineWidth',2)

legend( h, 'Data', 'Fit', 'Location', 'NorthEast' );
% Label axes
xlabel Time
ylabel Stress
grid on

%% This is using the fit parameters
for i = 1 : 4
        a(i) = fitresult.a;
        b0(i) = fitresult.b0;
        c0(i) = fitresult.c0;

        b1(i) = fitresult.b1;
        c1(i) = fitresult.c1;
        b2(i) = fitresult.b2;
        c2(i) = fitresult.c2;
        b3(i) = fitresult.b3;
        c3(i) = fitresult.c3;

        R2_Value(i) = gof.rsquare;     %gof -goodness of fit
end

for i = 1:4
    E0(i) = a(i)/Ep;
    E1(i) = b0(i)/Ep;
    n1(i) = E1(i)/c0(i);

    E2(i) = b1(i)/Ep;
    n2(i) = E2(i)/c1(i);

    E3(i) = b2(i)/Ep;
    n3(i) = E3(i)/c2(i);

    E4(i) = b3(i)/Ep;
    n4(i) = E4(i)/c3(i);

end

%%
```