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Supplemental Information

Endocytic Profiling of Cancer Cell Models Reveals Critical Factors Influencing LNP-Mediated mRNA Delivery and Protein Expression

Edward J. Sayers, Samantha E. Peel, Anna Schantz, Richard M. England, Maya Beano, Stephanie M. Bates, Arpan S. Desai, Sanyogitta Puri, Marianne B. Ashford, and Arwyn T. Jones

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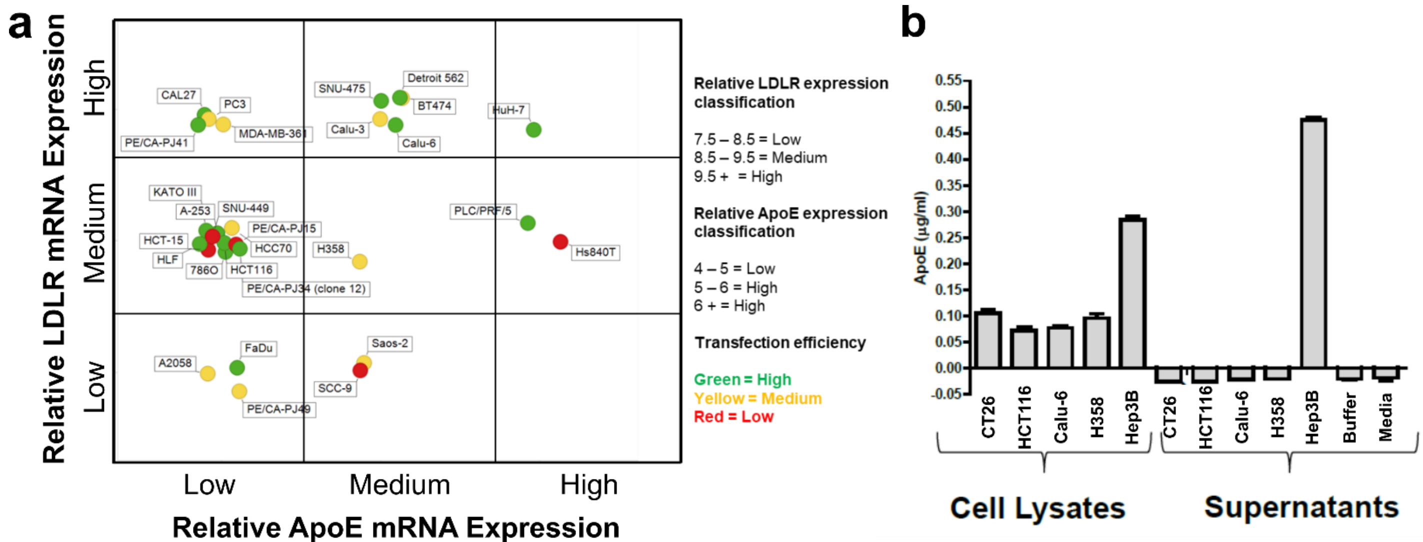
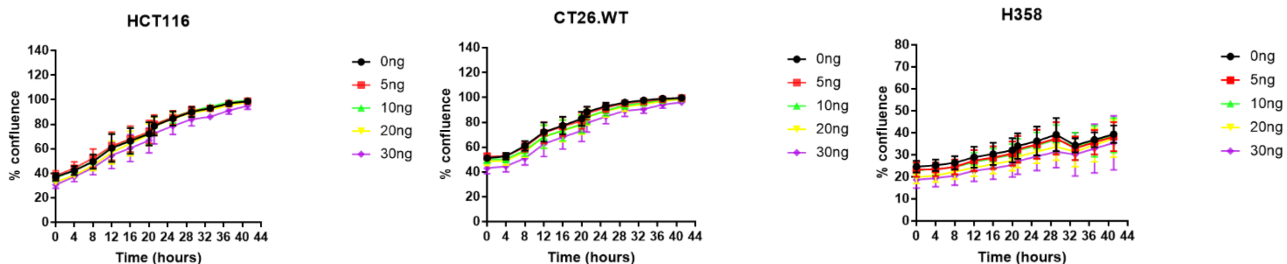
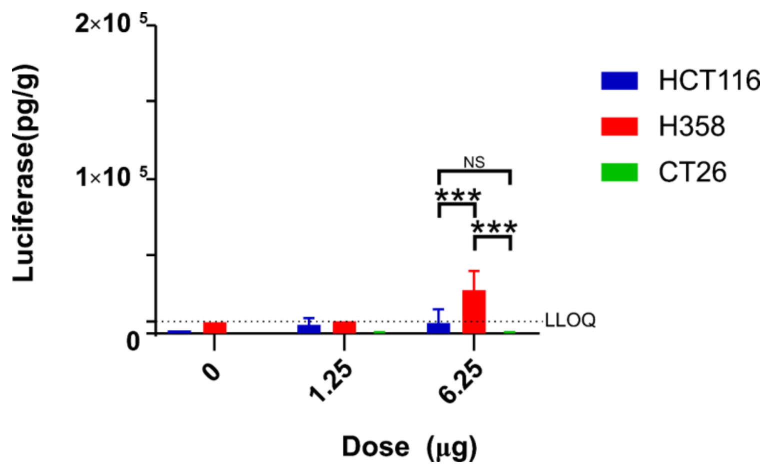
SUPPLEMENTARY FIGURES



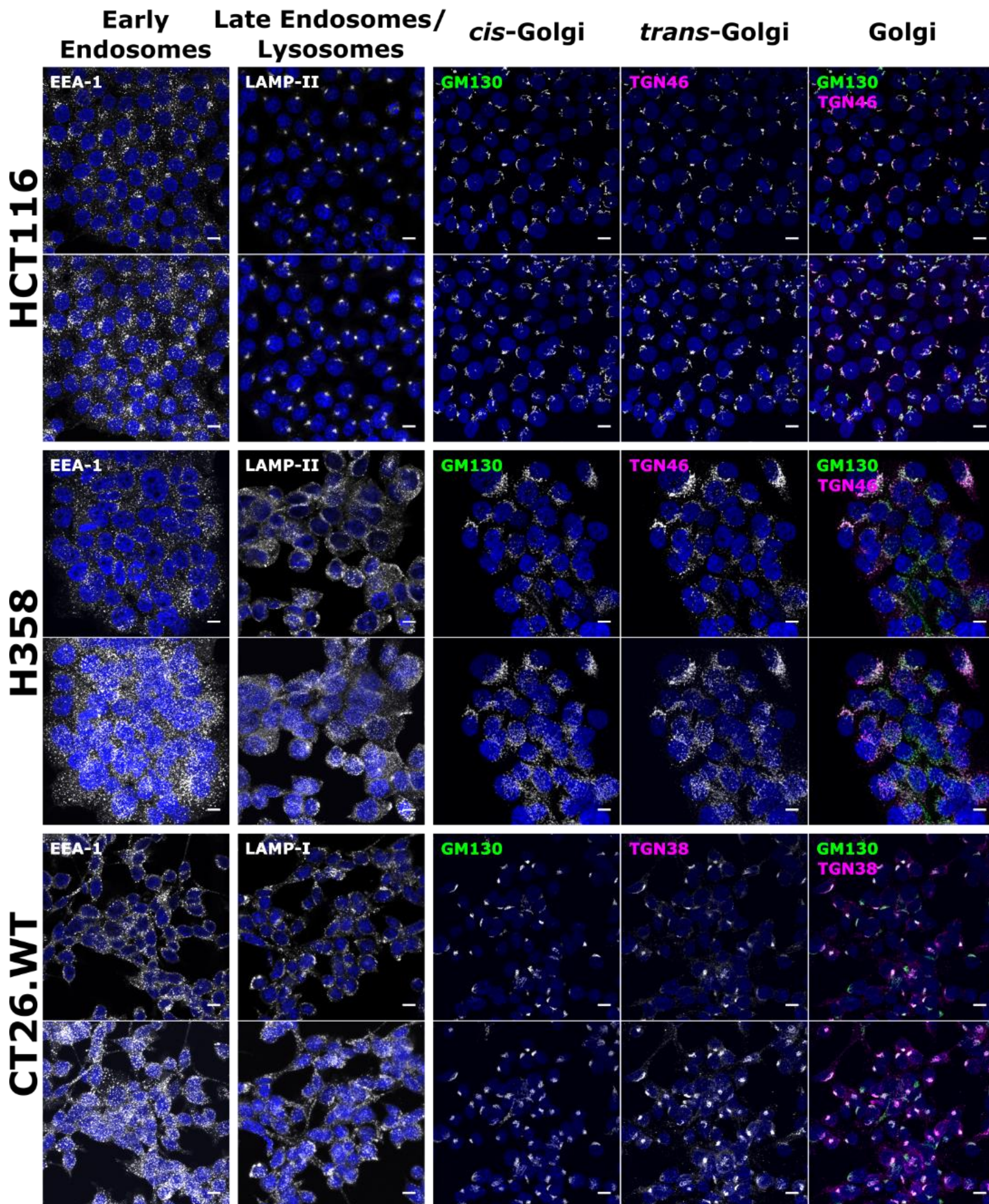
Supplementary Figure S1: LDLR and ApoE expression in the cell lines examined. Relative mRNA expression (a) of LDLR and ApoE using data from the Cancer Cell Line Encyclopaedia, Broad institute[1] (accessed May 2019) plotted with eGFP expression from the cell screen in Figure 1. High eGFP expressors are shown in green, moderate expressors in yellow and low expressors in red. Data for both CT26.WT and RPMI 2650 are not present in the CCLE database. ApoE protein expression (b) in both cell lysates and in the supernatant analysed by ELISA with liver cell line Hep3B included as positive control.



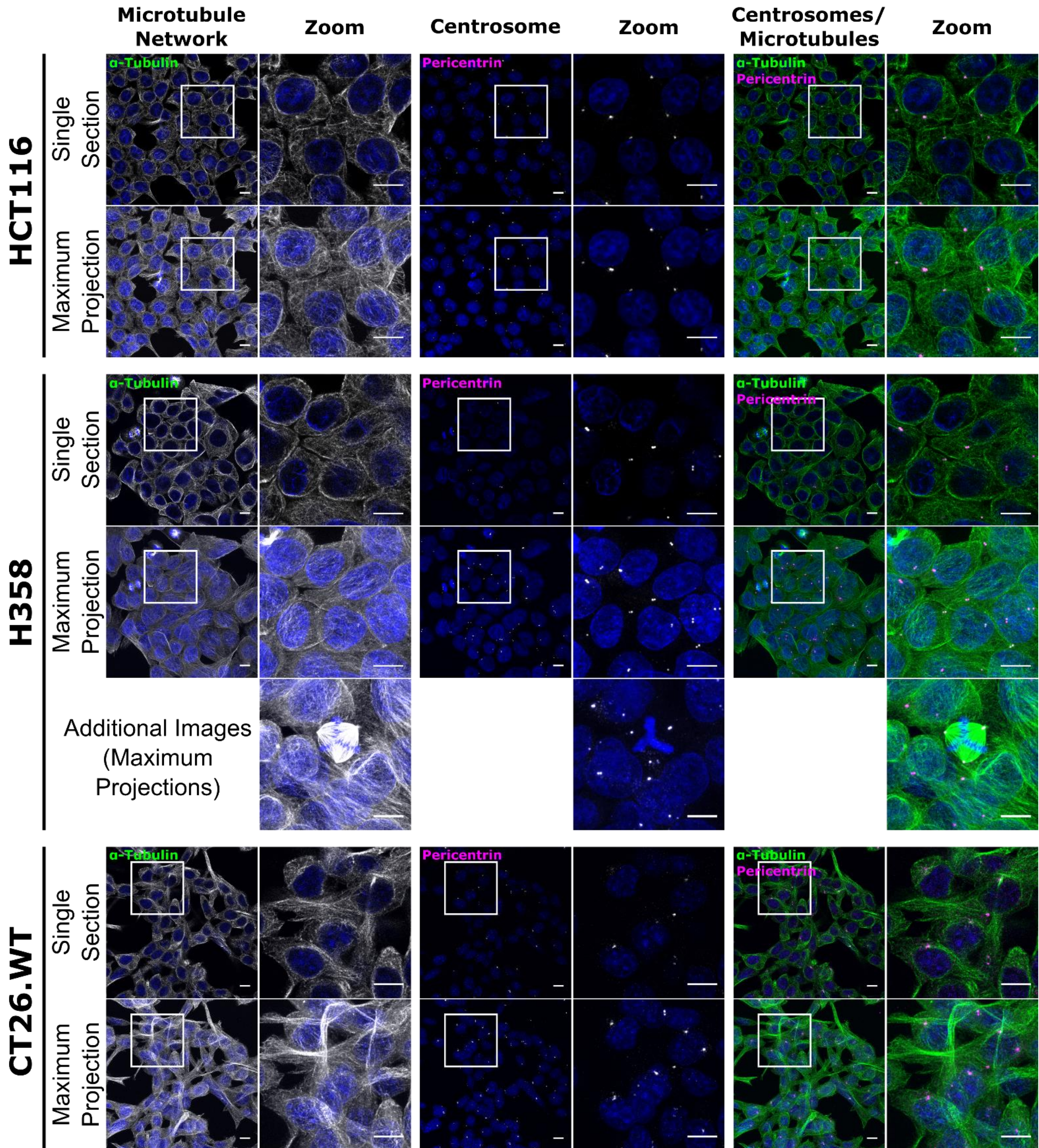
Supplementary Figure S2: Growth curves for three cell lines incubated with different amounts of LNP. Cells were incubated with LNP and their confluency assessed every 4 hr over 44 hr. Data represents 4 independent experiments performed in duplicate.



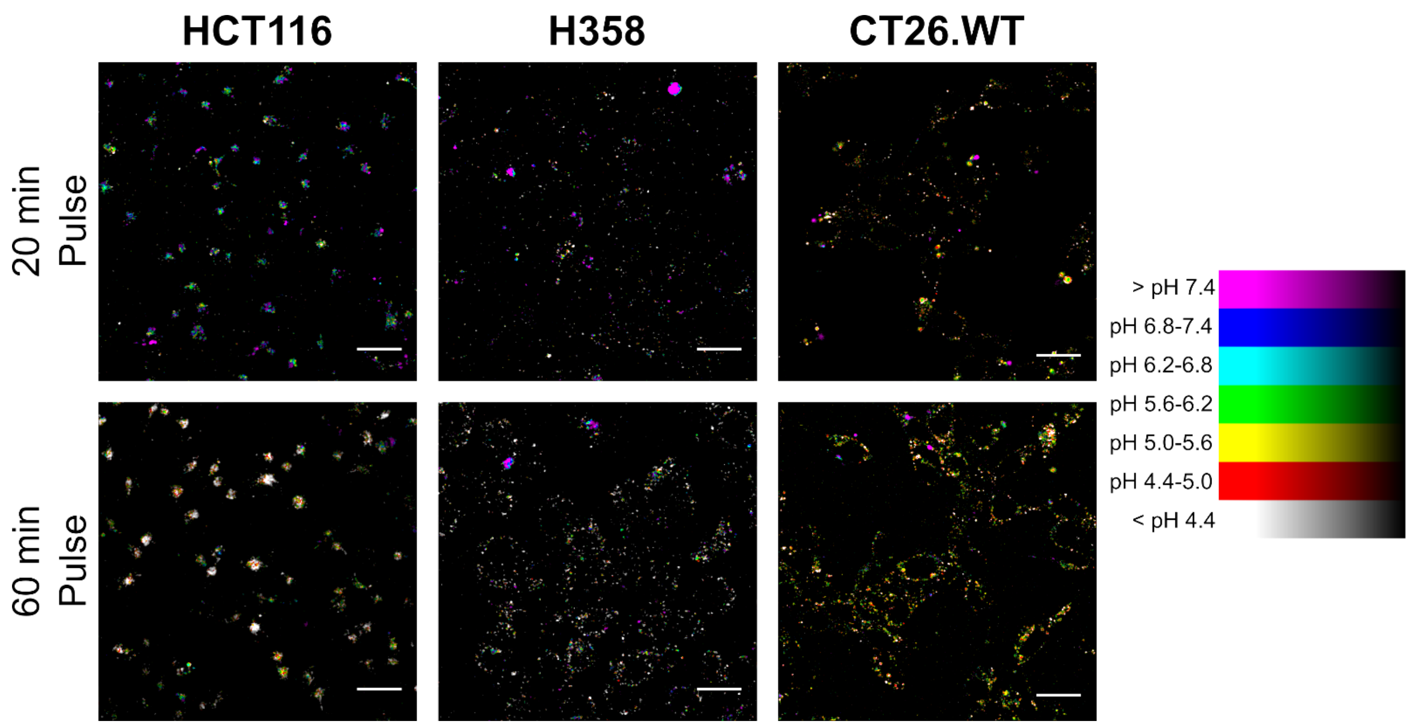
Supplementary Figure S3: Support to Figure 1, Luciferase expression in the liver of mice grafted with HCT116, H358 and CT26 cells. LNP containing luciferase mRNA were injected into the tumour sites of mice using a final volume of 30 µL per injection (LNP in PBS). The tumour (Figure 1 in main text) and liver were excised after 6 hrs and assayed for luciferase expression. LLOQ = lower level of quantification. P-values calculated using a two-way ANOVA, see ANOVA table in Statistics below, all other comparisons are not significant.



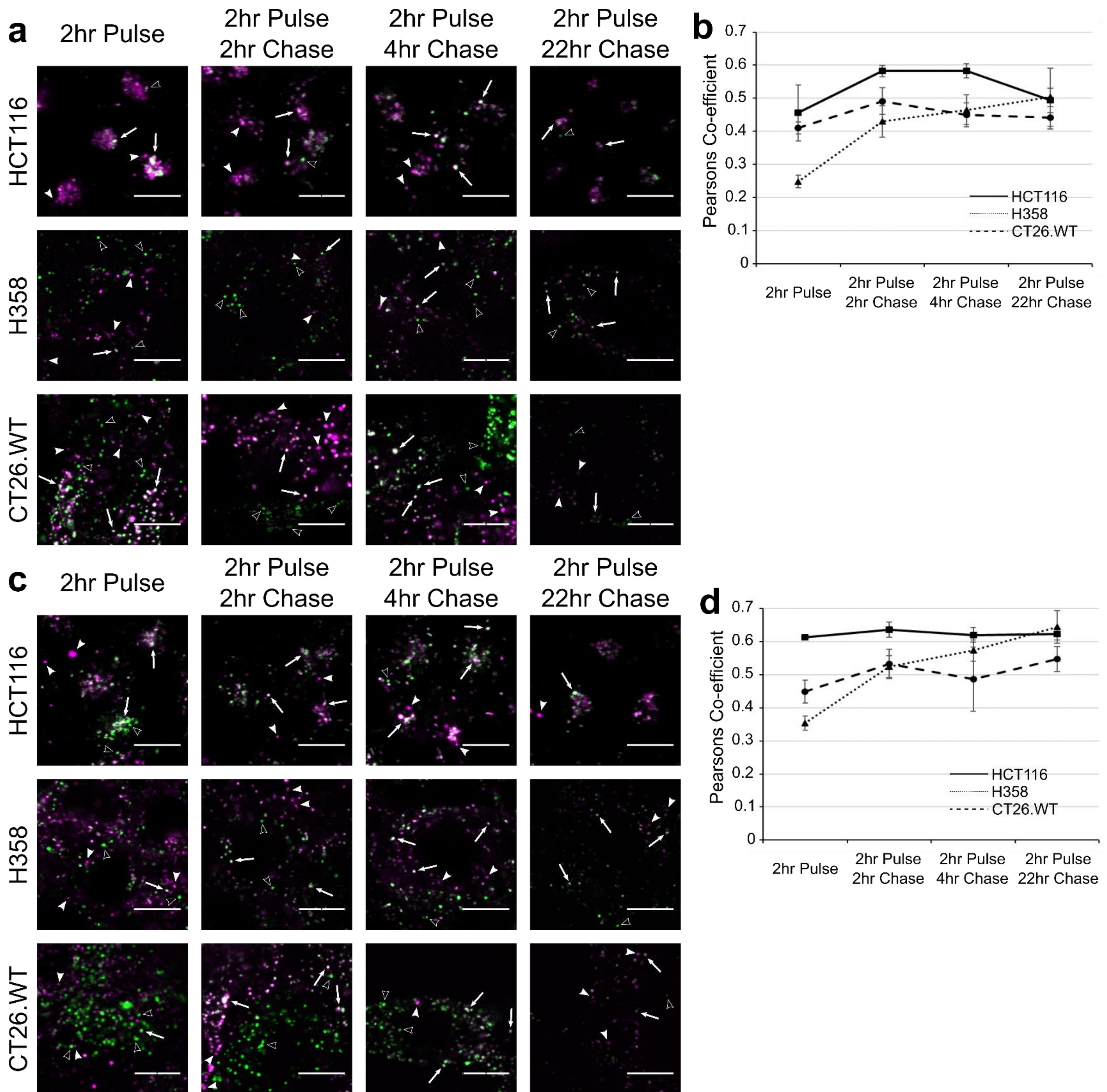
Supplementary Figure S4: Wide field of view to supplement Figure 2, Organelle Distribution in HCT116, H358 and CT26.WT Cells by immunofluorescence. Cells were grown for 48 hrs before fixing, labelling different compartments and imaging by confocal microscopy. Upper row represents single sections, lower row represents maximum projection images. Scale bar = 10 μm



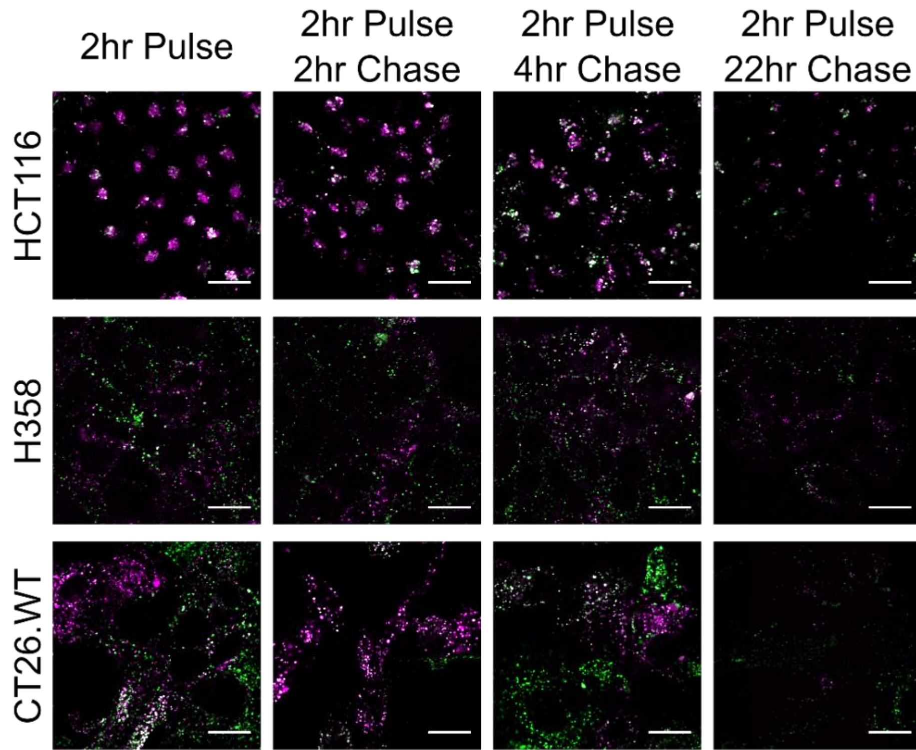
Supplementary Figure S5: Immunofluorescence highlighting centrosome and microtubules in HCT116, H358 and CT26.WT cells. Cells fixed in PFA were immunolabelled for pericentrin (centrosome, magenta) and α-tubulin (microtubules, green), with the nucleus stained using Hoechst 33342 (blue). Zoomed features are outlined by the white boxes. Scale bar = 10 μm.

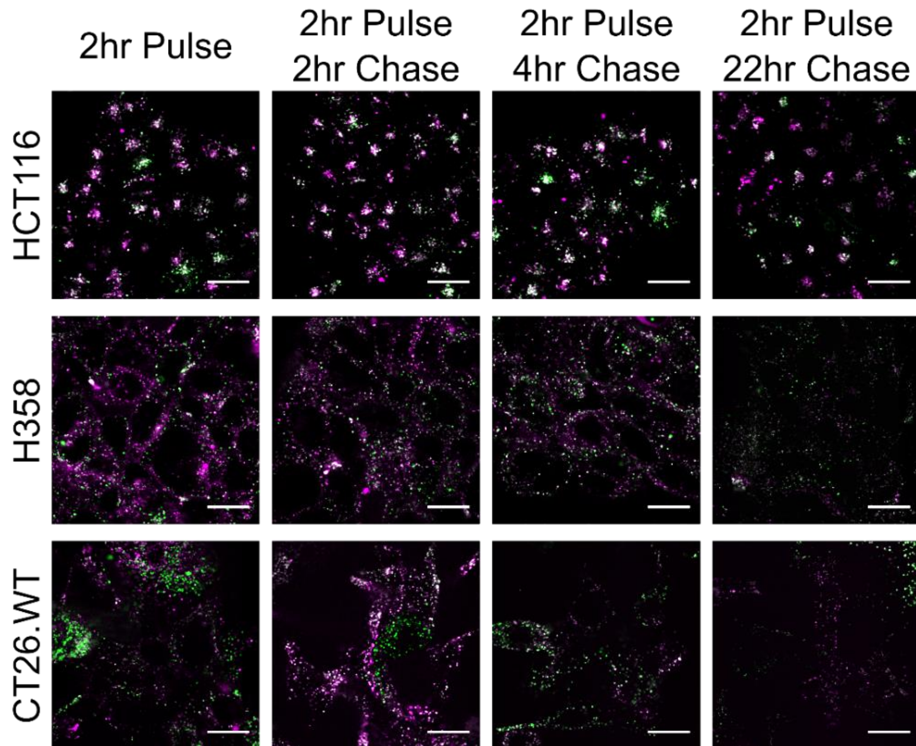


Supplementary Figure S6: Supplement to Figure 3a, enlarged field of view of pseudocoloured cells depicting pH. Cells were incubated with Fluo-Dex-Rhod for 20mins or 60mins before imaging by confocal microscopy. Images were analysed using an automated script (Supplementary Figure S10 and Scripts 1-2) and endosomal pH calculated against a calibration curve. Pixel colour represents the ratio between the pH sensitive and insensitive colourful with a gaussian blur applied to aid clarity. Scale bar = 10 μm

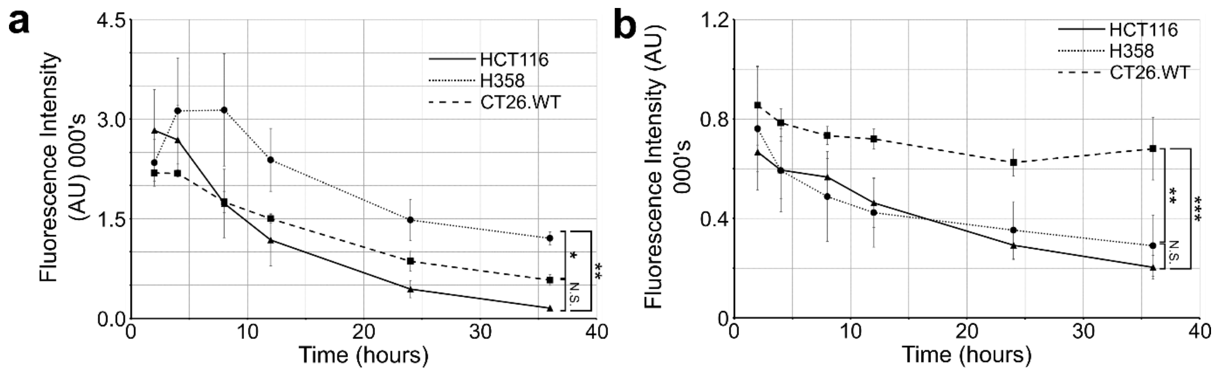


Supplementary Figure S7: Colocalisation of LNP and Dextran with lysosomes. Cells were incubated with (A) LNP or (B) Dex647 for 2hrs followed by a chase period of 0, 2, 4 and 22 hrs. Arrows indicate colocalization of the two probes (white/grey), solid arrow heads indicate non-colocalised LNP or Dex-647 (magenta) and hollow arrow heads indicate lysosomes containing only the pulse chased Dex-488 (green). Scale bars represent 10 µm. Intensities are comparable between time points but cannot be compared between cell lines. Zoomed out representative images showing more cells are below. Graph represent the amount of colocalization of (B) LNP or (D) Dex647 with the lysosome (Pearsons coefficient) and are the mean of three separate experiments. Error bars represent SEM.

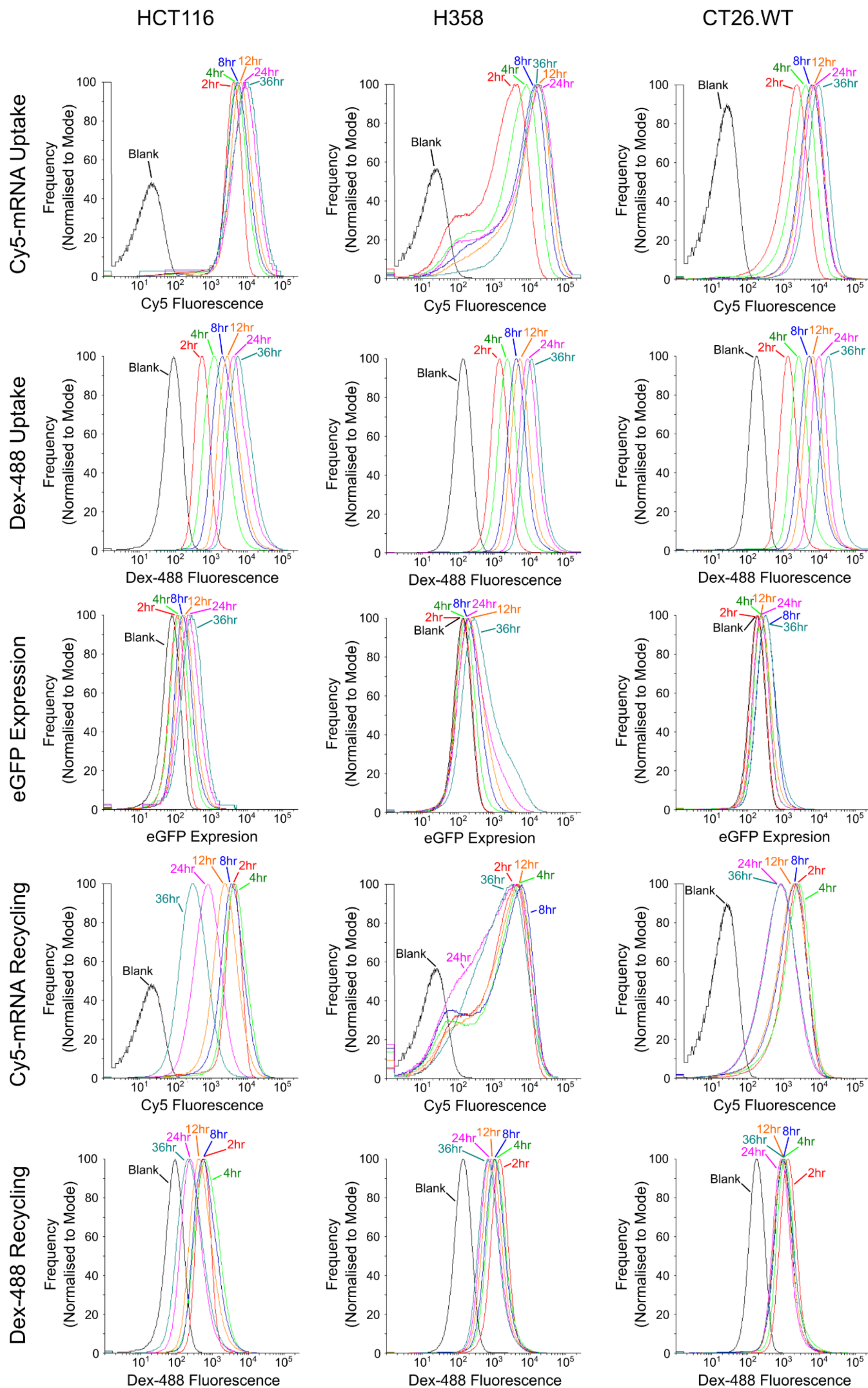




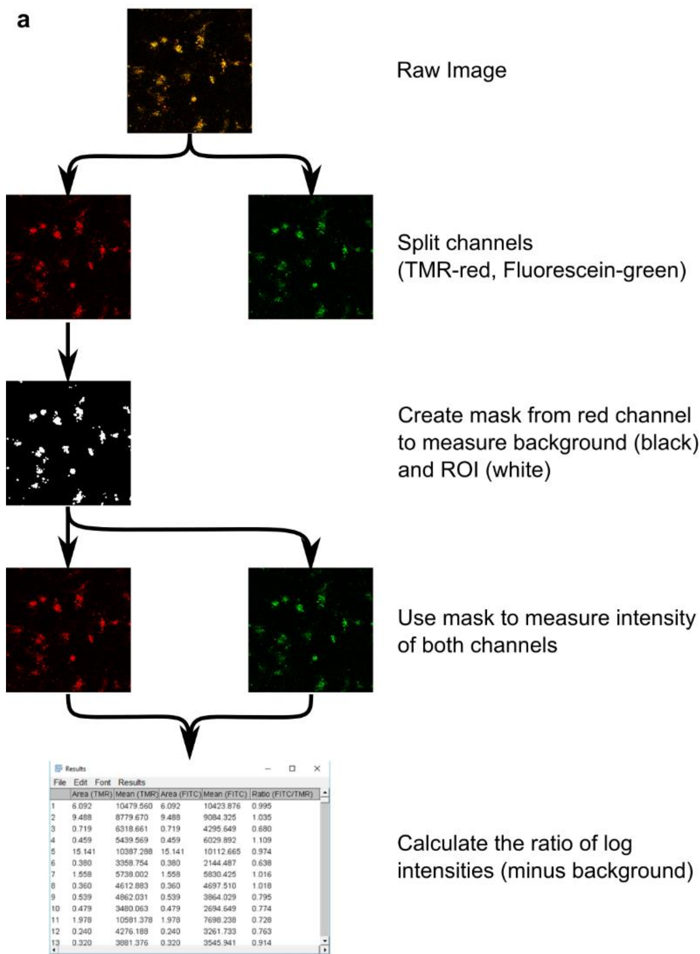
Supplementary Figure S8: Colocalisation of LNP and Dextran with lysosomal dextran over time. Cells were incubated with LNP (top) or Dex-647 (bottom) for 2hrs followed by a chase period of 0, 2, 4 and 22 hrs.

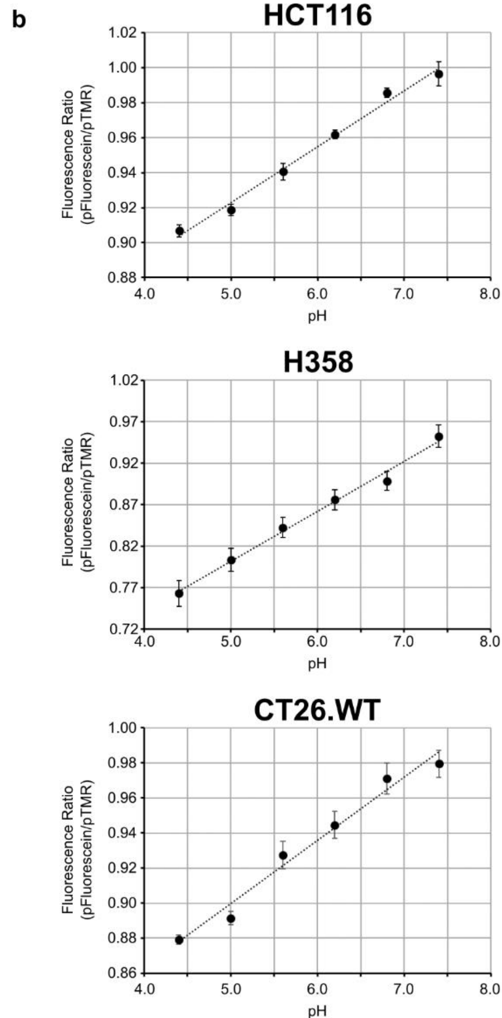


Supplementary Figure S9: Recycling of Cy5-mRNA and Dextran-Alexa488. Support for Figure 5, un-normalised flow cytometry data showing cell associated fluorescence of (a) Cy5-mRNA and (b) Dextran-Alexa488 in the three cell lines studied in detail. P-values calculated using two-way ANOVA, see Statistics below for further information.



Supplementary Figure S10: Histograms to supplement Figure 5. Representative histograms showing uptake and recycling of either Cy5-mRNA or Dex-488 and eGFP expression in HCT116, H358 and CT26.WT





Supplementary Figure S11: Measurement of endosomal pH and generation of calibration curve. (a) Endosomes were measured using the automated script "Script for measuring the fluorescence ratio between two fluorophores". Dual fluorescence images (1) were split into their component colours (2), from this a mask was created to highlight regions of high uptake (endosomes) that can be used to generate regions of interest "ROIs" (3), after dilation of the mask of 1 pxl, the background fluorescence was taken of the whole image. The intensity in each ROI of both the red and green channels was measured (4) and the log intensity ratio in each ROI was measured (5). (b) A calibration curve of the Fluorescence Ratio of intensity against pH is generated and is used to estimate pH in the ROIs generated after 20 and 60 min pulses. These values were also used to pseudo-colour the images according to pH.

SUPPLEMENTARY TABLES

Experimental	Fig 1a,b, S1	Fig 1c, S2	Figs 4-5, S6-S8		
Batch:	1	2	3	4	5
ζ-Average size (nm):	121†	84	83	94	84
PDI:	0.07	0.23	0.10	0.23	0.04
mRNA:lipid	20:1	20:1	10:1	10:1	10:1
mRNA source	Moderna	TriLink	TriLink	TriLink	TriLink
Encapsulation efficiency:	97%	97%	>99%	98%	96%

Supplementary Table S1: Characteristics of LNP formulations used in this study. †Batch 1 utilised 0.5% DMG-PEG 39.5% Cholesterol

Target	Catalogue number	Supplier	Dilution	Fixation method	Secondary
EEA1	SC-6415	Santa Cruz	1:200	PFA	Do α Go 488
LAMP-1	1D4B	DSHB	1:1000	Methanol	Ch α Mo 488
LAMP-2	H4B4	DSHB	1:1000	Methanol	Ch α Mo 488
TGN46	AHP500	Biorad	1:750	PFA	Do α Sh 555
TGN38	170-5060	Biorad	1:200	PFA	Do α Sh 555
GM130	610822	BD Bioscience	1:1000	PFA	Ch α Mo 488
Pericentrin	Ab4448	AbCam	1:2000	Methanol	Ch α Ra 647
Tubulin	T9026	Sigma	1:2000	Methanol	Ch α Mo 488

Supplementary Table S2: Further information on antibodies used within this study. Donkey, Goat, Chicken, Mouse, Sheep, Rabbit. All secondary antibody conjugates are Alexa fluor (Life technologies) and used at 1:1000.

pH	***3.8***	***4.4***	***5.0***	***5.6***	***6.2***	***6.8***	***7.4***
Citric acid (mM)	64.6	46.0	48.6	42.0	34.0	22.8	9.2
Sodium phosphate (mM)	71.2	88.2	103.2	116.0	132.4	154.6	181.6

Supplementary Table S3: concentrations of citric acid and sodium phosphate used for each pH buffer, values adapted from [2].

STATISTICS

Figure 1c Luciferase expression in three cell lines at three doses (Two-way ANOVA):

	Degrees Freedom	F (DFn, DFd)	P value
Interaction	4	F (4, 34) = 3.752	P=0.0124 *
Dose	2	F (2, 34) = 9.753	P=0.0004 ***
Cell Line	2	F (2, 34) = 10.55	P=0.0003 ***
Residual	34		

Figure 3c Fluorescence ratio of OG514:Cy5 in three cell lines over time (Two-way ANOVA):

	Degrees Freedom	F (DFn, DFd)	P value
Interaction	8	F (8, 29) = 2.997	P=0.0141 *
Time	4	F (4, 29) = 25.99	P<0.0001 ***
Cell Line	2	F (2, 29) = 18.03	P<0.0001 ***
Residual	29		

Figure 4b Colocalisation of LNP with lysosomes in three cell lines over time (Two-way ANOVA):

	Degrees Freedom	F (DFn, DFd)	P value
Interaction	6	F (6, 35) = 1.607	P=0.1744 NS
Time	3	F (3, 35) = 5.977	P=0.0021 **
Cell Line	2	F (2, 35) = 7.319	P=0.0022 **
Residual	35		

Figure 4d Colocalisation of dextran with lysosomes in three cell lines over time (Two-way ANOVA):

	Degrees Freedom	F (DFn, DFd)	P value
Interaction	6	F (6, 35) = 2.319	P=0.0545 NS
Time	3	F (3, 35) = 5.563	P=0.0031 **
Cell Line	2	F (2, 35) = 9.91	P=0.0004 **
Residual	35		

Figure 5a Uptake of Cy5-mRNA in three cell lines over time (Two-way ANOVA):

	Degrees Freedom	F (DFn, DFd)	P value		
Interaction	10	F (10, 42) = 3.153	P=0.0043 **	HCT116 v H358	P<0.0001 ***
Time	5	F (5, 42) = 25.87	P<0.0001 ***	HCT116 v CT26.WT	P=0.0001 ***
Cell Line	2	F (2, 42) = 33.80	P<0.0001 ***	H358 v CT26.WT	P=0.0048 **
Residual	42				

Figure 5b Normalised exocytosis of Cy5-mRNA in three cell lines over time (Two-way ANOVA):

	Degrees Freedom	F (DFn, DFd)	P value		
Interaction	10	F (10, 42) = 1.671	P=0.1200 NS	HCT116 v H358	P<0.0001 ***
Time	5	F (5, 42) = 30.36	P<0.0001 ***	HCT116 v CT26.WT	P=0.0457 *
Cell Line	2	F (2, 42) = 24.09	P<0.0001 ***	H358 v CT26.WT	P=0.0004 ***
Residual	42				

Figure 5c eGFP expression in three cell lines over time (Two-way ANOVA):

	Degrees Freedom	F (DFn, DFd)	P value		
Interaction	10	F (10, 42) = 2.047	P=0.0521 NS	HCT116 v H358	P<0.0001 ***
Time	5	F (5, 42) = 4.138	P=0.0038 **	HCT116 v CT26.WT	P<0.0001 ***
Cell Line	2	F (2, 42) = 20.14	P<0.0001 ***	H358 v CT26.WT	P=0.7067 NS
Residual	42				

Figure 5d eGFP expression following 2 h pulse in three cell lines over time (Two-way ANOVA):

	Degrees Freedom	F (DFn, DFd)	P value		
Interaction	10	F (10, 42) = 1.092	P=0.3902 NS	HCT116 v H358	P<0.0001 ***
Time	5	F (5, 42) = 1.271	P=0.2943 NS	HCT116 v CT26.WT	P<0.0001 ***
Cell Line	2	F (2, 42) = 17.80	P<0.0001 ***	H358 v CT26.WT	P=0.9262 NS
Residual	42				

Figure 5e Percentage of cells containing Cy5-mRNA over time (Two-way ANOVA):

	Degrees Freedom	F (DFn, DFd)	P value		
Interaction	10	F (10, 42) = 0.557	P=0.8387 NS	HCT116 v H358	P=0.1840 NS
Time	5	F (5, 42) = 4.427	P=0.0025 **	HCT116 v CT26.WT	P=0.0027 **
Cell Line	2	F (2, 42) = 6.375	P=0.0038 **	H358 v CT26.WT	P=0.2362 NS
Residual	42				

Figure 5f Percentage of cells expressing eGFP over time (Two-way ANOVA):

	Degrees Freedom	F (DFn, DFd)	P value		
Interaction	10	F (10, 42) = 3.424	P=0.0024 **	HCT116 v H358	P<0.0001 ***
Time	5	F (5, 42) = 11.01	P<0.0001 ***	HCT116 v CT26.WT	P<0.0001 ***
Cell Line	2	F (2, 42) = 86.33	P<0.0001 ***	H358 v CT26.WT	P=0.0025 **
Residual	42				

Figure 5g Uptake of Dextran-Alexa488 in three cell lines over time (Two-way ANOVA):

	Degrees Freedom	F (DFn, DFd)	P value		
Interaction	10	F (10, 42) = 6.837	P<0.0001 ***	HCT116 v H358	P=0.8921 NS
Time	5	F (5, 42) = 55.85	P<0.0001 ***	HCT116 v CT26.WT	P<0.0001 ***
Cell Line	2	F (2, 42) = 36.72	P<0.0001 ***	H358 v CT26.WT	P<0.0001 ***
Residual	42				

Figure 5h Normalised exocytosis of Dextran-Alexa488 in three cell lines over time (Two-way ANOVA):

	Degrees Freedom	F (DFn, DFd)	P value		
Interaction	10	F (10, 40) = 2.266	P=0.0328 *	HCT116 v H358	P=0.2574 NS
Time	5	F (5, 40) = 20.35	P<0.0001 ***	HCT116 v CT26.WT	P=0.0003 ***
Cell Line	2	F (2, 40) = 17.30	P<0.0001 ***	H358 v CT26.WT	P<0.0001 ***
Residual	42				

Supplementary Figure S2 Luciferase expression in three cell lines at three doses (Two-way ANOVA):

	Degrees Freedom	F (DFn, DFd)	P value
Interaction	4	F (4, 34) = 7.491	P=0.0002 ***
Dose	2	F (2, 34) = 12.34	P<0.0001 ***
Cell Line	2	F (2, 34) = 31.27	P<0.0001 ***
Residual	34		

Supplementary Figure 9a Exocytosis of Cy5-mRNA in three cell lines over time (Two-way ANOVA):

	Degrees Freedom	F (DFn, DFd)	P value		
Interaction	10	F (10, 42) = 0.8676	P=0.5695 NS	HCT116 v H358	P=0.0060 **
Time	5	F (5, 42) = 11.66	P<0.0001 ***	HCT116 v CT26.WT	P=0.9994 NS
Cell Line	2	F (2, 42) = 6.494	P=0.0035 **	H358 v CT26.WT	P=0.0115 *
Residual	42				

Supplementary Figure 9b Exocytosis of Dextran-Alexa488 in three cell lines over time (Two-way ANOVA):

	Degrees Freedom	F (DFn, DFd)	P value		
Interaction	10	F (10, 40) = 0.3809	P=0.9477 NS	HCT116 v H358	P=0.9477 NS
Time	5	F (5, 40) = 4.620	P=0.0020 **	HCT116 v CT26.WT	P=0.0005 ***
Cell Line	2	F (2, 40) = 10.03	P=0.0003 ***	H358 v CT26.WT	P=0.0021 **
Residual	42				

SUPPLEMENTARY METHODS

Preparation of pH Buffers

All buffers contained 5 mM NaCl, 115 mM KCl and 1.2 mM $MgSO_4$ (all Sigma Aldrich) in distilled water. In addition to this different pH buffers contained a pH dependent ratio of citric acid and sodium phosphate (both Sigma Aldrich) the final concentrations given in supplementary table S3.

After formulation of each pH buffer solution, buffers were equilibrated for pH (Jenway 3510) after machine calibration to +/- pH 0.05.

Measurement of Apolipoprotein E

Apolipoprotein E (ApoE) was quantified from both cell pellets and cell supernatants using a 96well ELISA kit purchased from Abcam. The kit recognises ApoE 2, 3 and 4 isoforms and quantification was performed as per manufacturer's instructions. Protein from each sample was quantified using the Thermo Scientific Pierce BCA Protein Assay Kit and equal amounts of protein (20µg) was loaded for each reaction. Absorbance at 450nm was read using an Envision 2104 Multi Detection Microplate Reader (Perkin Elmer). A standard curve of human ApoE was used to determine sample concentration with a detection range of 0-2µg/ml. Each condition was run in duplicate and triplicate readings were taken from each sample.

SCRIPTS

Script 1 for measuring the fluorescence ratio between two fluorophores

```
/* Macro designed by E. Sayers 18th August 2016
 *
 * This macro is designed for use with dual labelled probes, such as those utilised
 * in pH measurements.  The macro will determine the intensity of individual
 * endosomes from channel 3 (e.g. TMR) and use this as a mask to calculate the
 * intensity in channel 1 (e.g. FITC) and provide area, MFI. Log MFI, ratio and log ratio of MFI between
 * red and green.
 * Images should be displayed as a 3-channel multichannel image.
 * NB, if there is a measurement in channel 1 where there is no fluorescence in
 * channel 3 (mask), this will be measured as background.
 */

path = "C:\\Users\\User1\\saveFolder\\"

greenBackground = 0;     // rescinded constant, no longer in use
redBackground = 0;                              // rescinded constant, no longer in use

// Load all open images into an array
names = newArray(nImages);
for (i=0; i < names.length; i++)
{
   selectImage(i+1);
   names[i] = getTitle();
//    print(names[i]);
}

// Main macro body
for (i=0; i < names.length; i++)
{
                setBatchMode(true);

//Selects channel 3 and creates a mask from that channel
                selectWindow(names[i]);
                run("Split Channels");
                selectWindow("C3-" + names[i]);                          //mask image
                run("Duplicate...", "title=mask");
                run("Despeckle");
                setAutoThreshold("Moments dark");                        //moments threshold  most suitable under current conditions
                setOption("BlackBackground", true);
                run("Convert to Mask");
                run("Despeckle");                                                   //remove background
```

```
		run("Dilate");						//extend ROI 1 pxl

//Duplicates mask and calculates background measurements (anti-mask)
		run("Duplicate...", "title=mask2");
		selectWindow("mask2");
		run("Dilate");
		run("Create Selection");
		run("Make Inverse");
		roiManager("Add");
		selectWindow("C1-" + names[i]);
		roiManager("select", 0);
		getStatistics(bFitcArea, bFitcMean);			//Values for background
		selectWindow("C3-" + names[i]);
		roiManager("select", 0)
		getStatistics(bTmrArea, bTmrMean);
		print(names[i]);
		print("Area = " + bFitcArea + ", TMR = " + bTmrMean + ", FITC = " + bFitcMean);	//Output background in log

//Creates ROI for each endosome/endosome cluster
		selectWindow("mask");
		run("Analyze Particles...", "clear add");
		close("mask2");
		close("C2-" + names[i]);

//Remove ROIs containing saturated pixels
		selectWindow("C3-" + names[i]);
		tmrArray = newArray();
		n = roiManager("count");
		print("ROI before TMR removal = " + n);
		for (j=0; j<n; j++)						//Remove ROI with saturated red pixels
		{
			roiManager("select", j);
			getStatistics(tmrArea, tmrMean, tmrMin, tmrMax);
			if(tmrMax==65535)
				tmrArray = Array.concat(tmrArray, j);
			if(tmrMean<=redBackground)
				tmrArray = Array.concat(tmrArray, j);
		}

		for (j=(tmrArray.length-1); j>-1; j--)
		{
			roiManager("select", tmrArray[j]);
			roiManager("delete");
		}

		fitcArray = newArray();
		selectWindow("C1-" + names[i]);
		n = roiManager("count");
		print("ROI before FITC removal = " + n);

		for (j=0; j<n; j++)						//Remove ROI with saturated green pixels
		{
			roiManager("select", j);
			getStatistics(fitcArea, fitcMean, fitcMin, fitcMax);
			if(fitcMax==65535)
				fitcArray = Array.concat(fitcArray, j);
			if(fitcMean<=greenBackground)
				fitcArray = Array.concat(fitcArray, j);
		}

		for (j=(fitcArray.length-1); j>-1; j--)
		{
			roiManager("select", fitcArray[j]);
			roiManager("delete");
		}

//Measure intensity of each endosome or cluster in both red and green
		selectWindow("C3-" + names[i]);
		n = roiManager("count");
		print("ROI after removals = " + n);
		for (j=0; j<n; j++)						//Measures in channel 3
		{
			roiManager("select", j);
			getStatistics(tmrArea, tmrMean, tmrMin, tmrMax);
			setResult("Area (TMR)", j, tmrArea);
			setResult("Mean (TMR)", j, (tmrMean - redBackground));
			setResult("Log (TMR)", j, log(tmrMean - redBackground));
			setResult("Log (TMR-background)", j, log(tmrMean-bTmrMean));
		}
		selectWindow("C1-" + names[i]);
		for (j=0; j<n; j++)						//Measures in channel 1
		{
			roiManager("select", j);
			getStatistics(fitcArea, fitcMean, fitcMin, fitcMax);
			setResult("Area (FITC)", j, fitcArea);
			setResult("Mean (FITC)", j, (fitcMean - greenBackground));
```

```
			setResult("Log (FITC)", j, log(fitcMean - greenBackground));
			setResult("Log (FITC-background)", j, log(fitcMean-bFitcMean));
		}

//Calculate the ratios and the area adjusted mean ratios for the image
		areaSum = 0; ratioSum = 0; logRatioSum = 0; logBackRatioSum = 0;
		for (j=0; j<n; j++)						//Calculates the MFI ratio and log MFI ratio
		{
			ratio = getResult("Mean (FITC)", j)/getResult("Mean (TMR)", j);
			logRatio = getResult("Log (FITC)", j)/getResult("Log (TMR)", j);
			logBackRatio = getResult("Log (FITC-background)", j)/getResult("Log (TMR-background)", j);
			setResult("Ratio (FITC/TMR)", j, ratio);
			setResult("Log Ratio", j, logRatio);
			setResult("Log Ratio - Background", j, logBackRatio);

			ratioSum = ratioSum + (getResult("Ratio (FITC/TMR)", j) * getResult("Area (FITC)", j));
			areaSum = areaSum + getResult("Area (FITC)", j);
			logRatioSum = logRatioSum + (getResult("Log Ratio", j) * getResult("Area (FITC)", j));
			logBackRatioSum = logBackRatioSum + (getResult("Log Ratio - Background", j) * getResult("Area (FITC)", j));
		}

		updateResults();						//Loads data to table

//Output ratio and log ratio into ImageJ log
		print("Area adjusted ratio = " + (ratioSum/areaSum));
		print("Area adjusted log ratio = " + (logRatioSum/areaSum));
		print("Area adjusted log minus background ratio = " + (logBackRatioSum/areaSum));


//Saves the results table, mask and ImageJ Log
		selectWindow("mask");
		saveAs("tiff", path + "Results\\Masks\\" + names[i] + "\.tiff");
		selectWindow("Results");
		saveAs("results", path + "Results\\CSV\\" + names[i] + "\.csv");
		selectWindow("Log");
		saveAs("text", path + "Results\\Logs\\" + names[i] + "\.txt");

//Close everything
		close("C1-" + names[i]);
		close("C3-" + names[i]);
		close("mask");
		close(names[i] + ".tiff");
		selectWindow("Results");
		run("Close");
		selectWindow("Log");
		run("Close");

		setBatchMode(false);

}
//End
```

Script 2 for pseudocolouring ROIs (endosomes) according to pH

```
/* Macro designed by E. Sayers 19th September 2016
 *
 * This macro is designed so that ROIs can be false coloured according to their
 * pH. This is performed using a gaussian blur with a diameter of 5 pixels to
 * smooth the colouration of the image.  The macro retains the original pixel
 * intensity so relative intensities within an image can be compared along with
 * the pH. In order to pseudocolour the user is required to manually enter the
 * ratios obtained from the calibration curve into each if statement.
 */

{
   imageTitle = getTitle();

//Selects channel 2 and creates a mask from that channel
   getDimensions(imageWidth, imageHeight, no1, no2, no3);
   getPixelSize(unit,pxlWidth,pxlHeight,depth);
   run("Split Channels");
   selectWindow("C3-" + imageTitle);			//mask image
   run("Duplicate...", "title=mask");
   run("Despeckle");
   setAutoThreshold("Li dark");				//Li most suitable under current conditions
   setOption("BlackBackground", true);
   run("Convert to Mask");
   newTitle = "pHcolour " + imageTitle;
//  print(newTitle + " " + imageTitle);
   newImage(newTitle,"RGB black",imageWidth,imageHeight,1);

   setBatchMode(true);

   for(i=2;i<(imageWidth-2);i++)
```

```
    for(j=2;j<(imageHeight-2);j++)
    {
        selectWindow("mask");
        if(getPixel(i,j)==255)
        {
            if(getPixel(i-2,j-2)==255) xm2jm2=1; else xm2jm2=0;                  if(getPixel(i-1,j-2)==255) xm1jm2=4;  else xm1jm2=0;             if(getPixel(i,j-2)==255) xm0jm2=7;  else
xm0jm2=0;  if(getPixel(i+1,j-2)==255) xp1jm2=4;  else xp1jm2=0;              if(getPixel(i+2,j-2)==255) xp2jm2=1; else xp2jm2=0;
            if(getPixel(i-2,j-1)==255) xm2jm1=4; else xm2jm1=0;                  if(getPixel(i-1,j-1)==255) xm1jm1=16; else xm1jm1=0;             if(getPixel(i,j-1)==255) xm0jm1=26; else
xm0jm1=0;  if(getPixel(i+1,j-1)==255) xp1jm1=16; else xp1jm1=0;              if(getPixel(i+2,j-1)==255) xp2jm1=4; else xp2jm1=0;
            if(getPixel(i-2,j  )==255) xm2jm0=7; else xm2jm0=0;if(getPixel(i-1,j  )==255) xm1jm0=26; else xm1jm0=0;             if(getPixel(i,j  )==255) xm0jm0=41; else xm0jm0=0;
   if(getPixel(i+1,j  )==255) xp1jm0=26; else xp1jm0=0;          if(getPixel(i+2,j  )==255) xp2jm0=7; else xp2jm0=0;
            if(getPixel(i-2,j+1)==255) xm2jp1=4; else xm2jp1=0;if(getPixel(i-1,j+1)==255) xm1jp1=16; else xm1jp1=0;             if(getPixel(i,j+1)==255) xm0jp1=26; else xm0jp1=0;
   if(getPixel(i+1,j+1)==255) xp1jp1=16; else xp1jp1=0;          if(getPixel(i+2,j+1)==255) xp2jp1=4; else xp2jp1=0;
            if(getPixel(i-2,j+2)==255) xm2jp2=1; else xm2jp2=0;if(getPixel(i-1,j+2)==255) xm1jp2=4;  else xm1jp2=0;             if(getPixel(i,j+2)==255) xm0jp2=7;  else xm0jp2=0;
   if(getPixel(i+1,j+2)==255) xp1jp2=4;  else xp1jp2=0;          if(getPixel(i+2,j+2)==255) xp2jp2=1; else xp2jp2=0;
            totalG =   (xm2jm2 + xm1jm2 + xm0jm2 + xp1jm2 + xp2jm2 +
                              xm2jm1 + xm1jm1 + xm0jm1 + xp1jm1 + xp2jm1 +
                              xm2jm0 + xm1jm0 + xm0jm0 + xp1jm0 + xp2jm0 +
                              xm2jp1 + xm1jp1 + xm0jp1 + xp1jp1 + xp2jp1 +
                              xm2jp2 + xm1jp2 + xm0jp2 + xp1jp2 + xp2jp2);

            selectWindow("C3-" + imageTitle);
            tmr = getPixel(i,j);
            tmrBlur =       ((xm2jm2*getPixel(i-2,j-2)) + (xm1jm2*getPixel(i-1,j-2)) + (xm0jm2*getPixel(i  ,j-2)) + (xp1jm2*getPixel(i+1,j-2)) + (xp2jm2*getPixel(i+2,j-2)) +
                              (xm2jm1*getPixel(i-2,j-1)) + (xm1jm1*getPixel(i-1,j-1)) + (xm0jm1*getPixel(i  ,j-1)) + (xp1jm1*getPixel(i+1,j-1)) + (xp2jm1*getPixel(i+2,j-1)) +
                              (xm2jm0*getPixel(i-2,j  )) + (xm1jm0*getPixel(i-1,j  )) + (xm0jm0*getPixel(i  ,j  )) + (xp1jm0*getPixel(i+1,j  )) + (xp2jm0*getPixel(i+2,j  )) +
                              (xm2jp1*getPixel(i-2,j+1)) + (xm1jp1*getPixel(i-1,j+1)) + (xm0jp1*getPixel(i  ,j+1)) + (xp1jp1*getPixel(i+1,j+1)) + (xp2jp1*getPixel(i+2,j+1)) +
                              (xm2jp2*getPixel(i-2,j+2)) + (xm1jp2*getPixel(i-1,j+2)) + (xm0jp2*getPixel(i  ,j+2)) + (xp1jp2*getPixel(i+1,j+2)) +
(xp2jp2*getPixel(i+2,j+2)))/totalG;

            selectWindow("C1-" + imageTitle);
            fitcBlur =       ((xm2jm2*getPixel(i-2,j-2)) + (xm1jm2*getPixel(i-1,j-2)) + (xm0jm2*getPixel(i  ,j-2)) + (xp1jm2*getPixel(i+1,j-2)) + (xp2jm2*getPixel(i+2,j-2)) +
                              (xm2jm1*getPixel(i-2,j-1)) + (xm1jm1*getPixel(i-1,j-1)) + (xm0jm1*getPixel(i  ,j-1)) + (xp1jm1*getPixel(i+1,j-1)) + (xp2jm1*getPixel(i+2,j-1)) +
                              (xm2jm0*getPixel(i-2,j  )) + (xm1jm0*getPixel(i-1,j  )) + (xm0jm0*getPixel(i  ,j  )) + (xp1jm0*getPixel(i+1,j  )) + (xp2jm0*getPixel(i+2,j  )) +
                              (xm2jp1*getPixel(i-2,j+1)) + (xm1jp1*getPixel(i-1,j+1)) + (xm0jp1*getPixel(i  ,j+1)) + (xp1jp1*getPixel(i+1,j+1)) + (xp2jp1*getPixel(i+2,j+1)) +
                              (xm2jp2*getPixel(i-2,j+2)) + (xm1jp2*getPixel(i-1,j+2)) + (xm0jp2*getPixel(i  ,j+2)) + (xp1jp2*getPixel(i+1,j+2)) +
(xp2jp2*getPixel(i+2,j+2)))/totalG;
            ratio = log(fitcBlur(/log(tmrBlur);
            pxlInt = tmrBlur/65535;
            blue = round(255*pxlInt);
            green = blue*256;
            red = blue*65536;
            if(ratio>???)                    //Insert calibration value 7.4
            {
                selectWindow(newTitle);
                setPixel(i,j,(red+blue)); //magenta
            }
            else if (ratio>???)              //Insert calibration value 6.8
            {
                selectWindow(newTitle);
                setPixel(i,j,(blue)); //blue
            }
            else if (ratio>???)              //Insert calibration value 6.2
            {
                selectWindow(newTitle);
                setPixel(i,j,(blue+green)); //cyan
            }
            else if (ratio>???)              //Insert calibration value 5.6
            {
                selectWindow(newTitle);
                setPixel(i,j,(green)); //green
            }
            else if (ratio>???)              //Insert calibration value 5.0
            {
                selectWindow(newTitle);
                setPixel(i,j,(red+green)); //yellow
            }
            else if (ratio>???)              //Insert calibration value 4.4
            {
                selectWindow(newTitle);
                setPixel(i,j,(red)); //red
            }
            Else                             //Anything below the value for 4.4 will be coloured grey/white
            {
                selectWindow(newTitle);
                setPixel(i,j,(red+green+blue)); //white
            }
        }
    }

  setBatchMode(false);
  updateDisplay();

  selectWindow(newTitle);
  setVoxelSize(pxlWidth,pxlHeight,depth,unit);
```

```
    selectWindow("C3-" + imageTitle);
    close();

    selectWindow("C1-" + imageTitle);
    close();
}

//End
```

REFERENCES

1. Ghandi, M, Huang, FW, Jane-Valbuena, J, Kryukov, GV, Lo, CC, McDonald, ER, 3rd*, et al.* (2019). Next-generation characterization of the Cancer Cell Line Encyclopedia. *Nature* **569**: 503-508.
2. Dawson, RMC (1986). *Data for biochemical research*, Clarendon Press, Oxford.