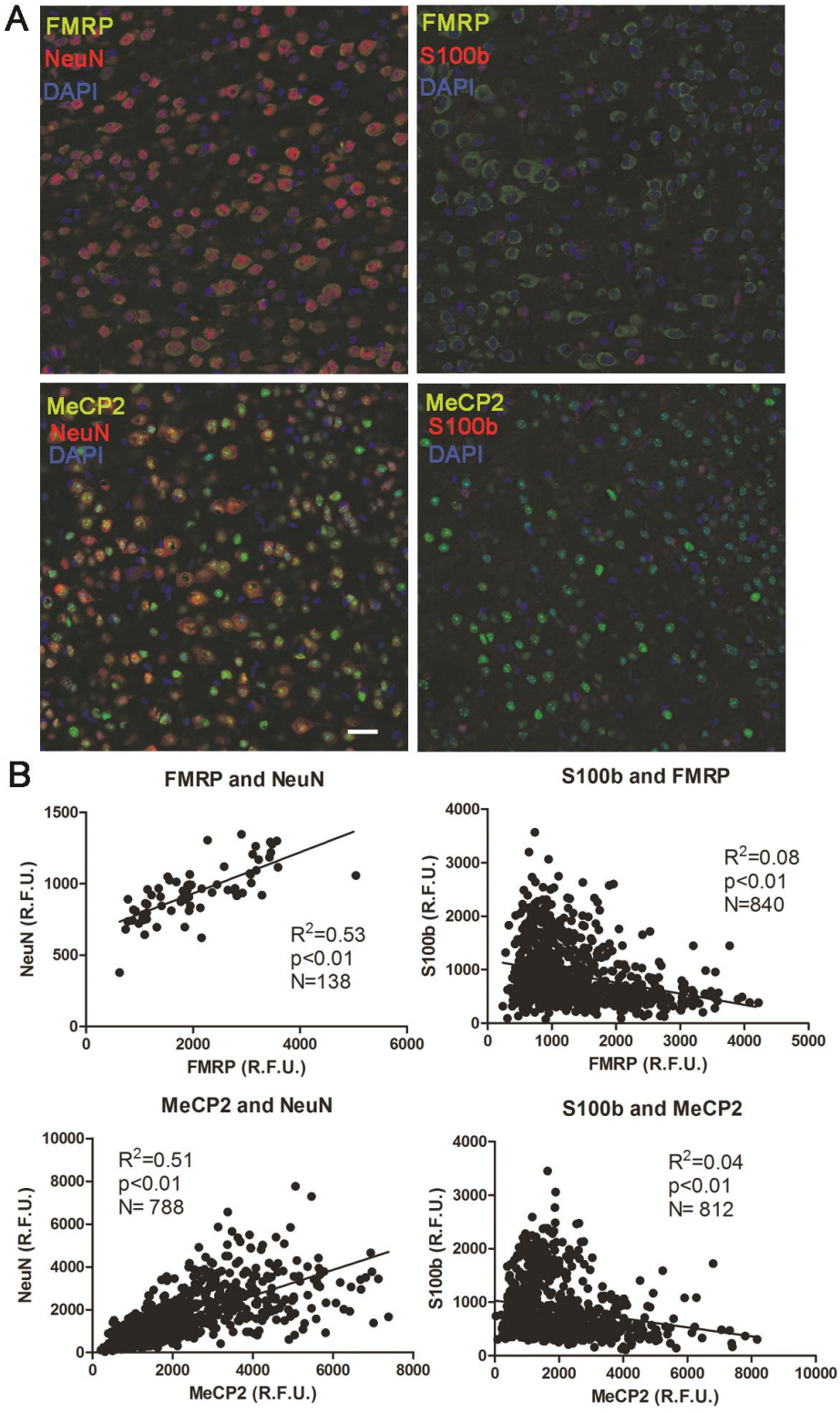
Supplementary information

Arsenault, et al., *Interregulation between Fragile X Mental Retardation Protein and Methyl CpG Binding Protein 2 in the Mouse Posterior Cerebral Cortex,* Human Molecular Genetics, 2020.

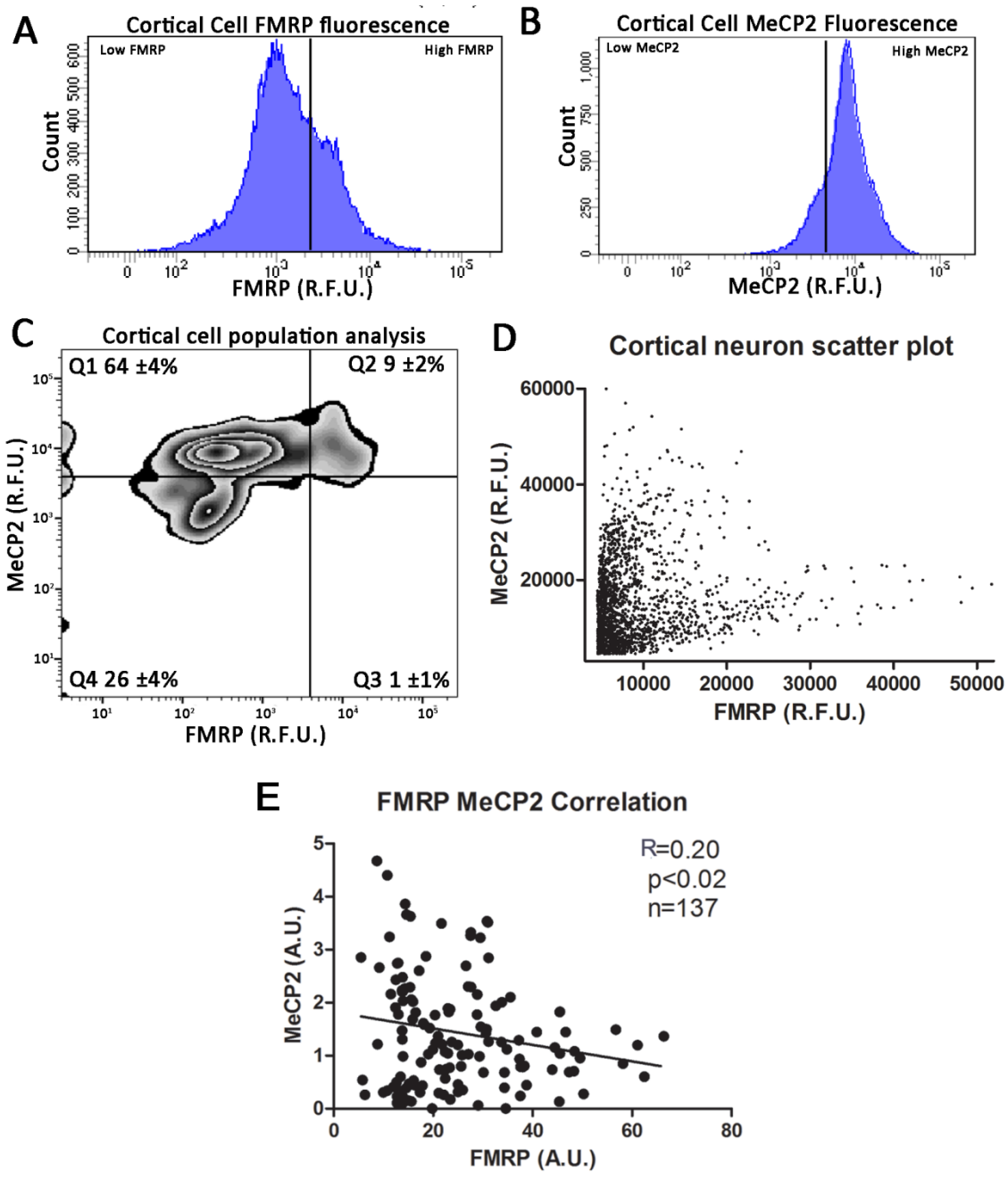
Supplementary Figures

Figure 1.



Automated MATLAB quantification of cells populations in the motor cortex labeled with anti-MeCP2, anti-FMRP, anti-S100β, or anti-NeuN. (A) Example photomicrographs of the motor cortex (top). White bar = 20 μm. (B) Scatter plot of quantified cells. FMRP and MeCP2 expression displayed a higher correlation with NeuN+ neurons compared to cells expressing the astrocyte marker S100β, confirming the neuronal lineage of the quantified cells (*i.e.* high MeCP2 and high FMRP cells are predominantly neurons). Correlation, *p* value, and number of quantified cells are indicated in each panel.

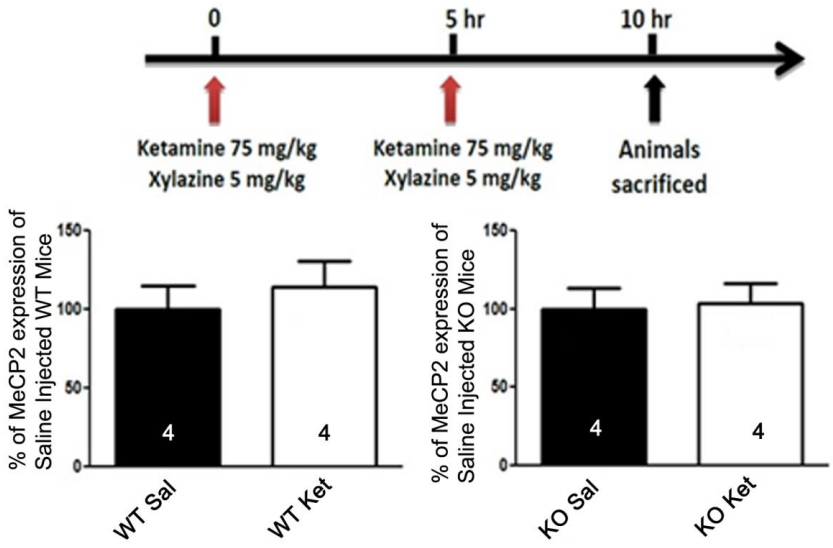
Figure 2.



Flow cytometry of cortical cells immunolabeled with anti-MeCP2 and anti-FMRP as well as manual image quantification. (A) Fluorescence histogram of FMRP and (B) MeCP2 staining intensity of single cells (G2). Cells were separated into low and high FMRP (A) and MeCP2 (B) populations as illustrated by the vertical line (arbitrary threshold). (C) Cortical cell

population analysis based on anti-FMRP and anti-MeCP2 immunofluorescence. Cell population averages from four independent experiments are shown as mean ± SEM. (D) The distribution of the high-FMRP and high-MeCP2 populations (Q2) was further analyzed. Neuronal MeCP2 protein expression, as a function of FMRP protein expression, displays a similar trend to the single cell fluorescence obtained by confocal microscopy quantification (see Fig. 2D-E). R.F.U.: Relative Fluorescence Units. (E) Cortical neuronal population from *Fmr1* KO mice transduced with AAV-FMRP quantified using Zen Software and manual selection of cells. Cells were plotted according to the relative fluorescence of anti-MeCP2 and anti-FMRP labeling. Flow cytometry and manual cell quantification displayed similar patterns as the unbiased quantification from MATLAB (Fig. 2D).

Figure 3.



Effects of acute sedation on MeCP2 expression. The injection strategy is shown at the top. Densitometric quantification of MeCP2 protein expression (mean ± SEM) in WT (left panel) and *Fmr1* KO mice (right panel) maintained under anesthesia (Ketamine) for 10 hours prior to euthanasia shows no difference in MeCP2 expression compared to freely moving saline (Sal) injected littermates. The MeCP2 protein expression was normalized to the saline group of each corresponding genotype. The numbers of mice analyzed are indicated within the bars. Protein expression normalized to GAPDH expression and control condition was set at 100%. Data are presented as mean ± SEM.

Supplementary information

Materials and methods (cont.)

AAV Vectors and AAV Vector Injections

Pups were immobilized by cryo-anesthesia then gently placed over a fiber-optic light to illuminate the transverse and midline sutures that were used to guide the injections. A 30-gauge needle was attached to a 5ml Hamilton syringe (Hamilton, Reno, USA) using a long fluoropolymer tube (internal diameter of 0.5mm; Western Analytical, Lake Elsinor, USA) for the injections. The needle was inserted at a depth of 2mm perpendicular to the skull surface, 1mm lateral to the sagittal suture, and 1mm rostral to the neonatal coronary suture. A volume of 1µl of either vector was injected into each lateral ventricle using a syringe pump at the rate of 1µl/min. Following the injection, the needle was maintained in place for 1 min before being slowly retracted to prevent backflow.

Qualitative and Quantitative Reverse Transcriptase Polymerase Chain Reaction of Forebrain and Cortical mRNA

Mouse cortices from two naive PND 30 WT male mice were pooled and homogenized with 3 mL of IP buffer (10 mM HEPES, 200 mM NaCl, 30 mM EDTA, 0.5% Triton X-100, pH 7.4, with the addition of Ribolock RNase inhibitor; Thermo Scientific) and 1X complete protease inhibitor cocktail (Roche). Following 3,000g centrifugation, the supernatant NaCl concentration was adjusted to 400 mM and centrifuged at 70,000g for 30 min. To remove non-specific mRNA binding to the beads, the solution was pre-cleared using a 1:1 mixture of protein A and Protein G magnabeads® (Genscript) for 1 h at 4°C. The supernatants were then incubated with 10 µg of anti-FMRP 17722 antibody (abcam) or, to exclude non-specific mRNA binding to the antibody,

goat IgG (CLCC10200; Cedarlane) for 2 h at 4°C under constant agitation. Protein A/G beads, blocked with 0.1 mg/mL of bovine serum albumin and 0.1 mg/mL of yeast tRNA (Thermo Scientific), were added and the mixture was incubated overnight. Then the supernatant was removed, and the beads were washed four times with ice-cold IP buffer. TRIzol® reagent (Thermo Scientific) was added to the supernatant or the beads, and RNA isolation was performed following the manufacturer's instructions. RNA was re-suspended in RNAse free H_2O and reverse transcribed using random nonamers (Sigma-Aldrich) and the superscript II reverse transcriptase (Thermo Scientific) according to the manufacturer's protocol. The following primers were used to detect the presence of specific mRNAs: for PSD-95 (forward 5′-AAGCCCCAGGATATGTGAACGG; reverse 5′-AGCCCAGACCTGAGTTACCCCTT), for MeCP2 transcript variant 1 (forward 5′-GTCCGGAAAATGGCCGCCGC; reverse 5′-TTCTGACTTTTCCTCCAGTCTCTCCTCC), and for MeCP2 transcript variant 2 (forward 5′-GGGATGTTAGGGCTCAGGGAGG; reverse 5′- TGAGCTTTCTGATGTTTCTGC). PCR amplifications were resolved on 2% agarose gel. For quantitative PCR, total mRNA was isolated from one hemisphere of the forebrain of 2-month old WT and *Fmr1* KO mice using the RNeasy RNA isolation kit (Qiagen, Toronto, Canada) as recommended by manufacturer. 2µg of total RNA was reverse-transcribed using random nonamer primers (Sigma) and Superscript II reverse transcriptase (Invitrogen) was used following manufacturer's protocol. Negative control immunoprecipitations were submitted to the same treated. 5µl of diluted cDNA was used as a template for PCR quantification.

Immunocytochemistry and Confocal Microscopy

Prior to experimentations, three different antibody dilutions were used to determine proper antibody titer and to avoid signal saturation (at 1:500, 1:1000, and 1:5000 dilutions, respectively). After overnight incubation, sections were washed five times with PBS and then secondary antibodies diluted in PBS containing 5% goat serum (Sigma-Aldrich, St Louis, USA) were applied.

The images were captured using a laser scanning confocal microscope (Zeiss 700, Carl Zeiss Canada, Toronto, Canada) at 20x objective magnification and compiled using the Zen 2010 Blue software (Carl Zeiss). Pinhole size (1 airy unit), signal gain, and laser intensity were maintained identical between samples. Settings were determined for each experiment using *Fmr1* KO mice slices (anti-FMRP immunoreactive control), as well as no-primary controls for both anti-FMRP and anti-MeCP2 (to eliminate non-specific secondary antibody labeling). MATLAB R2017a (9.2.0; Mathworks, Massachusetts, USA) was used to quantify single cells using a custom code, briefly, DAPI staining were used to confirm cells were in the proper focal plane for analysis, then a circle function was used to delineate individual cells and total intensities of the respective photomicrographs were used to compare immunoreactive intensities of each channel (*i.e.* MeCP2, FMRP, NeuN, and S100β). The histogram function was used to verify that the cellular immunoreactivity was not saturated. Manuel counting and flow cytometry was used to confirm whether the counting algorithm was optimal (See Suppl. Fig. 3).

Quantitative Western Blotting

After incubation with secondary antibody, following 3 washes with PBS, the membranes were incubated with HRP-conjugated goat anti-mouse or anti-rabbit secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA), and SuperSignal West Pico Chemiluminescent

Substrate (Thermo Scientific, Rockford, IL) was added to the membranes to reveal the bands. Images were exposed under an Alpha Innotech Fluorochem gel imager (Protein Simple; Toronto, ON, Canada). All gels were run in duplicates. Following exposure, PVDF membranes were stained with Coomassie Blue to assure contiguous transfer of protein onto the membrane.

Ketamine induced sedation

To tests the effects of motor activity on brain MeCP2 levels, mice were maintained under sedation using an intraperitoneal injection of a Ketamine (75mg/kg) and Xylazine (5mg/kg) solution for induction and a subsequent equal dose was administered 5h following the initial injection. Ten hours after administration (the lengthiest time approved by the ethical committee for induced sedation), the mice were euthanized and brain regions were harvested, processed, and analyzed by quantitative western blotting as indicated above.

Isolation of Cortical Cells and Flow Cytometry

Wild-type mouse cortical cells were extracted as described for neonatal rats (58). Briefly, fresh cortical tissue was dissected into Hank's Balanced Salt Solution (Thermo-Scientific) on ice. The tissue suspension was decanted to 1ml and digested using 10 units/ml of Papain (Worthington, Lakewood, USA) for 15 min at 37°C. Tissue was triturated by five, slow aspirations and ejections of the suspension using a 5ml syringe (BD Bioscience, Mississauga, Canada) attached to a 25 gauge needle (BD Bioscience). The cell suspension was passed through a 70μm Nylon Mesh filter (Fisher Scientific, Ottawa, Canada) and centrifuged at 600g for 10 min at 4°C. The supernatant was discarded and cells were washed twice with Hank's Balanced Salt Solution. The cell suspension was fixed with a final concentration of 2% PFA and gently rocked at 4°C for 20

min, then centrifuged and washed twice. Cells were permeabilized with 0.5% Triton X-100 and 5% goat serum in Hank's Balanced Salt Solution, then centrifuged and resuspended in 500µl of incubation buffer (5% goat serum in Hank's Balanced Salt Solution). Anti-FMRP 5c2 and polyclonal rabbit anti-MeCP2 2828 (Abcam, Toronto, Canada) were added to each sample at 1:250 dilutions and incubated for 1 h at room temperature. Samples were washed three times with incubation buffer, then incubated with secondary anti-mouse 594 (Jackson Immunoresearch Laboratories) and anti-rabbit 488 at 1:500 dilutions. Samples were washed three times and resuspended in Hank's Balanced Salt Solution.

Single cell suspensions were sorted using BD Fortessa II (BD Bioscience). Cells were initially separated by forward (FSC) and side scatter (SSC) and whole cells were gated (G1) for subsequent analysis. Single cells were then gated (G2) using height (FSC-H) and area (FSC-A). MeCP2 immunoreactive signal was detected using Blue 530/30 while FMRP was detected with Yellow-Green 610/20. FMRP and MeCP2 signal intensity was divided into low and high intensity (arbitrary threshold). High-MeCP2/High-FMRP cells (Q2) were selected for further analysis. Suspensions containing no primary antibodies were used as isotype controls and single fluorophore stained cells were used as compensation controls for each channel. Fluorescent intensity was calibrated between experiments using Ultra Rainbow Calibration particles (Spherotech, Inc., Lake Forest, USA).

MATLAB coding

The following code was used to quantify 2 fluorophore channel intensities from photomicrographs of DAPI stained cells. Cells at the periphery of the image were excluded from the analysis. Notes in the codes are indicated with a %.

```
clc
clear
close all
warning off

im1=imread('./ file 1 address.tif'); %Green
im2=imread('./ file 2 address.tif'); %Blue,DAPI-Nucleus
im3=imread('./ file 3 address.tif'); %Red

%Minimum and maximum area threshold value for blue channel
%These numbers can vary according to image sizes and resolution
B_area_min=200;
B_area_max=1000;

%Fluorescence threshold value for blue channel
B_K=1.0;

%Blue Channel
gim_B=rgb2gray(im2);
n = graythresh(gim_B);
%Determine cell boundaries based on the fluorescence threshold
bw_B= im2bw(gim_B, n*B_K);
figure; imshow(im2);title('Blue')
figure; imshow(bw_B); title('BlueGrayscale')
bw_B=imfill(double(bw_B));
%Select cells within the area thresholds
bw_B = xor(bwareaopen(bw_B,B_area_min,8), bwareaopen(bw_B,B_area_max,8));
%Merges the small gaps where the scanning identified two cells likely came from the same nucleus
se=strel('disk',2);
bw_B=imclose(bw_B,se);
figure;  imshow(bw_B);title('BlueWithNoiseDeleted')
figure; imshow(im2); hold on; contour(bw_B,[0 1],'r' );title('BoundariesForBlue')

%Green channel
gim_G=rgb2gray(im1);
n = graythresh(gim_G);
bw_G= im2bw(gim_G);
%figure; imshow(im1);title('Green')
%figure; imshow(bw_G); title('GreenGrayscale')
bw_G=imfill(double(bw_G));
bw_G = bwareaopen(bw_G,8);

%Red channel
gim_R=rgb2gray(im3);
n = graythresh(gim_R);
bw_R= im2bw(gim_R);
%figure; imshow(im3);title('Red')
%figure; imshow(bw_R); title('RedGrayscale')
bw_R=imfill(double(bw_R));
bw_R = bwareaopen(bw_R,8);

%Assign number to labeled blue nuclei and counts total
```

```
[L, num] = bwlabel(bw_B);


%According to the center of selected blue fluorescence (nuclei),
%Measure fluorescent from green and red channel accordingly
r_g=12; %Radius for green,
r_r=12; %Radius for red,
circle_bw_G=zeros(size(bw_B));
circle_bw_R=circle_bw_G;
 [m,n]=size( bw_B);
points=zeros(m*n,2); %location of the cell, row and column
r=0;
result_circle_BGR=[];
 for i=1:m
    for j=1:n
       r=r+1;
       points(r,:)=[i,j];
    end
 end

for i=1:num
 fprintf('%d ',i);
 very_bw=zeros(m,n);
  p_b=find(L==i); %Blue
   very_bw(p_b)=1;
       %Return the coordinates of the center of nucleus
       s = regionprops(very_bw,'Centroid');
       circle_points=s.Centroid;

  dists=dist( points,  circle_points');
  dists=reshape(dists,[m,n]);
  p_g=find(dists <=2*r_g); %Distance between 2 circles smaller than r_g
  p_r=find(dists <=2*r_r); %Distance between 2 circles smaller than r_r

  circle_bw_G(p_g)=1;
  circle_bw_R(p_r) =1;
  result_circle_BGR=[result_circle_BGR ;    ...
                      [ round( circle_points(2) ) ,( circle_points(1) ),   numel(p_b) ,  sum(gim_B(p_b )  ),  numel(p_g) ,
sum(gim_G(p_g )  ),    numel(p_r) ,    sum(gim_R(p_r )  )   ]  ];
end

figure
imshow(im1);hold on; contour(circle_bw_G,[0 1],'r' );title('CirclesInGreenWithLocationDecidedByNucleus')

figure
imshow(im3);hold on; contour(circle_bw_R,[0 1],'g' );title('CirclesInRedWithLocationDecidedByNucleus')
 fprintf('\n');
disp('Circles drawn from center of nucleus(blue), results shown in result_circle_BGR, row, column, blue area, blue
intensity, green area, green intensity, red area, red intensity');
```