Eunice Kennedy Shriver NICHD
National Institute of Child Health & Human Development

Uteroplacental Pathology and DOHaD: Important Gaps in Knowledge

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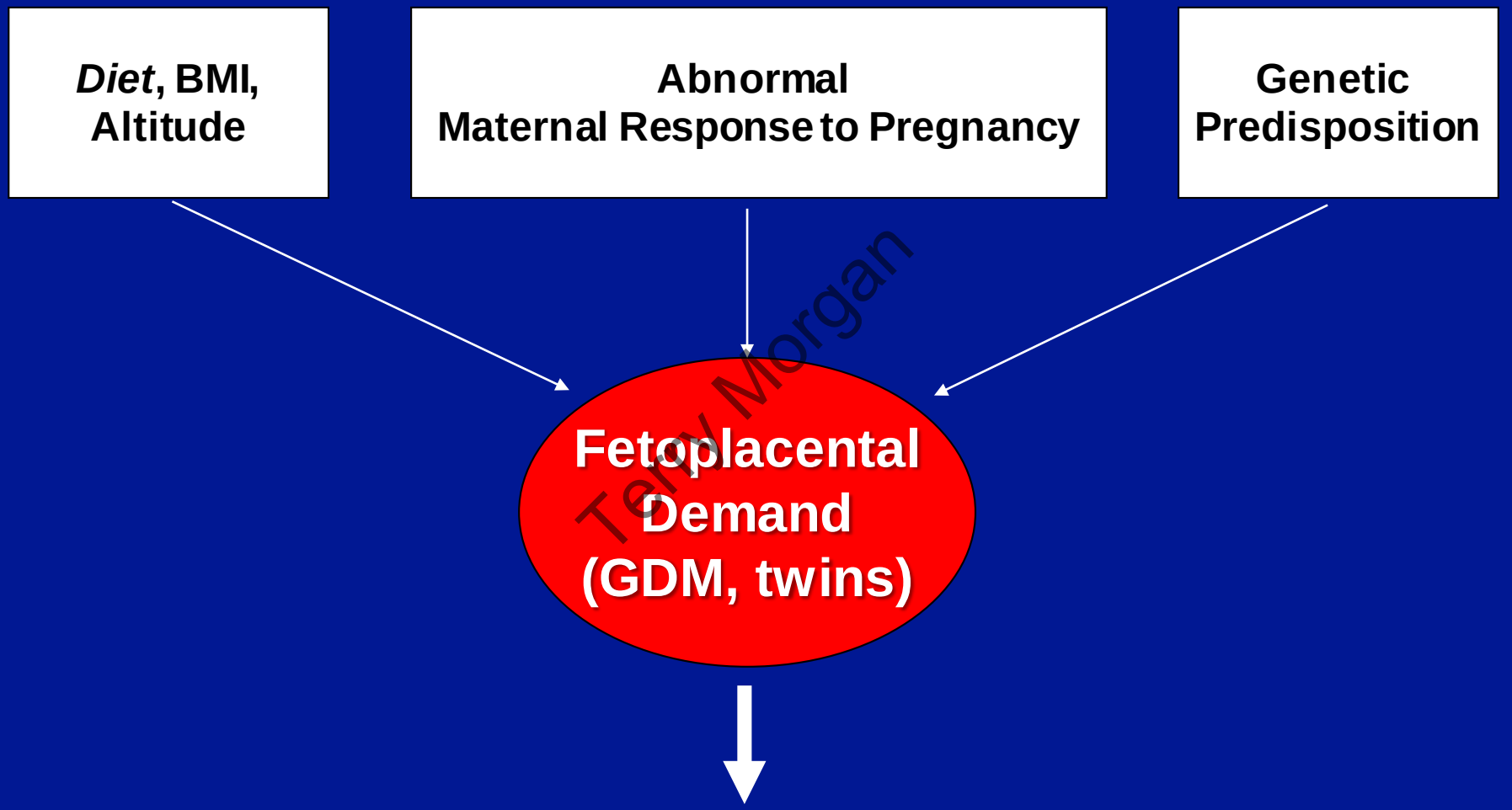
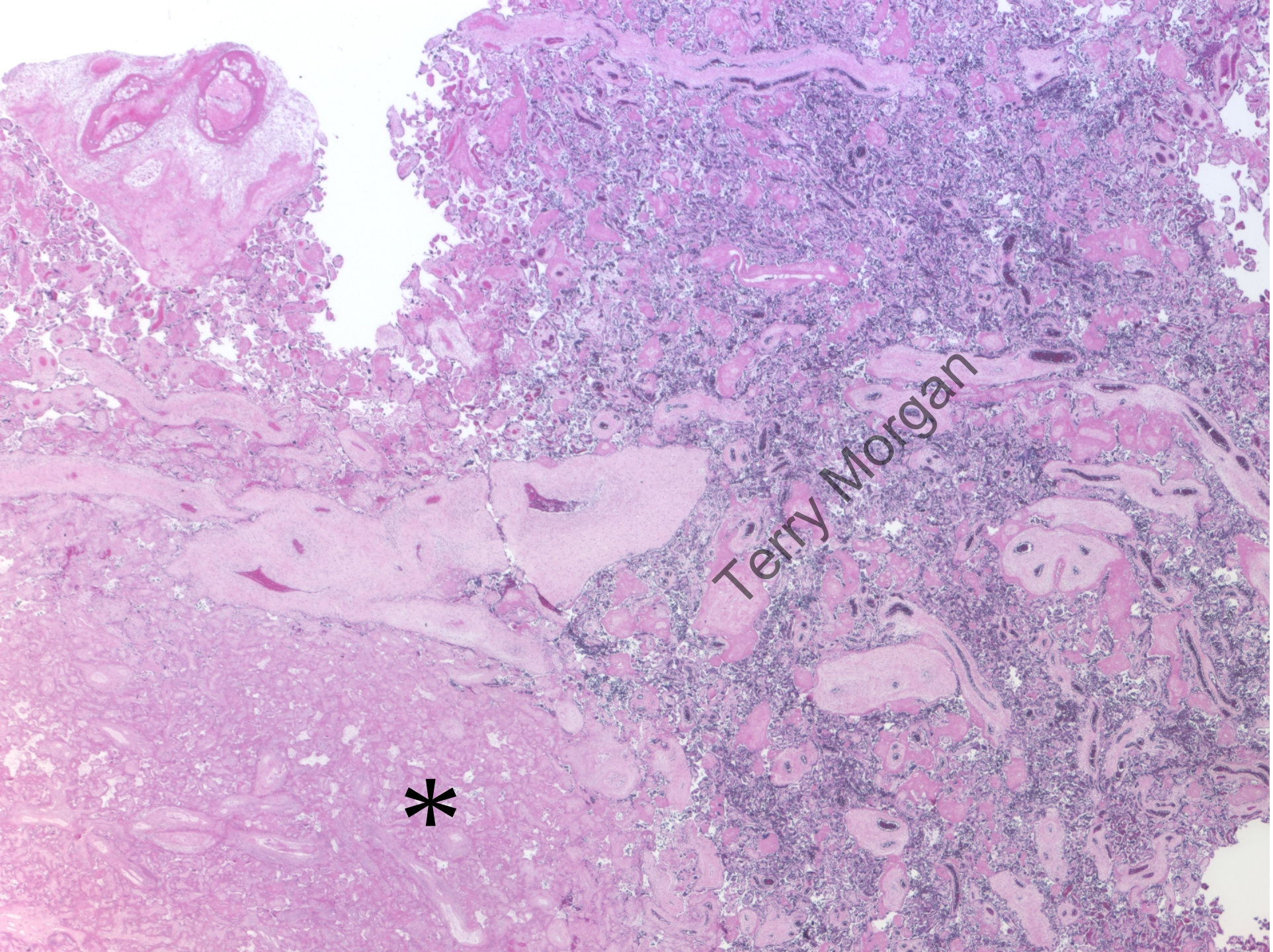
*** Dr. Morgan has no conflicts of interest to disclose**

Problem of Supply and Demand



Maternal Vascular Malperfusion and Clinical Outcomes

- What we know: Snapshots at delivery
- What we think: *Pathology begins in 1st trimester*
- Unknowns:
  - Causes vs Consequences?
  - *In vivo* "snapshots" throughout gestation linked to outcomes



Snapshot of Maternal Vascular Malperfusion (MVM) 34 weeks' PET/IUGR

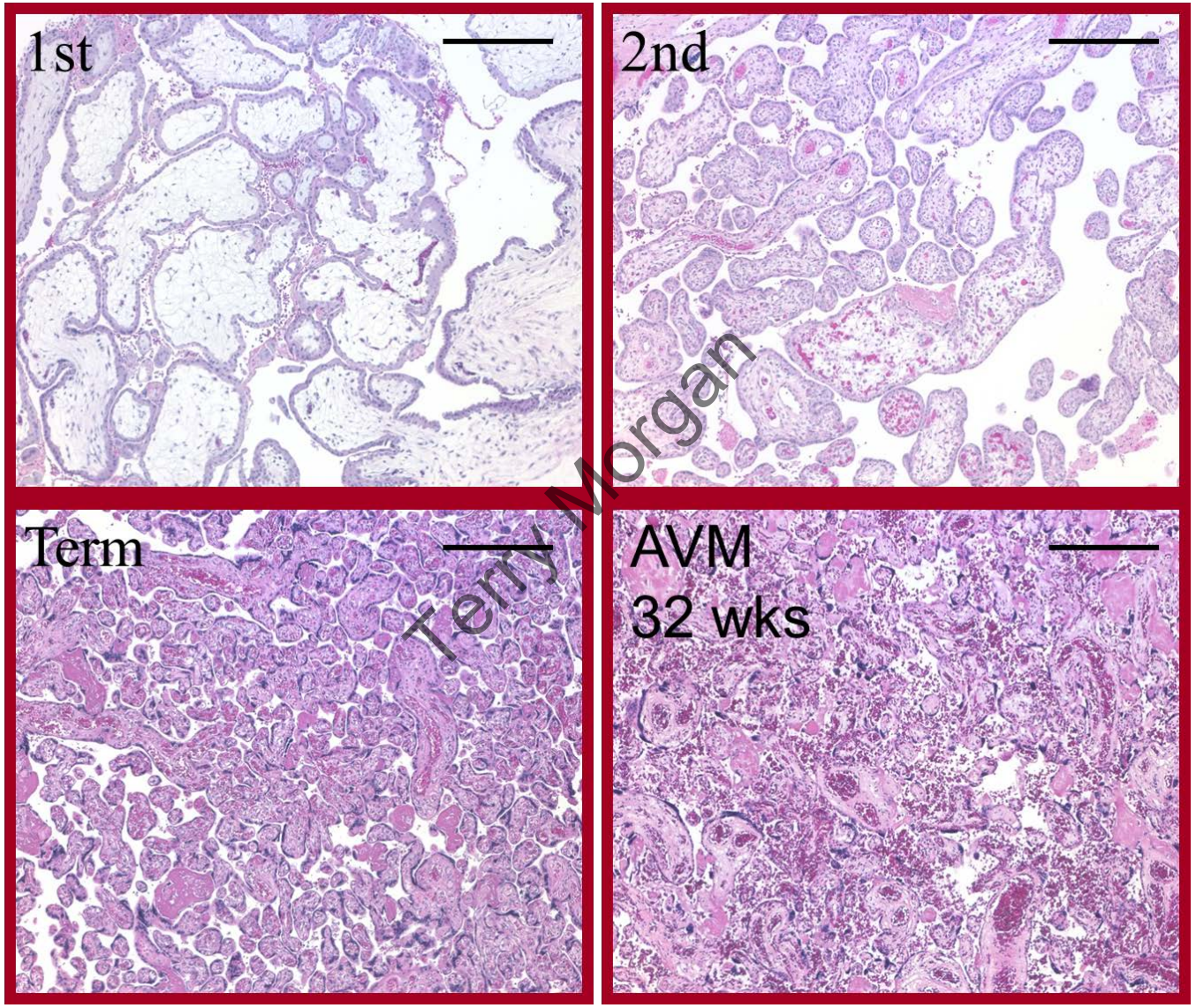
Amsterdam Placental Workshop Consensus Statement: Khong T, et al. Arch Pathol & Lab Med. 140:698-713

Dragon Tree

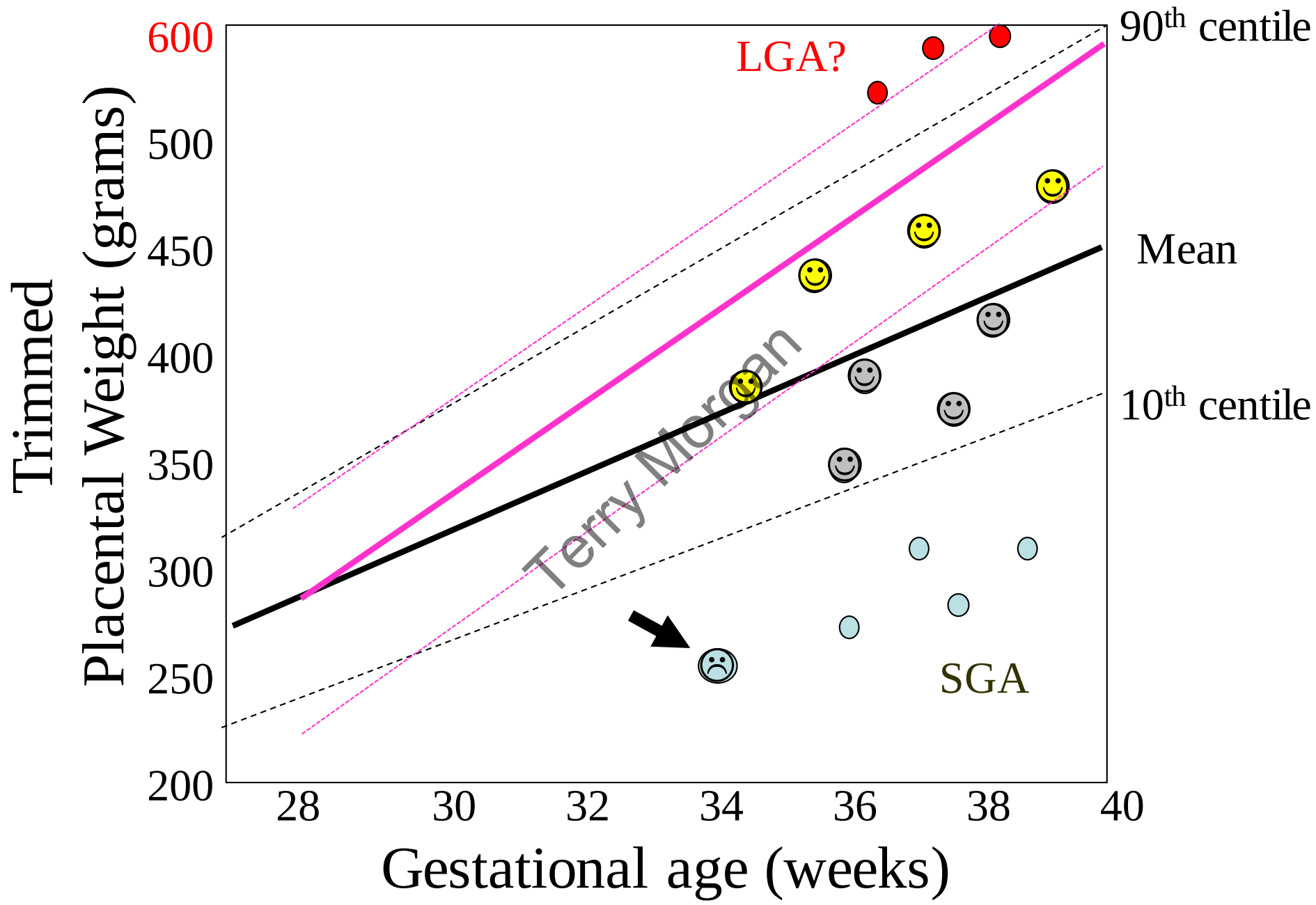
Socotra Island, East of Aden

Tree of Life/Knowledge

AVM is Reproducible < 38 weeks’



Khong T, et al. J Clin Pathol. 1995; 48:420-3
Morgan T. Am J Perinatol. 2016; 33:258-66
Amsterdam Placental Workshop Consensus Statement: Khong T, et al. Arch Pathol & Lab Med. 140:698-713



Naeye R. *Hum Pathol*. 1987; 18: 387-91: >20,000 cases

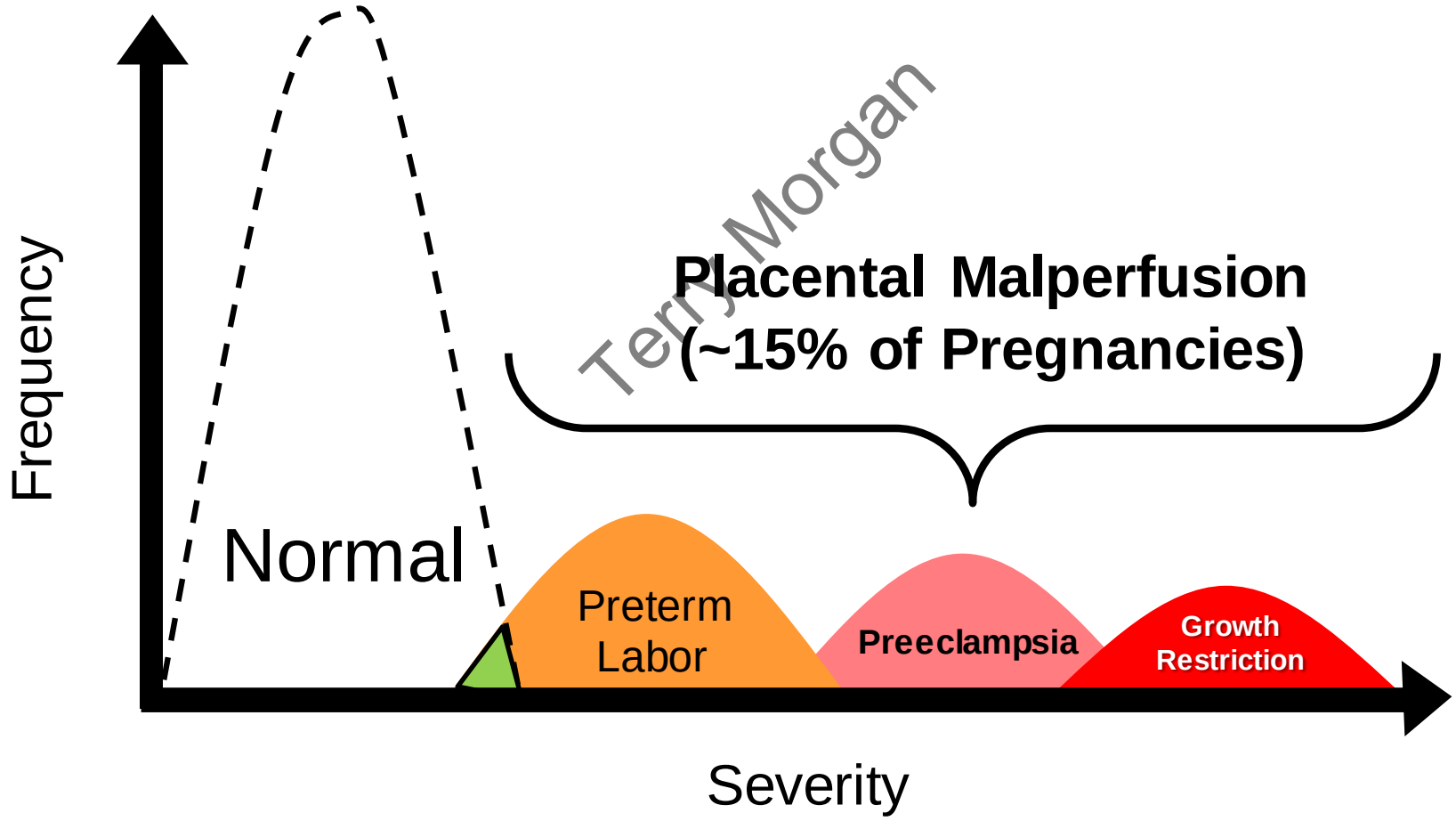
Pinar H. *Ped Path Lab Med*. 1996; 16:903: <200 cases

Table 2. **Gross and Histologic Findings in Preterm Birth: Oregon Health & Science University Experience**[a]

Variable	No. (%) of Cases			
	PTL with IAI (n = 68)	PTL without IAI (n = 179)	Preeclampsia (n = 121)	FGR (n = 29)
SGA placenta	0 [Reference]	0	12 (10)[b]	28 (97)[c]
Gross infarction	4 (6) [Reference]	21 (12)[d]	30 (25)[c]	10 (35)[c]
AVM	13 (19) [Reference]	106 (59)[c]	70 (58)[c]	16 (55)[b]

AVM=accelerated villous maturation; FGR=fetal growth restriction; IAI=intra-amniotic infection; PTL=preterm labor; SGA=small for gestational age.
[a]Gross and histologic data from the Department of Pathology, Oregon Health & Science University (OHSU) , diagnosed by a single placental pathologist (T.K.M.) scoring for small placentas (SGA), which were defined as less than the 10th percentile when using trimmed weight and adjusting for gestational age, gross infarctions (usually 1–2 cm in diameter), and AVM, which was defined as term villous morphologic findings with conspicuous syncytial knotting and perivillous fibrin deposition before 38 weeks' gestation. (9) PTL with or without IAI was defined by the presence or absence of histologic chorioamnionitis (Figure 1). Severe preeclampsia and late-onset FGR were defined clinically by the maternal fetal medicine faculty at the OHSU. Findings were compared by χ^2 analysis with the Fisher exact probability test when appropriate.
[b]$P < .01$.
[c]$P < .001$.
[d]$P < .05$.

Multifactorial Hypothesis



Should we Grade MVM?

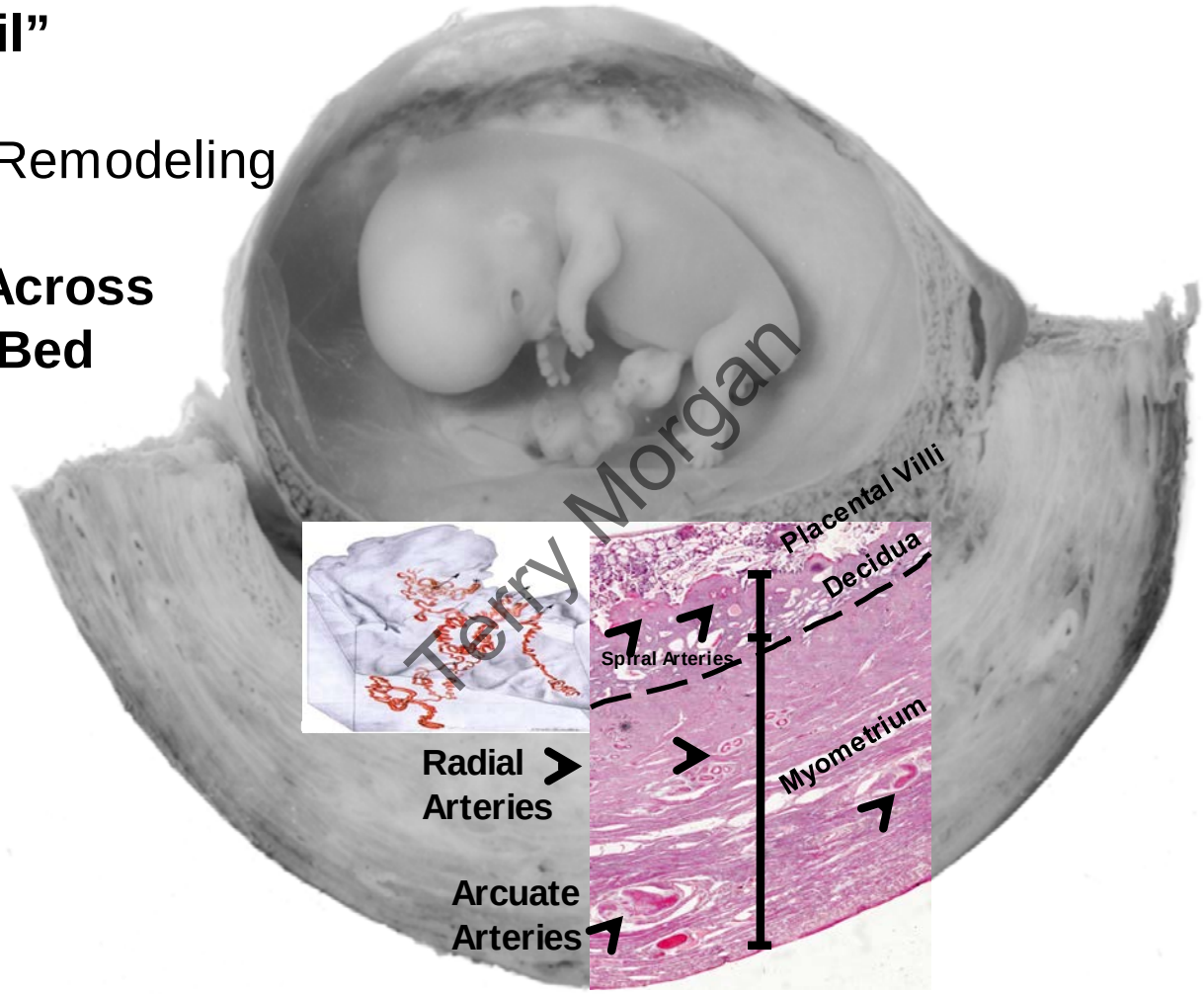
- What we know: Features associated with outcomes
  - "Idiopathic" PTL: AVM
  - Severe PET: AVM + Infarctions
  - Early onset IUGR: AVM + Infarctions + SGA placenta

- Unknown: Variable penetrance of an underlying disease process, or separate pathophysiologies?

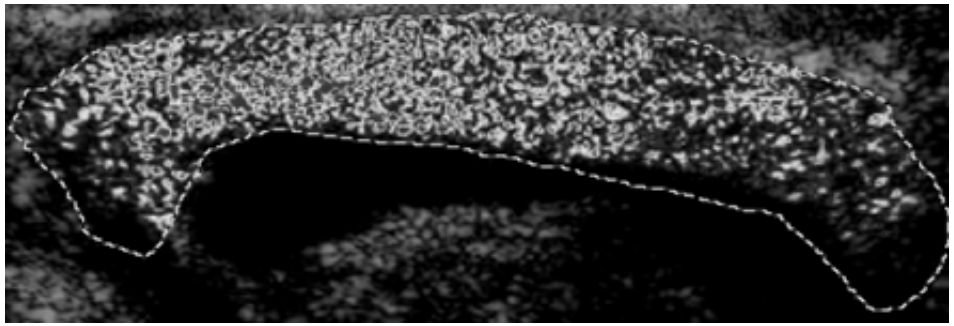
Spiral Artery Angiogenesis Is the “Soil”

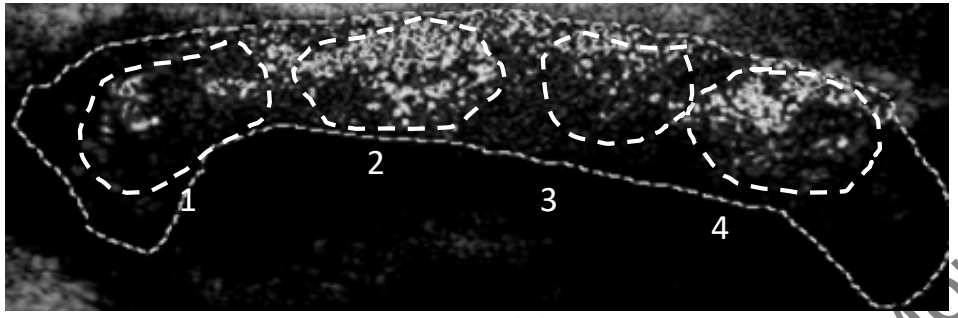
Upstream Remodeling

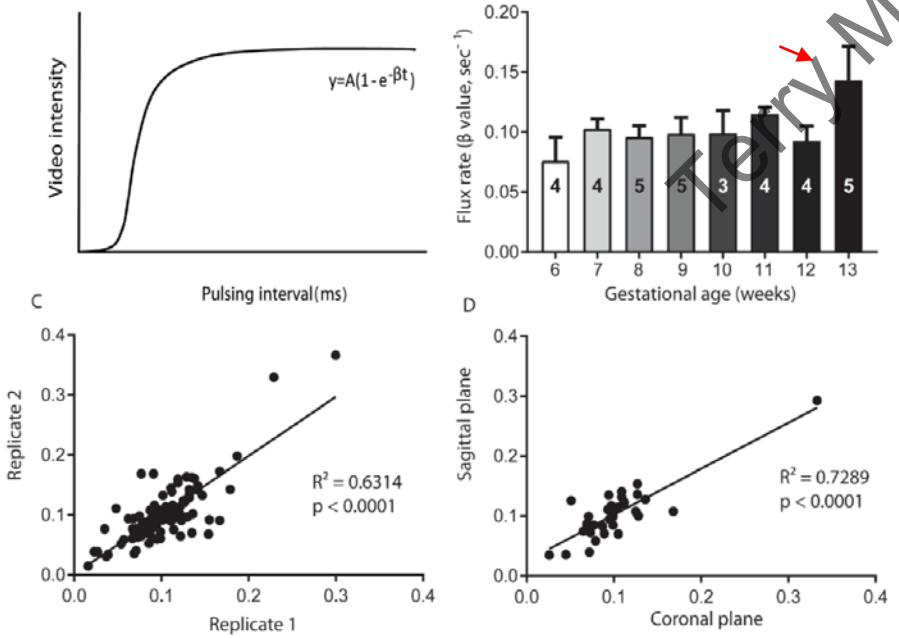
Variance Across Placental Bed



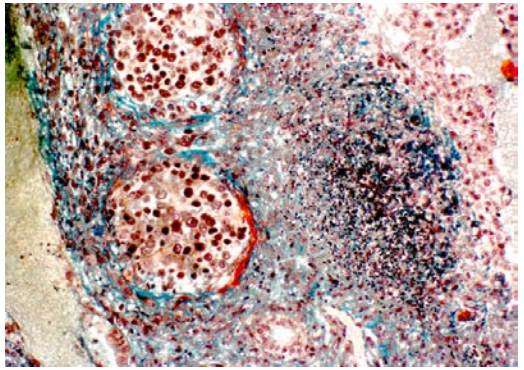
Adapted from Hamilton & Boyd, 1960 and Harris & Ramsey vascular reconstructions



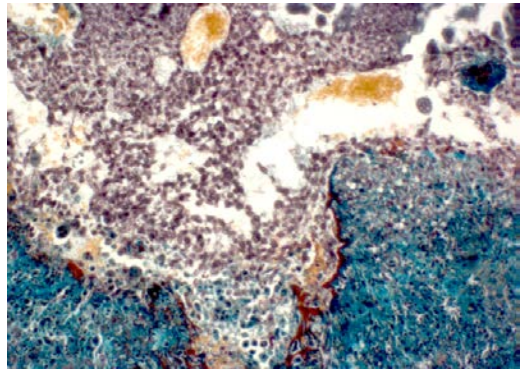




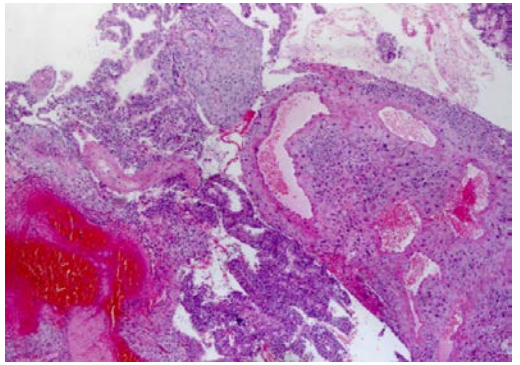
6 Weeks
Arterial Plugs



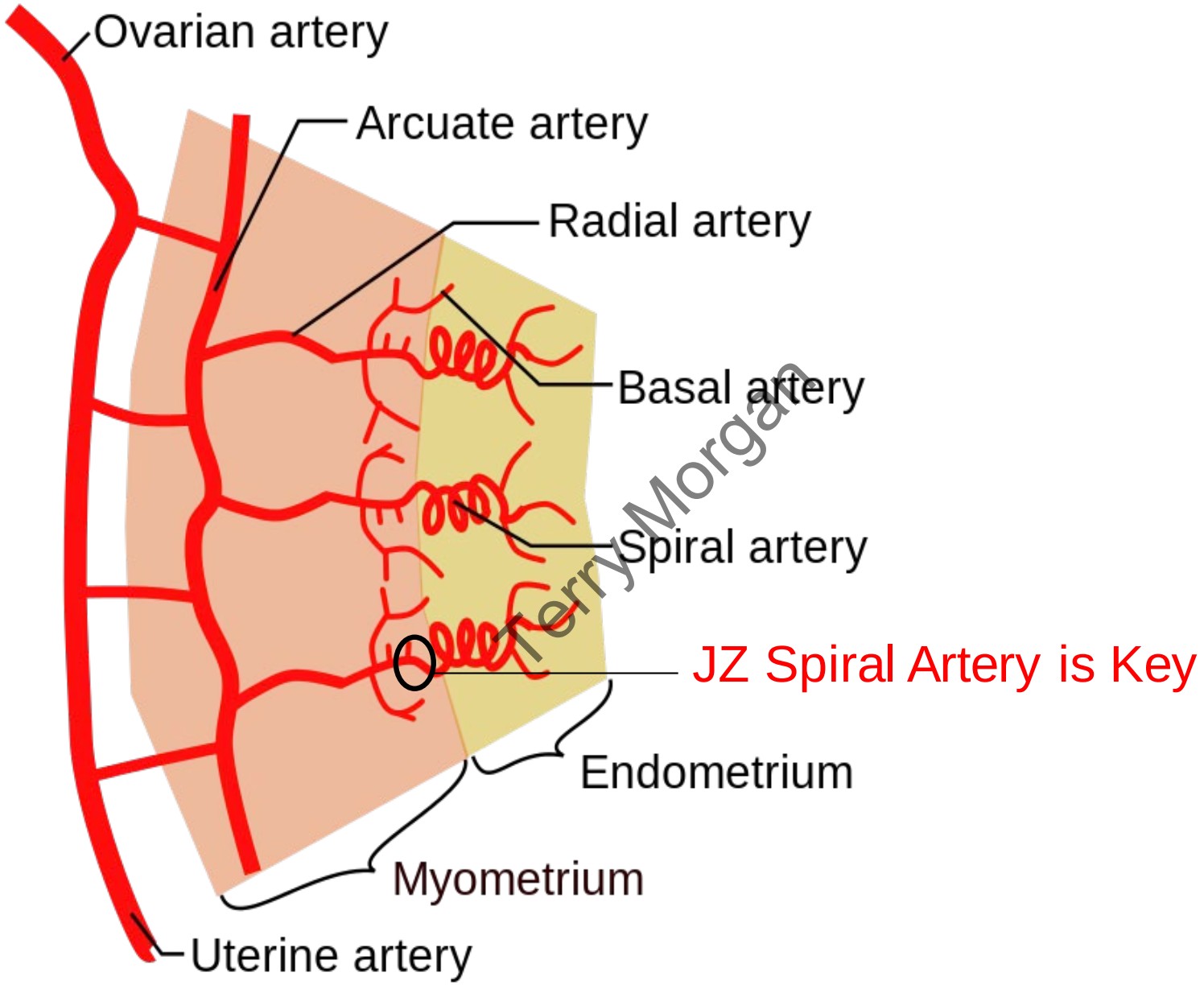
7 Weeks
Channels



10 Weeks
Wide Open



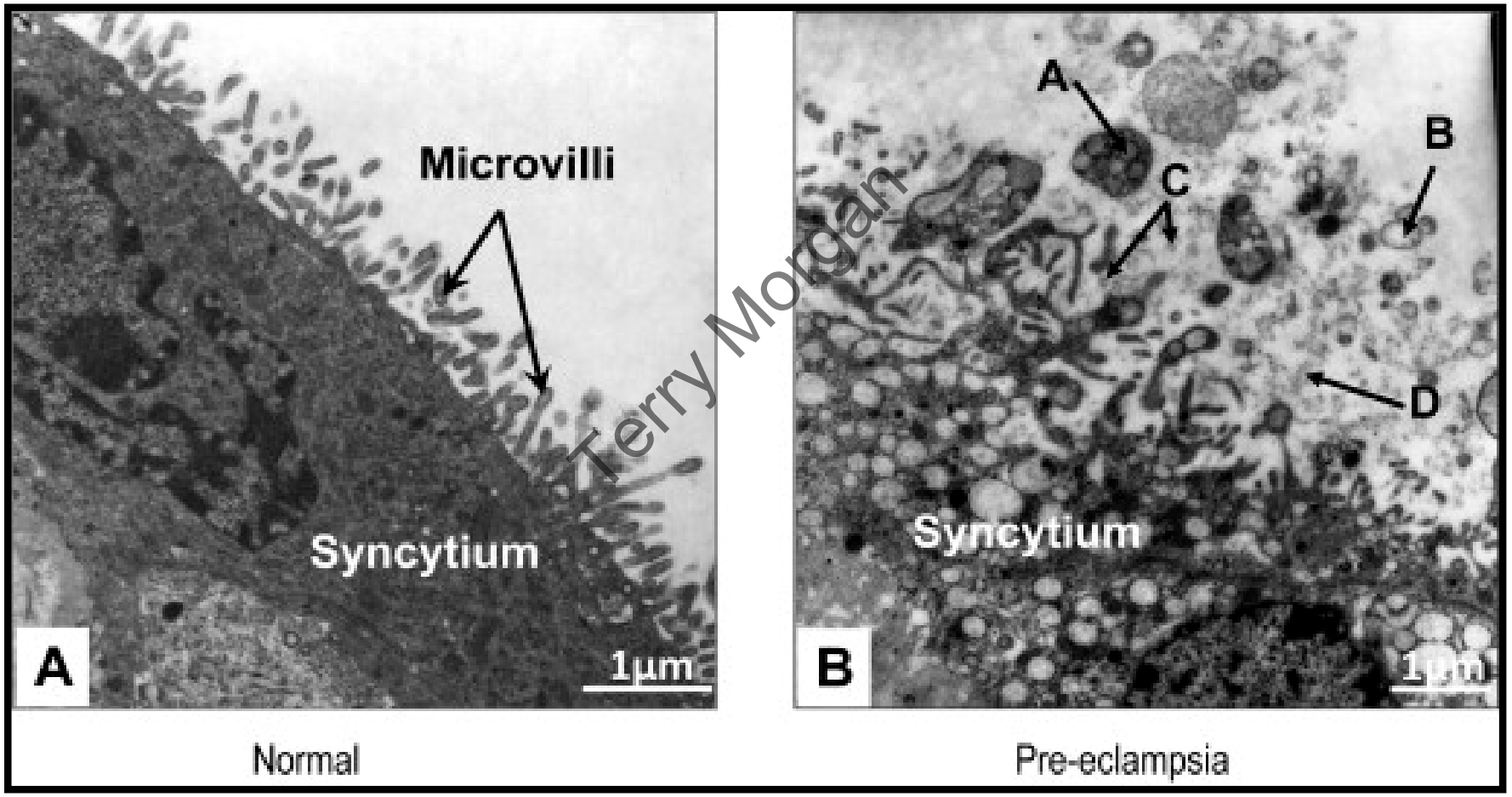
Roberts & Morgan, *et al*. Hum Reprod. 2017. 32(12): 2382-93



Allerkamp H, et al. Hum Reprod. 2021; 36(3): 571-86

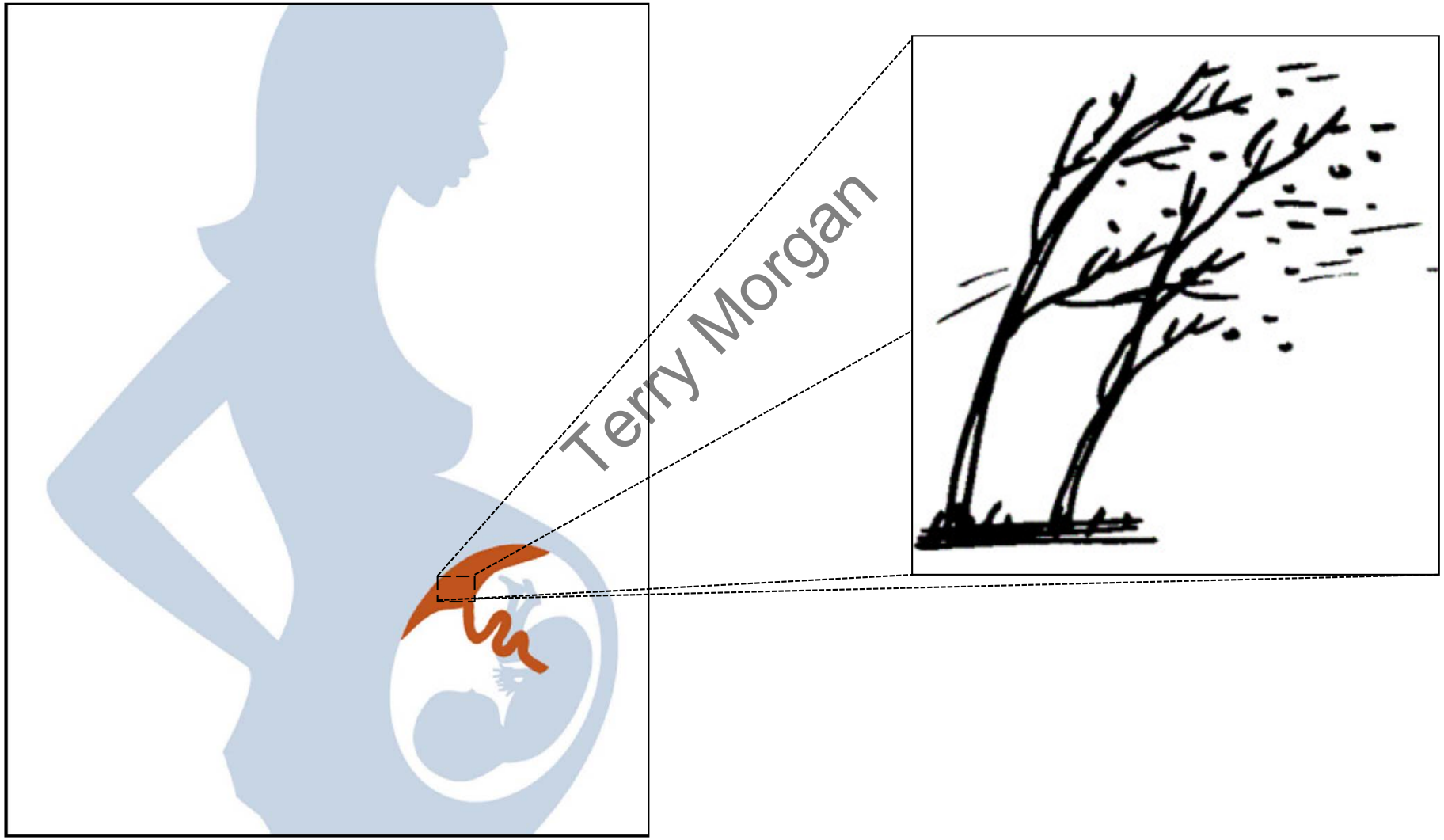
What is the Impact ?

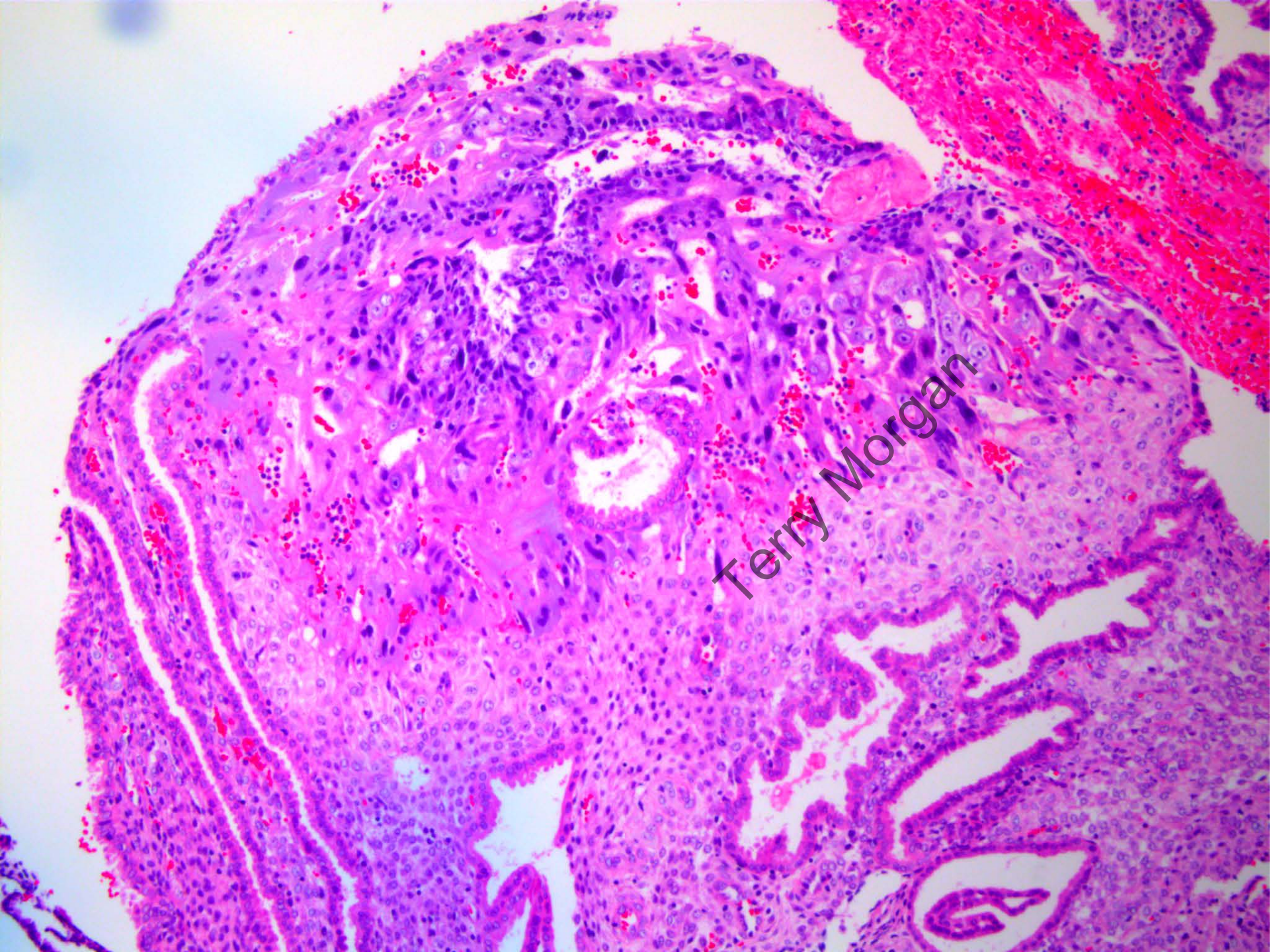
Maternal Vascular Malperfusion Causes Placental Damage



Placenta. 1980;1:61-76

Blood Sample Could Provide a "Liquid Biopsy of the Placenta"



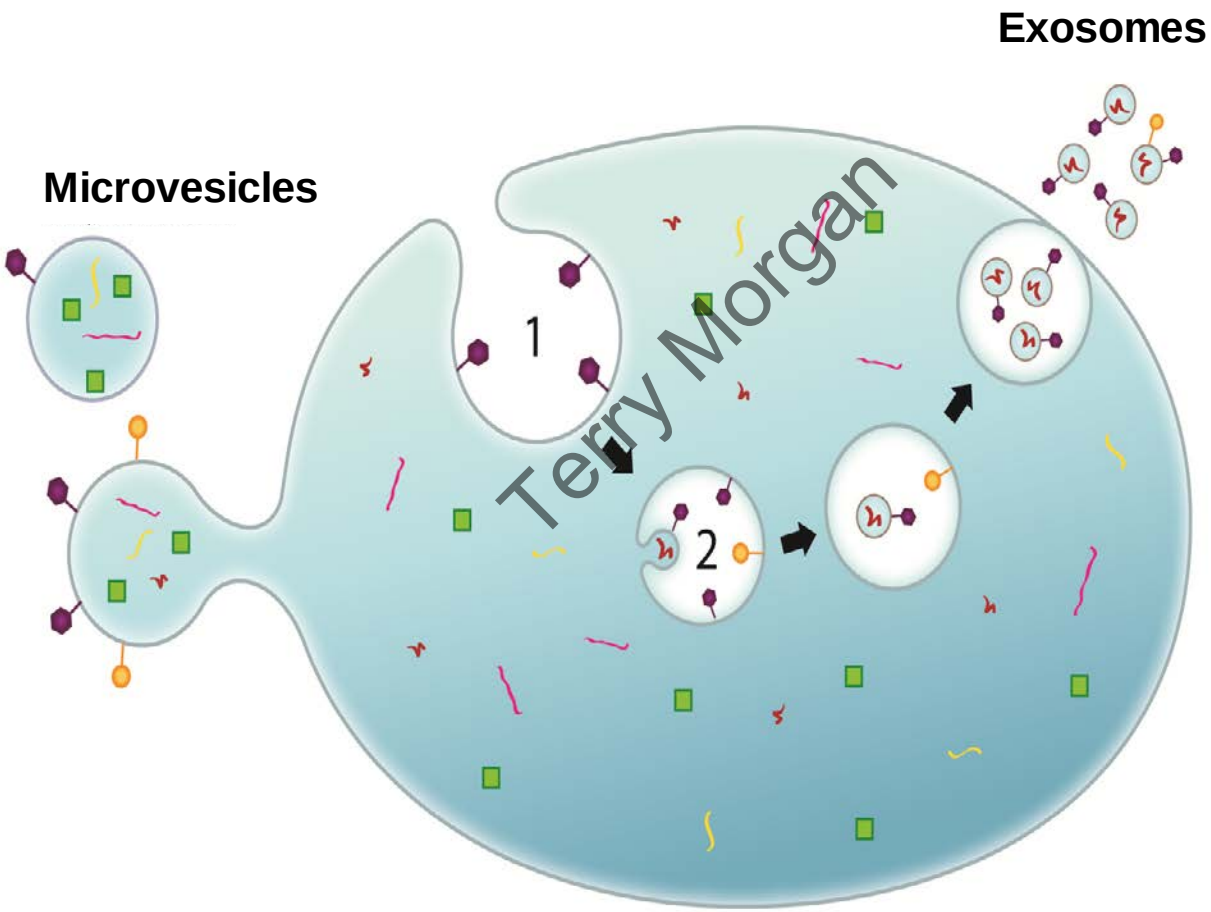


Placenta @ Day 10 Post Conception (1-2 mm in size)

hCG is detectable!

Are EVs detectable that may provide insights into health, microenvironment regulation, pregnancy outcomes and DOHaD?

Extracellular vesicles (EVs) are abundant (~10^{6-8}/ml) and maintain cell of origin surface markers

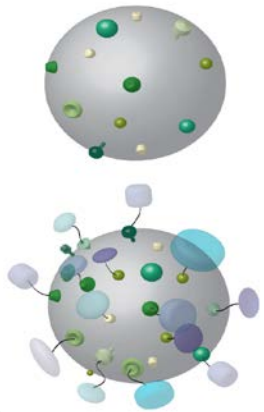


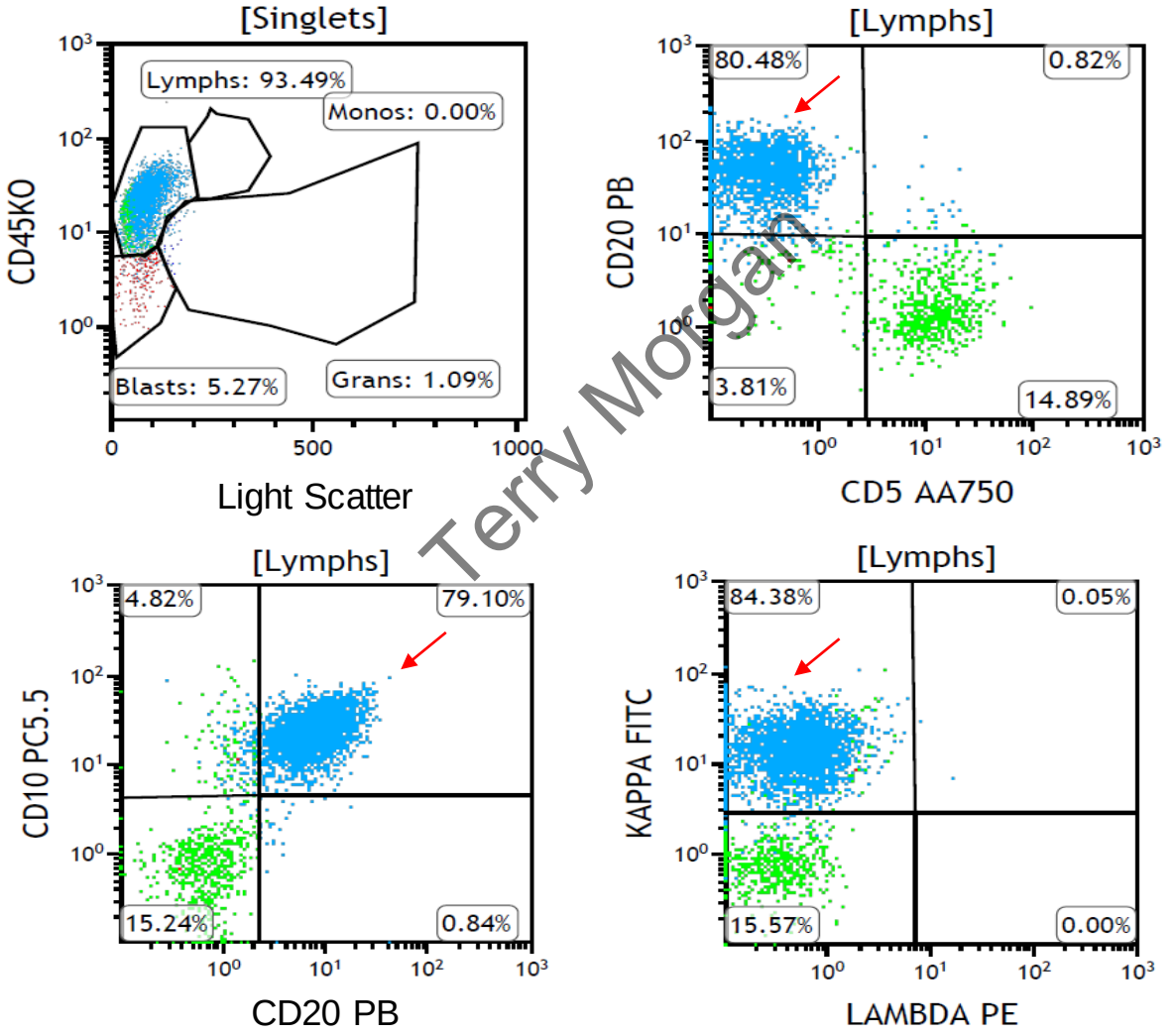
(*Trans Res*. 2018; 201:40-48. *AJP-Cell Phys*. 2019; 316:C264-73 **Nat Commun*. 2019; 10(1):4682.)

Flow cytometry is highly sensitive and specific

Antibody Panel

1. CD45
2. CD20
3. CD5
4. CD10
5. Kappa
6. Lambda

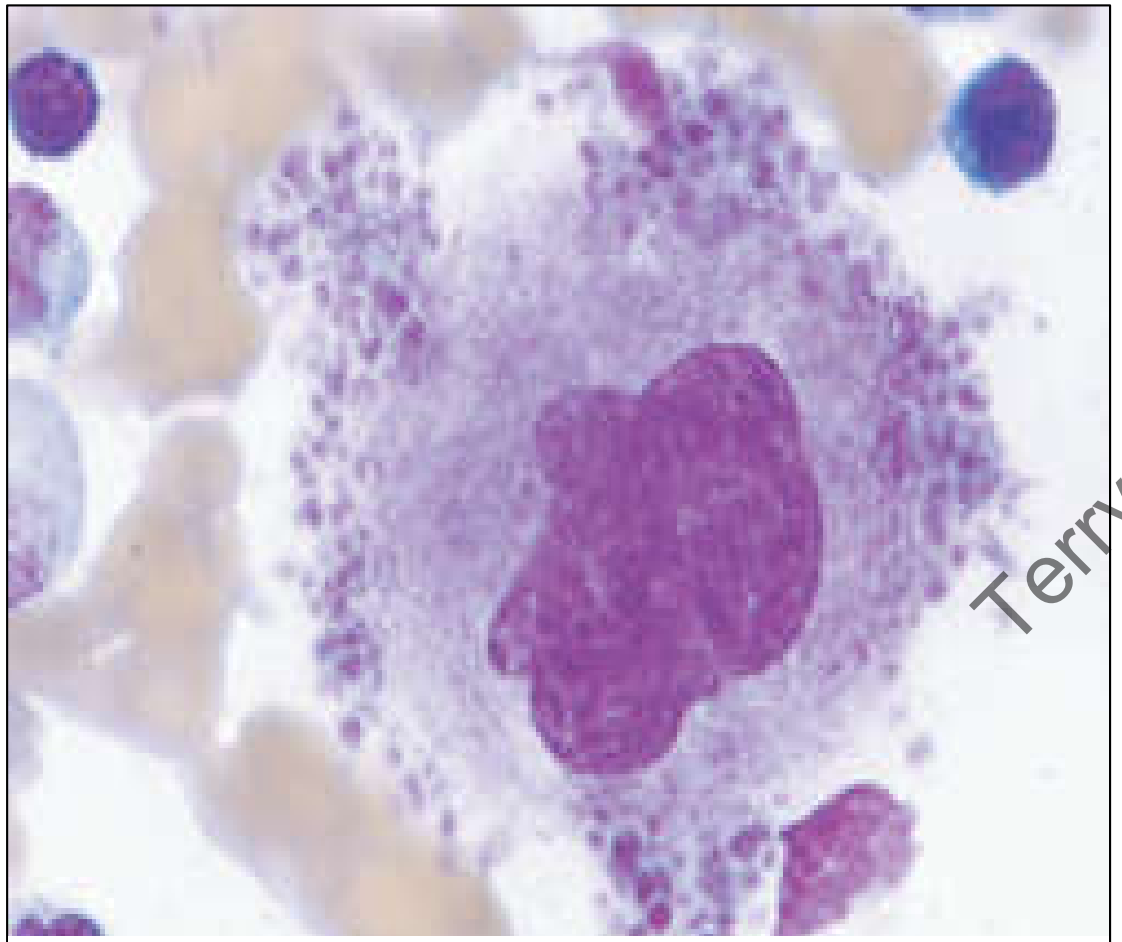




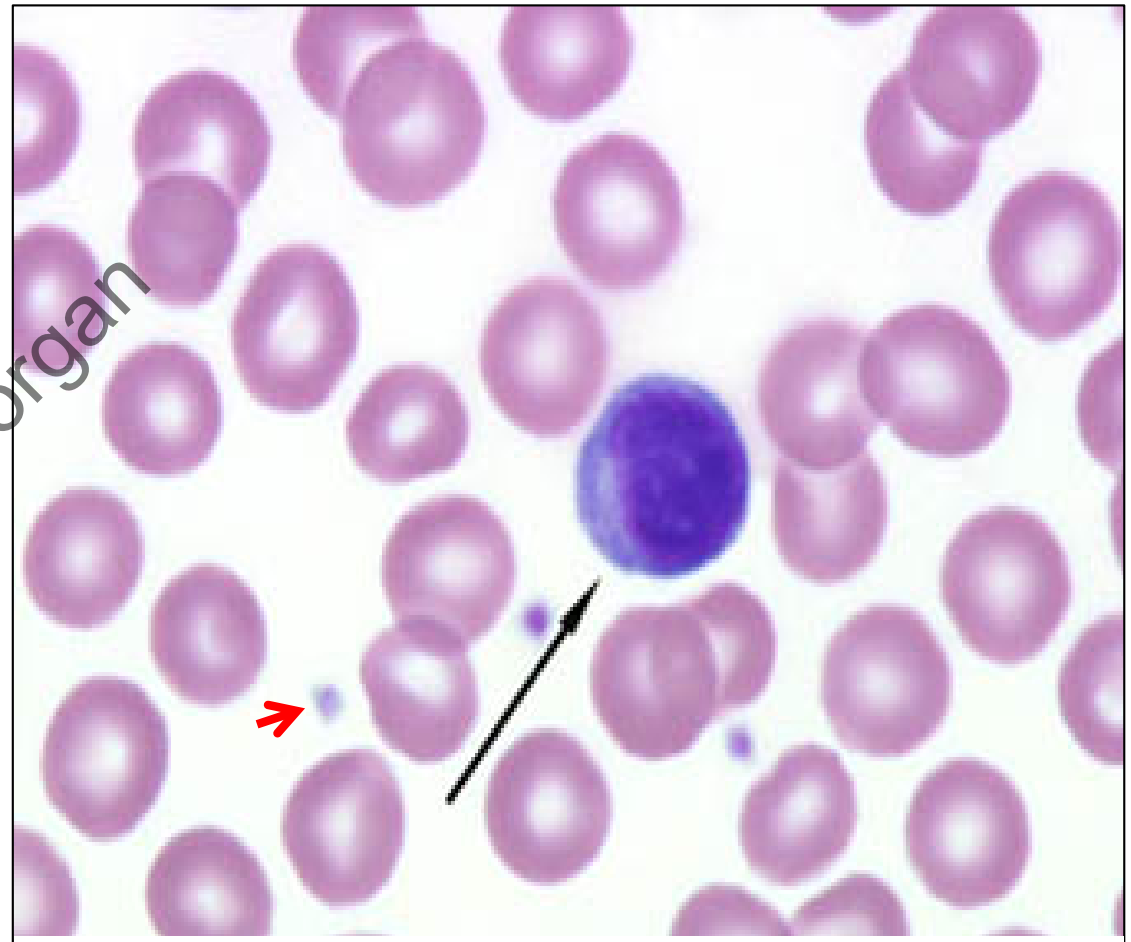
(e.g. lymphoma cells [arrows] in this biopsy)

<u>THE PROBLEM</u>: Circulating target cells are rare (<1/ml)

Extracellular Vesicles (EVs) are Small

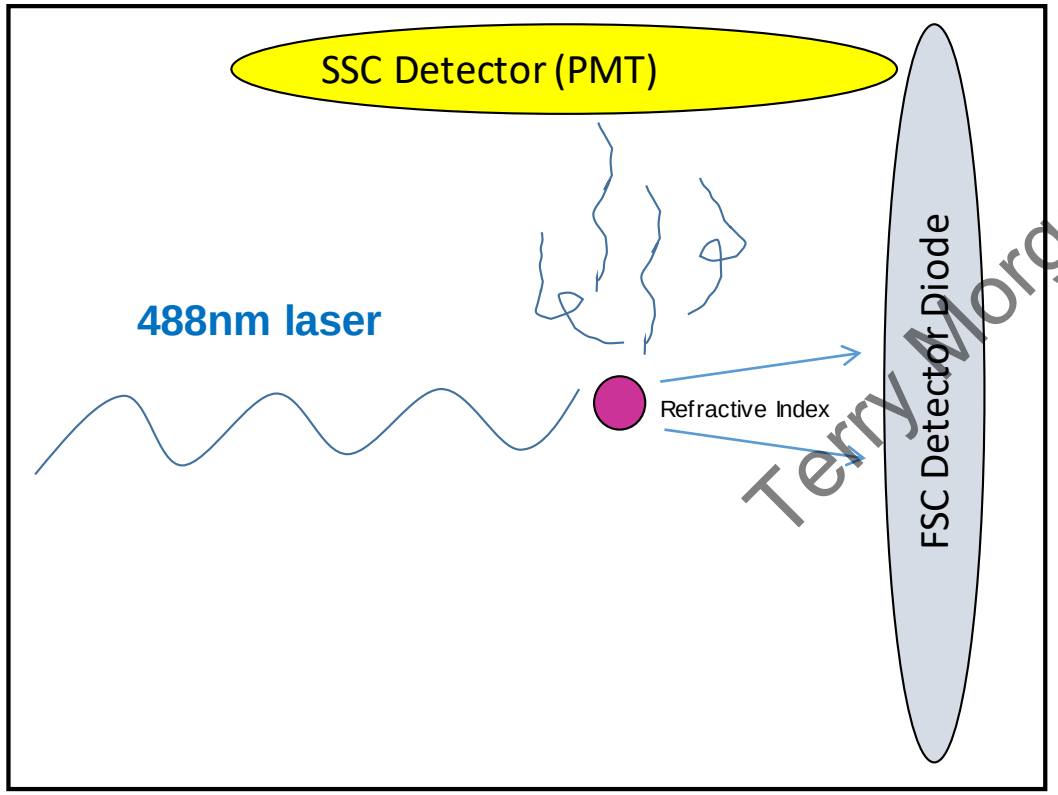


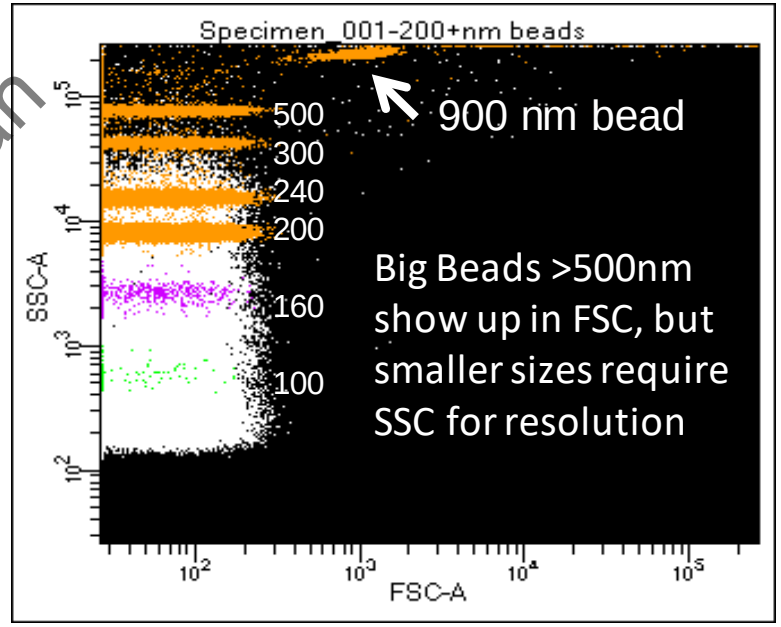
Platelets released from Megakaryocyte



Platelets are small (~ 1-3 um), but 10x bigger than EVs

Nanoscale Flow Cytometry
Side Scatter (SSC) is the Key

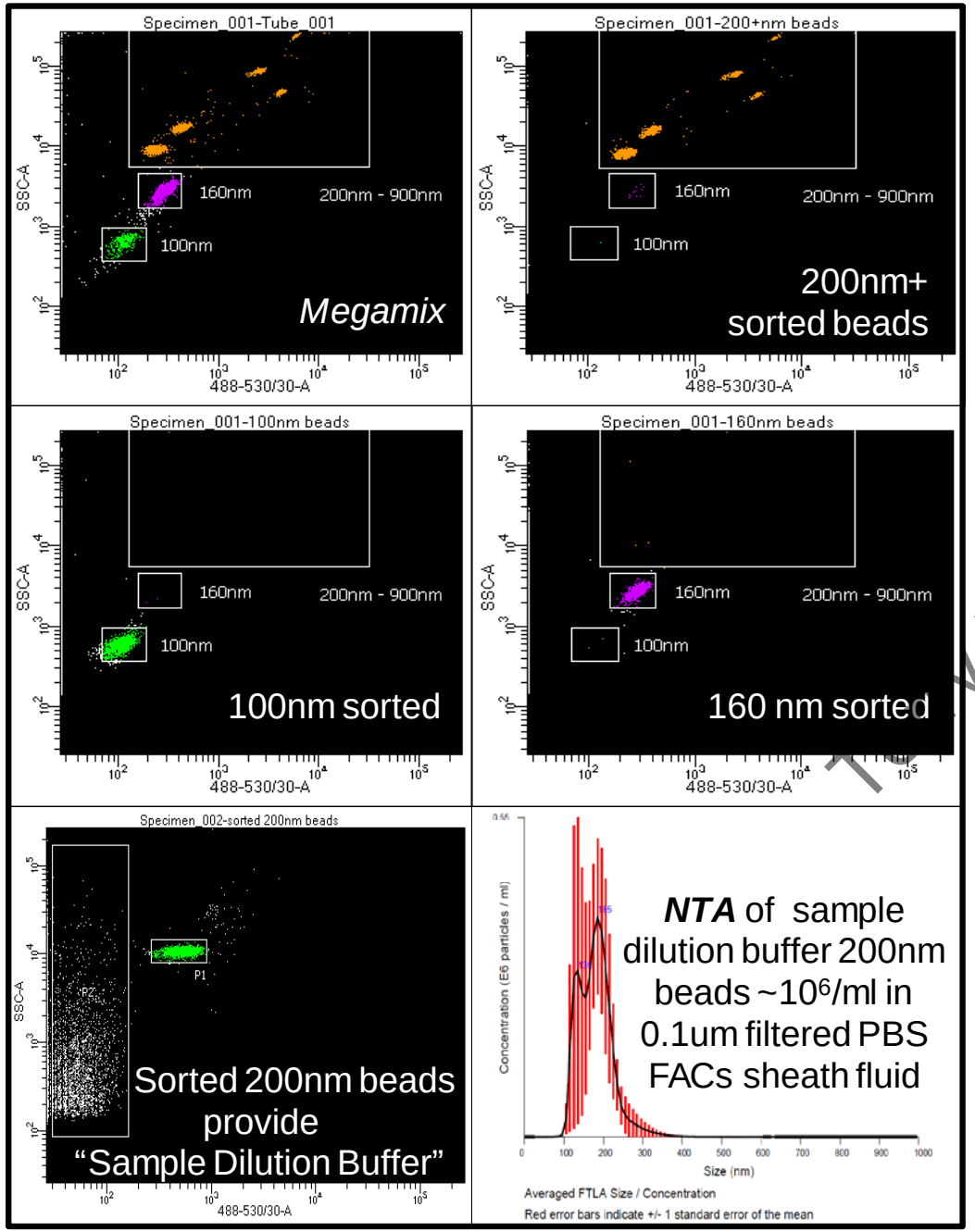


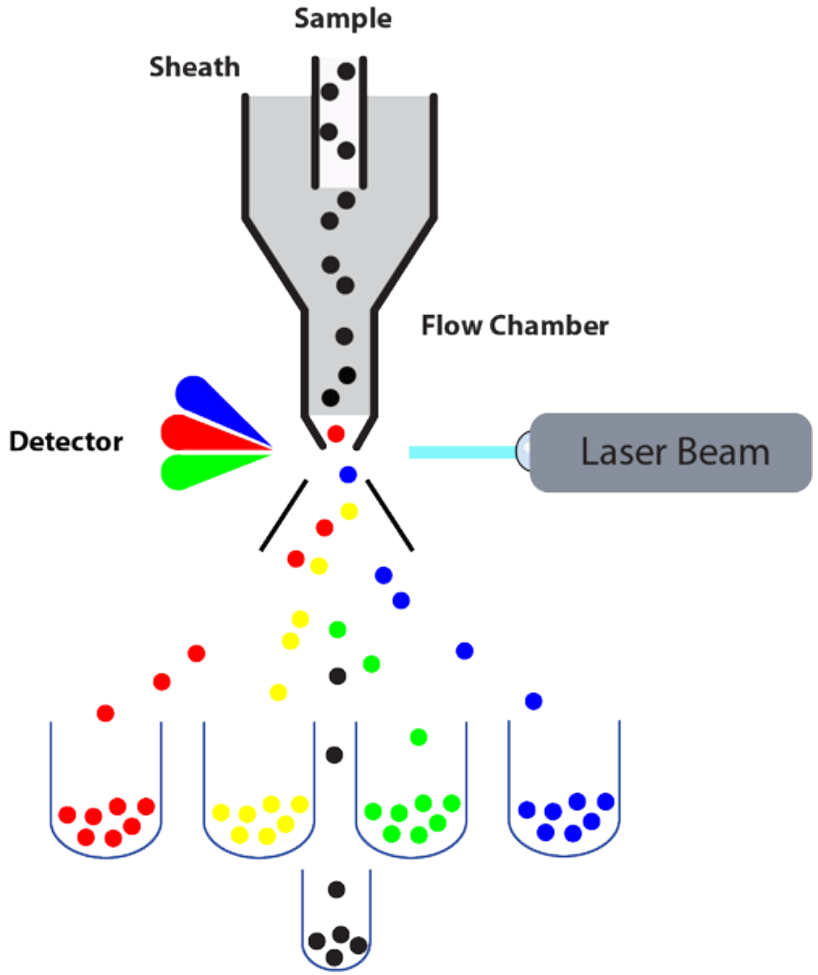


Light Scatter = $(2\pi^5/3)(D^6/\lambda^4)\,(RI^2\text{-}1/RI^2\text{+}2)^2$

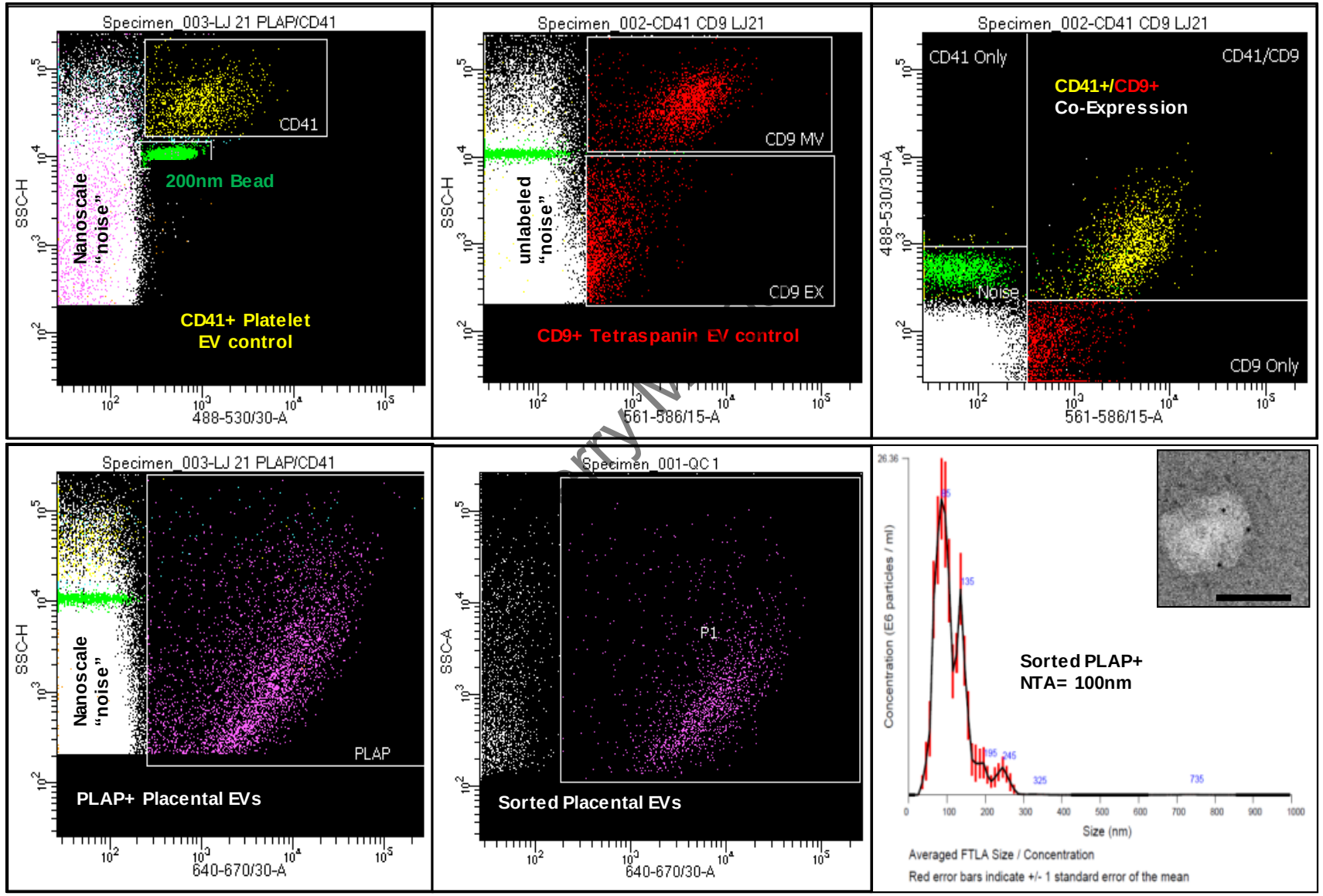
Refractive Index of Cell- and Size-specific EVs is currently a mystery

Validation by Gated Flow Sorting



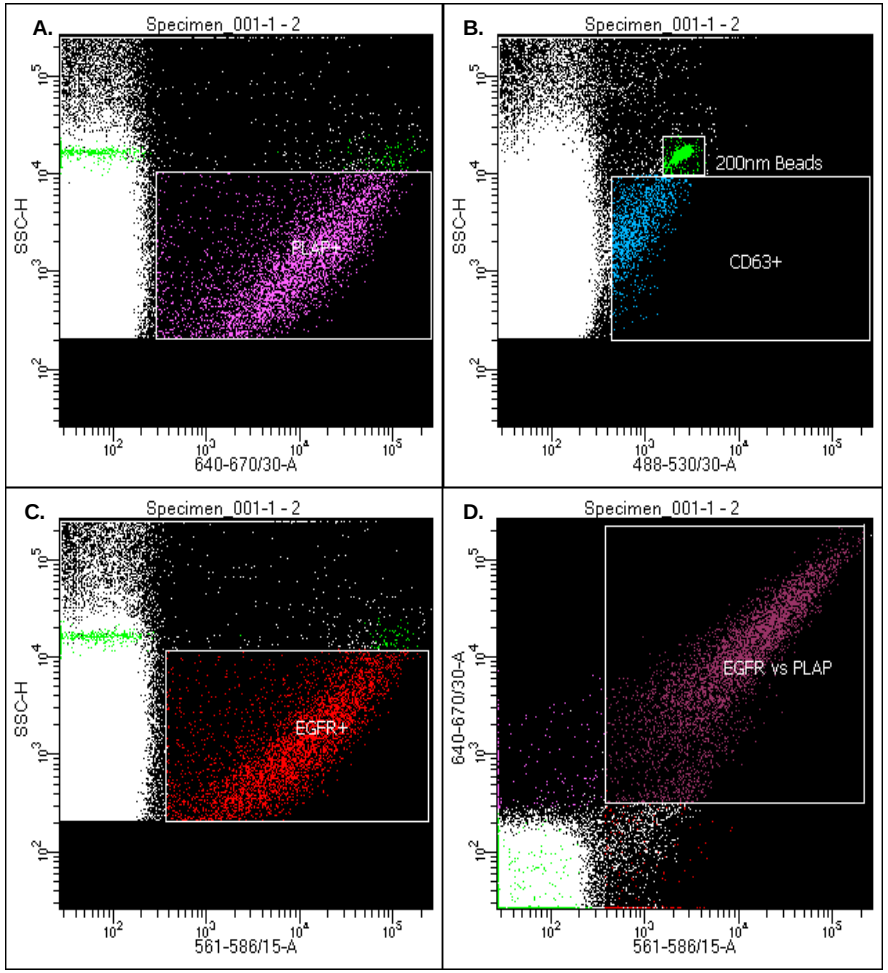


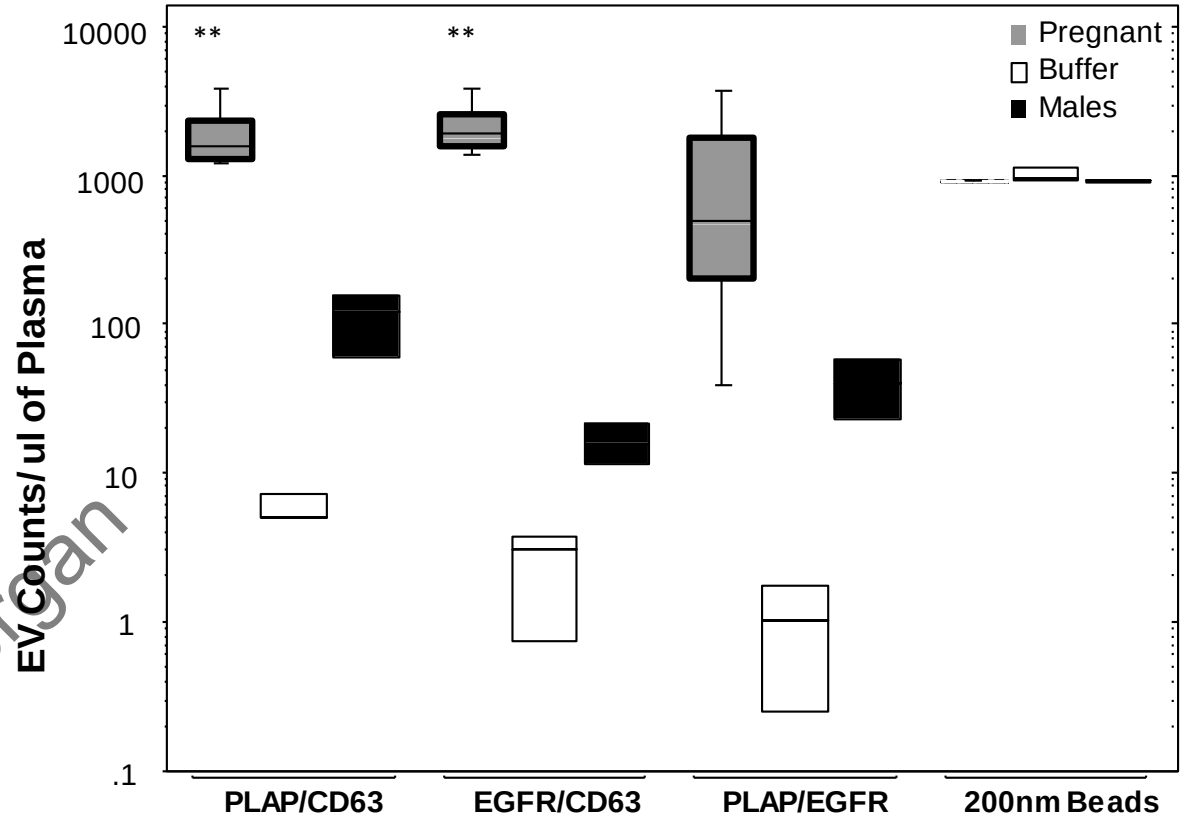
Internal Controls and Target Validation



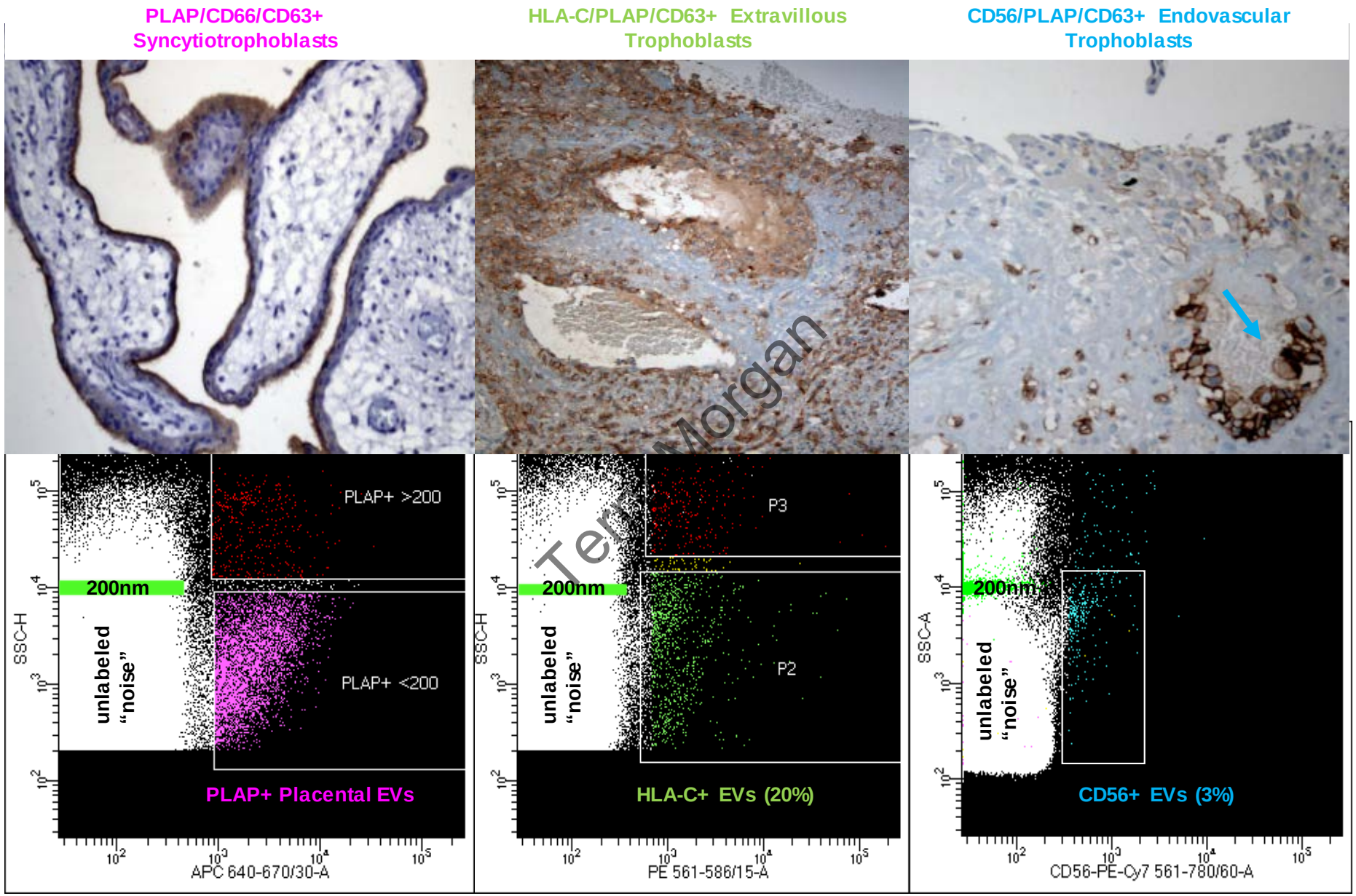
Adenoviral transduction of EGFR into pregnancy-adapted uterine artery endothelial cells remaps growth factor induction of endothelial dysfunction

Luca Clemente[a], Derek S. Boeldt[a], Mary A. Grummer[a], Mayu Morita[b], Terry K. Morgan[b], Greg J. Wiepz[c], Paul J. Bertics[d], Ian M. Bird[a,e,*] (Mol & Cell Endocrinol. 2020; 499:110590)

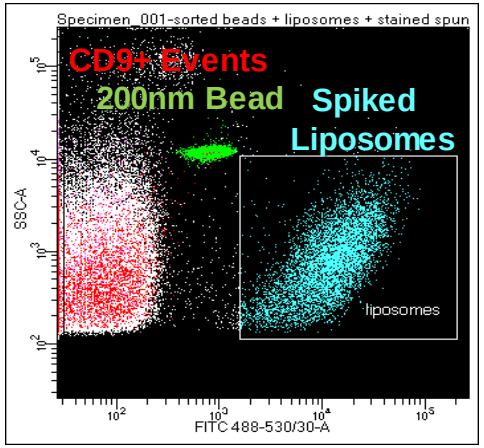


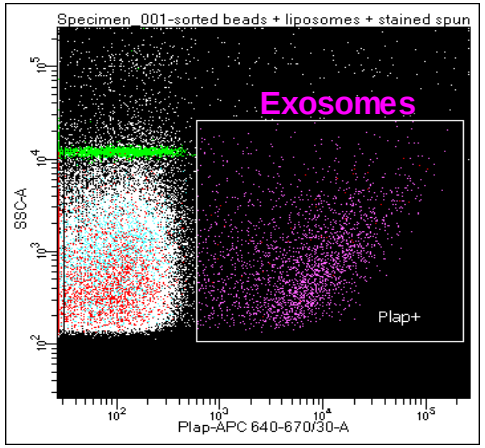


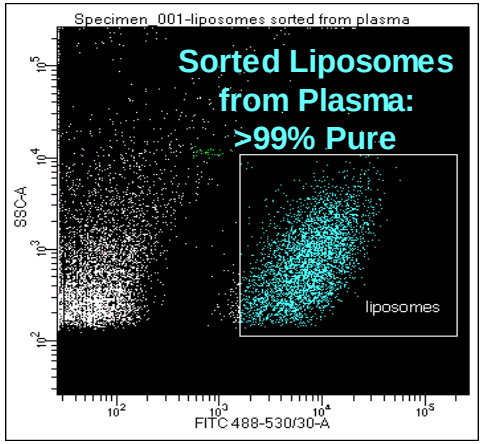
Extracellular Vesicles

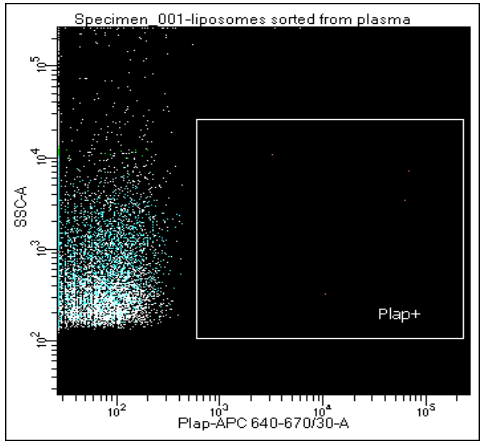


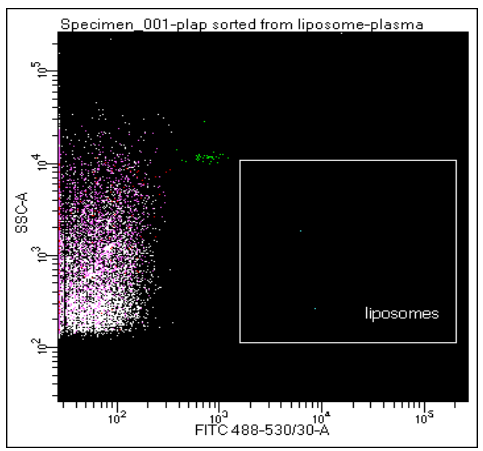
Morgan T & Myatt L: in preparation

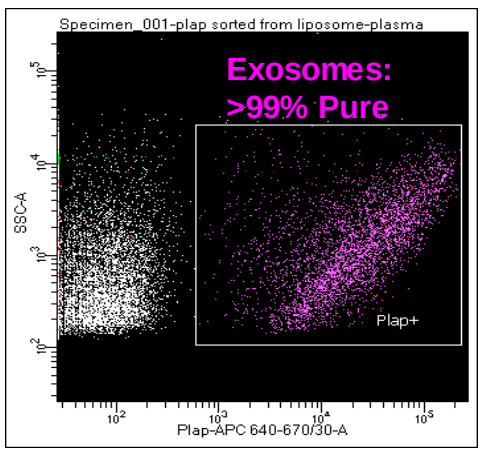


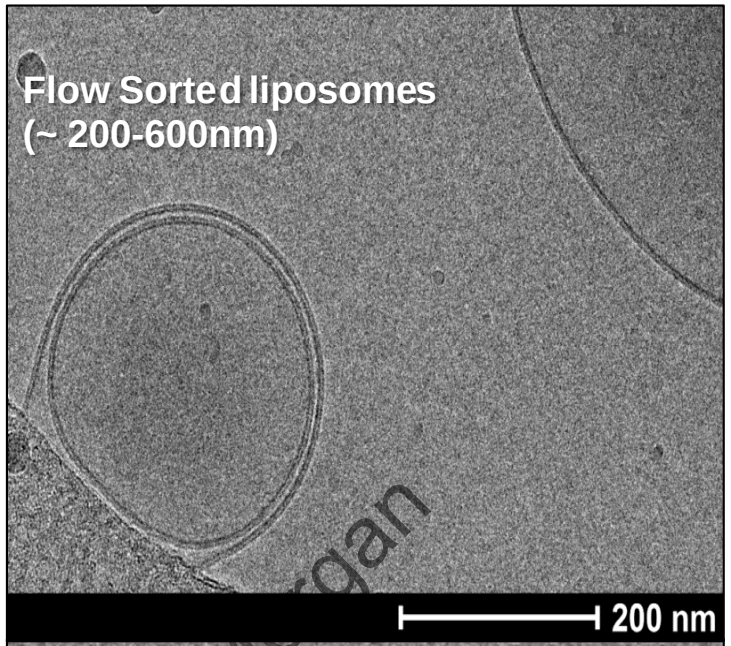


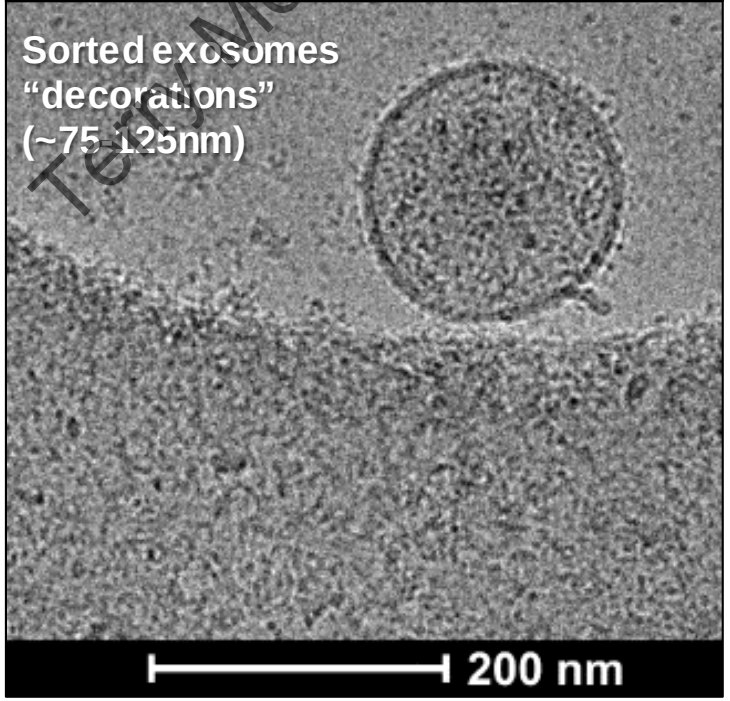










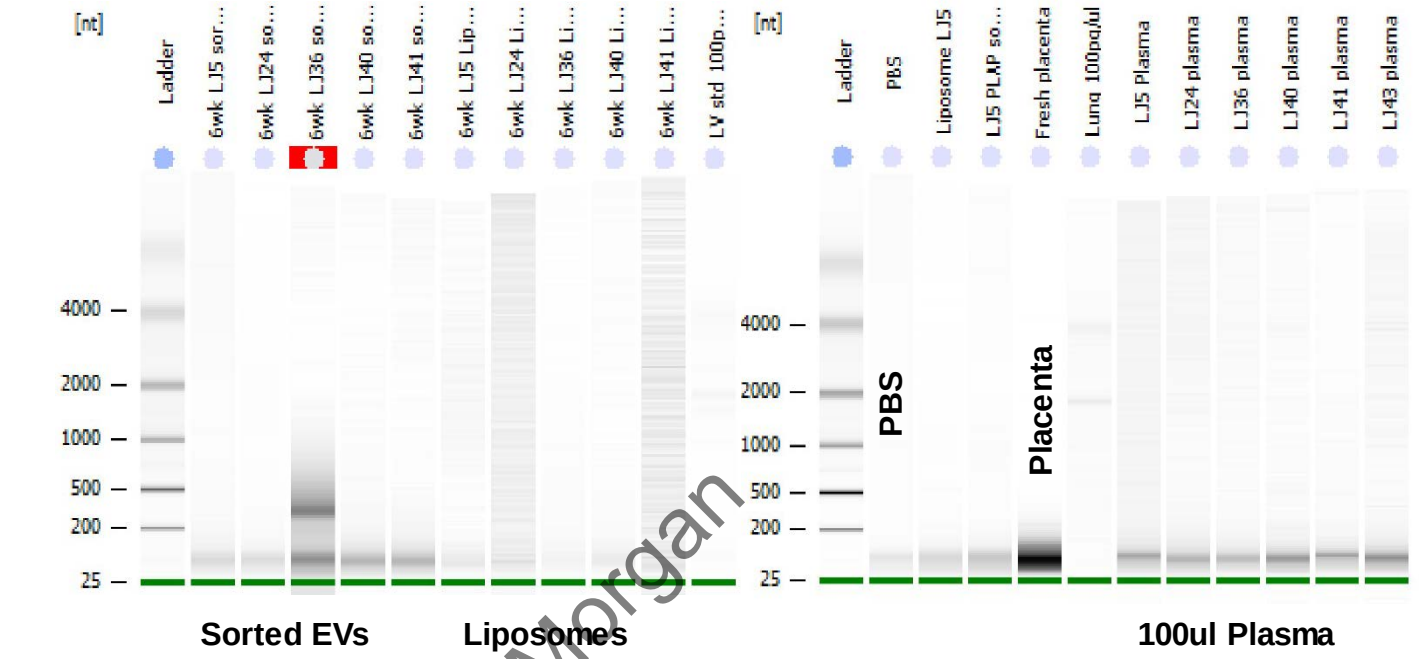


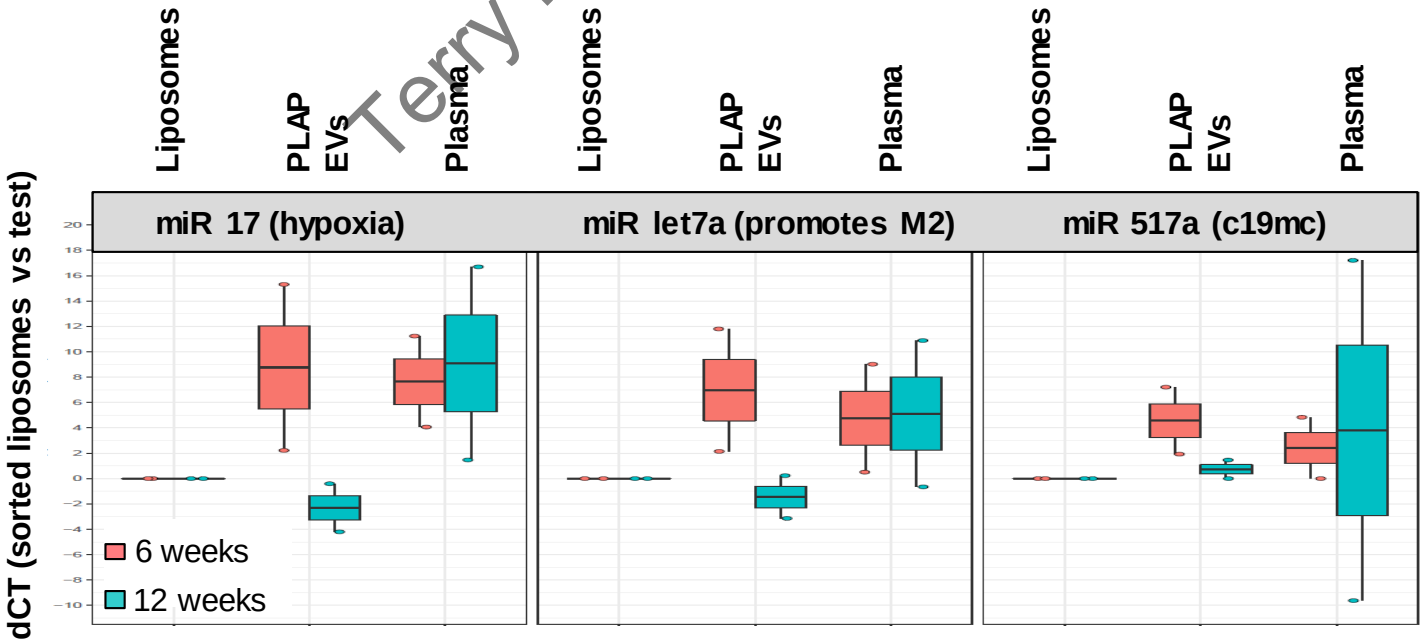
Liposomes are larger than expected relative to 200nm bead (polystyrene) because they have ~1.3 RI

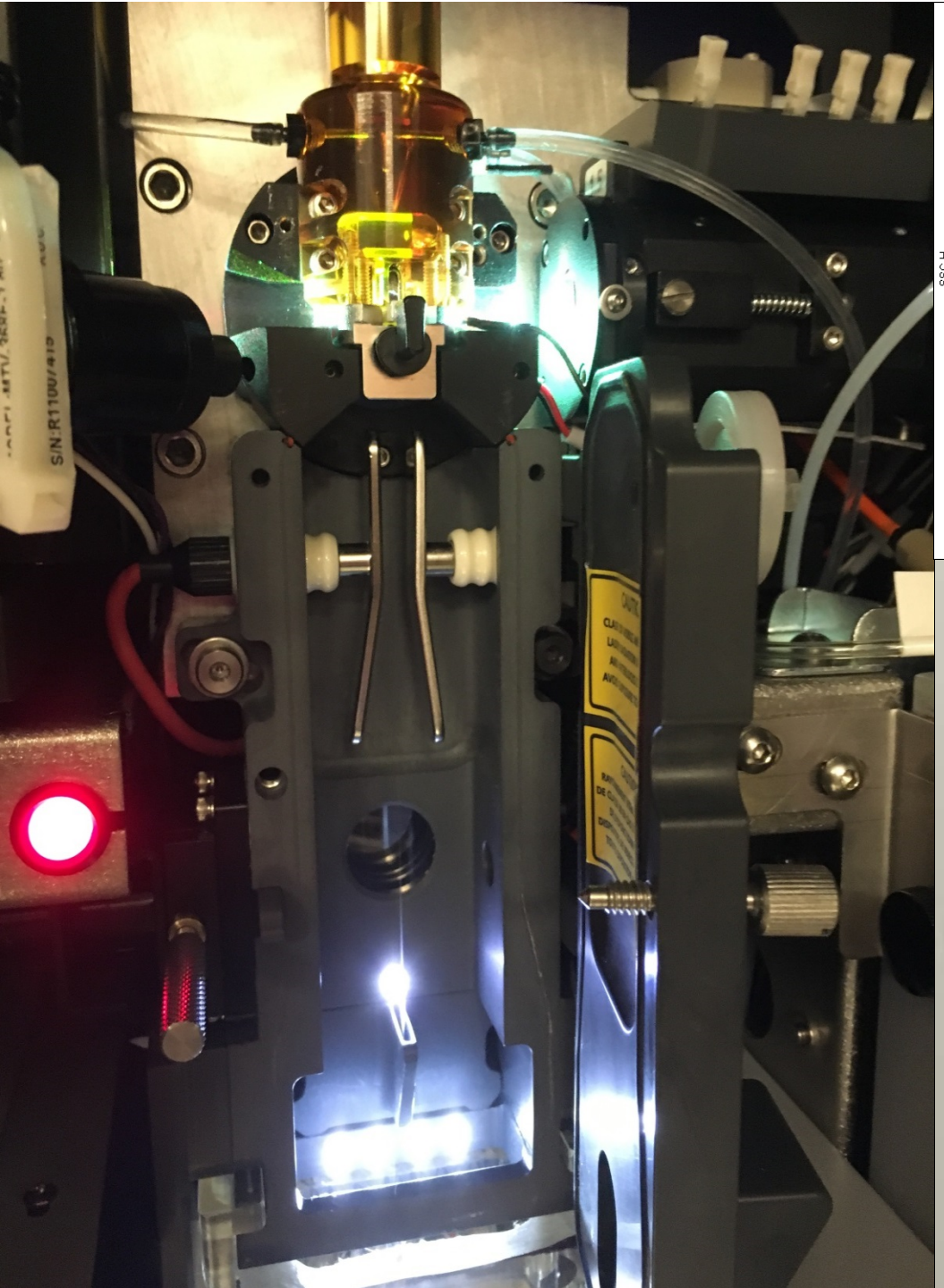
In contrast, flow sorted "exosomes" appear to be ~ 100nm in size

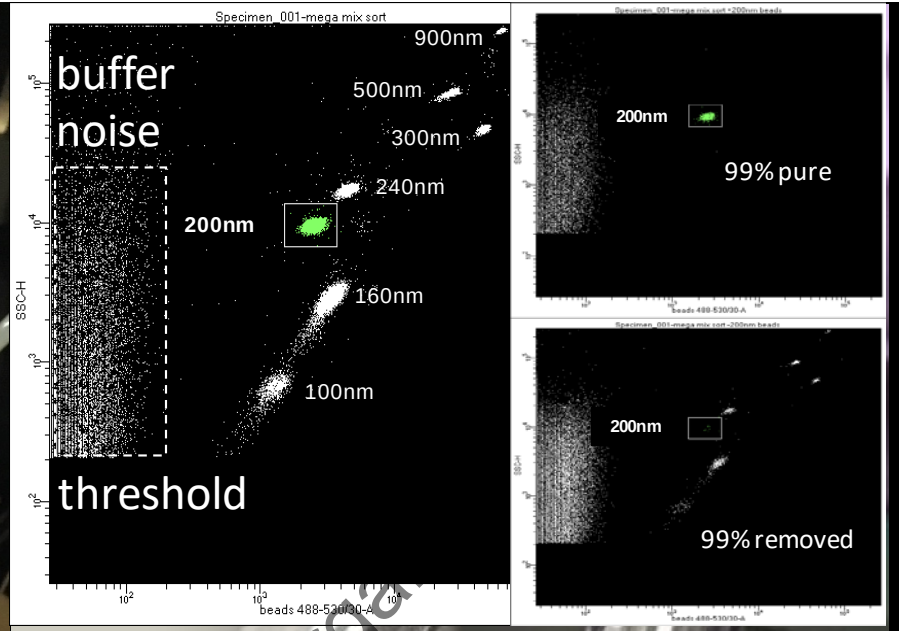
Could **dense exosomes** have RI > a cell & more like a virus ~1.5?

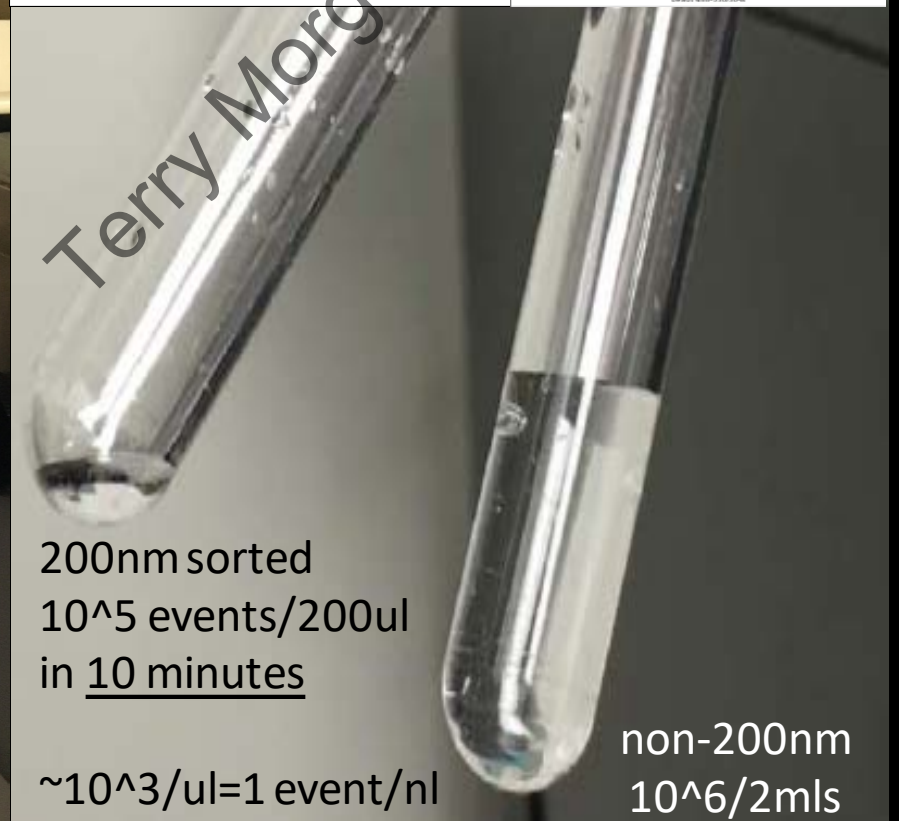
miRNA Analysis









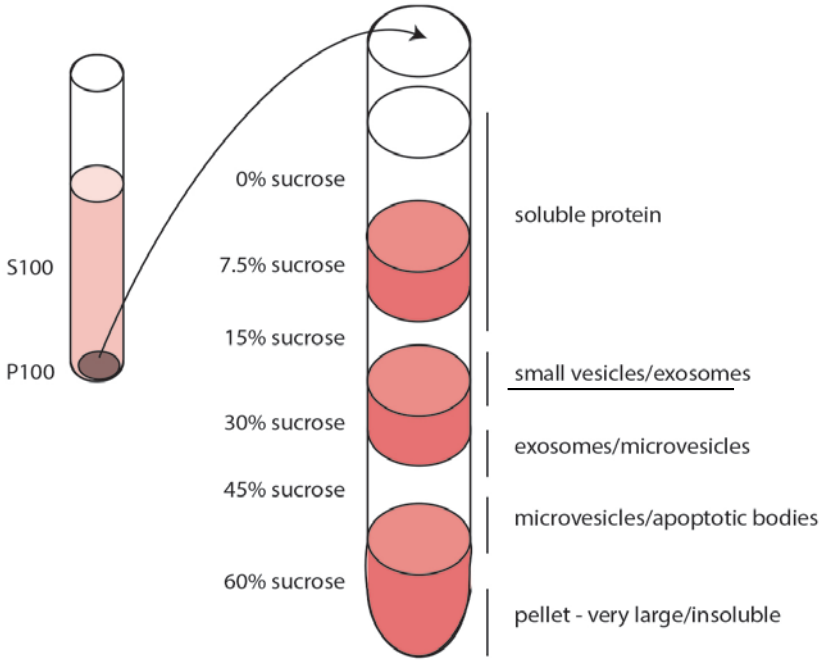


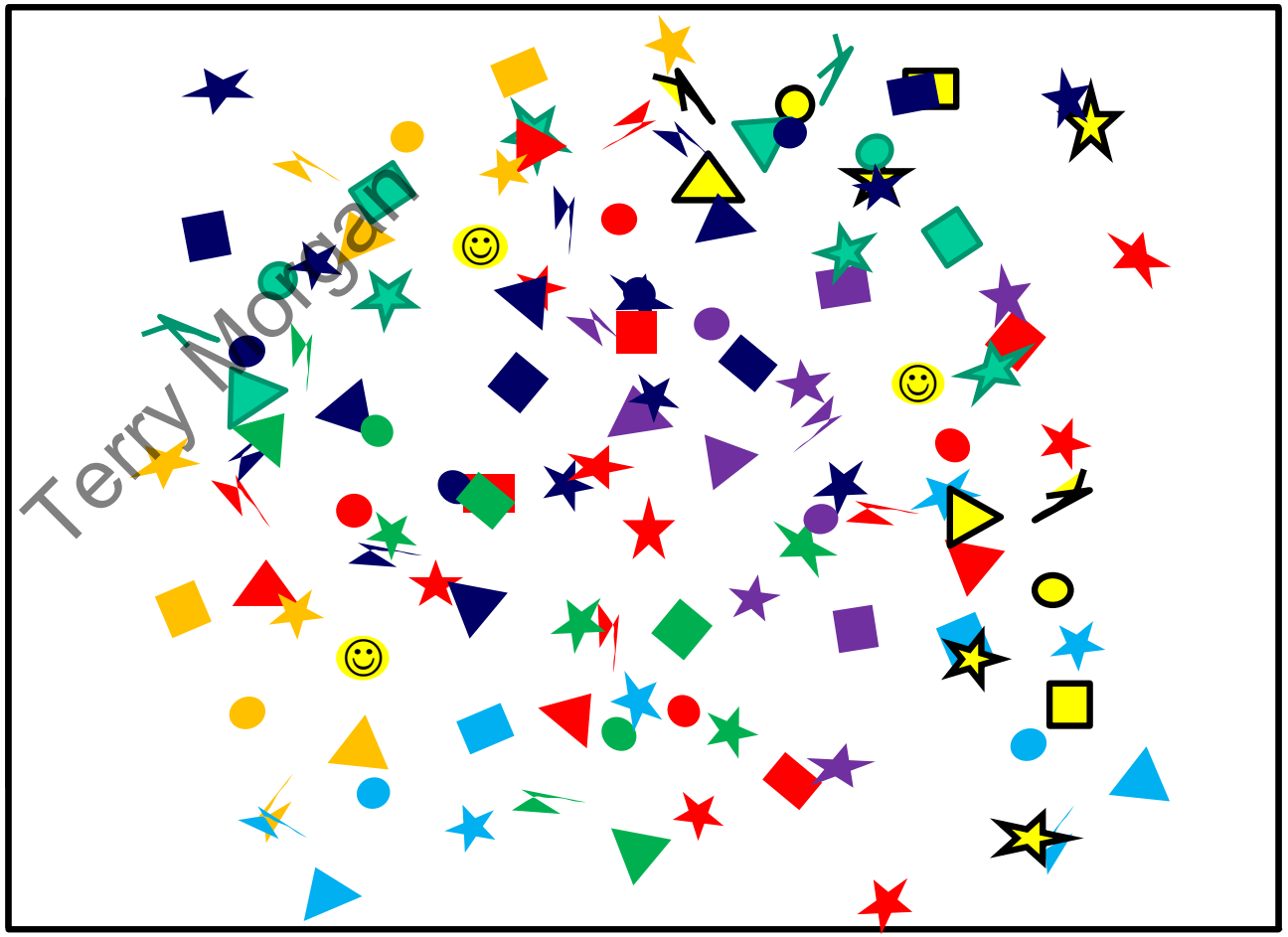
EV Target
Dilution in PBS
(~ 1 event/drop)

Major Limitation

DENSITY GRADIENT ULTRACENTRIFUGATION

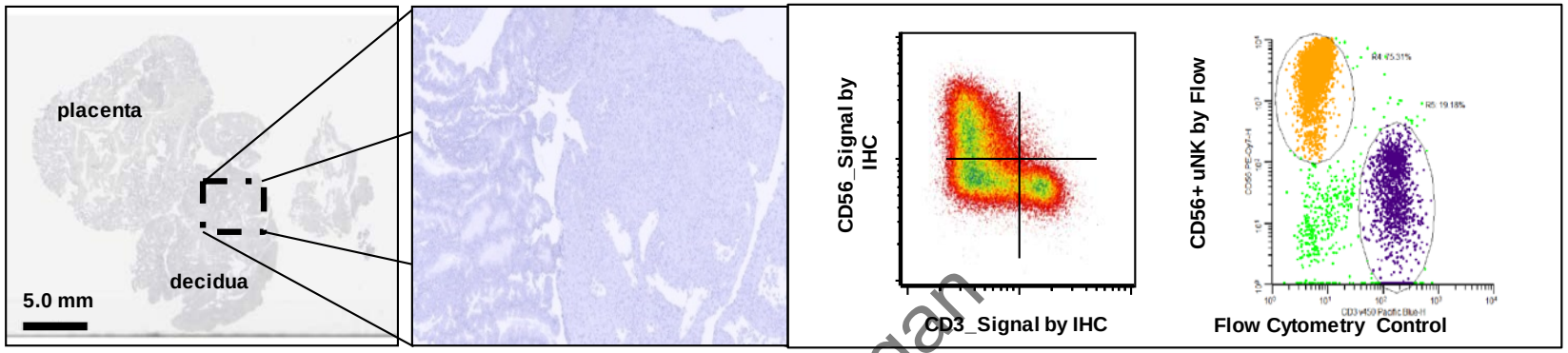
Most of the “exosome” fraction (1.12-1.19 g/ml) is not cell-specific

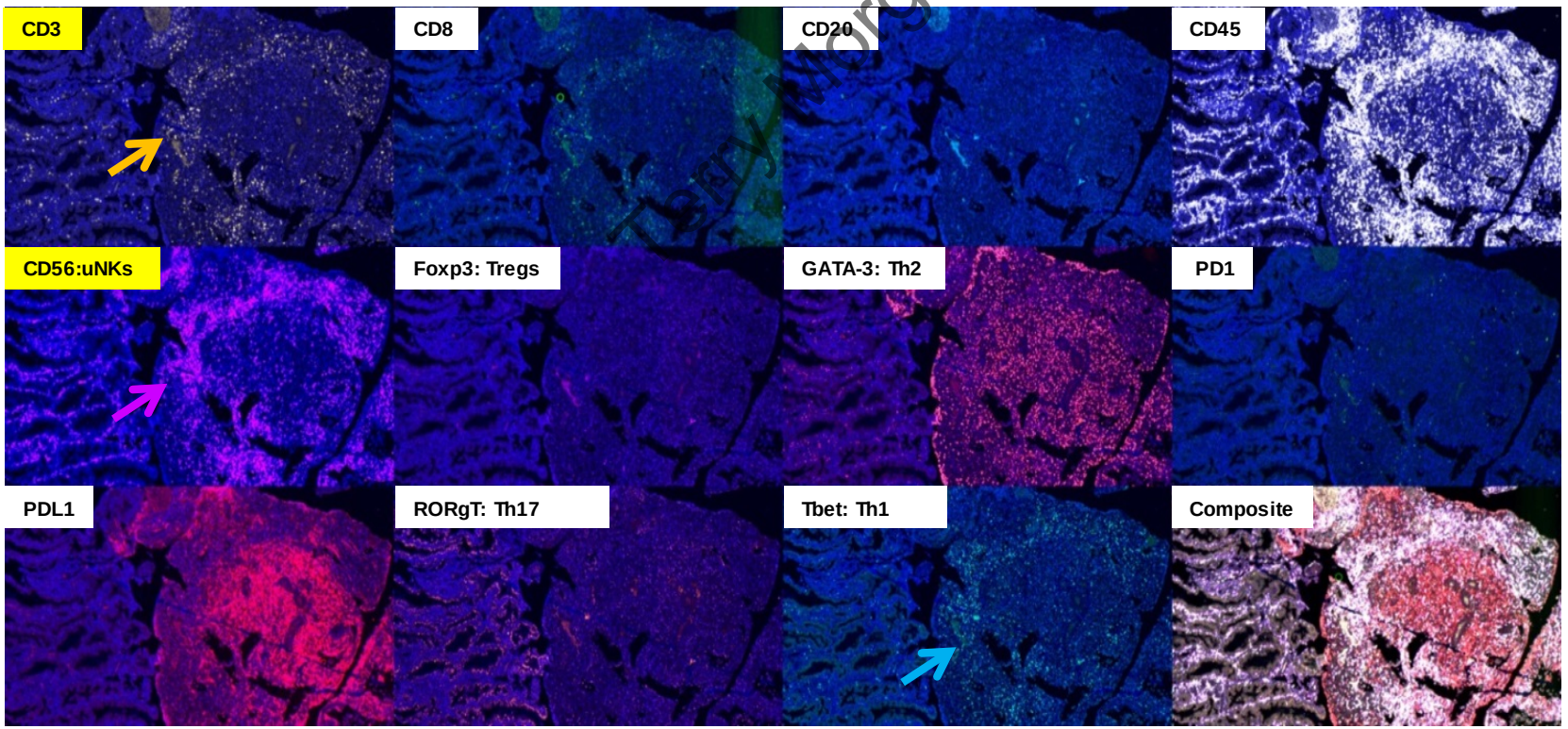




Transl Res. 2018 Nov;201:40-48.

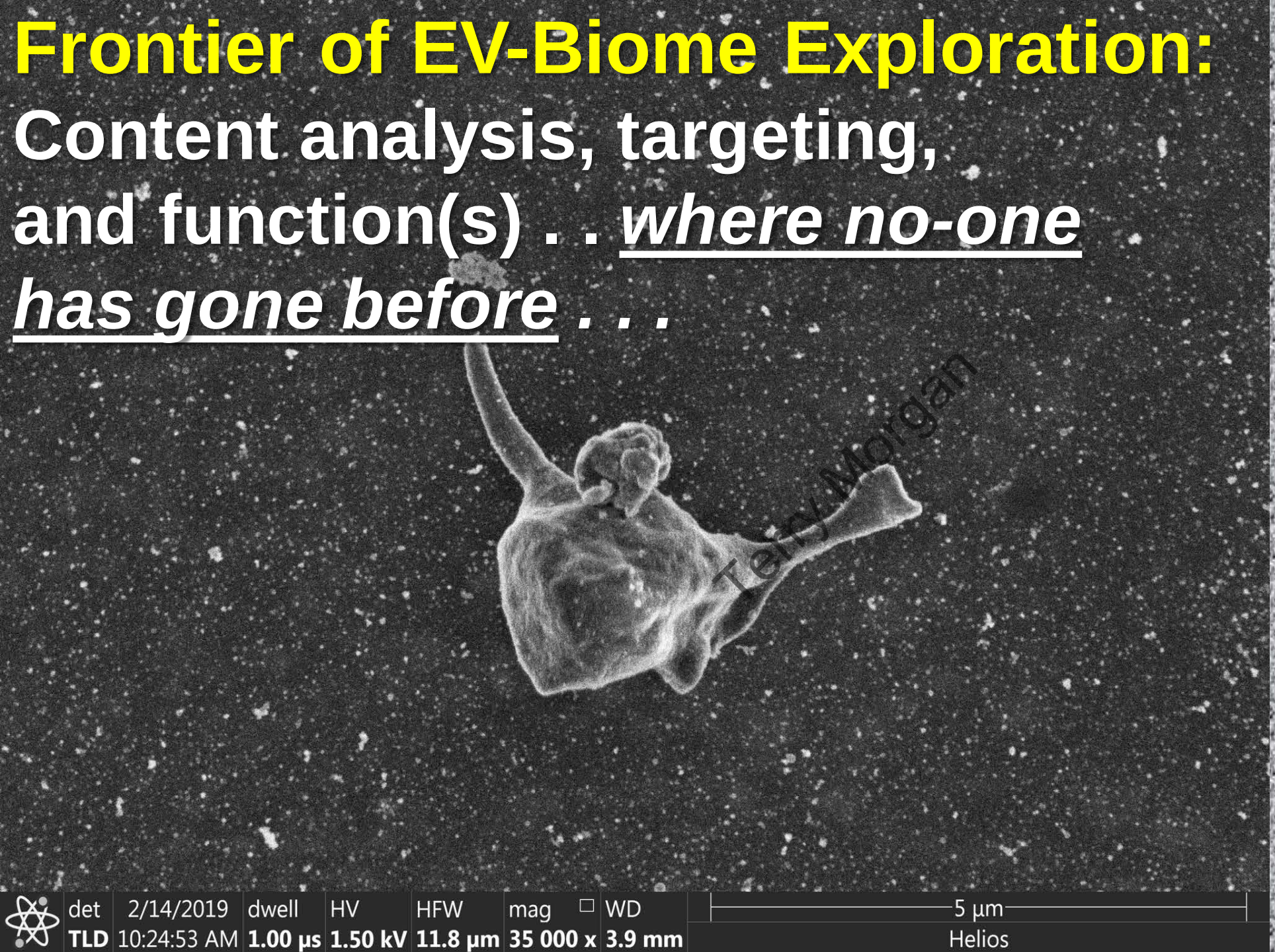
“Tumor” Microenvironment





Frontier of EV-Biome Exploration:

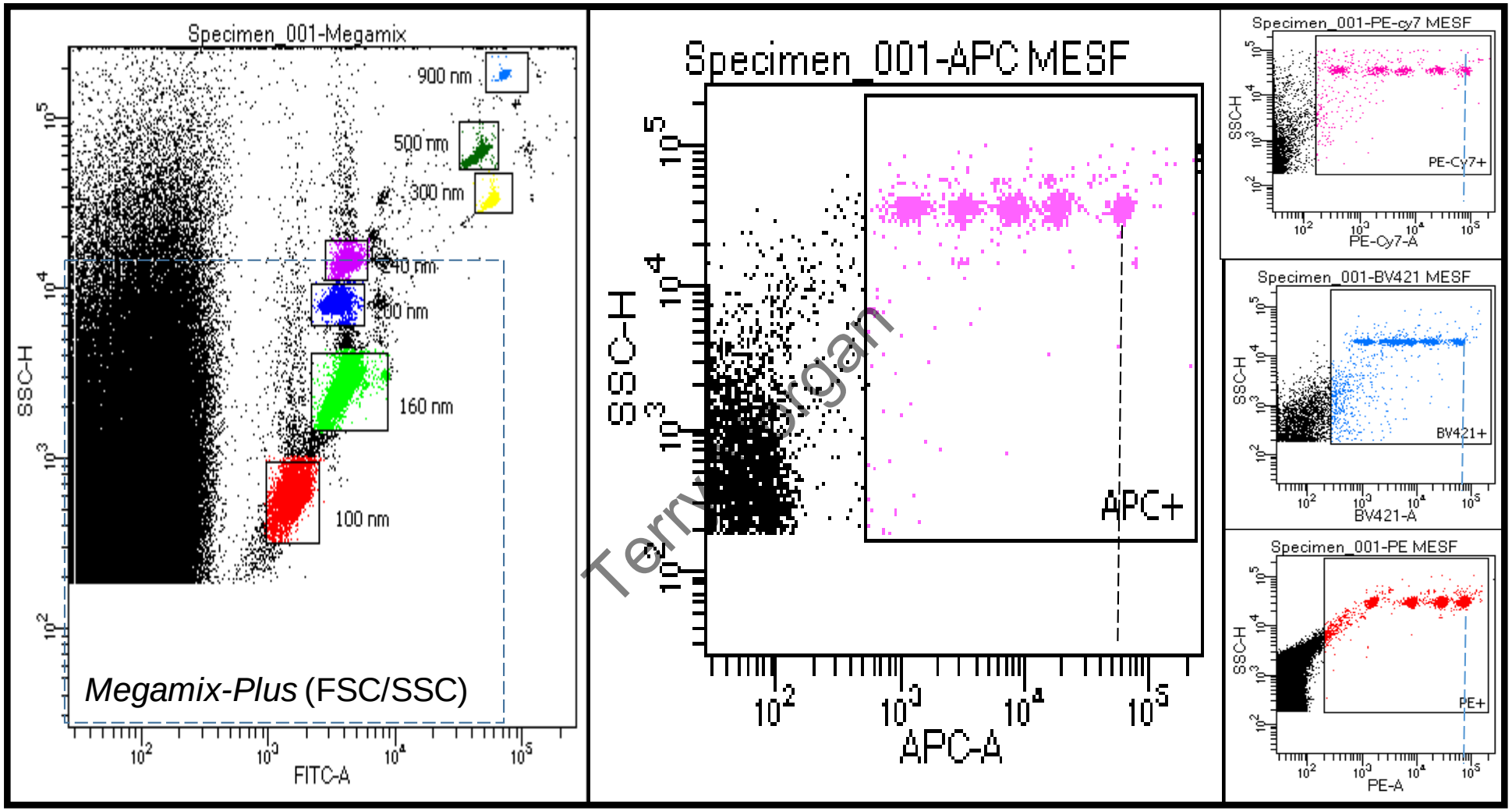
Content analysis, targeting, and function(s) . . *where no-one has gone before* . . .




Questions
Please
Terry Morgan

Nanoscale Flow Cytometry Standardization

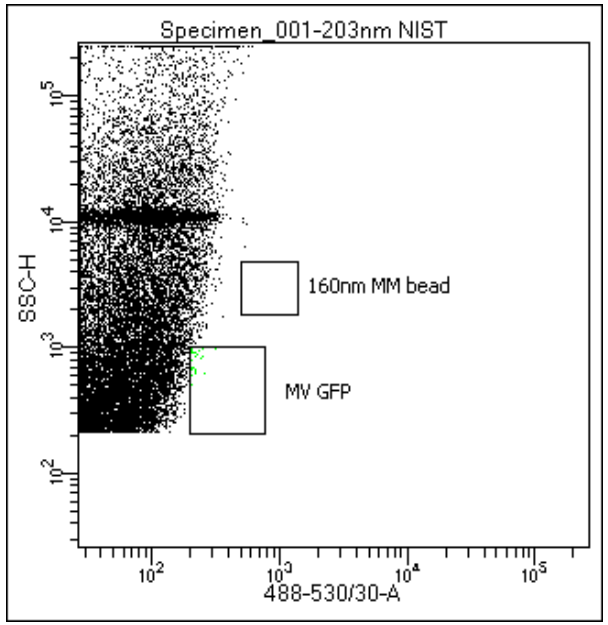
Exponential Scale

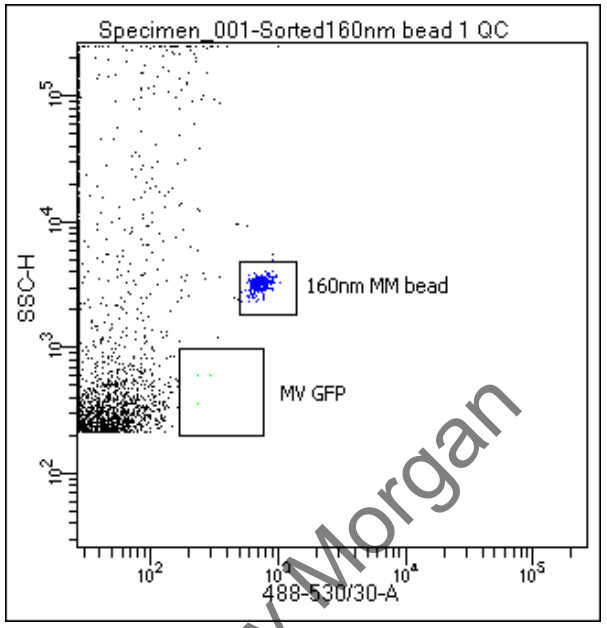


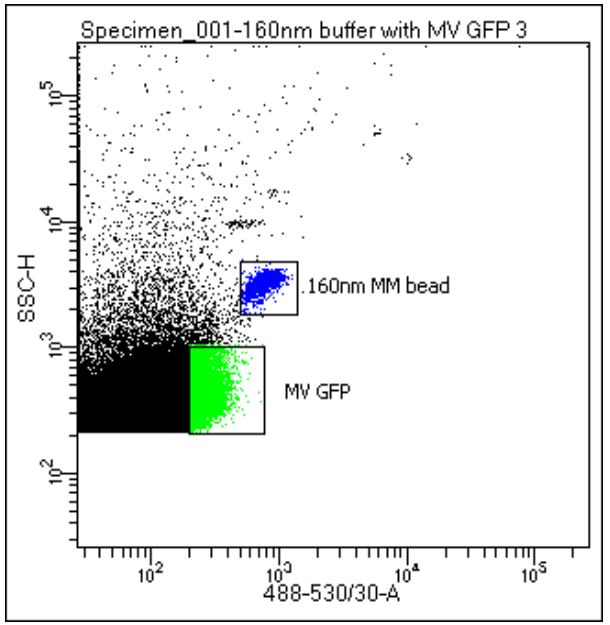
Y-axis standardized by voltage and polystyrene Megamix “sizing” beads putting 200nm bead at 10^4 SSC-H

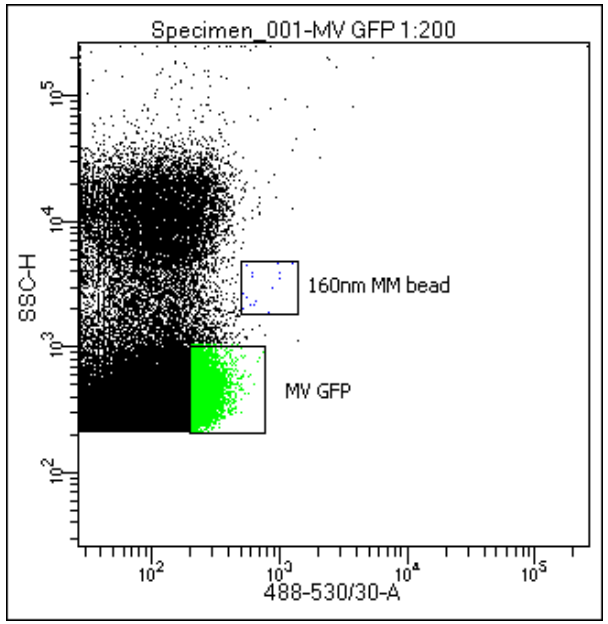
X- axis standardized by channel-specific laser voltage and BD-MESF beads with brightest at ~10^5 (e.g. FITC, APC, PE)

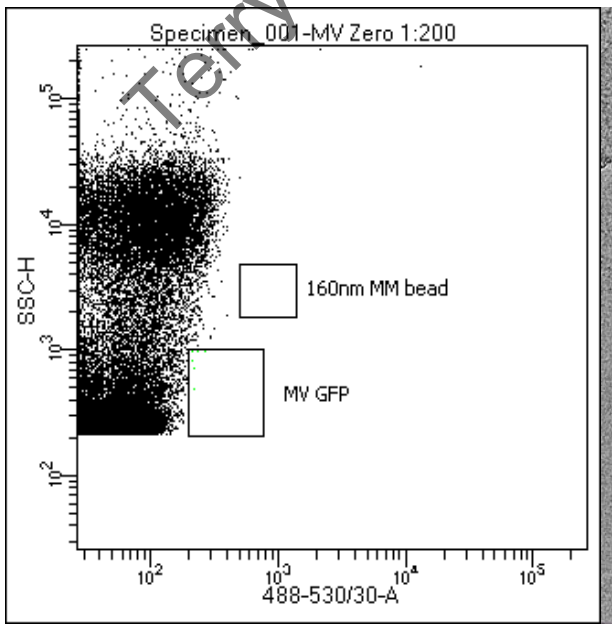
GFP-conjugated murine 100nm virus

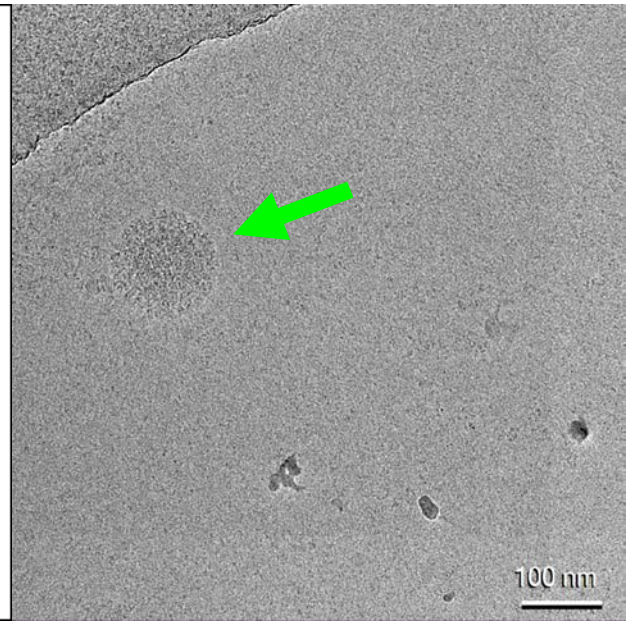




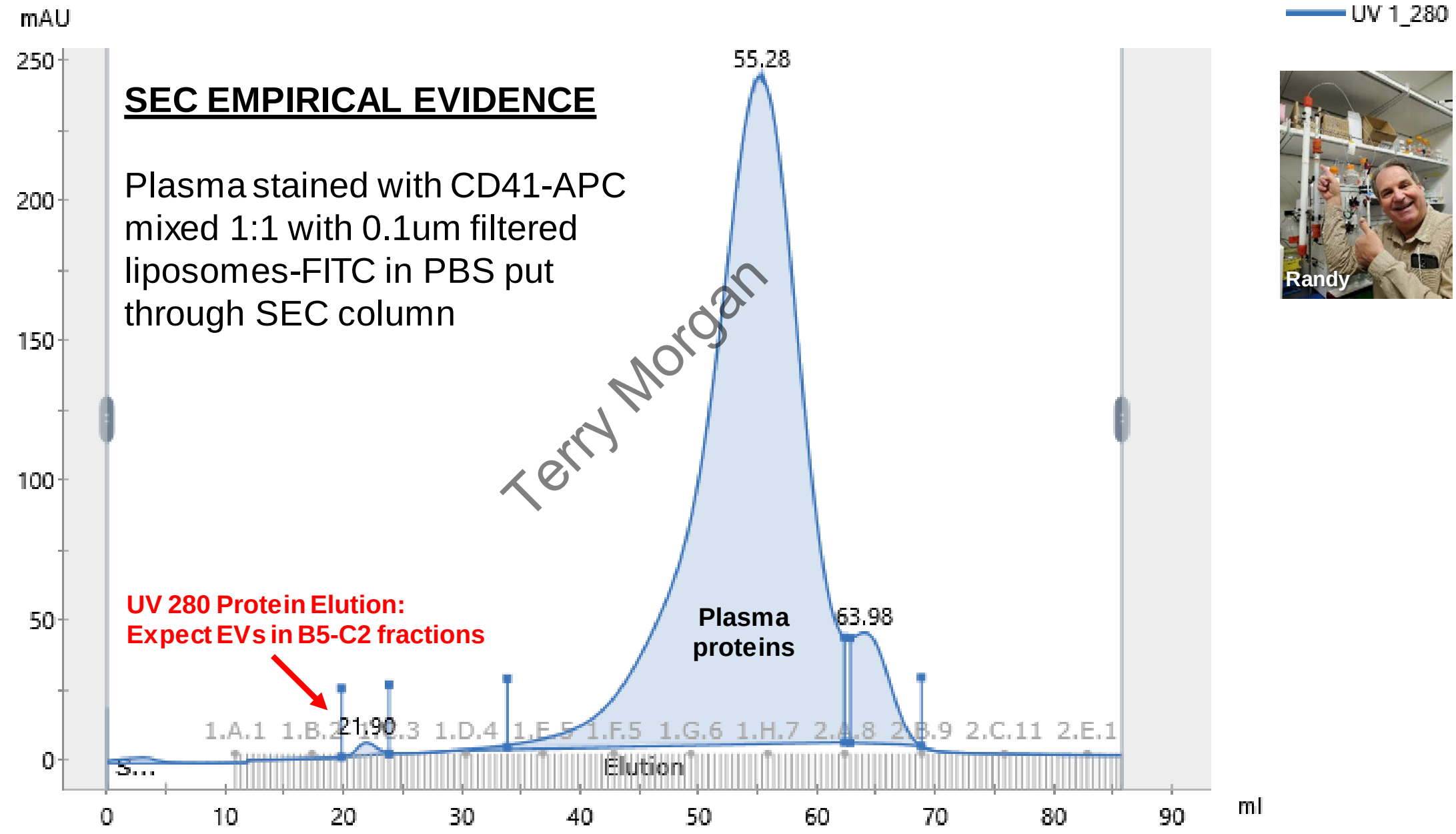




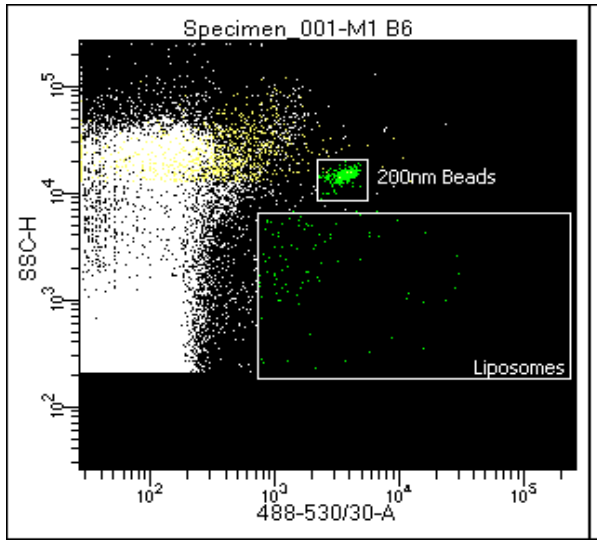


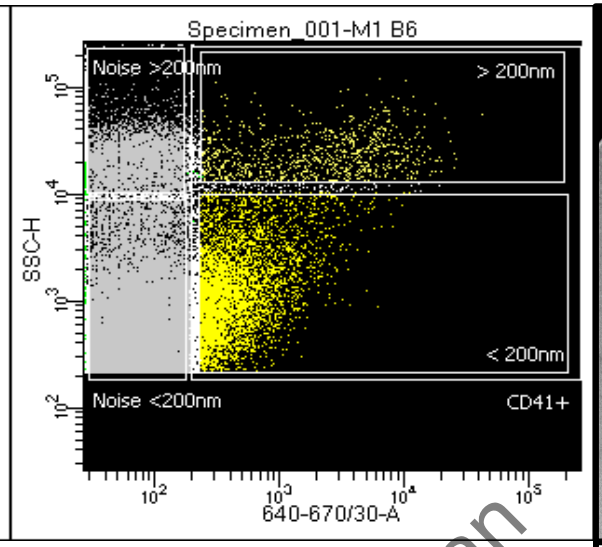


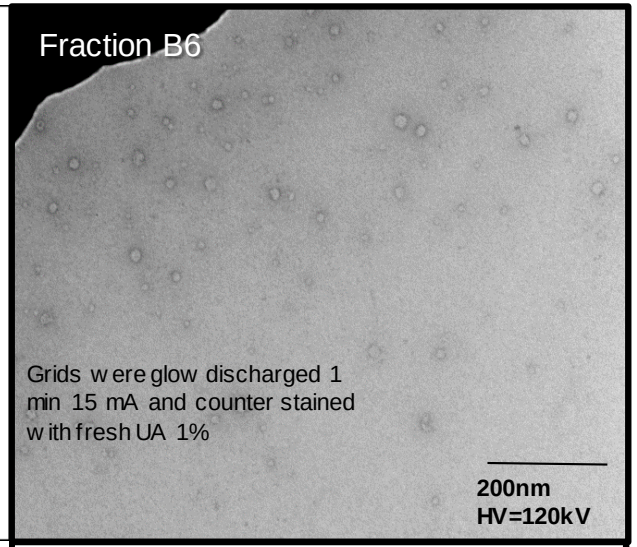
Terry Morgan


CL2B 16X40 EV 02072020 001
mAU
UV 1_280
250
200
150
100
50
0
55.28
63.98
21.90
SEC EMPIRICAL EVIDENCE
Plasma stained with CD41-APC mixed 1:1 with 0.1um filtered liposomes-FITC in PBS put through SEC column
Terry Morgan
UV 280 Protein Elution:
Expect EVs in B5-C2 fractions
Plasma proteins
1.A.1 1.B.2 1.C.3 1.D.4 1.F.5 1.F.5 1.G.6 1.H.7 2.A.8 2.B.9 2.C.11 2.E.1
Elution
0
10
20
30
40
50
60
70
80
90
ml

Randy





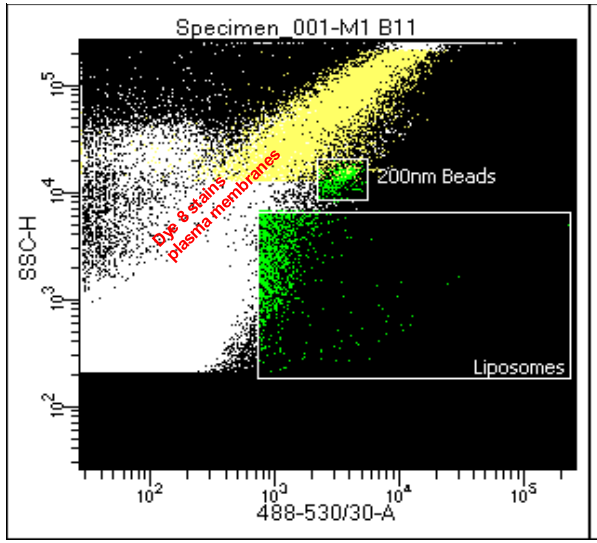


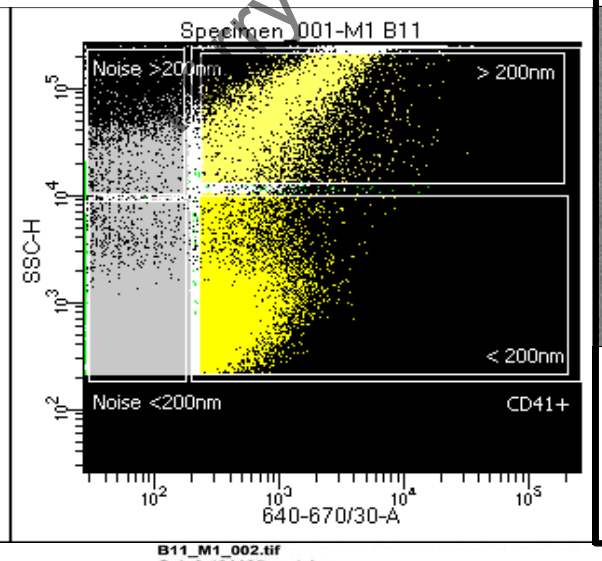
Tube: M1 B6

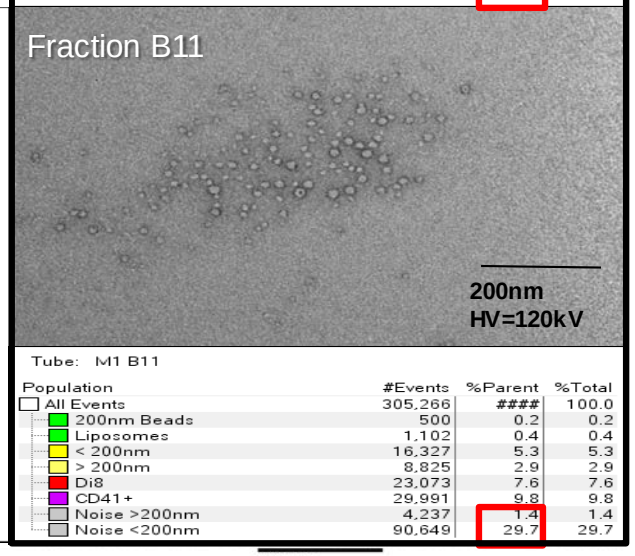
Population	#Events	%Parent	%Total
All Events	196,518	####	100.0
200nm Beads	500	0.3	0.3
Liposomes	89	0.0	0.0
< 200nm	4,423	2.3	2.3
> 200nm	821	0.4	0.4
Di8	1,137	0.6	0.6
CD41+	6,374	3.2	3.2
Noise >200nm	5,038	2.6	2.6
Noise <200nm	46,080	23.4	23.4

CD41+ MVs in B6 vs B11 fractions elute with Di-8 (stains plasma membranes)

***** Smaller elutes later on columns so expect more small EVs in B11> B6**







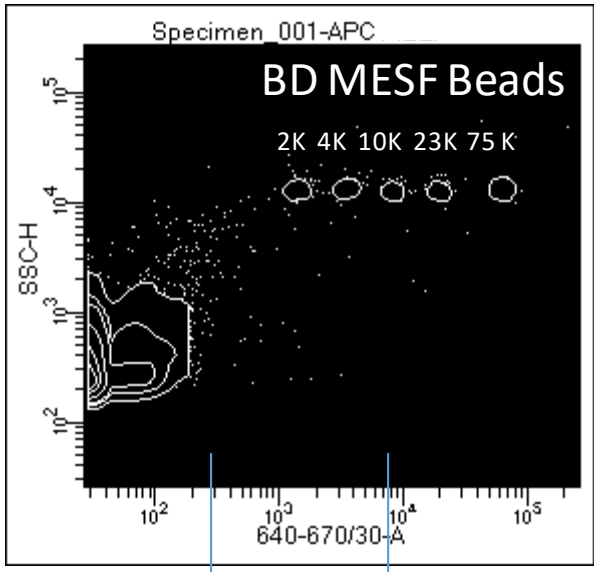
Tube: M1 B11

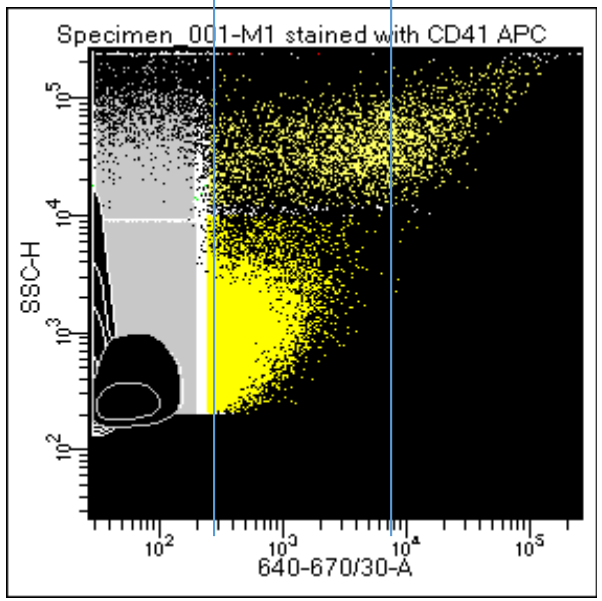
Population	#Events	%Parent	%Total
All Events	305,266	####	100.0
200nm Beads	500	0.2	0.2
Liposomes	1,102	0.4	0.4
< 200nm	16,327	5.3	5.3
> 200nm	8,825	2.9	2.9
Di8	23,073	7.6	7.6
CD41+	29,991	9.8	9.8
Noise >200nm	4,237	1.4	1.4
Noise <200nm	90,649	29.7	29.7

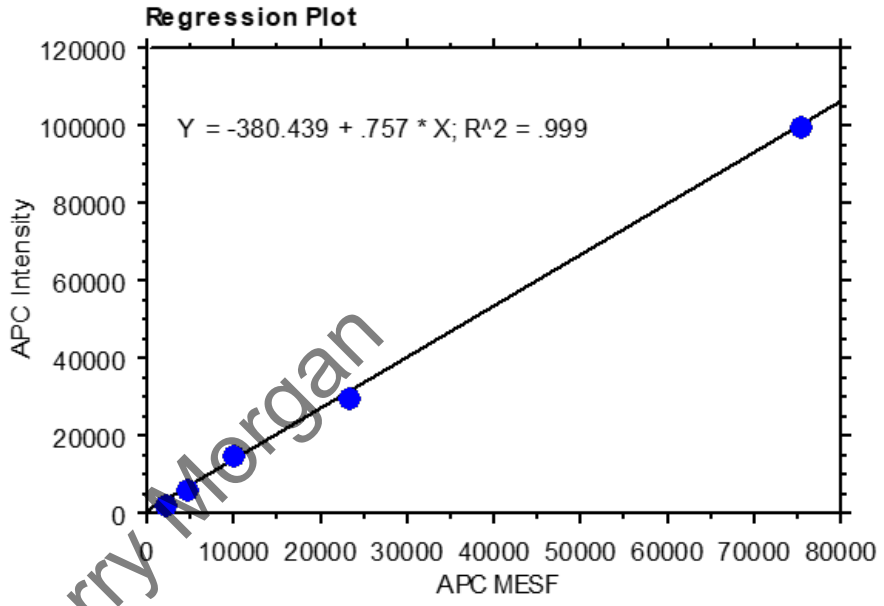
B11_M1_002.tif
Cal: 0.421135 nm/pix
12:15 2020-03-06

200 nm
HV=120kV
Direct Mag: 68000 x
MMC

How many antibodies/EV provided Fl/Ab ~ 1:1?







Expected number of CD41-APC antibodies on each "exosome" per regression plot equation from MESF beads is **~100 Ab/EV-sized "exosome"; whereas:**

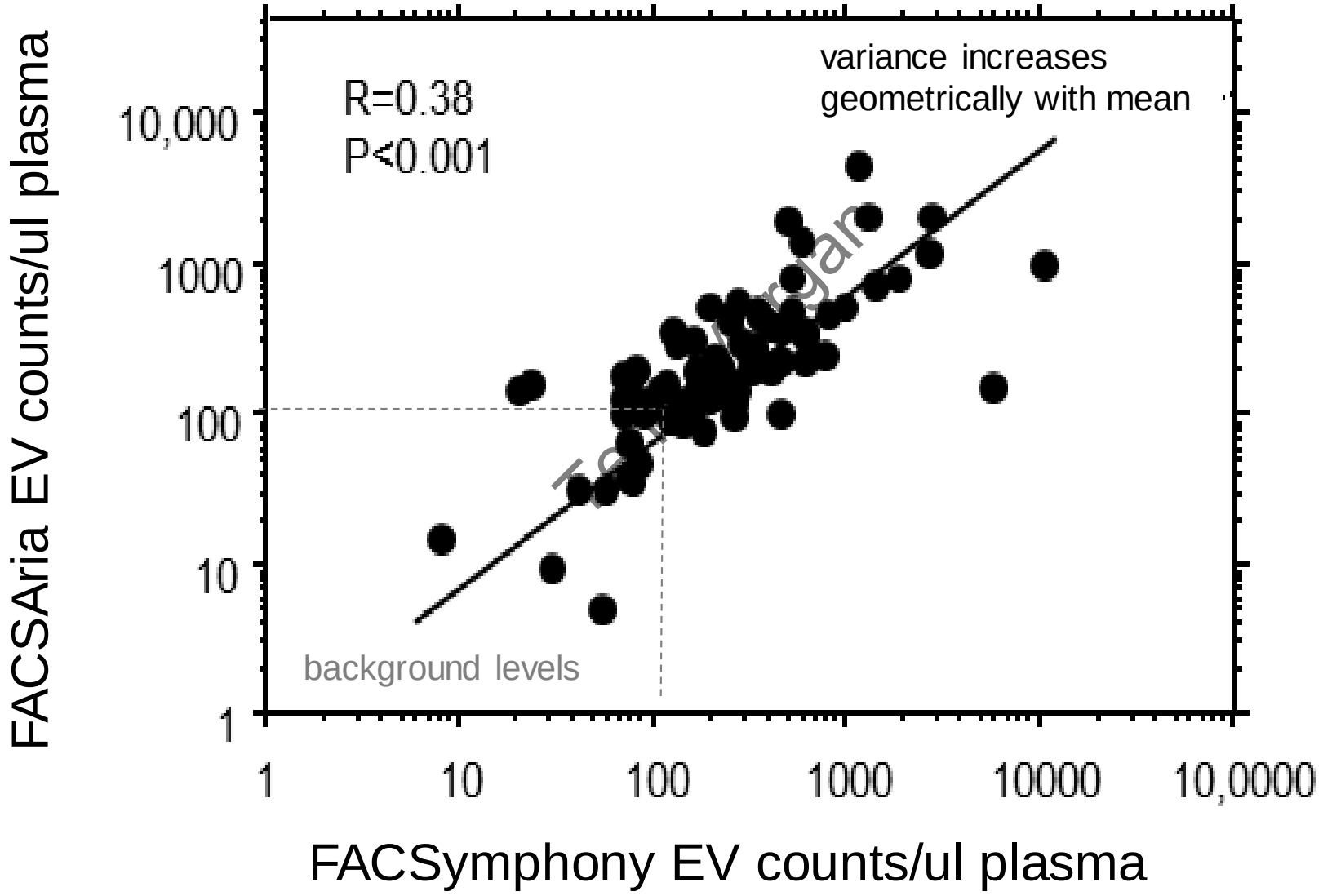
MVs have ~ 10,000 Ab/EV

Surface area ($4\pi r^2$) of a 100nm EV is **31,416nm**
Surface area of 350nm MV = **385,000nm**

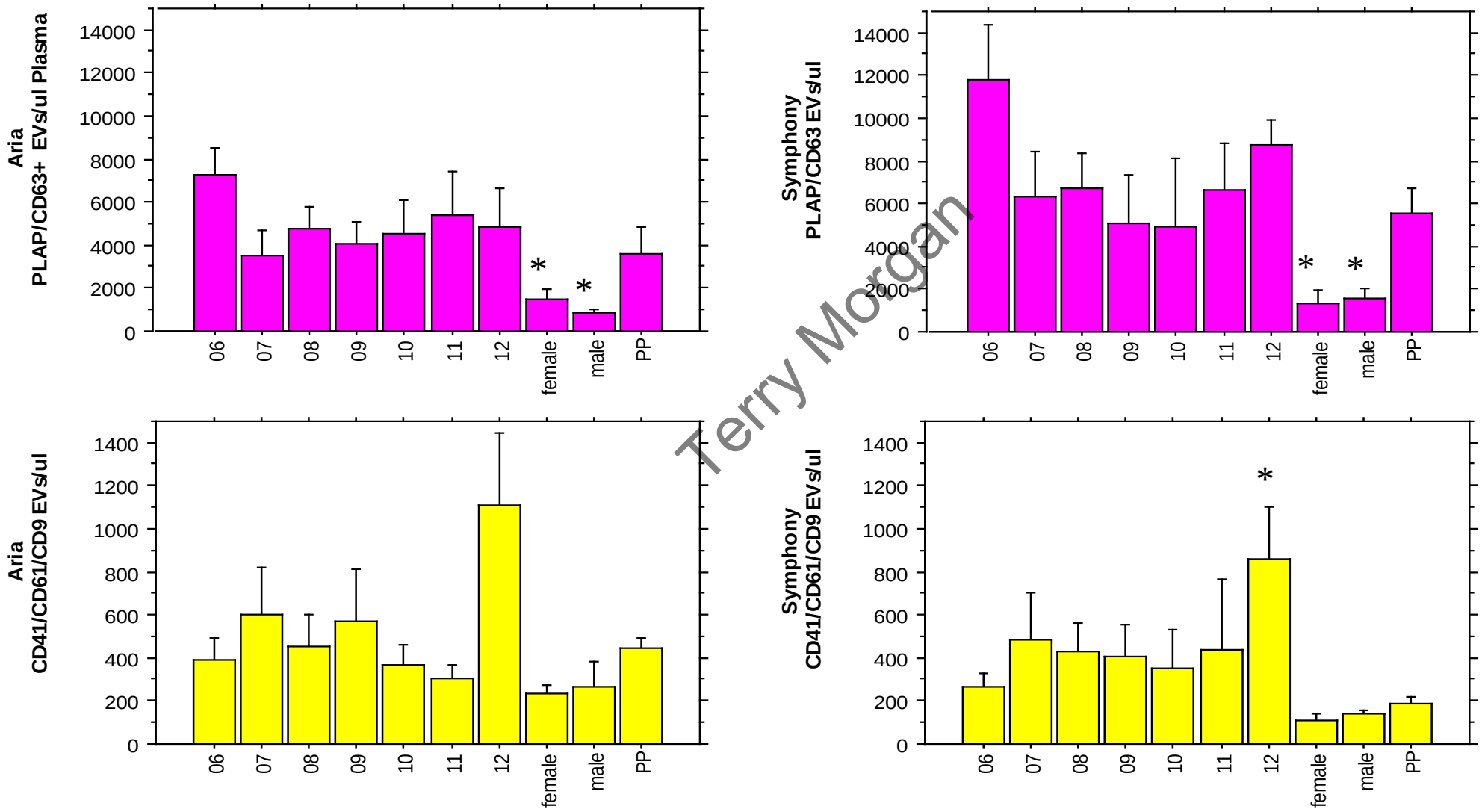
Antibody is ~ 10nm in diameter

We should be able to fit 100s if not 1000s of antibodies on an EV

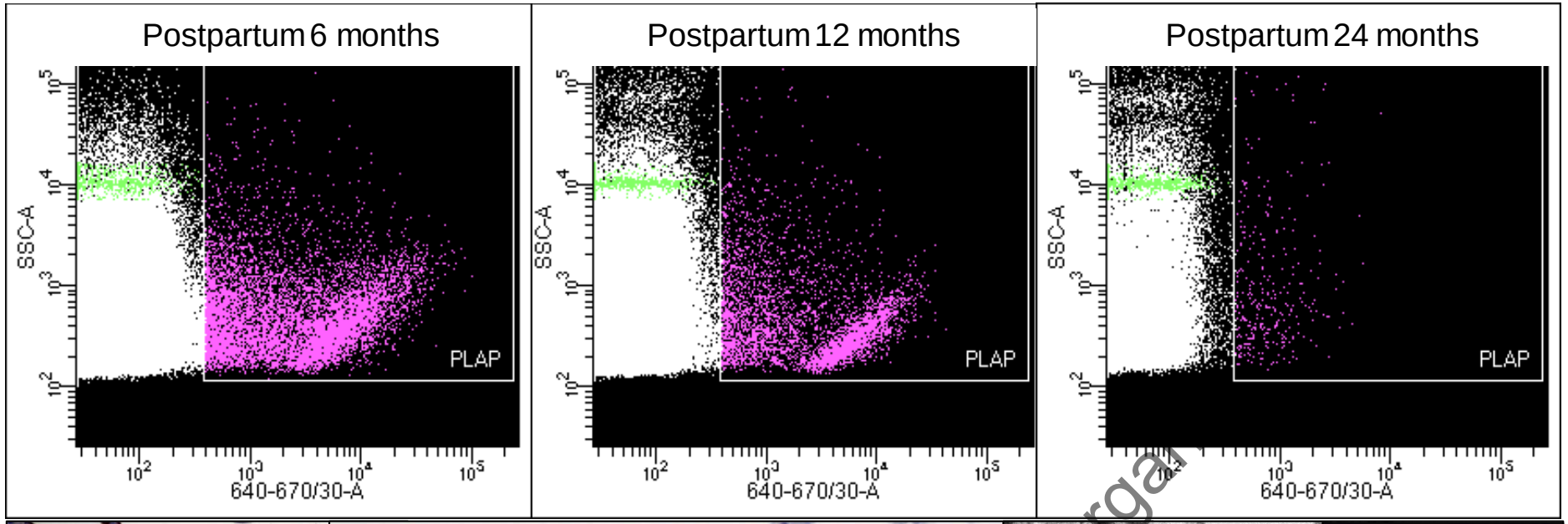
Reproducibilty Between Machines and Technicians Creating Independent Uniform Gates

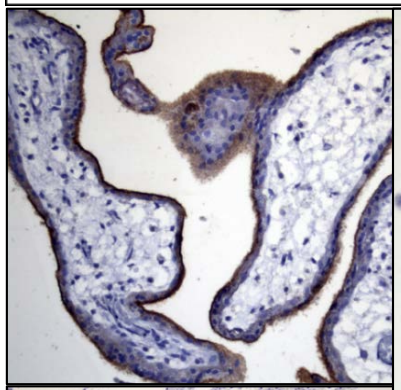


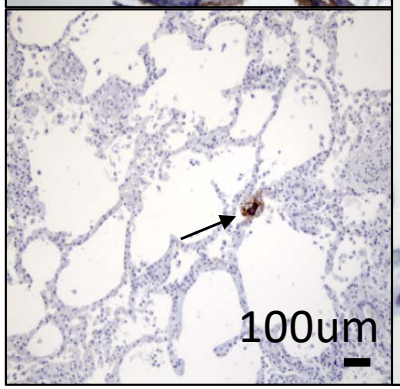
Reproducible Differences in Placental EV and Platelet EV Profiles

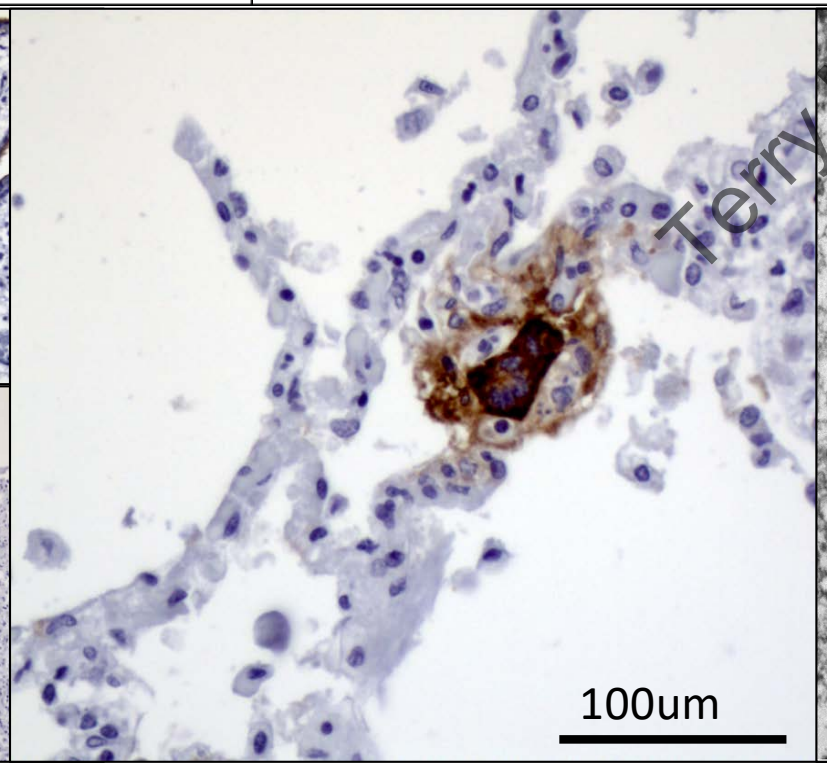


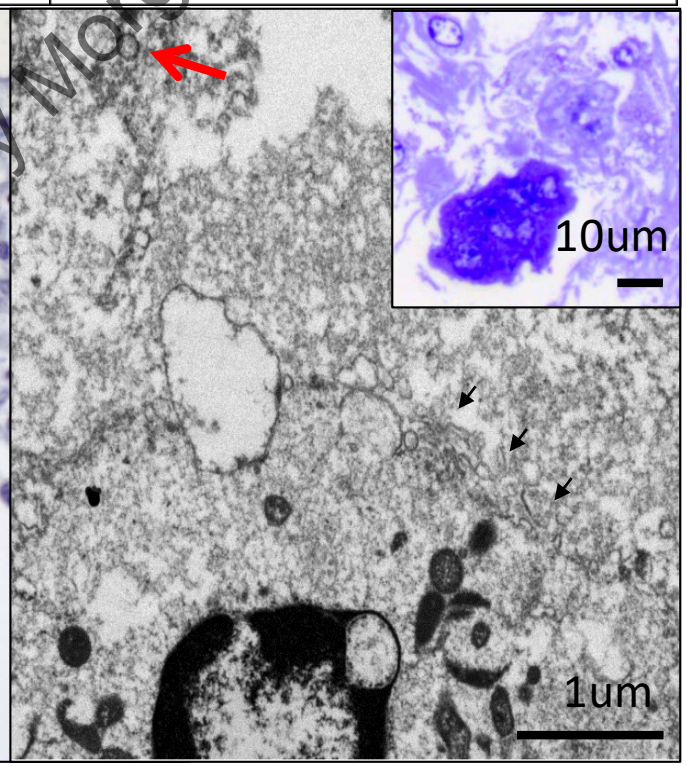
Roberts & Morgan, *et al*.
Hum Reprod. 2017. 32(12): 2382-93











Placental STB Metastases to **Mom's Lungs** Release PLAP+ EVs Into her blood:

Do they have a function?!