Science Webinar Series
Analysis of extracellular vesicles including exosomes by imaging flow cytometry
June 22, 2016

Participating experts

André Görgens, Ph.D.
Essen University Hospital
Essen, Germany
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Analysis of extracellular vesicles including exosomes by imaging flow cytometry

Science/AAAS Technology Webinar Series
June 22, 2016

University Hospital Essen
Institute for Transfusion Medicine
André Görgens, Ph.D.
Human Hematopoiesis

Multi-Parameter Flow Cytometry / Sorting

Görgens et al., Cell Reports 2013
Görgens et al., Cell Cycle 2013
Görgens et al., Stem Cell Reports 2014
Human Hematopoiesis

Multi-Parameter Flow Cytometry / Sorting

Görgens et al., Cell Reports 2013
Görgens et al., Cell Cycle 2013
Görgens et al., Stem Cell Reports 2014
Doeppner, Herz, Görgens et al., Stem Cells Transl Med 2015
Börger, Bremer, Görgens et al., ISBT Science Series 2016
Human Hematopoiesis

Multi-Parameter Flow Cytometry / Sorting

Specific markers and optimized methods required to resolve EV heterogeneity

Görgens et al., Cell Reports 2013
Görgens et al., Cell Cycle 2013
Görgens et al., Stem Cell Reports 2014
Doeppner, Herz, Görgens et al., Stem Cells Transl Med 2015
Börger, Bremer, Görgens et al., ISBT Science Series 2016
Extracellular Vesicles (EVs)

EVs are secreted by a wide range of cells from different species. EVs can be found in nearly any body fluid.
Putative physiological functions: antigen presentation, intercellular communication, shuttling of RNAs between cells …
Extracellular Vesicles (EVs) Heterogeneity

How can EVs/Exosomes be analyzed?
Methods for EV analysis

• Nanoparticle Tracking Analysis

• Electron Microscopy, Resistive Pulse Sensing, …

Size distribution
Particle concentration
Methods for EV analysis

- Nanoparticle Tracking Analysis
- Electron Microscopy, Resistive Pulse Sensing, ...
- Flow Cytometry
  - All methods currently available are limiting, including flow cytometry (size, signal intensity)
  - New & improved methods for EV analysis highly desired by the field
Submicron-Sized Polystyrene Beads as Calibrators?

Biocytex Megamix-Plus SSC/FSC beads

based on: „Set-Up of the CytoFLEX* for Extracellular Vesicle Measurement“, Prof. Dr. Andreas Spittler, Medizinische Universität Wien (Beckman Coulter White Paper)
www.beckmancoulter.de
Submicron-Sized Polystyrene Beads as Calibrators?

Biocytex Megamix-Plus SSC/FSC beads

Flow Cytometer Comparison:

- Beckman Coulter FC500
- Beckman Coulter Gallios
- Beckman Coulter Cytoflex
- ACEA Novocyte 3000
- BD Accuri C6
- BD FACS Aria IIIu
- Amnis ImageStreamX MkII

none of these were built for analysis of submicron particles!

based on: „Set-Up of the CytoFLEX* for Extracellular Vesicle Measurement“, Prof. Dr. Andreas Spittler, Medizinische Universität Wien (Beckman Coulter White Paper)

www.beckmancoulter.de
Analysis of Submicron-Sized Beads
Analysis of Submicron-Sized Beads
Imaging Flow Cytometry – Amnis ImageStreamX MkII

- **Immunophenotype**
  - CD133
  - Gated on CD34^+
  - CD45RA^-
  - CD45RA^+

- **Subcellular localisation**
  - Image of cells

- **Morphology**
  - Image of cells

**Amnis ImageStreamX MkII**
- 5 lasers
- up to 12 channels
- Automatic triggering on all channels

Amnis - part of MilliporeSigma a business of Merck KGaA, Darmstadt, Germany
Imaging Flow Cytometry – Amnis ImageStreamX MkII

Amnis - part of MilliporeSigma a business of Merck KGaA, Darmstadt, Germany
# ImageStreamX Fluorochrome Chart

## Excitation Laser (nm)

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### BRIGHTFIELD

- FITC, AF-488, GFP, YFP, DyLight488, PKH26, Syto13, SpectrumGreen, LysotrackerGreen, MitoTrackerGreen, QD625®
- PE, Cy3, AF546, AF555, DyLight549, DyLight594®, PKH67, DSRed, SpectrumOrange, MitoTrackerRedOrange
- PE-TexasRed®, ECD®, PE-AF610®, iAAtD®, RFP, QD625®
- AF588®, AF647®, AF647®, AF610®, DyLight594®, PE-TexasRed®, ECD®, TRITC®, PE-AF610®, RFP, mCherry®, iAAtD®, PI®
- PE-Cy5®, PE-AF647®, ParaCP®, ParaCP-Cys®, DRAQ6®, QD705®, aFluor625®
- PE-Cy5®, PE-AF647®, DRAQ6®, PE-Cy5®, PE-AF750®, QD800
- DAPI, Hoechst, PacificBlue, CascadeBlue, AF-405, aFluor405, DyLight405, CFP, LIVE/DEAD Violet
- ParaOrange, CascadeYellow, AF-430, QD525®
- QD625®, aFluor625®
- QD705®, aFluor650®
- QD800®
- AF647®, AF660, AF680, DRAQ6®, APC, Cy5, DyLight549, DyLight650, PE-AF610®, PE-Cy5®, ParaCP, ParaCP-Cys®
- APC-Cy7®, APC-AF750, APC, aFluor750, Cy7, AF750, DyLight800, aFluor850, PE-Cy7®, PE-AF750®

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Analysis of Microparticles Using Imaging Flow Cytometry
Submicron-Sized Polystyrene Beads as Calibrators

Amnis ISX MkII
Submicron-Sized Polystyrene Beads as Calibrators

24 nm beads (Thermo Scientific)

Amnis ISX MkII

✓ Beads

EVs? Exosomes?
CD63-eGFP EVs as Biological Calibrator for Flow Cytometry

Tetraspanins

CD53
CD63

HEK293T CD63-eGFP

Precipitation of the supernatant by ultracentrifugation

HEK + EGFP
HEK + CD53-EGFP
HEK + CD63-EGFP
CD63-eGFP EVs as Biological Calibrator for Flow Cytometry
CD63-eGFP EVs as Biological Calibrator for Flow Cytometry
CD63-eGFP EVs as Biological Calibrator for Flow Cytometry
Lysis Control

0.5% Nonidet NP-40
30 min

+ Detergent

0%
Evaluation of Acquisition Parameters
Evaluation of Acquisition Parameters
Optimization of EV Masking
Optimization of EV Masking
Detection of Single Vesicles vs. Swarm Detection

![Image showing detection methods with examples]

Table: Standard Channel Mask, Peak Mask, Morphology Mask, Intensity Mask

- **Standard Channel Mask**: Unique patterns for identification.
- **Peak Mask**: Highlighted areas showing peaks.
- **Morphology Mask**: Shown with morphological features.
- **Intensity Mask**: Differing intensities indicated.
Detection of Single Vesicles vs. Swarm Detection

Gated on eGFP+ (3.5x10^8 objects/mL)

Gated on eGFP+ (7.7x10^6 objects/mL)

Emission maps for different gates:
- 31650
- 32108
- 33300
- 45228
- 2657
- 3984
- 21674
- 3165
- 15287
- 84025
- 1672
- 22943

Intensity Mask

闸门设置和对比

"22943" 这个门被用红色方框标记，表示特殊关注。
Summary

- eGFP-positive biological calibrator to optimize imaging flow cytometry for EV analysis
  - Evaluation of acquisition parameters
  - Optimization of masking
  - Discrimination of single EV detection from swarm detection

Imaging Flow Cytometry...

- ... facilitates reliable detection & quantification of single, fluorescent EVs
- ... absolute quantification (objects/mL sample) of low volume samples (15 μL)
- ... high sensitivity, clear separation of signal from background/noise
- ... image-based features to optimize analysis post acquisition (i.e. exact discrimination of doublets or background)

- Range of linearity
- Analysis parameters
- Variability
- Comparison of different machines
How can we apply this protocol?

• Evaluation of EV isolation methods
• Validation of antibody staining protocols

• Validation of other specific EV probes (fluorescent dyes)
Re-Evaluation of Exosome Isolation Protocols
Re-Evaluation of Exosome Isolation Protocols

1. Complete Cell Culture Suspension
2. 2,000 x g SN
3. 10,000 x g SN
4. 0.22 µm Filtrate
5. UC Pellet

Results:
- 100% eGFP
- 76% eGFP
- 57% eGFP
- 40% eGFP
- 7% eGFP
Staining of EVs with Antibodies

- EVs + CD63-PE
- EVs + Isotype-PE
- PBS + CD63-PE

Staining at different dilutions:
- 1:50
- 1:25
- 1:10

Unstained control
Antibodies – Background Issues

**PBS** + antibody (buffer controls)

- Antibody A
- Antibody B
- Antibody C
Summary

- Optimized protocol for EV analysis including Exosomes using Imaging Flow Cytometry (Görgens et al, in preparation)
- Robust quantification of EV subsets
- Can be used with unprocessed samples
- Robust multiparametric surface analysis of single EVs
- CD63-eGFP EVs as a biological calibrator/tool for protocol optimization and validation

Robust high-resolution analysis of EVs subpopulations
- with specific, validated probes
- in all sample types/unprocessed samples
Acknowledgements

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Vera Rebmann

Coralie Guérin
Chantal Boulanger

Sebastian Seth
Christin Probst
Sherree Friend
Peter Rhein

Uta Erdbruegger
Joanne Lannigan

andre.goergens@uk-essen.de
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